Novel approaches in complement profiling; application in kidney transplantation

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DOI:
10.33612/diss.222088562

NOVEL APPROACHES IN COMPLEMENT PROFILING; APPLICATION IN KIDNEY TRANSPLANTATION

Rosa G.M. Lammerts

Financial support for this thesis was generously provided by Filtech Nederland BV, Erbe Nederland BV, de Nederlandse Transplantatie Vereniging, Hycult Biotech, XVIVO, de Nierstichting, Chiesi and the Rijksuniversiteit Groningen.

Printed by	Ipskamp Printing Enschede, the Netherlands
Design & layout	Bianca Pijl, www.pijlldesign.nl Groningen, the Netherlands
Illustration cover	Marja van der Linden, www.marjavanderlinden.com

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NOVEL APPROACHES IN COMPLEMENT PROFILING; APPLICATION IN KIDNEY TRANSPLANTATION

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

maandag 27 juni 2022 om 14:30 uur

door

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geboren op 11 juli 1989
te Zuidwolde (GR)

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Wouter Tobias van Haaften

Voor Hella

In memoriam

9 januari 1964 -7 januari 2019

Hella van der Lugt

Draden wikkelen
de kluwen;
genen van verledens
spinnen leven
nieuw

De wereld
dreunde verder
toen jouw hart zweeg;
maar in mij,
de stilte
vormend jouw beeld,
bewaar ik je.

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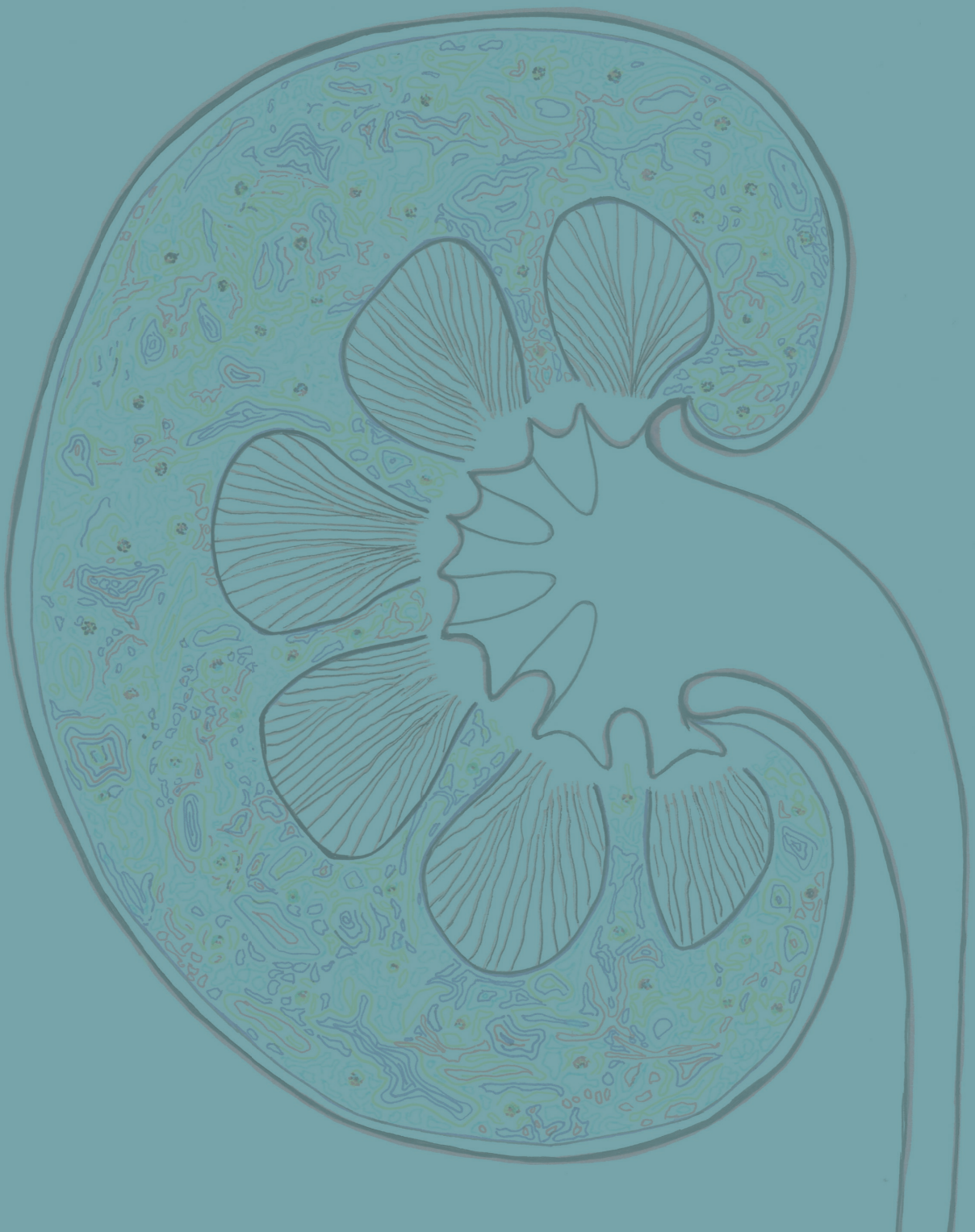

CHAPTER 1

General Introduction and aim

Introduction

The complement system

The complement system is an important part of the innate immune system. As an evolutionary ancient immunological entity, the complement system is found in vertebrae and invertebrae.
The complement system was discovered by Jules Bordet, more than one hundred years ago, as a distinguishable blood component that had the capacity to opsonise and lyse bacteria.[1] Since then, not only more complement factors were discovered , also the broad actions of complement started to be acknowledged. Now it is recognized that the complement system enhances the ability of antibodies and phagocytic cells to promote inflammation, clears damaged cells and microbes from an organism, and attacks the pathogen's cell membrane.[2] Heat stable antibodies, reacting specifically and with increasing activity with the immunizing organism were discovered; in contrast, the 'complementing' factor was constant in activity but was heat labile. Nowadays, we know that the antibody complexes formed in this reaction activate the classical pathway of the complement system (CP). The CP is amplified by the alternative pathway of the complement system (AP), followed by the formation of the membrane attack complex (MAC), also called C5b-9.[3,4] Complement activation involves three pathways (AP, CP and lectin pathway). This thesis focuses on the AP and the CP and their possible role in kidney diseases with proteinuria and in kidney transplantation.

Complement activation, regulation and inhibition

CP activation occurs when antibody-antigen complexes bind to C1q, the first complement protein of the cascade, leading to activation of the serine proteases C1r and C1s. C2 and C4 are then cleaved by the $C1q,r_2s_2$ complex and the C3-convertase is formed, also known as C4bC2a. The LP is initiated via a number of different pattern recognition receptors, that form macromolecular complexes with mannan-binding lectin serine proteases 1, 2 and 3 (MASP 1, 2 and 3). Upon activation these will cleave C2 and C4 and form the C3-convertase. Mannan binding lectin (MBL) and various members of the ficolin and collectin families are the most important LP-associated pattern recognition receptors.[5] The AP is activated by spontaneous hydrolysis of C3, representing a state of constant low-rate activation, which causes C3 to split into C3b and C3a. If C3b binds covalently to factor B, the C3-convertase C3(H20)Bb is formed. The C3-convertase cleaves additional C3b. C3b is protected against inactivation by factor I and H if surfaces favouring AP activation such as on bacterial cells and yeast cells, are present. Then a highly efficient C3-convertase is formed, C3bBb, which is particularly efficient when stabilized by properdin.[6] The AP amplifies the complement activation caused by the CP or the LP, and properdin enhances the half-life of the AP C3-convertase tenfold.[7] However, in the past decade data has accumulated stating that properdin can also act as a pattern recognition molecule on bacterial cells, necrotic, apoptotic, and on proximal tubular epithelial cells.[8] This notion was originally postulated in 1954 by Pillemer,[9] and rediscovered 50 years later.[10] However, other groups showed that the binding of properdin to endothelial cells is dependent on initial C3b binding, partly challenging the concept of properdin being a pattern recognition molecule.[11]

Each of the C3-convertases, either the CP C4bC2a or the AP C3bBb, can form a C5-convertase by association with C3b. C5-convertase catalyzes the cleavage of C5 into C5a and C5b. The latter interacts with C6, C7, C8 and C9, after which C5b-9 is formed. C5b-9 is a structure creating pores in membranes that leads to cell lysis. Besides the cytotoxic effects of C5b-9, proximal complement factors reinforce pro-inflammatory actions. For example, C1q, C3b, iC3b and C4d function as opsonins that are covalently linked to the target cell surface. By this strategic position, they function as recognition molecules for phagocytes. Leukocytes possess specific complement receptors on their cell membrane (CR3, CR4, CRIg and C1qR), allowing them to bind and engulf the opsonized target. The major complement opsonins are C3b and iC3b, the C3 cleavage products.[12] The potent effects of the complement system are tightly regulated and controlled by a family of natural fluid phase and membrane bound regulatory proteins, referred to as complement inhibitors.[13] They restrict propagation to invading pathogens or altered self-cells. For example, membrane-bound complement inhibitors like membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55), and CD59 are constitutively expressed on the endothelium.[14] CD46 binds to C4b and C3b, acting as cofactor for their breakdown by factor I to iC3b and C4c and C4d.[15] CD55 prevents the formation and accelerates the decay of C3 and C5 convertases, reducing the deposition of C3b and C5b-9 and preventing the generation of the anaphylotoxins C3a and C5a.[16] CD59 is a membrane glycoprotein, inhibiting MAC by incorporating into the formation of the complex at the level of C9.[17] Fluid phase complement regulators like C4b binding protein, factor H and factor I prevent complement overactivation by exerting decay-acceleration activity.[14]

Over the past decades, the understanding of the complement system has evolved from the traditional view as defence system against microbes, necrotic and apoptotic cells, towards its crucial and acknowledged role in the development and/or progression of disease processes. The latter includes diseases like paroxysmal nocturnal hemoglobinuria, angio edema, neurogenerative diseases, pregnancy complications, atherosclerosis and cardiovascular diseases. Moreover, the complement system has been shown to contribute to renal injury in various renal diseases and is known to play a central role in the immunological processes after kidney transplantation (Table 1).[2,18–21]

Table 1. The involvement of complement in kidney diseases.

Classical pathway	Lectin pathway	Alternative pathway
Transplantation[22]	Transplantation[23]	Transplantation[24]
Anti-GBM disease[25]	Ischemia reperfusion[26]	Ischemia reperfusion[24]
Lupus nephritis[26]	IgA nephropathy[27]	IgA nephropathy[28]
Membtanoproliferative glomerulonephritis[29]	Henoch-Schonlein purpura nephritis[30]	Henoch-Schonlein purpura nephritis[30]
	Diabetic nephropathy[31]	ANCA-associated vasculitis[32]
	Membranous nephropathy[33]	C3 glomerulopathy[29,34]
	Lupus nephritis[26]	Lupus nephritis[26]
		Hemolytic uremic syndrome[35]
		Membranoproliferative glomerulonephritis[29]

There are a number of complement inhibitors that have been applied in the clinic or are currently being tested. Theoretically inhibition of the complement system is possible at multiple levels; for example inhibition of only the classical pathway, the alternative pathway, the lectin pathway or the membrane attack comp lex. Most complement inhibitors are still in development and only two complement inhibitors are currently established in clinical use; eculizumab and C1-INH. The oral small molecule inhibitor avacopan is a C5aR antagonist, has recently received FDA approval for use as add-on treatment in ANCA-associated vasculitis and is currently entering clinical practice.[36] Eculizumab is an anti-C5 antibody that prevents complement activation downstream in the complement cascade.[37] Eculizumab has proved to be effective in complement driven diseases like aHUS.[38–40] C1-INH is an endogenous plasma derived C1 esterase inhibitor and used for the treatment of for example angio edema.[41] The wide variety of therapeutic compounds against different parts of the complement system is currently in development and clinical trials are ongoing for a number of complement related diseases. TNT009 is an anti-C1s antibody,[42] APT070 (mirococept) is a C3 convertase inhibitor,[43] AMY101 (Compstatin) and Pegcetacoplan are C3 inhibitors.[44,45] The main limitations of downstream inhibition of complement, might be that the targeted surfaces can still be opsonized with C3b and iC3b, resulting in breakthrough events.[14] For example, studies evaluating the efficacy of C5 inhibitors as a potential treatment for antibody-mediated rejection (ABMR) were insufficient in preventing ABMR occurrence in the long term.[37,46,47] Also, inhibiting the membrane attack complex comes with a higher risk for infections, e.g. neisseria meningitis. Inhibition of the AP could be an interesting addition to existing inhibitors. In addition to the complement components that are already targeted, properdin could be attractive as a therapeutic target in diseases like aHUS andC3G that are associated with overactivation of the

alternative pathway.[48] A novel AP inhibitor is a protein derived from the deer tick Ixodes scapulari and is called Salp20. Salp20 has been shown to inhibit the AP via the displacement of properdin causing an accelerated decay of the C3bBb complex and subsequent inhibition of the AP up t0 70%.[49,50] However, no experiments have been performed yet in inhibiting the AP activation on a renal cellular level.

Kidney transplantation – an immunological challenge

Kidney transplantation represents the best therapeutic option for patients with end-stage renal failure, providing both a better quality of life and a longer life expectancy,[51] for only a fraction of the costs of chronic hemodialysis.[52,53] The process of transplantation consists of sequential events that may affect the graft: (immunological) effects linked to cardiac or brain death in organ donors, the organ procurement procedures, the processes of preservation and reperfusion, immune responses after transplantation and the recurrence of native diseases. During this stepwise process, various immune responses can induce graft injury and contribute to loss of kidney function and eventually graft loss.[54–56] The current major challenge faced by kidney transplantation is to prolong the duration of graft function.[57,58] Increasing evidence indicates the previously largely unrecognized role of the complement system in the specific- and non-specific inflammatory reactions that occur before, during and after transplantation.[18,59–62]

Proteinuria-driven tubular complement activation in renal transplantation

The development of interstitial fibrosis and tubular atrophy (IF/TA) is a major problem after kidney transplantation, presenting as renal allograft dysfunction (occurring at least three months post-transplant) in the absence of active acute rejection, drug toxicity, or other diseases.[63] The clinical diagnosis of chronic allograft failure after kidney transplantation is usually made in the presence of a gradual deterioration of allograft function, manifested by a slowly rising serum creatinine concentration, worsening hypertension and increasing proteinuria.[64,65] Since the reduction of proteinuria is a major therapeutic goal in reducing the risk for progression of renal (allograft) injury, it is of utmost importance to understand the role of complement in the pathomechanism of proteinuria mediated kidney damage. Among other mechanisms, proteinuria contributes to the progression of kidney failure by leakage of albumin-bound lipids across the damaged glomerular filtration barrier, leading to lipoapoptosis after reabsorption by the downstream proximal tubule.[66] In parallel with this, complement may be harmful to renal proximal tubular epithelial cells (PTEC). PTEC play a crucial role in renal function by reabsorbing water and nearly all of the amino acids and glucose in the glomerular filtrate, while allowing other substances to be excreted in the urine.

PTEC can produce inflammatory mediators such as complement and during proteinuria PTEC can respond with a pro-inflammatory and profibrotic pattern.[67] Our group has shown that tubular heparan sulfate proteoglycans (HSPGs) form the docking platform for urinary properdin and subsequent AP activation on PTEC.[68] This is in line with the finding that properdin acts as a pattern recognition molecule binding to proteoglycans on macrophages, necrotic and apoptotic cells.[69] HSPGs are linear carbohydrates consisting of repeating disaccharide units (the glycosaminoclycans) attached to a protein core. HSPGs can be found on cell surfaces and in

basement membranes. These HSPGs are able to bind numerous cytokines, chemokines, growth factors and complement factors. The binding is related to the negative charge of the HSPGs and it is critically dependent on the density and distribution of the sulfate groups along the glycosaminoglycan chain.[70,71] After binding with complement factors, HSPGs regulate complement activation on cells. Both the AP inhibitor factor H and AP stabilizer/activator properdin can bind to HSPGs on the tubular epithelium under proteinuric conditions. Binding of properdin to the HSPGs on PTEC may be a crucial step in the process of complement mediated tubular epithelial injury in proteinuria.[68,72] In addition, it has been shown that heparin can directly bind numerous complement factors explaining its complement inhibiting potential, as heparin is a highly sulfated glycosaminoglycan and has a similar structure to heparan sulfates.[73,74] This suggests that blockade of the interaction of complement components with HSPGs by heparin-related compounds can be a potentially interesting therapeutic strategy. Thus, HSPGs on PTEC function as a docking location for complement factors and properdin might be a pivotal complement marker in the processes of complement mediated damage after kidney transplantation. Therefore, the presence of properdin and C5b-9 in the urine may represent one of the driving forces of proteinuria mediated allograft damage after transplantation and could serve as biomarkers for graft failure.

Antibody-mediated complement activation in kidney transplantation

In addition to the pathological role of complement during proteinuria-mediated damage, complement plays a crucial role in the process of antibody-mediated rejection (ABMR) after kidney transplantation. Accumulating evidence has identified ABMR as the main cause of kidney transplant loss.[75–78] In kidney transplantation, the first battle field of the recipient's immune response is the donor's endothelium, turning endothelial cells (ECs) into the primary site of the alloimmune response. ECs within the renal vasculature can become a target of circulating antibodies against HLA and non-HLA anti-EC antibodies (AECA's), that may result in ABMR. Such donor-specific antibodies (DSAs) can engage different effector functions with their Fc-portion and evoke complement-mediated injury (Figure 1).[79] In the process of ABMR deposition of complement C3 and C4 split products (C3d and C4d) on ECs are often seen,[80] although ABMR without C4d deposition is also recognized.[81] Alternatively, DSA can also directly elicit EC activation by cross linking HLA (Fc-independent actions).[82]

Historically the risk of ABMR has been underestimated in transplant medicine and immunosuppression was largely targeted to prevent T cell mediated rejection (TCMR). The potency of HLA as initiating graft destruction became clear with the recognition of hyperacute rejection in the 1960s,[83] however decades passed before ABMR was distinguished from the interstitial-epithelial changes seen in TCMR. This distinction was made on the basis of damage in the microcirculation associated with intracapillary inflammatory cells plus DSA.[84] The presence of HLA-antibodies have been significantly associated with worse long term allograft survival across all of the solid organ types.[85–88] The criteria for diagnosis of ABMR have become increasingly complex, with major modifications of the first Banff classification in 2003,[89] in 2013,[90] 2017,[91] and 2020.[92]

The process of antibody-mediated rejection

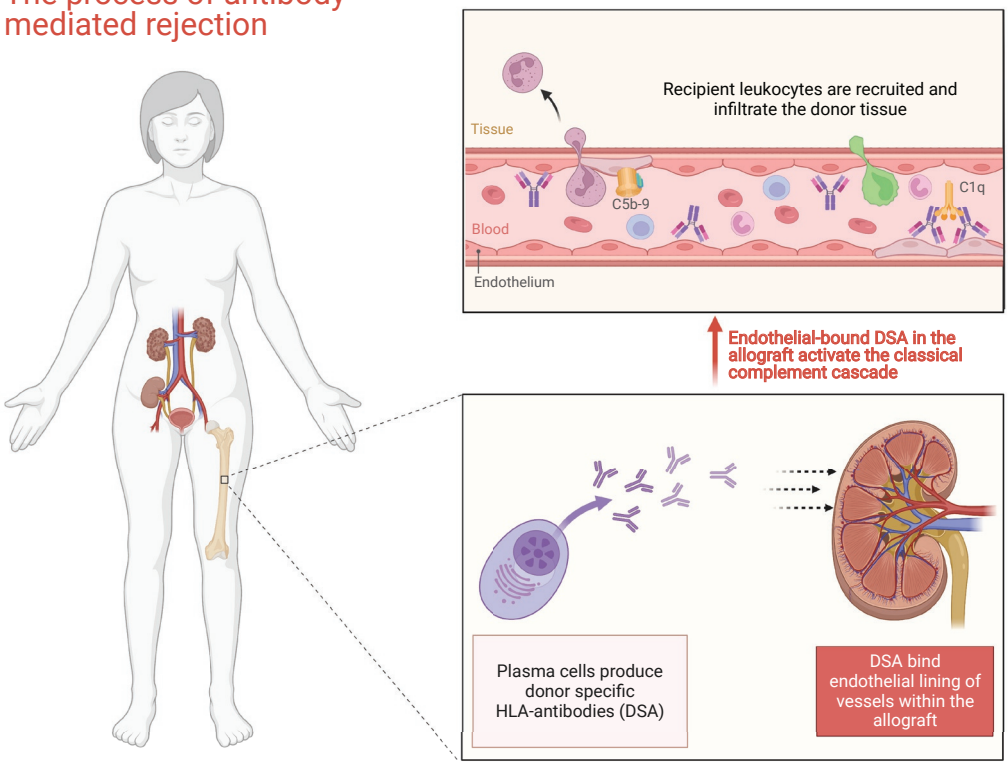

Figure 1. The process of antibody-mediated rejection.

Cross-match testing for HLA and non-HLA antibodies

Serum from patients requiring a kidney transplant is cross-matched with donor lymphocytes in the lymphocyte based complement-dependent cytotoxicity test (CDC), to predict the risk for ABMR. This assay was the first technique routinely used for HLA-antibody detection and for cross-matching. In the CDC, which is still routinely used, lymphocytes are used as target cells to detect complement-fixing IgM and IgG antibodies in the serum of a patient after the addition of rabbit complement. By using dithiothreitol (DTT), IgM and IgG antibodies can be differentiated. DTT reduces the disulfide bonds in the IgM pentamer, resulting in a negative reaction due to IgM. Serum samples are tested against lymphocyte panels that represent the spectrum of HLA-antibodies in the potential donor population. A positive CDC crossmatch, reflected by a substantial degree of donor lymphocyte lysis, is regarded as an absolute contra-indication for transplantation. The results are expressed as the percentage of the panel to which the sample has reacted (% panel-reactive antibody: %PRA). With the widespread implementation of the CDC in clinical practice, hyperacute rejection caused by DSAs has almost completely disappeared.[93,94] There are a number of limitations of the CDC technique. The sensitivity of the technique is dependent on viable lymphocytic target cells and the particular batch of rabbit complement that is used. Additionally, only complement-

fixing antibodies that react with lymphocytes are detected. There are more sensitive techniques than the CDC available these days, like solid-phase assays including the luminex single antigen bead assays (LSA) and the flow cytometry crossmatch.[95–97] In the LSA magnetic beads coated with HLA antigens instead of donor lymphocytes are used and can therefore be used for a virtual crossmatch.[98] This very sensitive technique enables the identification of non-cytotoxic anti-HLA DSAs and non-donor specific anti-HLA antibodies.[97,98] The significance of non-donor specific anti-HLA antibodies remains controversial and unresolved,[85,99–101] whereas pretransplant non-cytotoxic DSAs are associated with acute rejection and impaired graft survival.[102–106] Additional crossmatch assays using flow cytometry have been developed that provide an extra analysis in testing the relevance of DSAs that are detected by the LSA but not convincingly visible in the CDC. However, there are still patients who develop ABMR despite a negative lymphocyte CDC and notably HLA DSA-negative ABMR is recognized in the latest Banff criteria[81,92] This absence of DSAs might be due to HLA-independent DSA directed at non-HLA endothelial antigens or circulating DSA levels that are below detection levels but sufficiently deposit in the target organ to cause damage. Cross-matching with endothelial cells in addition to lymphocytes might tackle these limitations, broadening the prediction range of ABMR and deliver additional important diagnostic and mechanistic information.[107] Several antigen targets including angiotensin type 1 receptor (AT1R), laminin G-like domain 3 (LG3), endothelin type A receptor (ETaR) and vimentin have been reported as non-HLA target antigens. Newer non-HLA targets are continuously emerging.[108] Non-HLA antibodies against these targets are frequently identified as autoantibodies, yet can also occur as alloantibodies, directed against the renal ECs.[107,109] Multiple groups have tried to address the role and interaction of HLA and non-HLA antibodies with renal ECs. However, several obstacles hamper the clarification of the site-specific endothelial phenotypes, and their role in ABMR. Specifically, current EC-based cross-matching research is commonly using widely available primary EC like HUVEC, derived from immune-privileged fetal tissue and representing macrovascular venous endothelium. The latter aspects suggest that HUVEC do not adequately represent the immunological situation of adult microvascular endothelium.[110,111] Moreover, EC assays with well-established cell-lines like human arterial endothelial cells and human dermal microvascular endothelial cells have been described, neglecting the physiological and phenotypic heterogeneity of endothelium and underestimating the uniqueness of renal microvascular endothelium.[112–115] Despite the general notion that endothelial cells are crucial in HLA and non-HLA mediated ABMR, it remains difficult to draw kidney specific conclusions from existing EC research.[116–120] It is important to understand links between site-specific endothelial phenotypes and physiology and to determine how these links may be leveraged for disease monitoring and therapeutic gain in patients with ABMR. However, due to difficulties in obtaining primary cultures of renal-ECs, molecular characterization, transcriptional profiling and assay development with these cells has been hampered. In addition, the (commercially) available endothelial cell based cross-match tests are also based on endothelium from diverse origins, like peripheral blood endothelial precursor cells.[38] At present, there is a need for the development of coherent cross-match assays for the detection of relevant non-HLA antibodies in order to determine their clinical relevance in transplantation.[95]

Scope of the thesis

The present thesis attempts to expand the current knowledge on the role of complement after renal transplantation on a cellular level at the cell membrane of the epithelial and endothelial cells, with a major focus on the alternative and classical pathway, respectively.

Part A of this thesis will focus on the role of alternative complement activation after kidney transplantation.

We studied urinary complement activation in a cohort of kidney transplant recipients long-term after transplantation in Chapter 2. We hypothesized that the AP regulator properdin and terminal complement complex C5b-9 play an important role in graft failure and could serve as early biomarkers for late graft failure. Previous experimental work performed by our group showed that properdin can bind to HSPGs on the tubular epithelium under proteinuric conditions. On the other side, other groups showed that the binding of properdin to endothelial cells is dependent on initial C3b binding. This difference will be important for the development of therapeutic approaches. Therefore, in Chapter 3 we investigated whether the binding of properdin to tubular epithelial cells is dependent on initial C3b binding. In order to investigate the exact binding site of properdin on tubular cells, we also focussed on whether the binding of properdin to tubular epithelial cells is syndecan-1 mediated. In addition we looked into the molecular interactions of properdin with HSPG, C3b, and its inhibitor Salp20.

Part B of this thesis focuses on the role of classical complement activation after kidney transplantation.

As the complement system is involved at different cellular binding sites in the kidney after transplantation, we additionally moved from epithelial activation towards complement activation on an endothelial level. The endothelium is tremendously heterogeneous between different organs, but also when comparing different vascular beds within the same organ. In Chapter 4 we investigated, whether the kidney machine perfusate after organ donation could serve as a source for renal endothelial cells. We aimed to isolate endothelial cells from multiple human donors with different blood groups and HLA profiles, that can be utilized to study the specific inter-individual variation of endothelial biology and more specifically the endothelial susceptibility to complement activation upon binding of donor specific antibodies. In Chapter 5 we show the first application of these machine perfusion derived endothelial cells (MP-PRECs) described in Chapter 4 in a CDC assay. We show the clinical relevance of the MP-PREC cross-match test by demonstrating that therapeutical removal of antibodies against non-HLA endothelial specific molecules can be monitored by our renal endothelial cross-match test, resulting in successful re-transplantation. Over the last decade, several assays have been developed to detect and screen for non-HLA antibodies. . In Chapter 6 we give an overview of the current non-HLA antibodies assays and non-HLA cross-match assays for use in solid organ transplantation that are currently available, either in a research setting or commercially. In Chapter 7 we explored the link between systemic and local complement activation in kidney transplant recipients. The etiology of ABMR is still poorly understood and C5-complement inhibitors fail to be effective in long-term kidney

transplant recipients. We therefore aimed to study the role of the complement system in kidney transplant patients with ABMR, by quantifying complement activation products systemically in plasma and locally in kidney biopsies.

Finally, in **Chapter 8** the findings presented in this thesis are critically discussed and the possible implications for transplantation and other future perspectives are presented.

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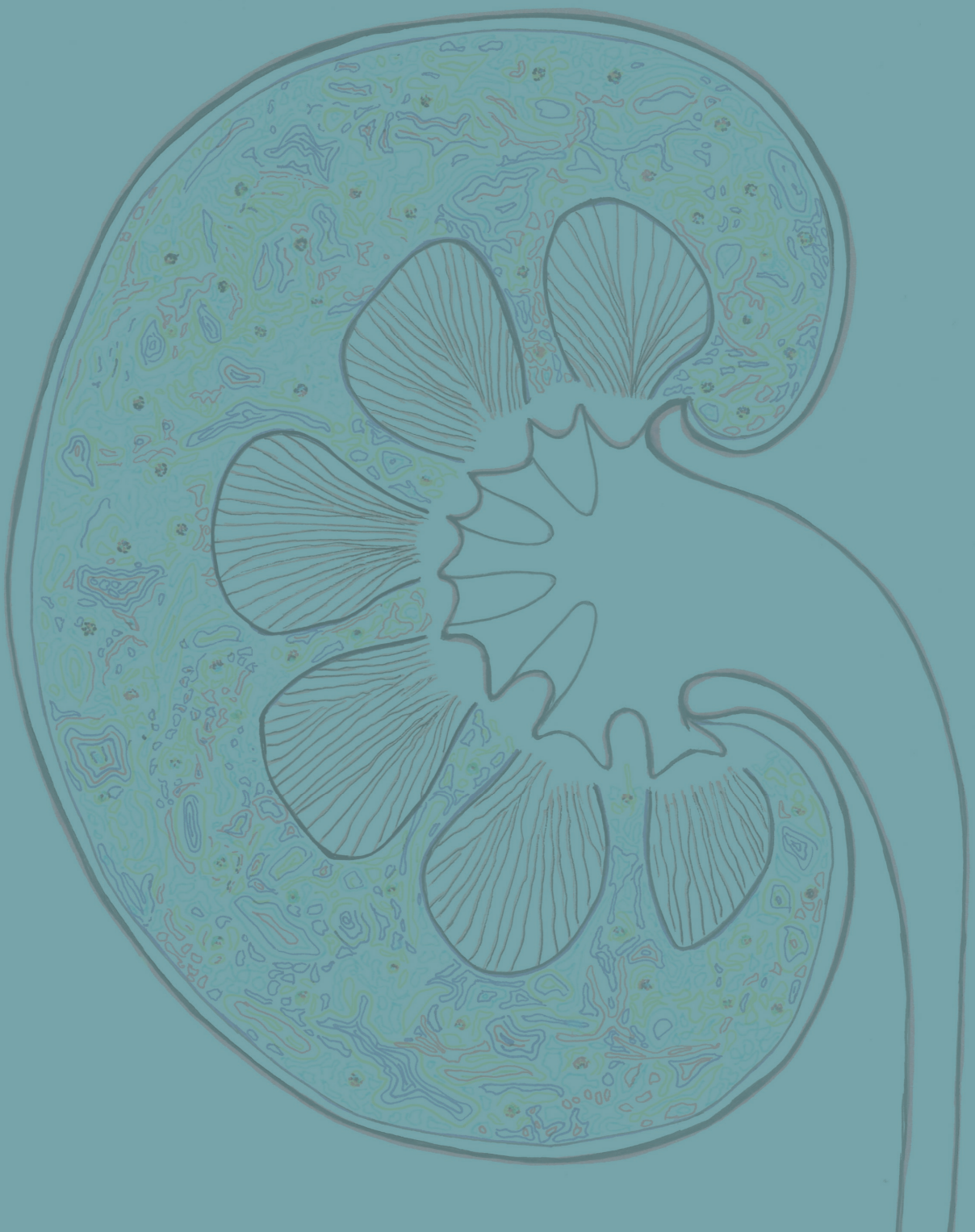
PART A – PROTEINURIA-DRIVEN ALTERNATIVE PATHWAY ACTIVATION IN KIDNEY TRANSPLANT RECIPIENTS



CHAPTER 2

Urinary properdin and sC5b-9 are independently associated with increased risk for graft failure in renal transplant recipients

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Frontiers in Immunology 2019; DOI: 10.3389/fimmu.2019.02511

Abstract

Introduction

The pathophysiology of late kidney-allograft failure remains complex and poorly understood. Activation of filtered or locally produced complement may contribute to the progression of renal failure through tubular C5b-9 formation. This study aimed to determine urinary properdin and sC5b-9 excretion and assess their association with long-term outcome in renal transplant recipients (RTR).

Methods

We measured urinary properdin and soluble C5b-9 in a well-defined cross-sectional cohort of RTR. Urinary specimens were taken from a morning urine portion, and properdin and sC5b-9 were measured using an enzyme-linked-immunosorbent assay (ELISA). Cox proportional hazard regression analyses were used to investigate prospective associations with death-censored graft failure.

Results

We included 639 stable RTR at a median [interquartile range] 5.3 (1.8-12.2) years after transplantation. Urinary properdin and sC5b-9 excretion were detectable in 161 (27%) and 102 (17%) RTR respectively, with a median properdin level of 27.6 (8.6-68.1) ng/mL and a median sC5b-9 level of 5.1 (2.8-12.8) ng/mL. In multivariable-adjusted Cox regression analyses, including adjustment for proteinuria, urinary properdin (HR 1.12; 95% CI 1.02-1.28; P=0.008) and sC5b-9 excretion (HR 1.34; 95% CI 1.10-1.63; P=0.003) were associated with an increased risk of graft failure. If both urinary properdin and sC5b-9 were detectable, the risk of graft failure was further increased(HR 3.12; 95% CI 1.69-5.77; P<0.001).

Conclusions

Our findings point towards a potential role for urinary complement activation in the pathogenesis of chronic allograft failure. Urinary properdin and sC5b-9 might be useful biomarkers for complement activation and chronic kidney allograft deterioration, suggesting a potential role for an alternative pathway blockade in RTR.

Introduction

Despite improvements in immunosuppressive therapy over the last decades, chronic and irreversible deterioration of a transplanted kidney graft remains a major problem and is responsible for disappointing outcomes in long-term graft survival.[1] Even though registry data can be used to define risk factors, chronic allograft failure pathophysiology remains complex and poorly understood, due to difficulty in distinguishing the contribution of several immunological and non-immunological factors.[2] Interstitial fibrosis/tubular atrophy (IFTA), presents itself as renal allograft dysfunction (occurring at least three months posttransplant) in the absence of active acute rejection, drug toxicity, or other diseases. Due to its multiple possible causes and complex etiology, classification of IFTA is still an ongoing process.[3,4] The clinical diagnosis is usually suggested by gradual deterioration of allograft function, manifested by a slowly rising serum creatinine concentration, worsening hypertension, and increasing proteinuria. Proteinuria is known to be a progression marker and a predictor for renal failure.[5,6] It is thought that proteinuria contributes to the progression of renal failure by various mechanisms. One of these mechanisms is suggested to be leakage of albumin-bound lipids across the damaged glomerular filtration barrier, leading to lipoapoptosis after reabsorption by the downstream proximal tubule.[7,8] Alongside this, activation of filtered or locally produced complement may be harmful to renal tubular cells and contribute to the progression of renal failure by initiating interstitial fibrosis.[9,10] Complement activation leads to the formation of C5b-9,11 which can be used as a clinical indicator of complement activation in native kidney diseases.[12,13]

Renal proximal tubular cells are known to activate complement via the alternative pathway (AP).[14] Gaarkeuken *et al.* showed that complement activation on tubular cells is mediated by properdin binding on the tubular brush border.[15] Our group identified tubular heparan sulfate as the docking platform for properdin and the consequent AP activation on tubular cells.[9] In proteinuric patients, urinary properdin excretion is associated with intrarenal complement activation and poor renal function.[16,17]

Although it has been established that there is a strong relationship between proteinuria, tubulo-interstitial injury and a poor prognosis in kidney disease, to our knowledge no studies have examined the role of urinary complement activation products in kidney transplantation outcomes.

We hypothesized that the AP regulator properdin and the terminal complement complex sC5b-9 play an important role in graft failure and could serve as early biomarkers for late graft failure. Hence, the aim of the present study is to investigate the role of properdin and sC5b9 in renal transplant recipients (RTR) in relation to the development of graft failure over time.

Methods

Study population

The study population consisted of a well-characterized and previously described cohort of 707 RTR.[18] In short, this cohort comprised RTR (aged ≥18 years) who visited the outpatient clinic of the University Medical Center Groningen (UMCG), Groningen, The Netherlands, between November 2008 and June 2011, and who had a functional graft for at least 1 year after transplantation. All patients provided written informed consent. Urinary morning samples were collected at inclusion

in the study and immediately placed on ice. The samples were centrifuged at 4°C at 4000 RPM for 15 minutes to remove components and debris, and the supernatants were stored at -80°C. They were not subjected to freeze/thaw cycles before analysis. There were 639 patients eligible for analysis after we excluded 67 patients with missing urinary samples which precluded the measurement of urinary properdin and sC5b-9 levels. Death-censored graft failure was defined as return to dialysis or re-transplantation. Kidney function was assessed by estimating glomerular filtration rate (eGFR) by applying the Chronic Kidney Disease Epidemiology Collaboration equation.19 Protein excretion of ≥0.5 gram per day was defined as proteinuria. The study was approved by the UMCG institutional review board (METc 2008/186), adheres to the Declarations of Helsinki and Istanbul and has NCT02811835 as ClinicalTrials.gov identifier.

Quantification of urinary properdin

Urinary properdin levels were assessed by a previously described sandwich ELISA,[9,17] with a detection limit of 1.2 ng/mL, a plasma intra-variation of <17% and an inter-variation of <20%. In brief, 96-well ELISA plates (NUNC MaxiSorp™, Sigma-Aldrich, Saint Louis, MO, USA) were coated overnight at 4°C with monoclonal anti-human properdin (Hycult HM2282, Uden, the Netherlands). Urinary samples were diluted 5 times in DPBS with 0.1% Tween and bovine serum albumin (PTB) and incubated for 1 hour at 37°C, followed by secondary antibody; polyclonal rabbit anti-human properdin-biotin (kindly provided by M.R. Daha, Leiden, The Netherlands) and detection with Streptavidin-HRP (Dako P0397, Glostrup, Denmark). Enzyme activity was detected using 2,2'azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (A1888, Sigma-Aldrich, Saint Louis, MO, USA). The optical density was measured at 415 nm using a microplate ELISA reader (Benchmark Plus, Bio-Rad, Veenendaal, The Netherlands). A standard curve was prepared using a serial dilution of zymosan activated serum in PTB with a known concentration of properdin. A reference sample, diluted in PTB with a known concentration of properdin was included as positive control. Potential background signal was assessed and corrected for, with PTB functioning as blank.

Quantification of urinary soluble C5b-9

Urinary sC5b-9 levels were assessed by a previously standardized and validated sandwich ELISA,[17,20] with a detection limit of 2.1 ng/mL, a plasma intra-variation of <13% and an inter-variation of <19%. In brief, 96-well ELISA plates (NUNC MaxiSorpTM, Sigma-Aldrich) were coated overnight at 4°C with monoclonal mouse anti-human C5b-9 (Dako M0777). Urinary samples were diluted 1.25 times and incubated for 1 hour at 37°C. Secondary antibody polyclonal goat anti-human C5 (Quidel Ca92121, San Diego, CA, USA), followed by tertiary antibody polyclonal mouse anti-goat IgG HRP (Jackson 205-035-108) were added. Enzyme activity was detected using 3,3',5,5'-tetramethylbenzidine. The optical density was measured at 450 nm using a microplate ELISA reader. The standard curve, reference sample, and the assessment of a potential background signal was prepared in the same way as the properdin ELISA, with a known concentration of sC5b-9.

Statistical analyses

Data were analyzed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL, USA) and R version 3.2.3 (Vienna, Austria). Data are expressed as mean ± SD for normally distributed variables and as median (25^{th}-75^{th} interquartile range [IQR]) for variables with a skewed distribution. Categorical data are expressed as number (percentage). Under normal conditions complement factors are not present in the urine. Therefore we defined urinary properdin and sC5b9 as a negative test when undetectable in the urine and as a positive test when detectable.

We evaluated between-group differences at baseline, comparing RTRs with versus without detectable properdin and sC5b-9 using Student t-test, Mann-Whitney U test, or Chi square test, as appropriate. To visualize the association between urinary properdin and urinary sC5b-9 excretion, we generated a restricted cubic spline plot based on linear regression analyses. Knots were placed on the 10^{th}, 50^{th}, and 90^{th} percentile of ln properdin. To visualize the association between urinary properdin and urinary sC5b-9 excretion with proteinuria, we generated restricted cubic spline plots based on linear regression analyses, with knots placed on the 10^{th}, 50^{th}, and 90^{th} percentile of ln proteinuria. Further, Kaplan Meier curves were used to depict the effect of the presence of urinary properdin and/or sC5b-9 on graft failure and all-cause mortality. Differences in survival rates were tested using the Cox-Mantel log-rank test. To study the prospective association with death-censored graft failure and all-cause mortality, we used Cox proportional hazards regression analysis. Prior to analyses, we first adjusted for statistically significant different parameters at baseline and for other known predictors of graft failure like HLA mismatches. First, death-censored graft failure was adjusted for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type (model 1). Additionally, adjustment was made for high sensitive-CRP (hs-CRP) (model 2); further adjustment for systolic blood pressure, and smoking (model 3); and final adjustment for proteinuria (model 4). Due to skewed distribution, hs-CRP, properdin, and sC5b-9 were natural log-transformed. To determine the optimal cut off value of urinary properdin and sC5b-9 for prediction of graft failure in RTR, the Youden index was used. Finally, we performed mediation analyses to assess whether sC5b-9 was a mediator in the association between properdin and graft failure. For this purpose, we used the method as stated by Preacher and Hayes, which is based on logistic regression.[21,22] These analyses allow for testing significance and magnitude of mediation. For all analyses, a two-sided P-value <0.05 was considered significant.

Results

Baseline characteristics

We included 639 RTR (age 53±13 years; 58% males at 5.3 (1.8-12.2) years after transplantation). Mean eGFR was 52.2±20.1 ml/min/1.73m^2, and urinary properdin excretion was detectable in 161 (27%) RTR with a median [interquartile range] properdin level of 27.6 (8.7-68.1) ng/mL. Urinary sC5b-9 excretion was detectable in 102 (17%) RTR with median sC5b-9 levels of 5.1 (2.8-12.8) ng/mL.

RTR with detectable urinary properdin were more frequently females (*P*<0.001), had significantly higher: body surface area (m^2) (*P*=0.004), creatinine (*P*=0.003), hs-CRP (*P*<0.001), frequency of proteinuria (≥0.5g/24h) (*P*<0.001), and received a deceased - donor kidney transplant (*P*=0.02). RTR with detectable urinary sC5b-9 were more frequently males (*P*=0.01), had higher levels of creatinine (*P*<0.001), a higher frequency of proteinuria (*P*<0.001) and a deceased-donor kidney transplant (*P*=0.02). An inverse association between eGFR and detectable properdin (P<0.001) and sC5b-9 levels (*P*<0.001) was detected at baseline. No significant differences were found at baseline in HLA mismatches, primary renal disease, history of delayed graft function, and rejection between patients with and without detectable urinary properdin or sC5b-9.

Detectable urinary properdin excretion was present in 11% and 16% of RTR with and without proteinuria, respectively. Detectable urinary sC5b-9 excretion was present in 9% and 8% of RTR with and without proteinuria, respectively (Figure 1). Urinary properdin was significantly associated with urinary sC5b-9 excretion in RTR in whom both complement products were detectable (β=0.25; *P*<0.001) (Figure 2). Urinary properdin and urinary sC5b-9 excretion were both significantly associated with proteinuria (β=0.26; *P*<0.001 and β=0.36; P<0.001 respectively) (Supplementary figure 1 and 2). Further demographics and clinical characteristics dichotomized into detectable or undetectable urinary properdin and sC5b-9 are specified in Table 1.

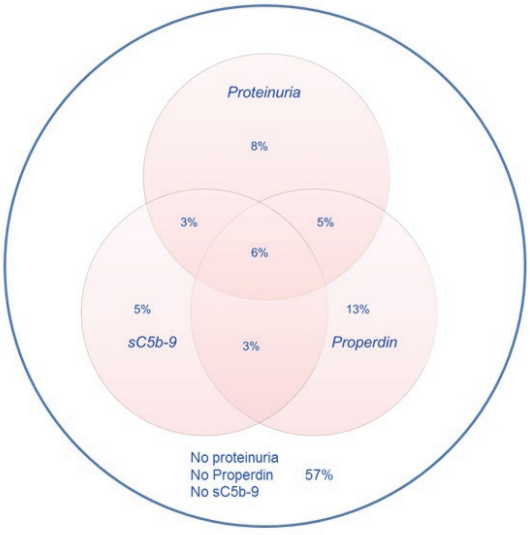

Figure 1. Prevalences of urinary properdin, sC5b-9 and proteinuria.

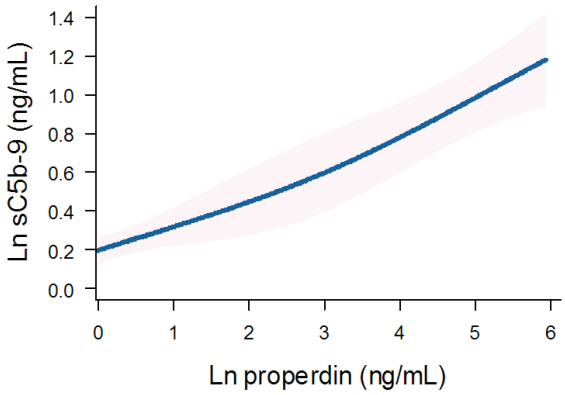

Figure 2. Association between urinary properdin and urinary sC5b-9 excretion in the RTR.
A restricted cubic spline is generated based on linear regression analyses. Knots are placed on 10th, 50th, and 90th percentile of ln properdin. Blue line represents coefficient, and pink band represents 95% confidence interval.

Table 1. Baseline characteristics according to detectable urinary properdin and sC5b-9 levels.

Variables	Urinary properdin		*P*-value	Urinary sC5b-9		*P*-value
	Not detectable (n=478)	Detectable (n=161)		Not detectable (n=537)	Detectable (n=102)	
Recipient						
Age (yrs)	53±13	53±13	.96	53±13	53±13	.88
Male sex (n, %)	305 (64)	66 (41)	<.001	298 (56)	70 (69)	.01
Body mass index, kg/m^2	26.5±4.2	26.7±5.0	.65	26.6±4.7	26.3±4.9	.44
Body surface area (m^2)	1.96±0.21	1.90±0.22	.004	1.95±0.21	1.94±0.22	.95
Alcohol use (n, %)	387 (82)	124 (77)	.28	441 (82)	74 (73)	.69
Current smoking (n, %)	55 (12)	20 (12)	.72	58 (11)	17 (17)	.06
Primary renal disease			.34			.95
Primary glomerular disease (n, %)	143 (30)	36 (22)		156 (29)	25 (25)	
Glomerulonephritis (n, %)	43 (9)	11 (7)		43 (8)	11 (11)	
Tubulo-interstitial disease (n, %)	48 (10)	25 (16)		63 (12)	11 (11)	
Polycystic renal disease (n, %)	95 (20)	36 (22)		109 (20)	22 (22)	
Dysplasia and hypoplasia (n, %)	19 (4)	6 (4)		22 (4)	4 (4)	
Renovascular disease (n, %)	29 (6)	8 (5)		30 (6)	7 (7)	
Diabetic nephropathy (n, %)	23 (5)	8 (5)		27 (5)	4 (4)	
Other or unknown cause (n, %)	78 (16)	31 (19)		87 (16)	18 (18)	
History of CV-disease (n, %)	58 (12)	23 (14)	.31	71 (13)	10 (10)	.65
Time since transplantation (years)*	5.3 (1.7-12.0)	6.1 (2.1-12.6)	.39	5.1 (1.9-11.6)	7.1 (1.7-15.0)	.07
Delayed graft function (n,%)	31 (7)	15 (9)	.27	36 (7)	10 (10)	.24
Rejection (n,%)	130 (27)	45 (28)	.82	143 (27)	32 (31)	.33
Diabetes mellitus (n, %)	109 (23)	38 (24)	.75	124 (23)	23 (23)	.84
Systolic blood pressure (mmHg)	136±17	135±18	.84	135±17	139±19	.05
Diastolic blood pressure (mmHg)	82±11	82±11	.64	82±11	85±11	.02

Laboratory measurements						
sC5b-9 (ng/mL)	0 (0-0)	0 (0-3.8)	<.001	0 (0-0)	5.1 (2.8-12.8)	
Properdin (ng/mL)	0 (0-0)	27.6 (8.7-68.1)		0 (0-0)	0 (0-32.4)	<.001
Hemoglobin (mmol/L)	8.3±1.1	7.9±1.0	<.001	8.2±1.1	8.1±1.2	.31
Total cholesterol (mmol/L)	5.1±1.1	5.2±1.1	.60	5.1±1.1	5.2±1.1	.49
eGFR (ml/min/1.73m^2)	54±20	47±21	<.001	54±20	44±21	<.001
Creatinine (µmol/L)	133±46	154±83	.003	132±48	172±91	<.001
Proteinuria (>0.5g/24h) (n, %)	74 (15)	65 (40)	<.001	83 (16)	56 (55)	<.001
hs-CRP (mg/L)	1.5 (0.6-3.7)	2.5 (1.0-7.6)	<.001	1.6 (0.7-4.3)	2.1 (0.8-6.1)	.09
Treatment						
ACE-inhibitors (n,%)	157 (33)	58 (36)	.47	176 (33)	39 (38)	.29
Bèta-blocker (n,%)	300 (63)	113 (70)	.08	346 (64)	67 (66)	.81
Calcium channel blockers (n,%)	117 (25)	39 (24)	.95	128 (24)	28 (28)	.44
Diuretic use (n,%)	189 (40)	72 (45)	.26	209 (39)	52 (51)	.02
Calcineurin inhibitor (n,%)	281(59)	92(57)	.57	315 (59)	60 (59)	.44
Sirolimus (n,%)	10(2)	2(1)	.33	11 (2)	1(1)	.50
Prednisolon, mg/24h (n,%)	468(99)	161(100)	.47	532(99)	101(99)	.53
MMF (n,%)	294(62)	87(54)	.10	328(61)	55(54)	.70
Azathioprine (n,%)	77(16)	41(26)	.68	93(17)	27(27)	.71
Donor						
Donor age (yrs)	46±18	43±15	0.07	43±15	42±16	0.29
Male sex donor (n,%)	232(49%)	90(56%)	0.11	280 (53%)	45 (46%)	0.19
Deceased type donor (n, %)	298(62%)	117(73%)	0.02	341 (63%)	77 (75%)	0.02
HLA mismatches (n,%)						
Class I						0.46
0 (n,%)	102(22)	27(17%)		103(19%)	26(25%)	
1 (n,%)	113(24%)	31(19%)		125(23%)	21(20%)	
2 (n,%)	169 (36%)	53 (33%)		191(36%)	33(32%)	
3 (n,%)	43 (9%)	20 (12%)		51(10%)	12(12%)	
4 (n,%)	21 (4%)	9 (6%)		27(5%)	3(3%)	
Class II						0.82
0 (n,%)	199 (42%)	60 (37%)		215(40%)	46(45%)	
1 (n,%)	198 (42%)	66(41%)		226(42%)	40(39%)	
2 (n,%)	47(10%)	14(9%)		52(10)	9(9%)	

*Normally distributed data are presented as means±standard deviation, skewed data as medians (interquartile range), and categorical data as number (percentage). P-values have been calculated by means of independent samples T-test, Mann-Whitney U test, or Chi-square test. Abbreviations: eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; ACE, angiotensin-converting enzyme; MMF, mycofenolaat mofetil; HLA, human leukocyte antigens. * time since transplantation at inclusion.*

Urinary properdin and Graft Failure

During a median follow-up of 5.3 (4.5-6.0) years, 75 (12%) RTRs developed death-censored graft failure. As depicted in the Kaplan Meier curves shown in Figure 3, RTR with both detectable urinary properdin and sC5b-9 had the highest risk of developing graft failure (*P*<0.001). RTR with urine in which either properdin or sC5b-9 was detectable, showed an intermediate risk with worse graft survival compared to RTR without detectable urinary properdin or sC5b-9 (Figure 3).

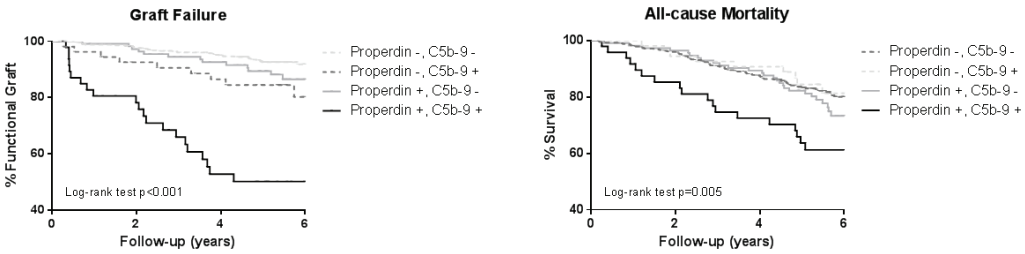

Figure 3. Kaplan-Meier analyses for percentage graft failure (A) and survival (B) according to no sC5b-9/no properdin, sC5b-9/no properdin, no sC5b-9/properdin, sC5b-9/properdin.

Log-rank tests showed that prevalence of graft failure and survival were significantly higher in the patients with urinary properdin and sC5b-9. Associations between survival and urinary properdin and sC5b-9 did not remain significant after adjustment for potential confounders.

In unadjusted Cox regression analysis, detectable urinary properdin was significantly associated with development of death-censored graft failure (HR, 3.08; 95% CI 1.95-4.85; *P*<0.001), in patients with neither urinary properdin or sC5b-9 as the reference group. In multivariable analyses, detectable urinary properdin remained associated with development of graft failure (HR, 2.30; 95% CI 1.37-3.82; *P*<0.001, Table 2), independent of adjustment for age, sex, primary renal disease, time since transplantation, eGFR, HLA mismatches, donor type, hs-CRP, systolic blood pressure, and smoking. However, the association between detectable properdin and graft failure became borderline significant after further adjustment for proteinuria (HR, 1.47; 95% CI 0.85-2.54; *P*=0.05).

When we assessed the association between properdin as a continuous variable and graft failure, findings were similar. After adjustment for potential confounders, urinary properdin as a continuous variable was significantly associated with graft failure (HR, 1.25; 95% CI 1.10-1.42; *P*<0.001) (Table 3). After adjustment for proteinuria, the association of properdin as a continuous variable with graft failure remained significant (HR, 1.12; 95% CI 1.02-1.38; *P*=0.008). The optimal cut-off (Youden index) of urinary properdin for prediction of graft failure was 2.35 ng/mL. At this cut-off value, there was a sensitivity of 59% and a specificity of 79% for prediction of graft failure.

Table 2. *Association of detectable properdin and detectable sC5b-9 with graft failure in renal transplant recipients.*

	Detectable properdin		Detectable sC5b-9		Both Properdin and sC5b-9	
Model	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Univariate	3.08 (1.95-4.85)	<.001	4.17 (2.63-6.63)	<.001	7.13 (4.30-11.83)	<.001
Model 1	2.35 (1.44-3.82)	.001	3.03 (1.86-4.96)	<.001	8.04 (4.74-13.63)	<.001
Model 2	2.27 (1.38-3.73)	.001	2.99 (1.83-4.89)	<.001	7.63 (4.46-13.10)	<.001
Model 3	2.30 (1.37-3.82)	<.001	3.09 (1.87-5.11)	<.001	6.75 (3.79-12.02)	<.001
Model 4	1.47 (0.85-2.54)	.05	2.16 (1.30-3.61)	.003	3.12 (1.69-5.77)	<.001

Model 1: *adjustment for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type;* ***model 2*** *= model 1 + adjustment for hs-CRP,* ***model 3*** *= model 2 + adjustment for systolic blood pressure, and smoking,* ***model 4*** *= model 3 + adjustment for proteinuria.*
Reference group defined as patients with neither urinary properdin or C5b-9, with a hazard ratio of 1.0.

Urinary sC5b-9 and Graft Failure

In unadjusted analysis, detectable urinary sC5b-9 was significantly associated with development of death-censored graft failure (HR, 4.17; 95% CI 2.63-6.63; *P*<0.001). In multivariable analyses, detectable sC5b-9 remained associated with the development of graft failure (HR, 3.09; 95% CI 1.87-5.11; *P*<0.001), independent of age, sex, primary renal disease, time since transplantation, eGFR, HLA mismatches, donor type, hs-CRP, systolic blood pressure, and smoking (Table 2). The association between detectable sC5b-9 and graft failure also remained after further adjustment for proteinuria (HR, 2.16; 95% CI 1.30-3.61; *P*=0.003).

When we assessed the association between sC5b-9 as a continuous variable and graft failure, findings were similar. sC5b-9 as a continuous variable was associated with risk of developing graft failure in the unadjusted analysis and in multivariable analyses, after adjustment for potential confounders, including proteinuria (HR, 1.34; 95% CI 1.10-1.63; *P*=0.004). (Table 3). The optimal cut-off (Youden index) of urinary sC5b-9 for prediction of graft failure was 2.88 ng/mL, there was a sensitivity of 48% and a specificity of 91% for prediction of graft failure.

Table 3. *Association of continuous natural log transformed properdin and sC5b-9 with graft failure in renal transplant recipients.*

	Ln properdin		Ln sC5b-9	
Model	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Univariate	1.36 (1.21-1.52)	<0.001	1.76 (1.51-2.06)	<0.001
Model 1	1.26 (1.11-1.43)	<0.001	1.61 (1.35-1.91)	<0.001
Model 2	1.25 (1.10-1.42)	0.001	1.61 (1.36-1.92)	<0.001
Model 3	1.25 (1.10-1.42)	0.001	1.63 (1.36 -1.96)	<0.001
Model 4	1.12 (1.02-1.28)	0.008	1.34 (1.10-1.63)	0.004

Model 1*: adjustment for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type;* ***model 2*** *= model 1 + adjustment for hs-CRP,* ***model 3*** *= model 2 + adjustment for systolic blood pressure, and smoking,* ***model 4*** *= model 3 + adjustment for proteinuria.*

Mediation analyses

Since properdin is involved in sC5b-9 complex formation via the alternative complement pathway,[9] we aimed to assess whether the association between properdin and graft failure was mediated by sC5b-9. In mediation analyses, sC5b-9 was found to be a significant mediator of the association between properdin and graft failure, 31% of the association between properdin and graft failure was explained by sC5b-9, the *P* value for indirect effect is <0.05 (Table 4).

Table 4. *Mediation analyses of the impact of sC5b-9 on the association between properdin and graft failure.*

Potential mediator	Outcome	Effect (path)*	Multivariable model**	
			Coefficient (95% CI)†	Proportion mediated§
C5b-9	**Graft failure**	Indirect effect (*ab* path)	.08 (.04; .13)	**31%**
		Total effect (*ab* + *c'* path)	.26 (.13; .37)	
		Unstandardized total effect‡	.22 (.07; .38)	

Adjusted for age, sex, primary renal disease, time since transplantation, and hs-CRP.

** The coefficients of the indirect ab path and the total ab + c' path are standardized for the standard deviations of the potential mediators and outcomes.*

*** All coefficients are adjusted for age, sex, eGFR, time since transplantation at inclusion, primary renal disease, donor type and proteinuria.*

**** The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.*

† 95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2000 bootstrap samples.

‡ Odds ratios for risk of outcomes can be calculated by taking the exponent of the unstandardized total effect.

Properdin, sC5b-9, and Mortality

In an unadjusted Cox regression analysis, detectable urinary properdin was significantly associated with an increased risk of mortality (HR, 1.58; 95% CI 1.11-2.25; *P*=0.01), whereas detectable urinary sC5b-9 was not significantly associated with increased risk of mortality(HR, 1.39; 95% CI 0.92-2.11; *P*=0.12). After adjustment for potential confounders, the association between properdin and mortality was abrogated, and the association between sC5b-9 and mortality remained non-significant (Supplementary Table 1).

In unadjusted Cox regression analyses, both urinary properdin and sC5b-9 as continuous variables were significantly associated with an increased risk of mortality (HR, 1.16; 95% CI 1.05-1.27; *P*=0.003 and HR, 1.26; 95% CI 1.08-1.48; *P*=0.004, respectively). However, after adjustment for potential confounders, the associations between properdin and mortality, and between sC5b-9 and mortality were lost (Supplementary Table 2).

Discussion

In this study, we show that the alternative pathway complement factor properdin and the terminal sC5b-9 complex are detectable in the urine of patients after kidney transplantation and are independently associated with chronic allograft failure. To our knowledge, this is the first report indicating urinary alternative complement pathway involvement in chronic renal allograft failure, independent of potential cofounders including eGFR and proteinuria.

It has long been recognized that patients with high-grade proteinuria are more likely to develop chronic renal failure than patients without proteinuria.[23,24] Urinary proteins elicit pro-inflammatory and pro-fibrotic effects that directly contribute to chronic tubulo-interstitial damage. Additionally, among multiple other pathways complement activation may be an important component leading to fibrogenesis in the kidney. In physiological conditions complement components are not filtered through the glomerular barrier, however complement components are present in the urine of patients with non-selective proteinuria.[25,26] Properdin positively regulates the AP of the complement system and is also a pattern recognition molecule for C3b that subsequently stabilizes the C3bBb complex and thus contributes to C5b-9 formation.[27,28,29,30] Tubular epithelial cells are especially susceptible to the effects of C5b-9 formation because they lack the membrane-bound complement regulators on the apical cell surface.[31] Recently, urinary complement measurements and their clinical value are of increasing interest in transplant medicine. Schröppel *et al*. showed not long ago the importance of anaphylatoxins C3a and C5a in donor urine and their association with delayed graft function[32], and van Essen *et al*. recently reviewed the detection of complement biomarkers in urine to monitor local injury in renal diseases, including properdin. [33]

In this study, we have shown a potential role of urinary properdin and sC5b-9 in the pathogenesis of chronic allograft failure. Our data show that graft survival is reduced in patients in whom properdin is present in the urine together with sC5b-9. Remarkably, in patients without overt proteinuria, we identified that properdin, sC5b-9 or both properdin and sC5b-9, were also associated with a worse graft survival. More importantly, not only the presence of properdin and sC5b-9 was significantly associated with graft failure, but also properdin and sC5b-9 were robustly associated with graft survival when analysed as continuous parameters, pointing towards a dose-dependent effect. There are several possible explanations for this association. Properdin is the only known complement protein that is not produced in the liver, but synthesized by various other cell types like monocytes, primary T cells, granulocytes, and endothelial cells.[34,35,36,37,38] Therefore, it is possible that locally produced properdin and/or filtered properdin with other filtered small complement components, causes intratubular C5b-9 activation leading to progressive renal disease without manifest proteinuria, defined as proteinuria >0.5g/24h.[39,40] In kidney transplant patients it is generally believed that small amounts of proteinuria, defined as < 0.5g/24h, are harmless.[41,42] Only persistent proteinuria, >0.5g/24h for at least 3-6 months is considered significant according to American Society of Transplantation guidelines, and low-grade proteinuria is often referred to as 'subclinical'.[43] However, low grade proteinuria may be less harmless than originally described. Halimi *et al*. showed a dose-dependent effect in transplant patients with low grade proteinuria (<0.5g/24h) in whom each 0.1g/24h difference in proteinuria increased the risk of graft loss by 25%.[44] In line with our findings in transplanted patients, Siezenga

et al. showed an association between urinary properdin and worse renal function in patients with diabetic nephropathy or glomerular disease.
Furthermore, the association of urinary properdin with urinary sC5b-9 was independent of the degree of proteinuria.[17]

The fact that adjustment for proteinuria > 0.5g/24h did not materially alter the prospective association in the prospective analysis of continuous properdin and sC5b-9 measurements, is supportive of our hypothesis that alternative pathway complement activation might be one of the driving forces of chronic graft failure. More importantly, after correction for other well-known predictors of graft failure, like HLA mismatches and donor type, the association remains. In mediation analysis, we showed that the association between properdin and graft failure was mediated to a considerable extent by urinary sC5b-9.

This may explain why RTR with properdin alone, or sC5b-9 alone in the urine have a better allograft survival compared to both properdin and sC5b9 in the urine. Therefore, it seems that AP complement activation plays an important role in the loss of allograft function of RTR. The possible mechanism of this effect at a tubular level is illustrated by the scheme presented in figure 4.

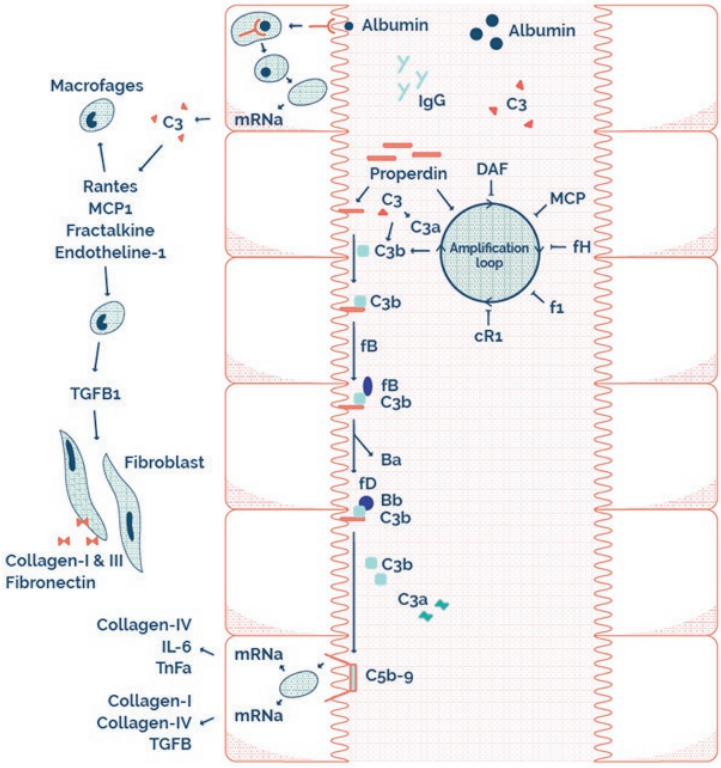

Figure 4. The possible mechanism on a tubular level illustrating tubular alternative pathway complement activation via properdin as pattern recognition molecule.

Interestingly, urinary properdin was more frequently detected in females and urinary sC5b-9 was more frequently detected in males. We can only speculate on the causes of these differences. Innate immune function may vary between males and females,[45] however a limited number of studies have investigated the influence of sex on the complement system[46,47,48,49] Properdin is encoded on the short arm of the X chromosome, and together with hormonal differences between males and females this could be explanations of the sexual differences in properdin.[50,51] However, in a healthy Caucasian population, Gaya da Costa *et al*. recently found decreased serum properdin and serum C9 in healthy human females compared to males.[49] In contrast, animal studies have shown that female mice have a similar serum complement cascade functionality at the level of C3 activation compared to male mice, but a strongly reduced level of serum C9, leading to an inability of female mice to promote inflammation through C5b-9.[46]

Multiple therapeutic modalities to inhibit complement pathway intervention are currently being developed. Our study points towards the potential for complement inhibition at the tubular level in proteinuric patients, which may improve long term outcome in patients with chronic allograft nephropathy.

The main strength of our study is that it comprises a large prospective cohort of stable RTR, in which several renal parameters as well as both urinary properdin and sC5b-9 were measured. In addition, end-point evaluation was complete in all participants despite the long follow-up period. We acknowledge several limitations of the study. First, no gold standard exists for the definition of urinary properdin and sC5b-9. In our study, we defined the detectability of properdin and sC5b-9 as urinary properdin and urinary sC5b-9. Second, complement activation may only be partially reflected by urinary properdin and sC5b-9 excretion, since the excretion may be altered by tubular complement binding and fixation. Third, possible residual confounding in this study cannot be excluded due to the observational status of this single center study. Furthermore, we do not have data on the presence of donor specific antibodies or protocol biopsies in this cohort. Thus, we cannot differentiate between general effects of glomerular filtration of complement products and a specific contribution of alloantibody mediated complement activation. Unfortunately our prospective cohort contained too few events of graft failure to perform analysis for the underlying cause of graft loss and their relation to urinary complement.

We identified that the presence of urinary properdin and sC5b-9 is independently associated with increased risk of late graft failure in RTR, compared to RTR without urinary properdin and sC5b-9. This suggests that urinary properdin and sC5b-9 can serve as useful biomarkers of immunological injury and kidney allograft deterioration. Importantly, urinary properdin and sC5b-9 was associated with graft failure independently of eGFR and significant proteinuria. We suggest that an important part of proteinuria mediated toxicity, is caused by the presence of complement in the primary urine and subsequent activation at the tubular surface. Further studies are needed to unravel the exact interplay between urinary properdin, sC5b-9 and the development of fibrosis, and moreover the potential for therapeutic interventions.

Acknowledgments

We thank H.M. van der Lugt for her contribution for the preparation of this manuscript, H.L. Lancaster for critical and careful reading of the manuscript and J. Potma for assistance in preparing figure 4.

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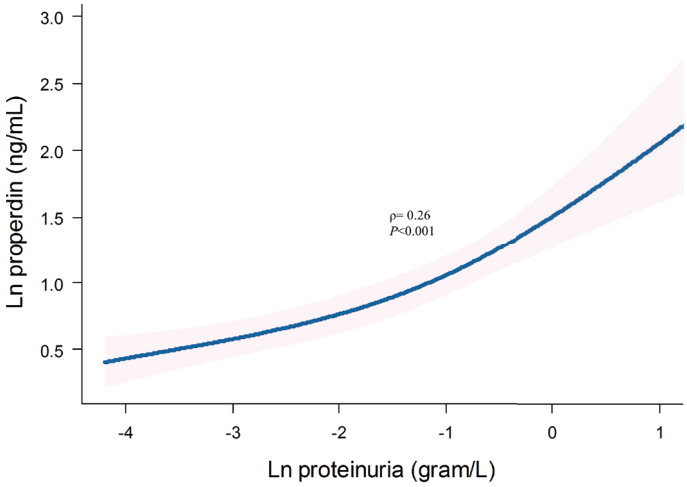
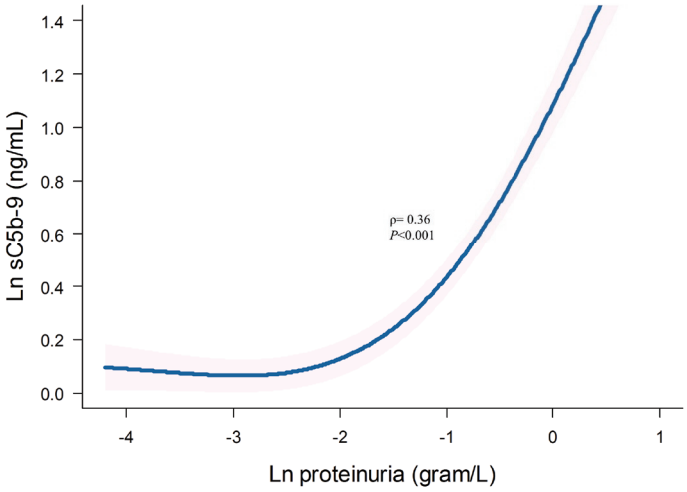
Supplementary results



Supplementary Figure 1. Association between urinary properdin and proteinuria excretion in the RTR.
A restricted cubic spline is generated based on linear regression analyses. Knots are placed on 10th, 50th, and 90th percentile of proteinuria. Blue line represents coefficient, and pink band represents 95% confidence interval.



Supplementary Figure 2. Association between urinary soluble C5b-9 and proteinuria excretion in the RTR.
A restricted cubic spline is generated based on linear regression analyses. Knots are placed on 10th, 50th, and 90th percentile of proteinuria. Blue line represents coefficient, and pink band represents 95% confidence interval.

Supplementary Table 1. *Associations between detectable properdin and detectable sC5b-9 with all cause mortality in renal transplant recipients.*

	Detectable properdin		**Detectable sC5b-9**		**Both Properdin and sC5b-9**	
Model	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Univariate	1.58 (1.11-2.25)	0.01	1.39 (0.92-2.11)	0.12	2.11 (1.28-3.46)	0.003
Model 1	1.43 (0.98-2.09)	0.07	1.06 (0.71-1.72)	0.66	1.87 (1.13-3.11)	0.02
Model 2	1.37 (0.93-2.00)	0.11	1.05 (0.67-1.64)	0.83	1.70 (1.01-2.86)	0.05
Model 3	1.30 (0.88-1.91)	0.19	1.00 (0.64-1.59)	0.99	1.20 (0.68-2.11)	0.53
Model 4	1.13 (0.75-1.70)	0.57	0.85 (0.53-1.33)	0.50	0.93 (0.52-1.66)	0.80

Model 1: *adjustment for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches and donor type;* ***model 2*** *= model 1 + adjustment for hs-CRP,* ***model 3*** *= model 2 + adjustment for systolic blood pressure, and smoking,* ***model 4*** *= model 3 + adjustment for proteinuria.*
Reference group defined as patients with neither urinary properdin or sC5b-9, with a hazard ratio of 1.0.

Supplementary Table 2. *Associations between urinary properdin and sC5b-9 as continuous variables with all cause mortality in RTR.*

	Ln properdin		**Ln sC5b-9**	
Model	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Univariate	1.16 (1.05-1.27)	0.003	1.26 (1.08-1.48)	0.004
Model 1	1.12 (1.01-1.24)	0.03	1.16 (0.97-1.38)	0.11
Model 2	1.10 (1.00-1.22)	0.06	1.14 (0.95-1.37)	0.15
Model 3	1.09 (0.99-1.21)	0.08	1.10 (0.91-1.33)	0.31
Model 4	1.06 (0.96-1.18)	0.27	1.01 (0.83-1.23)	0.92

Model 1: *adjustment for age, sex, primary renal disease, and time since transplantation at inclusion, eGFR, HLA mismatches and donor type;* ***model 2*** *= model 1 + adjustment for hs-CRP,* ***model 3***= *model 2 + adjustment for systolic blood pressure, and smoking,* ***model 4*** *= model 3 + adjustment for proteinuria*

CHAPTER 3

Properdin pattern recognition on proximal tubular cells is heparan sulfate/syndecan-1 but not C3b dependent and can be blocked by tick protein Salp20

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Frontiers in Immunology 2020; DOI: 10.3389/fimmu.2020.01643

Abstract

Introduction

Proteinuria contributes to progression of renal damage, partly by complement activation on proximal tubular epithelial cells. By pattern recognition, properdin has shown to bind to heparan sulfate proteoglycans on tubular epithelium and can initiate the alternative complement pathway (AP). Properdin however, also binds to C3b(Bb) and properdin binding to tubular cells might be influenced by the presence of C3b(Bb) on tubular cells and/or by variability in properdin proteins *in vitro*. In this study we carefully evaluated the specificity of the properdin – heparan sulfate interaction and whether this interaction could be exploited in order to block alternative complement activation.

Methods

Binding of various properdin preparations to proximal tubular epithelial cells (PTEC) and subsequent AP activation was determined in the presence or absence of C3 inhibitor Compstatin and properdin inhibitor Salp20. Heparan sulfate proteoglycan dependency of the pattern recognition of properdin was evaluated on PTEC knocked down for syndecan-1 by shRNA technology. Solid phase binding assays were used to evaluate the effectivity of heparin(oids) and recombinant Salp20 to block the pattern recognition of properdin.

Results

Binding of serum-derived and recombinant properdin preparations to PTECs could be dose-dependently inhibited ($P<0.01$) and competed off ($P<0.01$) by recombinant Salp20 (IC_{50}: ~125 ng/ml) but not by Compstatin. Subsequent properdin-mediated AP activation on PTECs could be inhibited by Compstatin ($P<0.01$) and blocked by recombinant Salp20 ($P<0.05$) . Syndecan-1 deficiency in PTECs resulted in a ~75% reduction of properdin binding ($P=0.057$). In solid-phase binding assays, properdin binding to C3b could be dose-dependently inhibited by recombinant Salp20> heparin(oid) > C3b.

Discussion

In this study we showed that all properdin preparations recognize heparan sulfate/syndecan-1 on PTECs with and without Compstatin C3 blocking conditions. In contrast to Compstatin, recombinant Salp20 prevents heparan sulfate pattern recognition by properdin on PTECs. Both complement inhibitors prevented properdin-mediated C3 activation. Binding of properdin to C3b could also be blocked by heparin(oids) and recombinant Salp20. This work indicates that properdin serves as a docking station for AP activation on PTECs and a Salp20 analogue or heparinoids may be viable inhibitors in properdin mediated AP activation.

Introduction

Proteinuria is caused by the passage of proteins through the damaged glomerular filtration barrier and is an independent prognostic factor for the progression of chronic renal failure to end stage renal disease.[1] Several mechanisms have been postulated on how proteinuria causes renal damage, one of them being via tubular complement activation. Evidence for involvement of the complement system in renal damage was already shown in 1985 by the finding of C3 deposits on the proximal tubular epithelial cells (PTECs) of nephrotic patients.[2] Ultrafiltration of complement factors under proteinuric conditions may lead to alternative pathway activation within the renal tubules. This may be explained by the absence of a number of complement regulatory proteins on the apical membrane including decay accelerating factor (DAF), complement receptor 1 (CR1), and membrane cofactor protein (MCP)[3,4] Complement regulatory protein CD59 is present on the brush border of proximal tubules, albeit weakly expressed.[4] As a result, failure to downregulate the complement cascade might lead to tubular epithelial damage under proteinuric conditions.

The complement system consists of three pathways; the lectin pathway (LP), classical pathway (CP) and alternative pathway (AP). The LP and CP are initiated by pattern recognition molecules (e.g. MBL and C1q), whereas the current conception of the AP is thought to be a purely auto-activating route, via the spontaneous or induced formation of fluid-phase AP C3 convertase.[5,6] The three pathways merge at the formation of a C3 convertase, the major enzymes of the cascade.[7,8]For the CP and LP this is the C4bC2a complex, whilst in the AP the C3bBb complex is formed. The C3bBb complex is relatively unstable in plasma and requires stabilization by properdin, the only known positive regulator of the complement system.[9] Properdin consists of seven thrombospondin repeat domains TSR0-TSR6 beginning at the N-terminus.[10] However, understanding the complex biology of properdin has proven to be difficult due to the different sources of properdin used in biochemical studies and also its intricate self-associations. Through head-to-tail interactions of monomeric subunits, properdin can form cyclic dimers, trimers and tetramers under physiological conditions.[11,12] Additionally, non-physiological high molecular weight polymers can also form during long term storage and freeze/thaw cycles.[12,13] Moreover, stored properdin in the granules of neutrophils, which is released upon cell stimulation, may be structurally different than serum properdin either in its multimeric structure or in its posttranslational modifications.[14,15]

In the AP auto-activating theory it was thought that stabilizing the C3bBb complex was the only function of properdin. However, in the past decade data has accumulated stating that properdin can act as a pattern recognition molecule on PTECs, apoptotic, necrotic and bacterial cells.[9] As ligands for properdin DNA and glycosaminoglycans have been proposed.[16,17] However this theory was questioned by Harboe and colleagues since they showed that properdin binding to granulocyte MPO, endothelial cells and Neisseria Meningitidis is completely dependent on initial C3b binding, raising doubt on the conclusions of formerly published work.[18] Their conclusion was based on properdin binding experiments in the presence or absence of Compstatin,[18] a circular peptide inhibiting the cleavage of C3 into C3a and C3b. On the other hand, studies by the group of Van Kooten *et al*. in mice demonstrated that properdin can be found in glomeruli of C3 knockout mice during anti glomerular basement membrane disease indicating that C3 is not essential for properdin binding to tissues.[19]

In proteinuric patients, AP activation has been linked to the presence of properdin on the PTEC brush border and *in vitro* binding of properdin and subsequent complement activation on HK-2 cells has also been shown. However, this is not the case for endothelial cells.[20] In addition, urinary properdin excretion is associated with renal complement activation and worsening renal function.[21,22] More recently, our group showed that the binding of properdin to HK-2 cells is dependent on heparan sulfates (HS), since pretreatment of the cells with heparitinase abolished the binding of properdin.[23] Moreover, competition experiments with heparin and non-anticoagulant heparinoids could reduce the binding of properdin to HK-2 cells, showing the treatment potential of heparinoids in AP mediated proteinuric damage.[24] Co-localization of properdin with syndecan-1 on PTECs in an adriamycin induced nephropathy model suggested a role for the heparan sulfated proteoglycan (HSPG) syndecan-1 in the tubular binding of properdin.[23] Syndecan-1 is a major membrane spanning HSPG in epithelial cells and has been shown to be upregulated on tubular epithelium in renal disease.[25] Our group has previously shown that syndecan-1 expression on tubular epithelium correlates with activation of renal repair mechanisms,[26] and that syndecan-1 deficiency in human tubular epithelial cells leads to reduced proliferation.[25] However a direct role for syndecan-1 in complement activation has never been described.

Although the AP has been shown to play a role in numerous diseases, no specific inhibitor for the AP is yet available. Salp20 is a protein derived from the deer tick Ixodes scapulari and has been shown to inhibit the AP via the displacement of properdin from the alternative pathway C3 convertase. This causes an accelerated decay of the C3bBb complex and subsequent inhibition of the AP by up to 70%.[27,28] *In vivo*, treatment with Salp20 in mice showed a reduction of AP mediated damage in ovalbumine-induced asthma, elastase-induced abdominal aortic aneurysm and after intraperitoneal injections with LPS.[29] However to the best of our knowledge, no experiments have been performed which assess the inhibition of the pattern recognition of properdin using Salp20. Therefore, in this study we investigated the interactions of properdin with PTECs, followed by AP activation and the inhibitory effects of Compstatin, Salp20 and heparinoids. To investigate the binding capacity of properdin from different sources and subsequent AP activation, experiments were performed with either normal human serum as a source of properdin, biochemically purified properdin and recombinant full length properdin.

Methods

HK-2 cells

The immortalized human kidney proximal tubular epithelial cell line HK-2 was obtained from ATCC (Manassas VA, USA). Cells were cultured in DMEM/F12 medium 1:1 (Invitrogen, Carlsbad, CA, USA), supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (All purchased from Sigma, Zwijndrecht, The Netherlands), and 50 U/ml penicillin, 50 µg/ml streptomycin and 25 mM Hepes (All purchased from Invitrogen, Carlsbad, CA, USA).

Syndecan-1 knockout cell line

Production of the syndecan-1 knockout HK-2 cell line by shRNA technology has been described before.[30,31,32] To confirm syndecan-1 knockdown and to evaluate the binding of properdin to wild type HK-2 cells or syndecan-1 knockout HK-2 cells, syndecan-1 expression and properdin binding on wild-type and syndecan-1 deficient HK-2 was determined by flow cytometry. Cells were plated in 6-wells cell culture plates at 37°C and were detached using cell dissociation solution (C5789, Sigma®, Zwijndrecht, The Netherlands), 900 µL/well at 37°C until cells were detached. Cells were collected in 4.5 mL tubes containing 2 mL cell medium, centrifuged at 300xg for 5 minutes at 4°C and washed with ice-cold phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) (FACS buffer) (Sigma®, Zwijndrecht, The Netherlands). Cells were subsequently incubated with Alexa Fluor®647 mouse anti-human anti-syndecan-1 (CD138; Bio-Rad/AbD Serotec, California, USA) antibody in FACS buffer on ice, or purified properdin (human factor P, Millipore, Cat 341283-250 1.1mg/mL) followed by rabbit anti-human properdin, prepared as described before,[20] and goat anti-rabbit FITC (Southern Biotech, Birmingham, USA) in FACS buffer on ice in the dark. After washing, cells were resuspended in 300 µL of FACS buffer and analysed in a FACSCalibur™ (FACSCalibur, Becton Dickinson, New Jersey, USA). The purified properdin used was stored at -80°C and thawed only once for the experiments. Non-relevant mouse IgG served as an isotype control. Experiments were independently repeated 4 times. The percentage reduction was calculated as reduction in%=100–((MFIKO x100)/MFIwildtype).

Binding of properdin to HK-2, alternative pathway complement activation and inhibition by Compstatin

Properdin binding to the immortalized human kidney proximal epithelial cell line HK-2 was tested using properdin from various sources. Normal human serum (NHS) from a healthy volunteer with normal classical, alternative and lectin pathway activity, as detected by functional ELISA and determined before, was used as a serum properdin source.[33] Concentrations of 5%, 20% and 50% NHS diluted in DMEM/F12 culture medium were tested. Also purified properdin (human factor P, Millipore, Cat 341283-250 1.1 mg/mL) and recombinant full length properdin, produced in HEK E+ cells resulting in the same N-linked glycanation as plasma properdin, were used. Recombinant properdin had the physiological 1:2:1 ratio and did not contain non-physiological aggregates after purification. The recombinant properdin was aliquoted and stored at -80°C and thawed only once for the experiments.[34]

Confluent HK-2 were cultured on a 6 well tissue culture plate and incubated for 36-48 hours with 10 µg/ml Compstatin that was not exposed to freeze-thaw cycles before (a kind gift from professor J.D. Lambris, University of Pensylvania, Philadelphia, PA, United States) to prevent eventual C3b deposition. Cells were detached using cell dissociation solution (C5789, Sigma®, Zwijndrecht, The Netherlands), 900 µl/1mL at 37°C, collected in 4.5 mL tubes containing 2 mL cell medium and centrifuged twice at 250g for 6 minutes at 20°C. For Compstatin mediated inhibition assays, cells were incubated with heat inactivated NHS in a serial dilution of 5%, 20% or 50% NHS, purified properdin or recombinant properdin in the presence or absence of 10 µg/ml Compstatin for 30 minutes at 37°C. Hereafter, cells were centrifuged for 6 minutes at 250g at 20°C. To detect bound properdin, cells were incubated with rabbit anti-human properdin,[20] followed by goat anti-rabbit FITC (Southern Biotech, Birmingham, USA) in FACS buffer on ice in the dark.

To explore alternative pathway complement activation the same procedure as described above was followed. Subsequent to the incubation of properdin from NHS, purified properdin or recombinant properdin, cells were washed twice at 250g without a break for 6 minutes at 20°C and were incubated in the presence or absence of 5% serum as a complement source for 45 minutes at 37°C. After incubation, cells were washed once with 2 mL 20°C FACS buffer at 250g for 6 minutes at 4°C and once with 2 mL 4°C FACS buffer at 250g without a break for 6 minutes at 4°C.

To detect activated C3, cells were incubated with mouse anti-human activated C3 recognizing C3b, iC3b and C3c fragments (Clone bH6, HM2168S, Hycult biotech, Uden, The Netherlands) for 30 minutes on ice. Cells were washed twice with ice cold FACS buffer, centrifuged at 250g for 6 minutes at 4°C, and incubated goat anti-mouse FITC (Purchased from Southern Biotech, Birmingham, USA) for 30 minutes on ice in the dark. Propidium iodide 1 µg/ml (Molecular Probes, Leiden, The Netherlands) was added just before measuring to be able to exclude apoptotic and necrotic cells. Properdin binding and activated C3 deposition on viable non-apoptotic cells were analysed in a FACSCalibur™ (FACSCalibur, Becton Dickinson, New Jersey, USA). Results are from 6 independent experiments.

Inhibition of binding of properdin to C3b

Competition ELISA was used to evaluate whether heparin-albumin (200 kDa) , unfractionated heparin (15-18 kDa) , low molecular weight (LMW)-heparin (4.5 kDa) , C3b (185 kDa) and recombinant Salp20 (48 kDa) inhibit the binding of properdin to immobilized C3b. Heparin-albumin was from Sigma-Aldrich (Saint Louis, MO, USA). According to the data sheet, this artificial proteoglycan contained 4.8 moles heparin per mole albumin, with a protein content of 55%. Maxisorp 96-well flat bottom microtiter plates (U96 from Nunc International, Amsterdam, The Netherlands) were coated overnight with 1 µg/ml C3b in PBS at 4°C. C3b was purified as described before.[35] After washing in PBS, wells were blocked with 1% BSA in PBS for 1 h at 37°C. A concentration range of heparin-albumin, unfractionated heparin, LMW-heparin, C3b or recombinant Salp20 was pre-incubated with 62.5 ng/ml properdin (Millipore, Billerica, Massachusetts, USA) in PBS, 0.05% Tween and 1% BSA for 15 minutes at room temperature. Thereafter the co-incubated heparin-albumin, unfractionated heparin, LMW-heparin, C3b and recombinant Salp20, together with properdin, was incubated on the C3b coated plate for 1 hour at 37 °C. Binding of properdin was detected with biotinylated rabbit anti-human properdin 1:3000 diluted in PBS, 0.05% Tween and 1% BSA. After washing, streptavidin HRP (DAKO, Glostrup, Denmark) 1:5000 was added to the plate and incubated for 1 hour. Substrate reaction was performed with 3.3',5.5'-tetramethylbenzidine substrate (Sigma, Zwijndrecht, The Netherlands) for 15 minutes in the dark, and the reaction was stopped by adding 1.5 N H2SO4. Absorbance was measured at 450 nm in a microplate reader. All incubations were carried out in a volume of 100 µl/well. The experiment was independently repeated 3 times.

Dose dependent block and dose dependent competition of properdin binding to HK-2 by recombinant Salp20

To evaluate whether recombinant Salp20 can inhibit the binding of properdin to the immortalized human kidney proximal epithelial cell line HK-2, cells were cultured in 6 well tissue culture plates. Cells were detached with cell dissociation solution as described previously, transferred into a 5 mL FACS tube with medium and centrifuged at 200g for 7 minutes at 20°C. After washing, cells were incubated with a serial dilution of recombinant Salp20 of 0 ng/mL, 125 ng/mL 250 ng/mL, 500 ng/mL, 1000 ng/mL or 8000 ng/mL recombinant Salp20 together with 10µg/mL purified properdin for 30 minutes at 37°C.

To evaluate whether recombinant Salp20 can dose-dependently compete off bound properdin and the activation of C3 and C5b-9 on HK-2, cells were also cultured in a 6 well tissue culture plates. Cells were detached with cell dissociation solution as described before, transferred into a 5 mL FACS tube with medium and centrifuged at 200g for 7 minutes at 20°C. Cells were incubated with or without 10µg/mL purified properdin (Millipore, Cat 341283-250 1,1 mg/mL) for 30 minutes at 37°C. Cells were washed twice at human factor P, 200g for 7 minutes at 20°C and incubated with a serial dilution of pre-incubated recombinant Salp20 of 0 ng/mL, 32 ng/mL, 125 ng/mL and 500 ng/mL together with 5% NHS for 1 hour at 37°C.

To detect bound properdin, activated C3 or neoantigen C9 (as a measure of C5b-9 formation), cells were incubated with either rabbit anti-human properdin, mouse anti-human activated C3 (Clone bH6, HM2168S, Hycult biotech, Uden, The Netherlands), or with mouse anti-human neoantigen C9 (Clone WU13-15, HM2264, Hycult biotech, Uden, The Netherlands), for 30 minutes on ice. Cells were washed with ice cold FACS buffer, centrifuged at 250g for 6 minutes at 4°C, and incubated with goat anti-rabbit FITC or goat anti-mouse FITC (both purchased from Southern Biotech, Birmingham, USA) for 30 minutes on ice in the dark. Propidium iodide 1 µg/ml (Molecular Probes, Leiden, The Netherlands) was added just prior to measurement in order to exclude apoptotic cells. Properdin binding and activated C3 deposition on non-apoptotic cells were analysed in a FACSCalibur™ (FACSCalibur, Becton Dickinson, New Jersey, USA). Experiments were repeated independently two times. The percentage effect was calculated based on the control data (0 ng/mL Salp20 + 10µg/mL purified properdin) in median fluorescence intensity (MFI), % inhibition = 100-(MFI test result/ MFI control) x 100.

Statistics

GraphPad Prism version 7.02 was used for statistical analyses. Data was examined by one-way ANOVA and the Mann-Whitney-U test and the Wilcoxon rank sum test were used as appropriate for the different experiments. A P value <0.05 was considered statistically significant.

Results

Properdin from various sources binds with PTECs *in vitro* and functions as a docking station for alternative pathway complement activation

Properdin binding to PTECs using properdin present in normal human serum, purified properdin and recombinant properdin was tested to investigate differences in properdin binding and complement activation between different preparations. Incubation for 30 minutes with 50% normal human serum (NHS), purified properdin and recombinant properdin, resulted in substantial binding of properdin detected by flow cytometry. Untreated samples served as a control. Means in median fluorescence intensity (MFI) were 35, 158, 68 and 7 respectively. Binding of all preparations was statistically different (P=0.02, P=0.005, P=0.049 respectively) when compared as fold change (fold change = MFI treated cells / MFI untreated cells) to the untreated control. No statistically significant differences were found between recombinant properdin versus either purified properdin or NHS (P=0.24 and P=0.11). A significant difference was found between 50% NHS versus purified properdin (P=0.049) (Figure 1A and B). In the presence of NHS as a complement source, deposition of activated C3 followed the same pattern (Figure 1C and D). Means in MFI were 31, 72, 16 and 6 for NHS, purified properdin, recombinant properdin and the untreated sample, respectively. Activated C3 deposition were statistically different (P=0.005, P=0.01, P=0.01 respectively) when compared as fold change (fold change = MFI treated cells / MFI untreated cells) to the untreated control for all preparations. No statistically significant differences were found for activated C3 deposition between purified properdin versus recombinant properdin (P=0.07), NHS versus recombinant properdin (P=0.06) or NHS versus purified properdin (P=0.17). As properdin may serve as the docking station for AP complement activation and to unravel the role of properdin in C3 activation on PTEC, we decided to further investigate complement activation on PTEC with and without C3 inhibitor Compstatin.

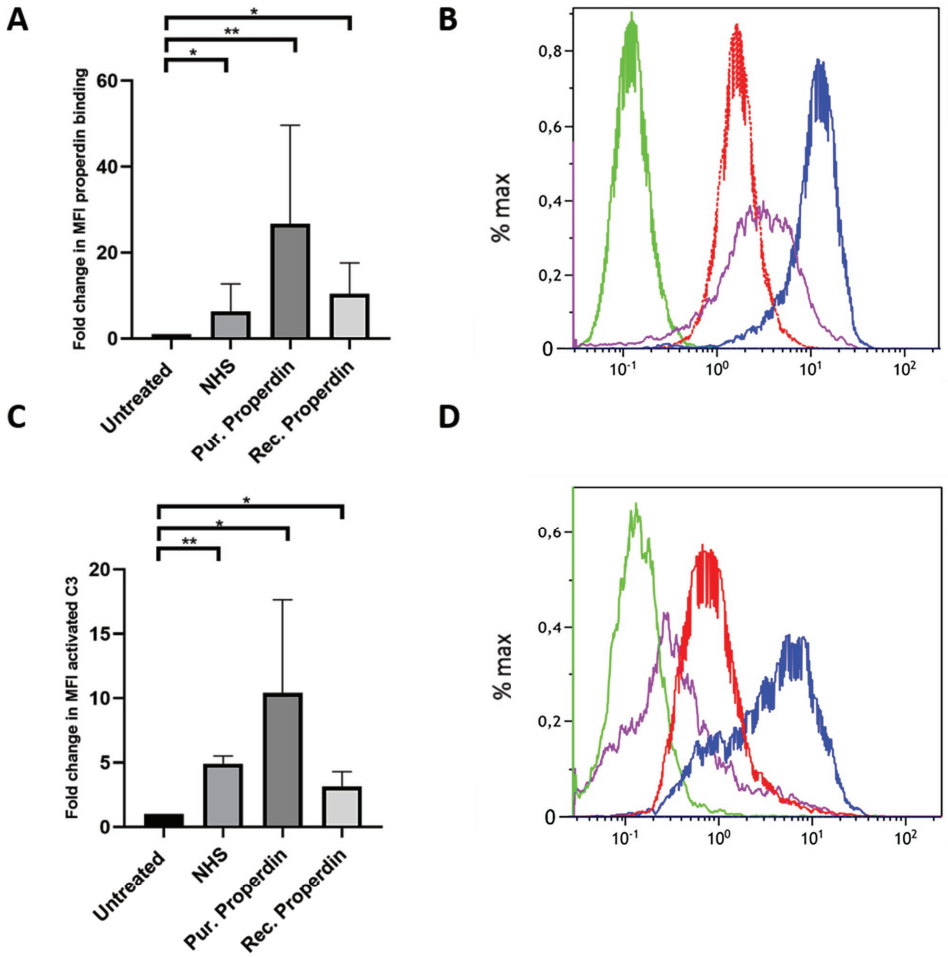


Figure 1. Properdin from various sources bind with PTEC in vitro and functions as docking station for alternative pathway complement activation.

*A. Properdin present in 50% normal human serum (NHS), purified properdin and recombinant properdin show binding with PTEC in comparison to the negative control. Data presented as fold change compared to the untreated control and analysed by the Wilcoxon trank sum test (*P<0.05, **P<0.01, ***P<0.001). Asterisks above the capped lines denote significant differences between the untreated samples and the properdin binding from different sources (n=10).*

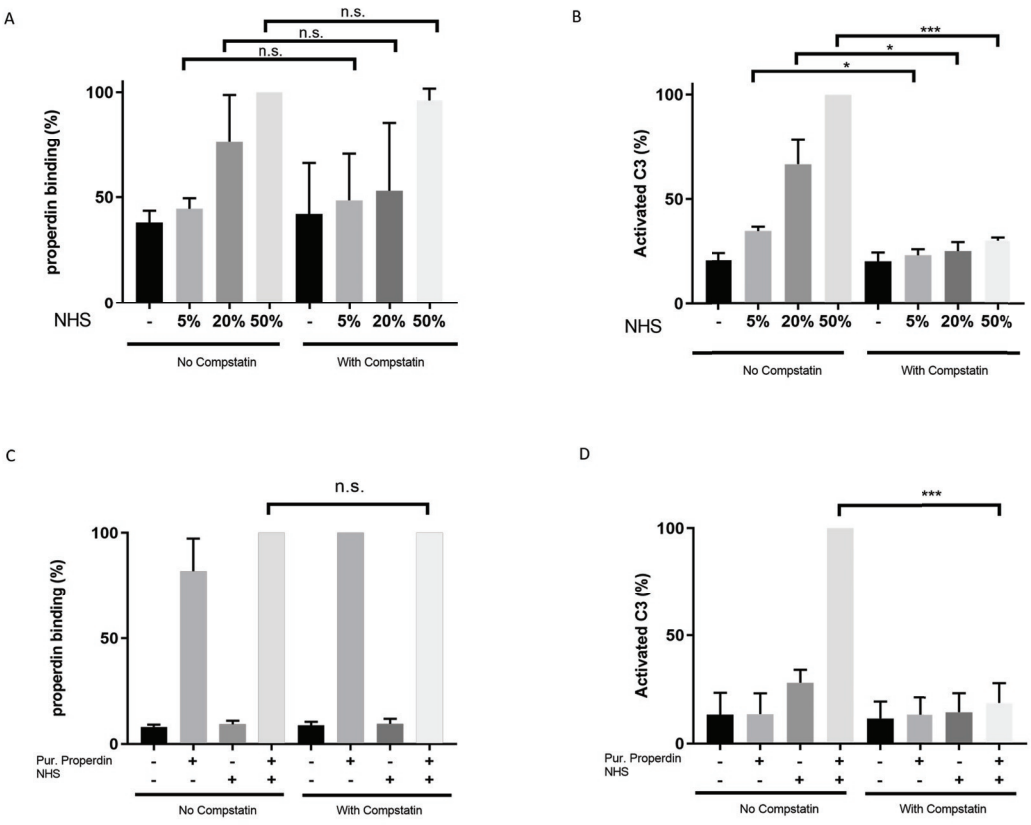
B. Representative flow cytometry experiment for properdin binding from 50% NHS (red line), purified properdin (blue line) and recombinant properdin (purple line) in comparison the untreated sample (green line). Data represented as % max (data normalised for the peak at 100%).

C. Complement C3 activation via properdin from NHS, purified properdin and recombinant properdin shows complement activation via the alternative pathway. Data presented as fold change compared to the untreated control and analysed as described in figure 1A (n=6).

D. Representative flow cytometry experiment for complement C3 activation via NHS (red line), purified properdin (blue line), recombinant properdin (purple line) and the untreated sample (green line).

AP activation but not properdin binding to PTECs can be inhibited by Compstatin

HK-2 cells were incubated with Normal Human Serum (NHS) as a source of properdin in increasing concentrations of 5%, 20% and 50%. Binding of properdin from serum to HK-2 was not affected by pre-incubation and co-incubation with the C3 inhibitor Compstatin, demonstrating that properdin binding with HK-2 cells is independent of the presence of C3b (5% NHS; *P*=0.82, 20% NHS; *P*=0.49 and 50% NHS; *P*=0.42) (Figure 2A). Measurement of activated C3 deposition on HK-2 cells after incubation with NHS confirmed the functionality of Compstatin, since no increase in activated C3 deposition was seen during co-incubation with Compstatin (5% NHS; *P*=0.04, 20% NHS; *P*=0.04 and 50% NHS; *P*=0.0002) (Figure 2B). This implies that properdin might act as a pattern recognition molecule on PTECs.



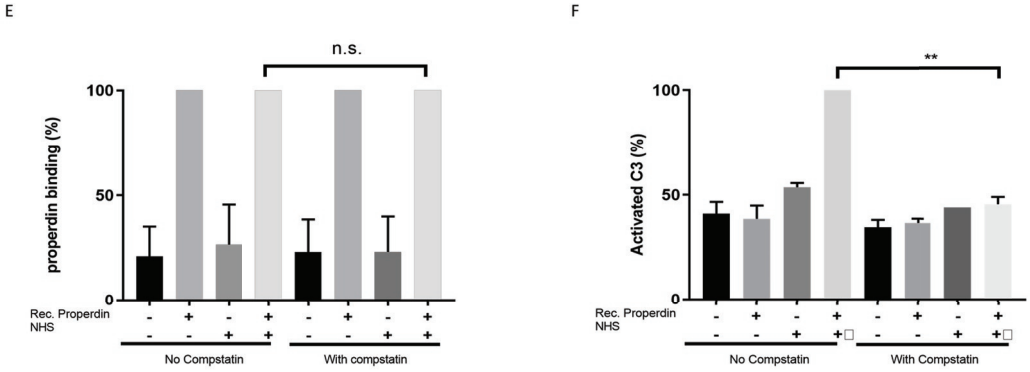


Figure 2. Properdin binding with PTECs in vitro is not mediated via C3b.

A. Properdin binding from serum is not influenced by co-incubation with compstatin. Bars represent a serial dilution of 5%, 20% and 50% NHS.

B. C3 complement activation mediated by properdin from serum is completely inhibited by compstatin.

C. Properdin binding purified from plasma is not influenced by compstatin.

D. C3 complement activation by purified properdin is completely inhibited by compstatin.

E. Recombinant properdin binding is not influenced by compstatin.

*F. C3 complement activation by recombinant properdin is inhibited by compstatin. All graphs are normalized by the sample without compstatin but with NHS (A&B) or purified properdin with NHS (C&D) or recombinant properdin with NHS (E&F). Data were analysed by the Mann-Whitney U test with an option of multiple comparison (*P<0.05, **P<0.01, ***P<0.001). Asterisks above the capped lines denote significant differences between the sample without Compstatin but with NHS (A&B) or purified properdin with NHS (C&D) or recombinant properdin with NHS (E&F) and the same sample with Compstatin. N=6 for all experiments.*

Similarly, when purified properdin or recombinant properdin was used as a source of properdin, pre-incubation and co-incubation of properdin with Compstatin did not affect the binding of properdin on HK-2 cells, further strengthening the finding that properdin binding to PTECs is independent of prior C3b deposition (P=0.23 and P=0.50 respectively) (Figure 2C and E). Co-incubation of serum with Compstatin after incubation of HK-2 cells with purified properdin or recombinant properdin resulted in the inhibition of activated C3 deposition, verifying the C3 inhibitory potential of Compstatin (P<0.0001 and P=0.002 respectively) (Figure 2D and F). In order to confirm the absence of C3 components (including C3b) on the PTEC cell surface to which properdin in NHS can bind, PTEC were stained for activated C3 (recognizing the cleavage fragments of C3b, iC3b and C3c) before and after NHS treatment with and without pre-incubation with Compstatin. (Supplemental figure 1). C3 components were not detectable on untreated PTEC when compared to the background staining (P=0.48). In conclusion, flow cytometry showed that properdin binds to HK-2 cells independent of C3b and regardless of properdin source.

Binding of properdin to proximal tubular epithelial cells is partly mediated by syndecan-1

In former studies it was shown that heparitinase I treatment of HK-2 cells obliterated properdin binding, while immunofluorescent staining showed co-localization of properdin with syndecan-1 *in vivo* on tubular epithelium under nephrotic conditions.[23] To identify the binding site of properdin on tubular cells, we tested properdin binding capacities of syndecan-1 silenced cells by short hairpin RNA technology. Stably transfected HK-2 Synd1-/-cells showed ~80% reduction in syndecan-1 expression (Figure 3A). The HK-2 Synd-1-/-cells show a ~70% reduced properdin binding potential compared to HK-2 WT cells(*P*=0.057) (Figure 3B and C).

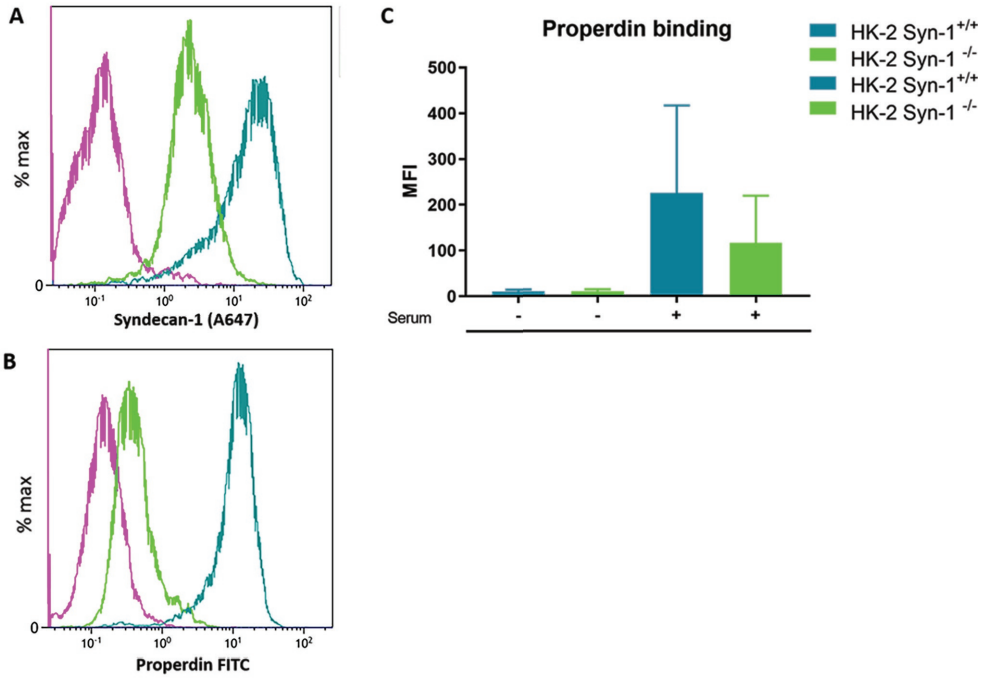


Figure 3. Properdin binding with PTEC in vitro is largely via syndecan-1 associated heparan sulfate.

A. A representative experiment showing high expression of syndecan-1 in PTEC wild type (turquoise) and >80% less expression of syndecan-1 in syndecan-1 KO PTEC (Green).

B. A representative experiment showing strong properdin binding to PTEC wilde type (turquoise) and ~75% less properdin binding to syndecan-1 KO PTEC (Green). The purple colour represents the antibody binding of the isotype control. X-axis is a logarithmic scale, MFI noted in the figure. Data represented as % max (data normalised for the peak at 100%)

C. Syndecan-1 deficient cells show a reduction in properdin binding compared to HK-2 WT cells the difference was borderline significant (n=4, P=0.057). Data is expressed as mean ± SEM.

AP activation and properdin binding to PTECs can be inhibited by recombinant Salp20

It has been described that Salp20 (a deer tick protein) functions as a properdin-blocking agent, displacing properdin from the C3-convertase.[27] To evaluate whether Salp20 inhibits the binding of properdin to HSPGs, resulting in inhibition of properdin's pattern recognition capacity of HSPGs, we co-incubated recombinant Salp20 with properdin in absence of activated C3 and measured the binding of properdin to heparin-albumin. Increasing concentrations of recombinant Salp20 showed dose dependent inhibition of properdin to heparin-albumin with an IC_{50} of 18 ng/ml (Figure 4A). Thus showing that Salp20 indeed inhibited the binding of properdin to an HSPG analogue, abolishing pattern recognition of HSPGs by properdin.

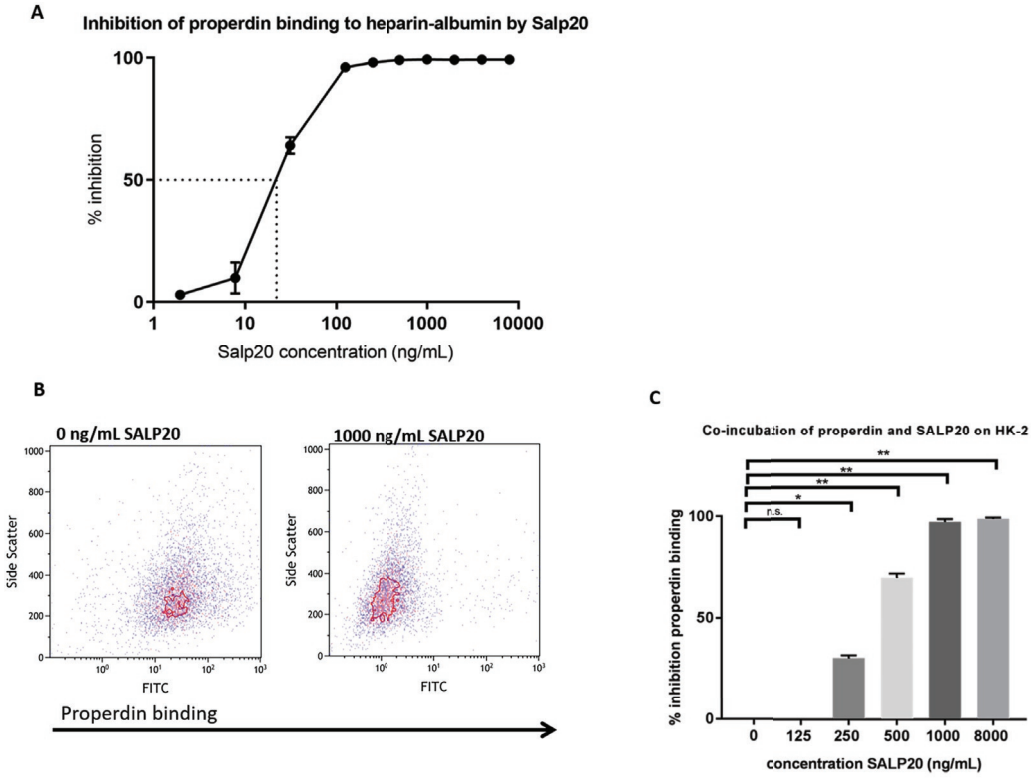


Figure 4. Properdin binding to heparin-albumin in ELISA and to HK-2 cells can be dose- dependently blocked by recombinant Salp20.

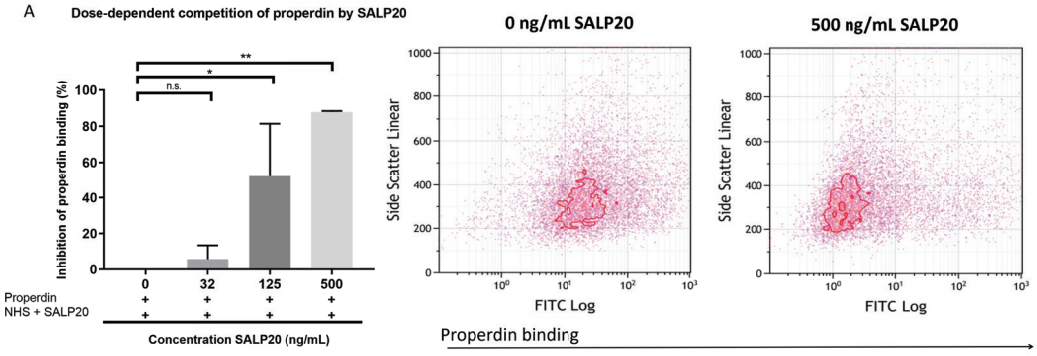
A. Pre-incubation of properdin with Salp20 dose-dependently reduced properdin binding to immobilized heparin-albumin. Dotted line in figure A represents the IC_{50}. Experiments were independently repeated in duplicate.

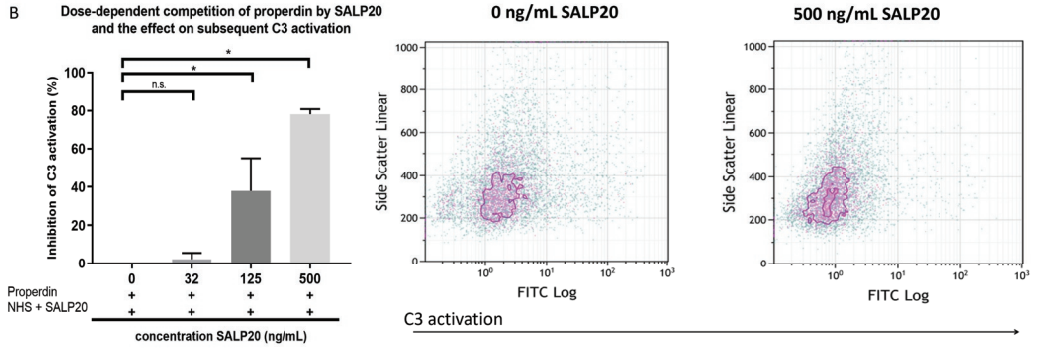
B. A representative flow cytometry experiment shows that properdin co-incubation with 1000 ng Salp20 reduces properdin binding to HK-2 in comparison to properdin incubation with HK-2 without SALP20.

*C. Quantitative analysis of multiple experiments (n=3) shows that recombinant Salp20 significantly blocks the binding of properdin to HK-2 per dose. Data were analysed by Mann-Whitney U test with an option of multiple comparison (*P<0.05, **P<0.01). Asterisks above the capped lines denote significant differences between the untreated samples and the recombinant Salp20 inhibition with different concentrations.*

Recombinant Salp20 was also tested for properdin binding and AP inhibitory potential on HK-2 cells by flow cytometry. Incubation of 10 µg/mL purified properdin, in the absence of activated C3 conditions, led to properdin deposition on the HK-2 cells, while recombinant Salp20 led to a dose dependent reduction in binding of purified properdin to the cells (Figure 4B and C). An inhibitory effect of 70% was achieved when incubating the cells with 500 ng/ml recombinant Salp20 (P=0.01) and an inhibitory effect of 98% (P=0.0003) when incubating the cells with >1000 ng/ml recombinant Salp20 (Figure 4C).

The dose dependent capacity of recombinant Salp20 to displace cell-bound properdin was also tested by flow cytometry. After pre-incubation with purified properdin, HK-2 cells were incubated with an increasing concentration of up to 500 ng/ml of Salp20 and NHS. Recombinant Salp20 dose-dependently displaced bound properdin with an inhibitory effect of 90% with 500 ng/ml Salp20 (P=0.003) (Figure 5A). Recombinant Salp20 was also effective in the inhibition of AP activation, shown by dose-dependent reduction of activated C3 and C5b-9 deposition. Concentration dependent reduction of activated C3 and C5b-9 deposition by recombinant Salp20, showed a similar pattern compared to properdin binding (Figure 5B and C). The maximum concentration of recombinant Salp20, 500 ng/ml, resulted in an inhibition of 80% in activated C3 deposition (P=0.02) and 90% in C5b-9 deposition (P=0.006) compared to the non-inhibited control.





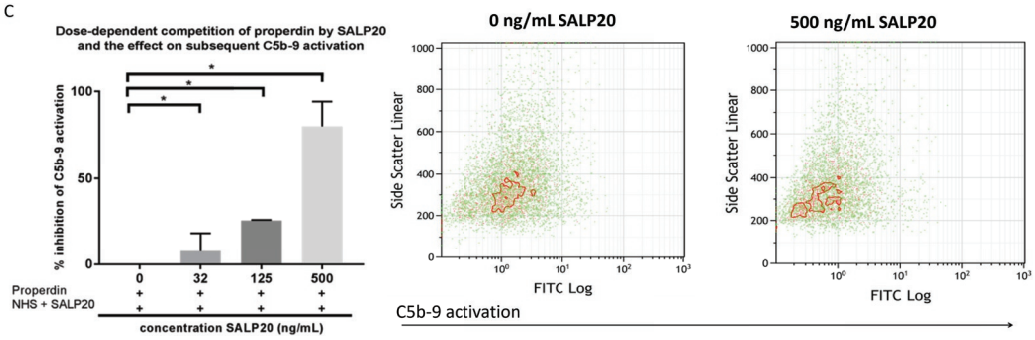


Figure 5. Recombinant Salp20 dose-dependently competes with HK-2 bound properdin, thereby preventing AP complement activation.

A. PTECs were incubated with purified properdin followed by incubation with NHS and increasing concentrations of Salp20. Bars show quantitative analysis of multiple experiments (n=2). Density plots of a representative experiment show the shift in PTEC-bound properdin in the presence of 500 ng/ml Salp20.

B. C3 activation can be dose dependently inhibited by recSALP20. The same experimental set up as in A, but now C3 activation was detected.

*C. C5b-9 activation can be dose dependently inhibited by Salp20. The same experimental set up as in A and B, but now C5b-9 activation was detected. Data were analysed by Mann-Whitney U test with an option of multiple comparison (*P<0.05, **P<0.01). Asterisks above the capped lines denote significant differences between the untreated samples and the recombinant Salp20 inhibition with different concentrations.*

Inhibition of properdin to C3b by heparins, C3b and recombinant Salp20

In the experiments described up to now we evaluated the properdin – syndecan-1/HS interaction as a focus point for intervention. In a last series of experiments, we evaluated the properdin-C3b interaction as a target point. Thus we set out a series of competition experiments using the potential dose-dependent inhibitory activity of heparins, C3b or recombinant Salp20 on properdin binding to immobilized C3b. The results of the binding assay showed that heparin-albumin, unfractionated heparin, C3b and recombinant Salp20 inhibit properdin binding to C3b in a dose-dependent manner. (IC_{50} 8.6 ng/mL for heparin-albumin, 63.4 ng/mL for unfractionated heparin, >1,000 ng/mL for LMW-heparin, 6484 ng/mL for C3b and 33.5 ng/mL for recombinant Salp20). Also, the inhibitory capacity depends on the size of the heparin products (IC_{50} 0.000043 nmol/mL for heparin-albumin, 0.004 nmol/mL for unfractionated heparin, >22.2 nmol/mL for LMW-heparin). Heparin has a lower IC_{50} compared to C3b (IC_{50} 0.004 nmol/mL versus IC_{50} 0.035 nmol/mL). Recombinant Salp20 has a lower IC_{50} compared to heparin (IC_{50} 0.0007 nmol/mL versus IC_{50} 0.004 nmol/mL respectively) (Table 1). These data show that unfractionated heparin and recombinant Salp20 not only compete for properdin binding with heparan sulfates, but also for properdin binding with C3b.

Table 1. Inhibition of properdin binding to C3b; IC50, Concentration causing 50% properdin binding inhibition expressed in ng/mL and nmol/mL.

Coating	Inhibitor	IC50 ng/mL	IC50 nmol/mL
C3b	Heparin-albumin	8.6 ng/mL	0.000043 nmol/mL
	Unfractionated heparin	63.4 ng/mL	0.004 nmol/mL
	LMW-heparin	>1000 ng/mL	>22.2 nmol/mL
	C3b	6484 ng/mL	0.035 nmol/mL
	Salp20	33.5 ng/mL	0.0007 nmol/mL

IC_{50} was calculated by non-linear regression with curve fitting in GraphPad Prism.

Discussion

In this study we provide evidence that properdin functions as a pattern recognition protein on PTECs where the binding is largely mediated via syndecan-1 associated heparan sulfate and is C3b-independent. Furthermore, we show that the tick protein Salp20 effectively blocks the heparan sulfate mediated pattern recognition by properdin, pointing towards the potential for therapeutic interventions at the tubular level in proteinuric conditions.

It has long been assumed that properdin, next to its stabilizing role of the alternative C3 convertase, could act as a pattern recognition molecule. We have shown previously that during proteinuria, properdin recognizes and binds to heparan sulfate proteoglycans (HSPG) on tubular epithelial cells.[23] Our results in this study using the C3 inhibitor Compstatin, show that Compstatin can inhibit complement activation and therefore C3b deposition, but cannot preclude the deposition of properdin on PTECs. We display that this finding does not materially differ between properdin sources, including recombinant properdin. The latter confirms that our finding is robust, since different properdin isolates might differ in purity, conformation, multimerization, post-translational modifications, and the eventual presence of other co-purified or properdin-bound proteins. However, properdin in purified preparations is prone to aggregation which can be avoided to a certain extend by storage at 4°C for up to two weeks without any additional freeze/thaw cycles.[12,36] Our properdin preparations were exposed to one freeze/thaw cycle and our preparations could therefore contain non-physiological aggregates. However, since unpurified properdin present in serum essentially showed the same binding to PTEC as purified and recombinant properdin, it is unlikely that properdin aggregates importantly contributed to our findings. Therefore, irrespective of the source, all experiments indicate a real C3b-independent binding of properdin to heparan sulfates (HS), most likely present on the PTEC cell membrane as the polysaccharide side chains of syndecan-1. These findings are in agreement with experimental studies in C3 knockout mice demonstrating C3 deposition in glomeruli of mice with anti-GBM disease, although the authors did not address the glomerular cell type to which properdin binds.[19]

Conversely, *Harboe* and collegues recently showed that properdin binding on endothelial cells and *Neisseria meningitidis* is dependent on initial C3 deposition.[18] Consequently, our findings reopen the discussion whether properdin is a true pattern recognition molecule of the alternative pathway (AP). Pattern recognition of properdin has been indicated in other properdin interactions as well. Properdin binding to DNA and glycosaminoglycans on late apoptotic cells and necrotic cells has been suggested to be independent of initial C3 deposition.[16,17] Glycosaminoglycans and DNA share a strong negative charge, while properdin is strongly positively charged. Therefore the interaction of properdin with glycosaminoglycans (and DNA), is based on charge - charge interactions, as we previously analysed in detail.[23]

Syndecan-1 and properdin co-localize on PTECs under proteinuric conditions.[23] We present that syndecan-1 may be a ligand of properdin, using a syndecan-1 deficient HK-2 strain. Syndecan-1 is a major membrane spanning HSPG in epithelial cells and the interaction of properdin with sulfated glycosaminoglycans has been long known. In this study we found a reduced binding of properdin in syndecan-1 deficient HK-2 cells when compared to HK-2 wild type cells. It is likely that properdin not only binds to syndecan-1 but also to other epithelial HSPGs of which syndecan-1 is the most important properdin binding HSPG. Properdin consists of seven non-identical trombospondin-1 repeats (TSR), and literature has shown that a fragment consisting of TSR 4 & 5 forms the binding site for glycosaminoglycans, but also for C3b.[30] Earlier work already showed that trypsin treatment of properdin, cleaving the TSR5 in half, results in an inability to bind C3b while the glycosaminoglycan binding remains intact. This suggests that TSR5 is the principal C3b binding site for properdin that receives a co-operative contribution from TSR4.[30,37] Recent structural studies revealed TSR5 to be the dominant C3b binding domain with some contribution of TSR6.[34] Taken together, these studies showed that the binding site for C3b and glycosaminoglycans on properdin could be different, but are more likely very close. We show that inhibition of properdin to C3b by different heparinoid products is size dependent; the bigger the better, suggesting that larger heparins sterically hinder the C3b binding site on properdin as well. We also show that the deer tick protein Salp20 can inhibit both the binding of heparin-albumin and C3b to properdin. Salp20 has previously been shown to displace properdin from the alternative C3 convertase, resulting in accelerated decay of the convertase.[27] Our results confirm that recombinant Salp20 can inhibit the binding of properdin to C3b and thereby reduce the AP activation on PTECs. However, we also show that recombinant Salp20 can inhibit the binding of properdin to heparin-albumin and to HS on PTECs, indicating a double inhibitory role for Salp20 in properdin mediated AP activation, namely inhibition of the active C3 convertase and inhibition of the initial pattern recognition function of properdin. The results further strengthen the data shown by others that C3b and glycosaminoglycans have a closely related binding epitope on properdin.[27] Nevertheless, further molecular docking studies are needed to unravel the exact glycosaminoglycan-binding domain of properdin.

It has been demonstrated before that Salp20 can inhibit the AP of complement in multiple disease models.[29,38] This could be of major importance in the development of therapeutic modalities for tubular damage in proteinuric renal diseases. Our results demonstrate that in this setting, recombinant Salp20 may not only block the binding of properdin to proximal tubular epithelial cells, but also compete off properdin that was already bound to the cells and

avoid AP activation, even when the initial pattern recognition step had already formed. Since properdin-deficient humans do not show a severely compromised immune function, apart from an increased risk for meningitis for which vaccination is possible, blocking properdin seems a relatively safe approach.[39,40] There are some *in vitro* and *ex vivo* studies describing properdin competing composites and properdin blocking antibodies in humans, however unexpected results in animal models teach us that not all lessons have yet been learned.[30,41,42,43,44,45,46] Bansal-Gupta *et al*. described a properdin targeting monoclonal antibody, showing exclusive AP blocking activity by influencing the interaction of C3 with properdin in an *in vitro* model.[41] In addition, Pauly *et al*. reported the development of an anti-properdin monoclonal antibody that showed up to fifteen times more efficiency in blocking the complement cascade when compared to anti-Ba or anti-C5 antibodies in human blood samples.[42] Currently one anti-properdin antibody is at the stage of a phase 2 clinical trial.[43] However, properdin doesn't spill the beans that easily. In contrast to the studies described above, a protective role for properdin was described in two separate C3 glomerulopathy (C3G) mouse models. This is remarkable, as C3G occurs as a result of overactivity of the AP, leading to glomerular injury. Nonetheless, mice knocked out for properdin (and small amounts of truncated factor H) showed an injury exacerbation with increased accumulation of C3 along the glomerular basal membrane.[45,46] In conclusion, discovering the role of properdin remains challenging and not fully elucidated.

Salp20 is a new kid on the block in the field of properdin inhibition and since Salp20 is a tick protein, it would be expected to be strongly immunogenic. Therefore, prior to testing in animal models, small non-immunogenic molecule analogues of the Salp20 binding region should be produced and tested *in vitro* and *in vivo* for their AP inhibiting potential. Next to the inhibitory effect of Salp20, we have also shown in previous work that heparinoids compete for properdin binding with heparan sulfates on PTECs,[24] and in that study we showed that non-anti-coagulant heparins also compete for properdin binding with C3b. This is promising for AP-driven diseases such as tubular activation secondary to proteinuria. Finally, we also show that Compstatin does not inhibit the binding of properdin to PTEC, but does prevent subsequent AP complement activation. Therefore, Compstatin holds promise in blocking undesired complement activation in numerous pathogenic conditions. A possible mechanism of AP complement inhibition on a tubular level by Compstatin and Salp20 is depicted in Figure 6. Overall, our study demonstrates the inhibitory effects of Compstatin, non-coagulant heparins and recombinant Salp20 at the level of proximal tubular epithelial cells. These results might be of great importance for reducing proteinuria induced AP activation and tubular injury.

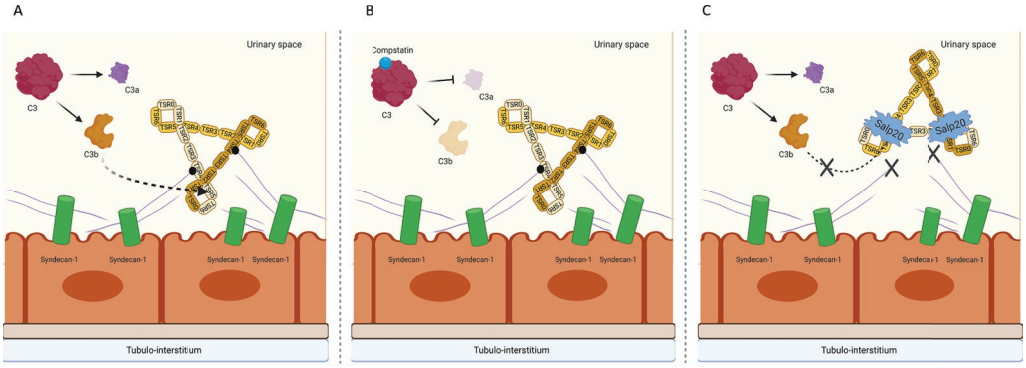


Figure 6. Proposed mechanism on a tubular epithelial level of properdin binding to syndecan-1/heparan sulfate and interruption of AP complement activation by C3 cleavage inhibiting peptide Compstatin and properdin inhibitor Salp20.

A. Properdin is depicted in its trimeric form since this is the most abundant form of properdin. The binding of syndecan-1 is most likely to the trombospondin-1 repeat (TSR) 4 domain of properdin, receiving a cooperative contribution of TSR5. C3b most likely binds to TSR5, with a cooperative function of TSR4 and TSR6.

B. In the presence of Compstatin, C3 cleavage is inhibited. However, properdin is able to bind to heparan sulfate proteoglycan syndecan-1.

C. In the presence of Salp20, C3b can not bind to the TSR5 domain of properdin, nor can properdin bind to syndecan-1.

Figure created with BioRender.com.

Acknowledgments

We thank H.M. van der Lugt for her support for the preparation of this manuscript and Dr J.D Lambris for his generous gift of Compstatin. We also thank R. van den Bos and Dr. P. Gros (Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands) for their helpful gift of recombinant properdin and recombinant Salp20 and their support during the preparation of this manuscript.

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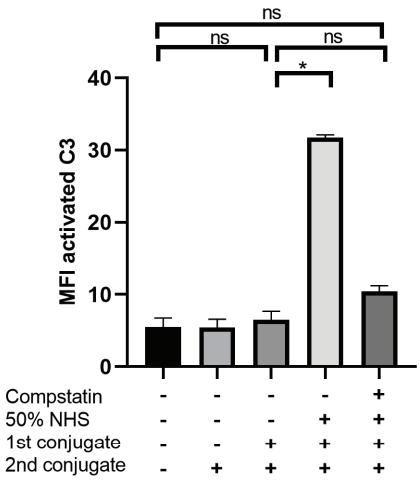
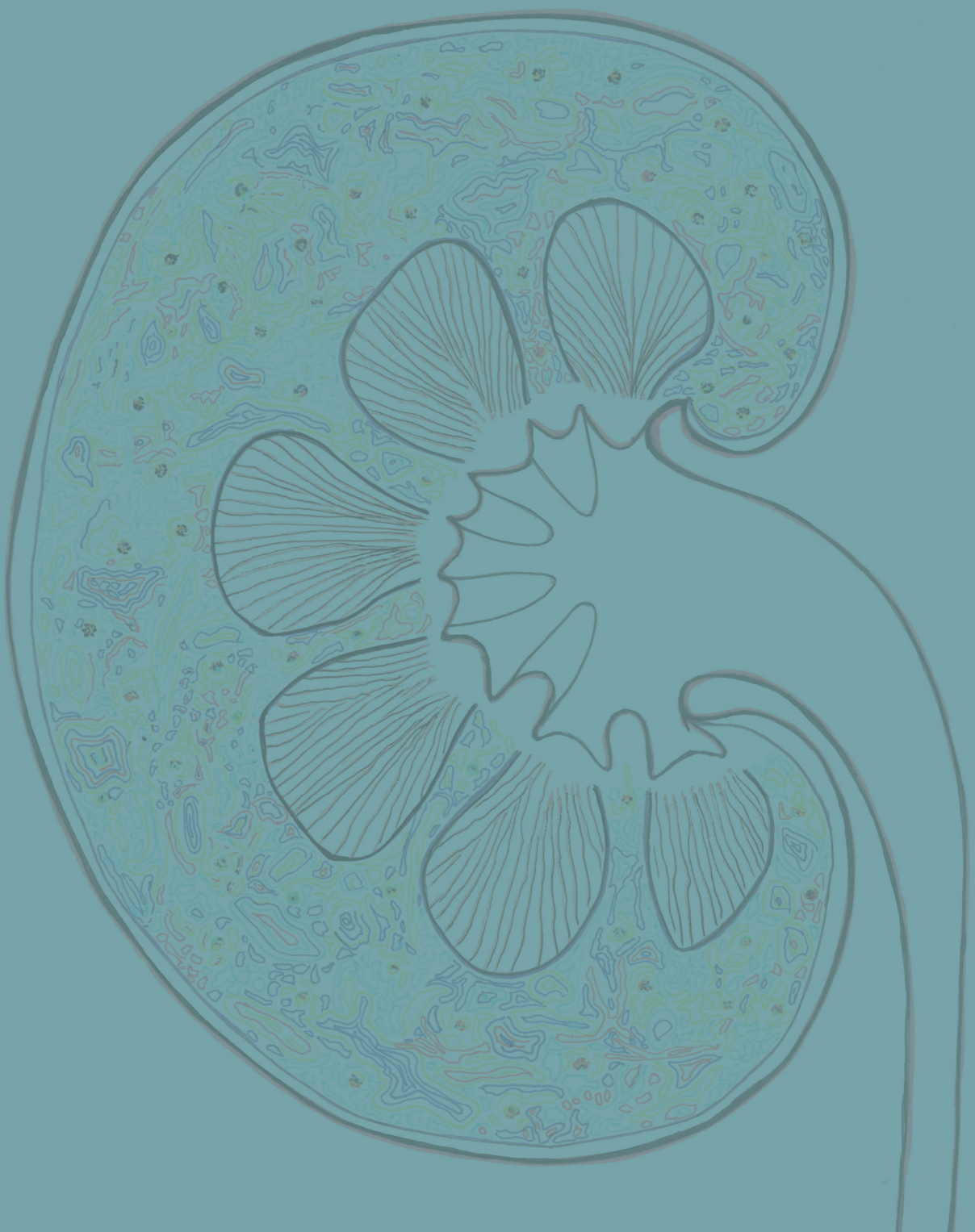
Supplementary results



Supplementary Figure 1. C3 components C3b, iC3b and C3c (activated C3) are not detectable on the cell membrane of untreated PTEC.

PTEC were treated with NHS with and without pre-incubation with Compstatin. FACS staining for activated C3 on untreated PTEC (- - + +) does not show presence of C3 compared to the background staining without detecting antibody (- - - -) (P=0.48). Data presented as mean fluorescence intensity (MFI). Asteriks above the capped lines denote significant differences.

PART B – ANTIBODY-MEDIATED COMPLEMENT ACTIVATION IN KIDNEY TRANSPLANT RECIPIENTS



CHAPTER 4

Machine perfused donor kidneys as a source of human renal endothelial cells

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American Journal of Physiology - Renal Physiology 2021; DOI: 10.1152/ajprenal.00541.2020

Abstract

Renal endothelial cells (ECs) play crucial roles in vasorelaxation, ultrafiltration, and selective transport of electrolytes and water, but also in leakage of the glomerular filtration barrier and inflammatory processes like complement activation and leukocyte recruitment. In addition, they are target cells for both cellular and antibody mediated rejection in the transplanted kidney. In order to study the molecular and cellular processes underlying EC behavior in renal disease, well characterized primary renal ECs are indispensible. In this report we describe a straightforward procedure to isolate ECs from the perfusion fluid of human donor kidneys by a combination of negative selection of monocytes/macrophages, positive selection by CD31 Dynabeads and propagation in endothelial specific culture medium. Thus, we isolated and propagated renal ECs from 102 donor kidneys, representative of all blood groups and major HLA class I and II antigens. Obtained ECs were positive for CD31 and von Willebrand Factor, expressed other endothelial markers such as CD34, VEGFR-2, TIE2, and PV-1 to a variable extent, and were negative for monocyte marker CD14 and lymphatic endothelial marker podoplanin. HLA class II was either constitutively expressed or could be induced by IFN-γ. Furthermore, as a proof of principle, we showed the diagnostic value of this renal endothelial biobank in renal endothelial specific cross matching tests for HLA antibodies.

Introduction

The endothelium covers the inner surface of all blood vessels in our body and therefore resides at the critical interface between blood and tissue. The endothelium plays an important role in innate and adaptive immunity and is involved in various disease states, either as a primary determinant of pathophysiology or as a responder to secondary stage damage.[1,2,3] No two endothelial cells (EC) are alike, though a few universal phenotype markers are known; including CD31 and CD34. The endothelium has different functions, corresponding to the region of the body where it is situated.[3] A number of studies have shown molecular heterogeneity at the level of the endothelium, e.g. serial analysis of gene expression has uncovered several transcripts that are selectively expressed in glomerular ECs, but not on aortic ECs.[44] Also within the kidney, heterogeneity in the endothelium is impressive, as shown by single cell mRNA profiling.[5,14]

An important corollary of endothelial heterogeneity in the kidney is the unique response of each vascular segment to pathophysiological processes.[5,53] In kidney transplantation, the first immunological barrier that the recipient's immune system encounters is the donor's endothelium, turning ECs into the primary target of the alloimmune response.[47] ECs within the renal vasculature thus become target of circulating antibodies against human leukocyte antigens (HLA) and anti-EC antibodies (AECAs), resulting in antibody-mediated rejection (AMR).[13] AMR is a leading cause of graft dysfunction and inferior outcomes after transplantation, and is often unresponsive to current therapies.[17] Vascular inflammation, injury, and complement deposition along the microvascular branches are diagnostic criteria in AMR, but the pathophysiology of the vascular damage is not fully understood.[22,27] Consequently, AMR remains a diagnostic and therapeutic challenge.

Multiple groups have tried to address the role of endothelial responses to HLA and non-HLA antibodies, however several hurdles hamper the clarification of endothelium-antibody interplay. EC research is commonly based on widely available primary EC cultures like Human Umbilical Vein Endothelial cells (HUVEC): macrovascular venous endothelium derived from immune-privileged fetal tissue which is not representative of adult endothelium.[7,16] In addition, EC cross matches and renal related EC assays with cells like Human Arterial Endothelial Cells (HAEC) and Human dermal microvascular endothelial cells (HMEC-1), do not take the physiological and phenotypic heterogeneity of endothelium into account, and may underestimate the distinction of renal microvascular endothelium.[4,32,36,41]To our knowledge, only one renal glomerular endothelial cell line (CiGeNC)[42] is widely used, and Delville *et al*. recently developed a non-anti-HLA anti-EC antibodies (AECAs) cross match assay using CiGeNC. They confirmed specific renal microvascular EC responses and revealed substantial differences in activation profiles between macrovascular and microvascular ECs in response to AECAs.[13] However, from cross matching comparison studies it appeared that it is both the nature of the heterogenous mixtures of antibodies, and the particular set of antigens on the target cells that determines the effectiveness of antibody-antigen binding and complement activation.[15] Since CiGeNC are derived from only one donor with a specific blood group, HLA and endothelial antigen expression profile, the clinical relevance of cross matching potential recipients with this cell line is limited. Therefore, we investigated whether kidney machine perfusate after organ donation could serve as a source for renal specific ECs, thereby creating the possibility to isolate ECs from multiple human donors with different blood groups and HLA profiles.

Materials and Methods

Isolation of Machine Perfusion derived Primary Renal Endothelial Cells (MP-PRECs)

Studies were performed with cells derived from machine perfusates after deceased-donor kidney donation. The study was approved by the UMCG institutional review board, adheres to the Declaration of Helsinki and Istanbul, and has NCT0327841 as ClinicalTrial.gov identifier. Hypothermic machine perfusion is currently the standard storage method for deceased-donor kidneys in the Netherlands, using either the LifePort 1.1 (Organ Recovery Systems, Zaventem, Belgium) or the Kidney Assist-transporter (Organ Assist, Groningen, the Netherlands). Both machines are pressure controlled (set at a mean of 25 mmHg),[54] and use University of Wisconsin machine perfusion solution.[29] After removing the kidney from the machine, the remaining perfusion fluid was resuspended. For the LifePort disposable, the filter was retrogradely flushed to retrieve cells caught in the filter. Perfusion fluid was collected in sterile 500 mL containers (Corning® SIGMA, Cat#CLS431123) and centrifuged at 4°C for 15 minutes at 300 g. After removal of the supernatant, cells were washed with culture medium (Medium 200 (GIBCO, Cat#M-200-500, Grand Island, NY, USA) containing Low Serum Growth Supplement (LSGS, GIBCO, Cat#S-003-K, Grand Island, NY, USA). After a second washing step with culture medium, plastic based negative selection was performed to remove monocytes, by incubating the cells for one hour at 37°C in an uncoated T75 culture flask.[11]

After one hour, non-adherent cells were distributed in a single well of a 6-well culture plate (10 cm^2) pre-coated with 1% gelatin (Sigma Aldrich, Cat#G1890-100G, Darmstadt, Germany). The cells were incubated at 37°C and culture medium was changed every two days.

CD31 immunomagnetic beads (Dynabeads® CD31, Invitrogen, ref.11155D, Landsmeer, The Netherlands) were used for positive selection of renal ECs from the mixture of cultured cells. After ~two weeks of culturing the number of ECs was estimated by eye, based on confluence and morphology of the cells in culture. Next, the amount of CD31 Dynabeads solution was estimated aiming at 4 beads per cell. Beads were washed and diluted in bead wash buffer (SigmaAldrich, A1595, Damstadt, Germany) with 0.1% Bovine Serum Albumine (BSA) (SigmaAldrich, A9647-100G) supplemented with gentamycine (BE02-012E, Lonza, Basel, Switzerland). Cells were detached using trypsin-EDTA (SigmaAldrich, Cat#59430C), which was subsequently neutralized using trypsin neutralizer solution (GIBCO, R-002-100, Grand Island, NY, USA). The cells were centrifuged (4°C, 1500 RPM, 7 minutes), supernatant was removed, and cells were incubated with CD31 Dynabeads in 200µl bead wash at 20°C for 20 minutes on a roller bank. Thereafter the cells-CD31 Dynabeads mixture was placed in a magnet for 2 minutes, followed by removal of supernatant and two additional washing steps of the beaded cells with bead wash buffer. Once the MP-PRECs were purified they were cultured in M200 medium with low serum growth supplement. To visualize all cells present in the primary perfusate, cytospins were made directly after isolation and before plastic based negative selection, containing 1.000.000 cells/mL. Differentiation of white blood cell populations in the primary perfusate was analyzed using a Sysmex XN series analyser (Sysmex, Kobe, Japan) and by May Grünwald-Giemsma staining. Detailed protocols of the isolation techniques are added as appendices. (Appendix 1 and appendix 2).

Flow cytometry

Cells were stained by flow cytometry directly after isolation; non-adherent and adherent cells were stained after plastic based negative selection and MP-PRECs were stained at 7 and 21 days after positive CD31 Dynabeads selection. Before the staining procedure, excess erythrocytes in the perfusion fluid from the donor were lysed using lysis buffer (155 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA, Pharmacy UMCG, Groningen, The Netherlands). Details on antibodies and conjugates used for flow cytometry are given in Table 1. All antibodies were diluted in 1% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS) and then incubated for 30 minutes on ice. Isotype and conjugate controls were included for all stainings. HUVECs obtained from the UMCG endothelial facility, were used as control for CD31 expression.[10] Cell culture contamination with peripheral blood mononuclear cells (PBMC) was examined with PBMC markers CD14 and L-selectin.[11,19] For measurement of HLA class I and II expression, cells were stimulated for 48 hours with 50ng/mL IFN-γ in M200 mediumat 37℃. The percentage IFN-γ induced HLA expression was calculated as an increase in % = 100 – [MFI unstimulated cells x 100)/MFI stimulated cells]. Cells were analyzed using a FACSCalibur equipped with CELLQuest software and Kaluza software (Kaluza 2.1 Becker Coulter).

Table 1. *Details on the primary antibodies and conjugates used in Flow cytometry and (confocal) immunofluorescence staining procedures.*

Technique	Marker	Antibody	Conjugate
Flow cytometry	CD31	PE conjugated mouse anti-human CD31 (ready to use, *IQ* products, IQP-552R, Groningen, The Netherlands)	
Flow cytometry	CD45	PerCP-Cy™5.5 conjugated mouse anti-human CD45 (Clone HI30, BD Biosciences, San Jose, USA)	
Flow cytometry	CD14	Alexa Fluor® 700 conjugated mouse anti-human CD14 (Clone M5E2, BD Biosciences, cat. 557923, San Jose, USA)	
Flow cytometry	Tie2/TEK	Mouse anti-human Tie2/TEK (1:25, Clone 33.1 (Ab33), Biolegend, San Diego, CA, USA)	Goat anti-mouse FITC (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands)
Flow cytometry	HLA I	Mouse anti-human HLA A-B-C (1:100, clone W6/32, Bio-Rad, Kidlington, UK)	Goat anti-mouse IgG FITC (1:100, Southern BioTech, cat. No. 1031-02, Birmingham, USA)
Flow cytometry	L-selectin	Mouse anti-human CD62L (1:100, Cat. 555542, BD Pharmingen™, San Jose, USA)	Goat anti-mouse IgG FITC (1:100, Southern BioTech, cat. No. 1031-02, Birmingham, USA)
Flow cytometry Immunofluorescence	HLA II	APC conjugated mouse anti-human HLA-DR (1:50, clone L243, BD Biosciences, cat. 347403, San Jose, USA)	
	Isotype control		PE conjugated Rabbit anti-mouse IgG1 (1:10 dilution of IW Products $F(ab)_2$ *IQ* products, IQP-190R, Groningen, The Netherlands)
Immunofluorescence	Von Willebrand factor	Sheep anti-human von Willebrand Factor (1:1000, AHP062, Bio-Rad, Kidlington, UK)	Donkey anti-sheep Alexa 488 (A11015, Invitrogen, Eugene, USA)
Immunofluorescence	Podoplanin	Mouse anti-human podoplanin (1:100, clone D2-40, DAKO, ref. M3619)	donkey anti-mouse alexa 647 (1:250, DAKO, P0447)
Immunofluorescence	VEGFR-2	Rabbit anti-human VEGFR-2 (1:200, clone D5B1, Cell Signaling Technology, 9698)	Goat anti-rabbit IgG-HRP + tetramethylrhodamine tyramide reagent (Tyramide-TRITC) (1:50, FPI051, Perkin Elmer LAS Inc, Boston MA)
Immunofluorescence	PV-1	Mouse anti-human PV-1 (1:50 MON60001-1)	Donkey anti-mouse alexa 647 (1:250, DAKO, P0447)
Immunofluorescence	Activated C3	Mouse anti-human activated C3 (HM2168, Hycult, Uden, The Netherlands)	Goat anti-mouse IgG FITC (Cat. No. 1031-02, SouthernBioTech, Birmingham, USA)

HLA, human leukocyte antigen; PV-1, plasmalemmal vesicle-associated protein-1; Tie2/TEK, tyrosine kinase transmembrane receptor; VEGFR-2, vascular endothelial growth factor receptor-2.

Immunofluorescence

Endothelial phenotyping was performed on MP-PRECs from different donors (n=8). Cells were grown on Lab-Tek® chamber slidesTM (ref.177445 ThermoFisher Scientific, Groningen, The Netherlands) coated with 1% gelatin for 30 minutes. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, then incubated with 0.5% Triton X-100 in PBS for 10 minutes and blocked with 2% Bovine Serum Albumin (BSA) for 30 minutes. All primary and secondary antibodies were diluted with 1% BSA in PBS and then incubated for 1 hour at room temperature. Details on antibodies and conjugates used for immunofluorescence are given in Table 1. All sections were counterstained for 10 minutes with DAPI (0.001 mg/mL in PBS, Sigma, ref. D954). Pictures were taken and evaluated on a Leica DM4000B equipped for immunofluorescence, and with a TissueFaxs using Tissue Quest (Tissuegnostics, medical & Biotech solutions, Vienna, Autria) as digital quantification software.

RT-qPCR

For characterization of endothelial specific gene expression, RT-qPCR was performed. RNA was isolated from MP-PRECs at day 0 and day 7 after CD31 Dynabeads selection, using the RNease Micro Plus Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands). Taqman assays (Applied Biosystems, Nieuwekerk aan den IJssel, The Netherlands) were used to perform gene expression analysis. Human-specific primers were selected to amplify CD31, CD34, von Willebrand Factor, PV-1, VEGFR-2, HLA-DR, Angiotensin II receptor type 1 (AGTR1), ACTA2 (α2SMA), PDGFB for (myo-)fibroblasts and/or mesangial cells, and NPHS1 (Nephrin) for podocytes (Table 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. PCR amplification was performed in a ViiA 7 real-time PCR System (Applied Biosystems) according to the manufacturer's protocol. Duplicate real-time PCRs were performed for each sample and the obtained threshold cycle (CT) values were averaged. Gene expression reactions were normalized to the averaged expression of the housekeeping gene, yielding the ΔCT value. Relative gene expression levels were calculated by $2^{-\Delta CT}$.

Table 2. Primers.

Species	Gene	Human assay ID
Homosapiens	GAPDH	Hs99999905_m1
Homosapiens	PECAM1 (CD31)	Hs00169777_m1
Homosapiens	CD34	Hs00990732_m1
Homosapiens	Von Willebrand Factor	Hs00169795_m1
Homosapiens	PV1 (PLVAP/MECA32)	Hs00229941_m1
Homosapiens	VEGFR2 (KDR)	Hs00176676_m1
Homosapiens	HLA-DR	Hs99999917_m1
Homosapiens	AGTR1	Hs00258938_m1
Homosapiens	α2SMA (ACTA2)	Hs00909449_m1
Homosapiens	NPHS1 (Nephrin)	Hs00190446_m1
Homosapiens	PDGFB	Hs00234042_m1

a2-SMA, a2-smooth muscle actin; AGTR1, angiotensin II receptor type 1; HLA, human leukocyte antigen; NPHS1, nephrin; PDGF-B, platelet-derived growth factor-B; PECAM-1, platelet endothelial cell adhesion molecule-1; PV-1, plasmalemmal vesicle-associated protein-1; VEGFR-2, vascular endothelial growth factor receptor-2.

Proof of principle: Complement activation using MP-PRECs

Complement activating capacity of the MP-PRECs was evaluated by classical complement activation. In a custom EC crossmatch assay, cells were incubated with human monoclonal anti-HLA antibodies (A2/A28 IgG1 and Bw6 IgG1, a kind gift from Dr. F. Claas, Leiden, The Netherlands), 25% human serum containing HLA antibodies directed against HLA antigens expressed on the MP-PREC cell membrane, and 25% heat inactivated (1 hour at 56°C) blood group incompatible serum diluted in M200 medium for 45 minutes. 25% heat inactivated blood group compatible serum and serum containing HLA antibodies not directed against HLA antigens expressed on the MP-PREC cell membrane were used as controls. After two washes with M200 medium, 20% pooled AB serum from healthy volunteers was used as a complement source, and cells were incubated for 30 minutes before two washes with 1% BSA. Complement activation was measured using anti-human activated C3 (recognizing the cleavage fragments of C3b, iC3b and C3c) (Table 1). Immunofluorescent pictures were taken and analyzed as described before.

Results

Isolation of Machine Perfusion derived Primary Renal Endothelial Cells (MP-PRECs)

From January 2017 until July 2019, 102 donor kidney perfusates were isolated after deceased kidney donation according to the procedure depicted in Figure 1. Of the 102 EC isolations, 41% were from the Kidney Assist perfusion device and 59% from the LifePort (Table 3). MP-PRECs equipped with a large variety of HLA-types (Table 4) and blood groups (Table 5) were obtained, which were representative of the general population when compared to the percentage of antigens within a pool of 10,000 recent organ donors.[55] Further clinical characteristics are specified in Table 3. After successful CD31 Dynabeads selection, 27% of cultures became infected and 25% stopped proliferating between day 7 and 21. Success rate (defined as ˜1 x 10^6 MP-PRECs yield to perform an experiment with/store in liquid nitrogen) was 39% (40 isolates).

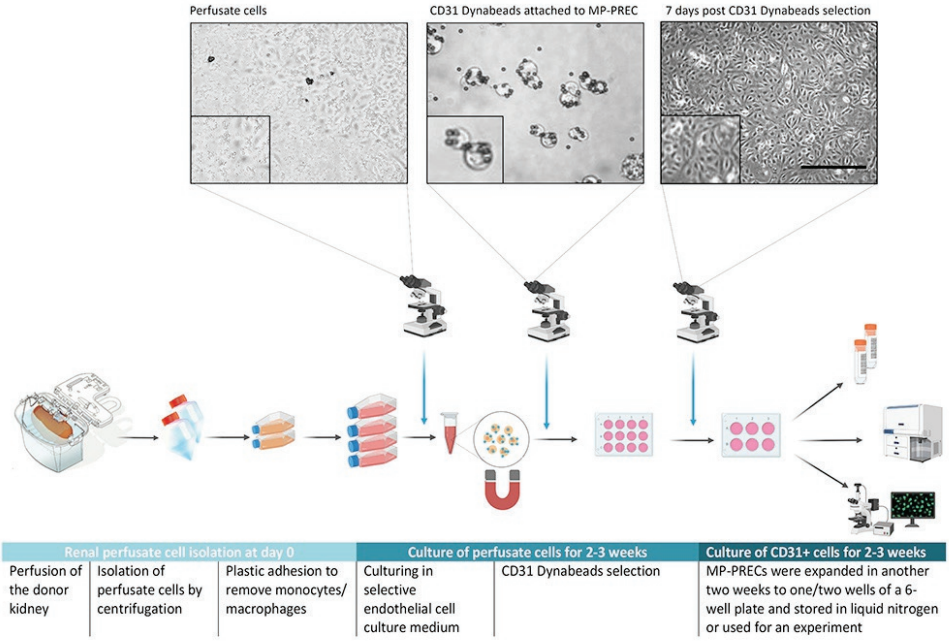

Figure 1. Isolation procedure of Machine Perfusion Derived primary Renal Endothelial Cells (MP- PRECs). Scale bar represents 200 µm. Figure created with Biorender.com.

Table 3. Baseline characteristics of post-mortem organ donors at time of death.

Variable	Value
Age, years	59 ± 17
Male sex (n,%)	56 (54.9)
Height, cm	175 ± 14
Weight, kg	80 ± 20
Body mass index, kg/cm^2	25.50 ± 5
Mean arterial pressure, mmHg	91 ± 21
Donor type DBD (n,%)	31 (30.4)
Hypotension at ICU (n,%)	23 (22.5)
Time hypotension at ICU, min	0 ± 0
Hypertension in medical history (n,%)	28 (27.5)
Cause of death (n,%)	
Circulational	20 (19.6)
Respirational	2 (2.0)
Trauma	14 (13.7)
Cerebrovascular accident	29 (28.5)
Subarachnoidal bleeding	18 (17.6)
Subdural hematoma	2 (2.0)
Suicide	8 (7.8)
Euthanasia	2 (2.0)
Medical complication	1 (1.0)
Meningitis	1 (1.0)
Not specified	5 (4.9)
Smoking at time of death (n,%)	48 (47.1)
Diabetes Mellitus (n,%)	4 (3.9)
Kidney Preservation (Kidney Assist/Life port)	42/60

Normally distributed data are presented as means ± SD, skewed data are presented as medians (interquartile ranges), and categorical data are presented as number (percentage) (n, %). DBD, donor after brain death; ICU, intensive care unit.

Table 4. Frequency of most common HLA alleles in the MP-PREC databank in comparison to the frequency in the Eurotransplant databank (https://www.etrl.org/).

Allele	Percent Eurotransplant n=10000	Percent Biobank n=102
A1	27%	26%
A2	51%	51%
A3	27%	31%
A9	22%	22%
A19	24%	18%
A24	18%	18%
B7	23%	26%
B12	23%	22%
B35	19%	17%
B44	23%	22%
Bw4	61%	48%
Bw6	85%	89%
Cw3	25%	38%
Cw4	23%	23%
Cw6	18%	15%
Cw7	50%	59%
Cw9	25%	18%
Cw10	25%	18%
DR2	30%	27%
DR5	28%	17%
DR6	30%	31%
DR51	30%	27%
DR52	67%	68%
DR53	40%	37%
DQ1	68%	78%
DQ2	35%	35%
DQ3	57%	61%
DQ5	35%	34%
DQ6	42%	43%
DQ7	37%	30%

MP-PREC, machine perfusion-derived primary renal endothelial cell.

Table 5. Frequency of bloodgroup A, B, O and AB in the MP-PREC databank in comparison to the frequency in the Eurotransplant databank (https://www.etrl.org/).

Bloodgroup	Percent Eurotransplant n=10,000	Percent Biobank n=102
A	43%	46%
B	12%	5%
O	40%	45%
AB	5%	4%

MP-PREC, machine perfusion-derived primary renal endothelial cell.

For all isolations, the time between isolation of cells from perfusate and CD31 Dynabeads selection (Passage 0 - Passage 1) was 18.1 ± 3.5 days. The first passage thereafter (Passage 1 - Passage 2) was 8.3 ± 3.4 days after re-seeding, at an average of 60,000 cells per cm^2 (Supplementary Table 1). MP-PRECs were stored in liquid nitrogen or utilized for phenotyping and/or antibody-mediated complement activation experiments between passages 3-7.

May Grünwald-Giemsa and flow cytometry of perfusate cells

Five-pas lymphocyte differentiation and May Grunwald Giemsa staining on cytospins (n=2) of perfusate cells showed a mixture of leukocytes of which ~30% were polynuclear cells (neutrophils) and ~70% were mononuclear cells (Figure 2 and Supplementary Table 2). FACS scatter plots revealed three clearly identifiable cellular groups, presumably representing neutrophils, lymphocytes and monocytes, next to ECs. Within the monocytic cellular group, a minority of the cells were CD31+, CD14- and CD45-. These likely represent the endothelial cells (Supplementary Figure 1).

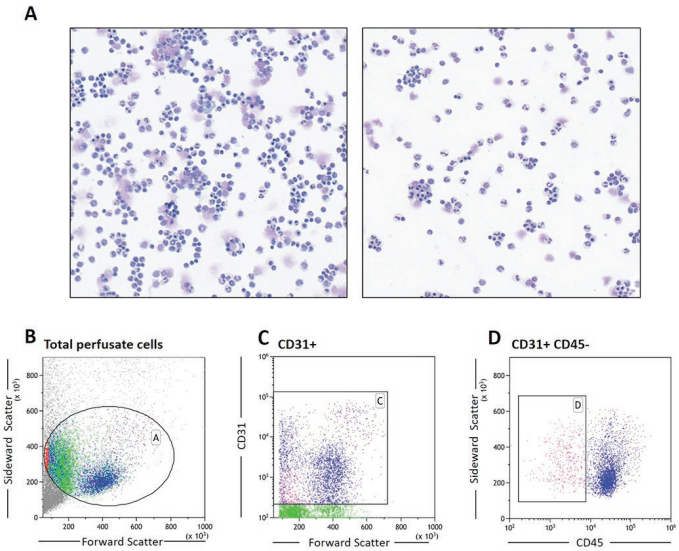

Figure 2. May Grunwald-Giemsa staining and immunofluorescence double staining on cytospins of perfusate cells.

(A) Representative pictures of May Grünwald-Giemsa stainings on cytospins of perfusate cells from two different donors show monocytes, lymphocytes and neutrophils. (B) Flow cytometry showing the total amount of cells in the perfusate fluid after kidney perfusion. 39% of the perfusate cells are CD31⁺ (C) of which 11% is CD31⁺ and CD45- (D).

Since some leukocytes express CD31 antigens, albeit to a much lower extent compared to ECs,[56] they could be contaminating cells in MP-PREC cell cultures. Flow cytometry showed that 39% of the total perfusate cells are $CD31^+$, of which 11% are CD31 positive and CD45 negative ($CD31^+$, CD45-) (Figure 2B-D). This indicates that ± 4% of the cells in the perfusate are in fact ECs.

Effective removal of CD31 CD14 double positive cells from perfusate cells by plastic adherence

Since May Grünwald-Giemsa staining of perfusate cells showed possible MP-PREC culture contamination by $CD31^+$ leukocytes, we performed a negative selection technique to remove contaminating cells from the same hematopoietic lineage as ECs, in particular monocytes. As monocytes adhere to plastic within 30 minutes of incubation while ECs do not, plastic based negative selection was used.[11] Cells from the perfusate, before and after negative selection were evaluated by flow cytometry. 20% of the cells before selection expressed high levels of CD31 and CD14 identifying them as monocytes, and 39% of the cells expressed only CD31). With plastic based negative selection, $CD14^+CD31^+$ adhered to the plastic (Figure 3) and CD14-$CD31^+$ remained non-adherent. Therefore, the selection resulted in the depletion of $CD14^+CD31^+$ from the original cell suspension. A subset of the cells expressed neither CD14 nor CD31. These CD14-CD31-cells are small in size in the forward scatter and show a low granularity in the sideward scatter, and likely represent lymphocytes that will be removed from the cell culture after CD31 Dynabeads selection.

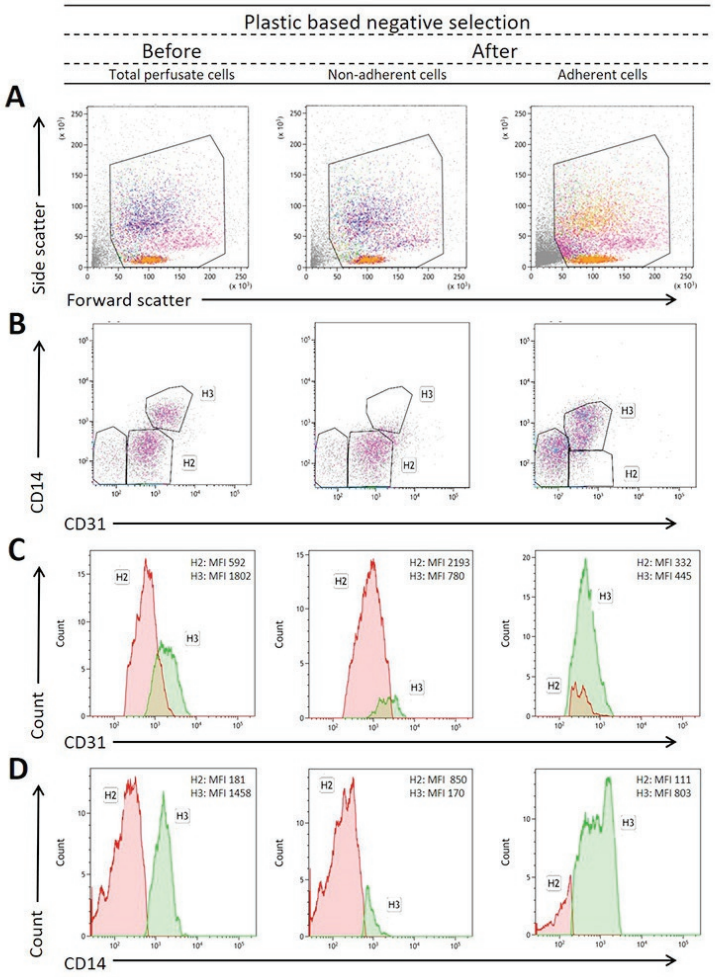

Figure 3. Expression of CD31 and CD14 on perfusate cells, and (non-)adherent cells after plastic based negative selection.

(A) Flow cytometric analysis shows the forward scatter versus the side scatter of cells within total perfusate cells, non-adherent cells and adherent cells, respectively. (B) CD31 versus CD14 scatter plots of viable cells within the total perfusate, non-adherent cells and adherent cells, respectively. Cells in region H2 express CD31 alone, cells in region H3 express both CD31 and CD14. (C) Single positive CD31 cells in region H2 and H3 within the total perfusate, non-adherent and adherent cells, respectively. (D) Single positive CD14 cells in region H2 and H3 within the total perfusate, non-adherent and adherent cells, respectively. Mean fluorescence intensities (MFI) are indicated in the figure.

MP-PRECs regain morphological EC appearances and retain CD31 expression after prolonged time in culture

CD31 is a pan EC marker expressed on the EC membrane, and is suitable for isolation of MP-PRECs using CD31 Dynabeads selection. This procedure did not affect growth of the MP-PRECs, since MP-PRECs could be cultured after CD31 Dynabeads procedure with beads still attached (Figure 4A). After approximately 7 days and 2-3 medium replacement steps, the beads had fully detached from the cell membrane and MP-PRECs showed features of primary culture ECs with the formation of 'cobblestone' monolayers (Figure 4B). At 7 and 21 days after CD31 Dynabeads selection and culture, a distinct peak of MP-PRECs with CD31 cell surface expression was found, comparable to that of HUVEC (Figure 4). 7 days after CD31 Dynabeads selection and culture, the mean CD31 MFI was 98.2 ± 14.9, in comparison to a mean CD31 MFI of 88.3 ± 22.6 at 21 days after CD31 Dynabeads and culture (*P*=0.49) (Figure 4).

Since CD31 is expressed on the cell membrane of all ECs and rarely on any other cell type,[56] overgrowth by contaminating cell types was investigated by flow cytometry analysis of cultured MP-PRECs for CD31 cell surface expression, and for PBMC surface expression markers CD14 and L-selectin. At 28 days after CD31 Dynabeads selection, no CD14 and L-selectin cell surface expression was found (Supplementary Figure 2).

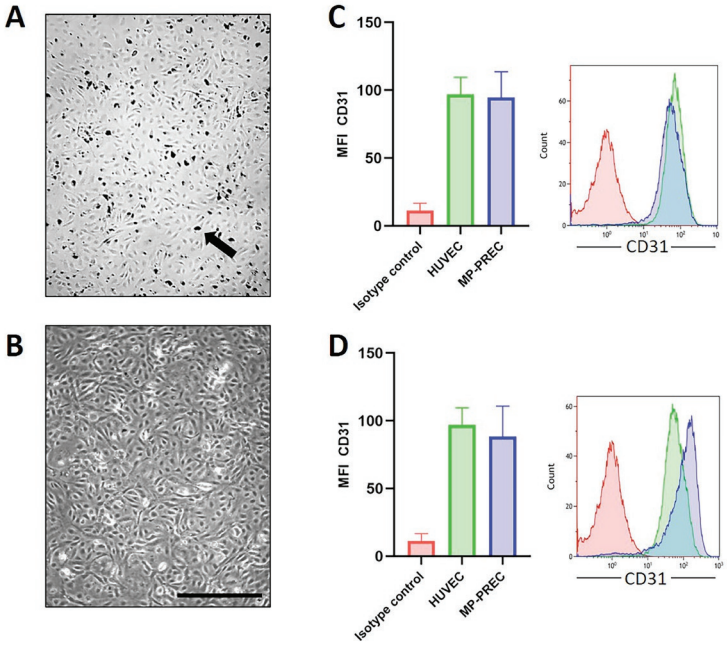

Figure 4. Machine Perfusion – Primary Renal Endothelial Cells (MP-PRECs) maintain endothelial cell appearances. Light microscopy of cultured MP-PRECs at 3 (A) and 7 (B) days after CD31 Dynabeads isolation. Black arrow points to a cluster of beads, scale bar represents 200 µm. CD31 expression on HUVEC and MP- PREC was determined by flow cytometry at 7 (n=4) (C) and 21 (n=4) (D) days after culturing.P values are indicated in the Figure.. Histograms of a representative experiment show that MP-PREC (blue) express CD31 comparable to Human Umbilical Cord Endothelial Cells (HUVEC) (green). The isotype control is indicated in red.

EC markers expressed by MP-PRECs show variation between donors

Since flow cytometry showed that MP-PRECs express CD31 on the cell membrane, different EC markers were selected as glomerular, peritubular, microvascular and macrovascular EC markers for further endothelial phenotyping. CD31, CD34 and Tie2/TEK were selected as general endothelial restricted markers,[6,51] von Willebrand factor (vWF) was selected as a macrovascular marker,[40,50] vascular endothelial growth factor receptor 2 (VEGFR-2) as a glomerular and peritubular microvascular marker,[43] PV-1 as a purely peritubular microvascular marker,[39] and podoplanin as a lymphatic endothelial marker.[49]

Using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR), expression of EC markers CD31, CD34, vWF, PV-1, VEGFR-2, and HLA-DR were assessed on two different MP-PREC isolates: directly after CD31 Dynabeads selection (Day 0) and after 7 days of culturing. CD31 mRNA expression levels remained stable throughout culture in both isolates, vWF increased over time, whereas CD34, PV-1, and VEGFR-2 remained relatively low or were lost over time. HLA-DR remained either present or not expressed (Figure 5A). No AGTR1 mRNA expression was found (Data not shown). Using RT-qPCR, four MP-PREC isolates were tested for contamination with mesangial cells, (myo-) fibroblasts and podocytes. All four cell cultures were negative for nephrin at day 0, day 1 and day 7 after CD31 Dynabeads selection, excluding contamination with podocytes. Three out of four cell cultures were negative for ACTA2 and PDGFB mRNA expression 7 days after CD31 Dynabeads isolation, excluding contamination with mesangial cells and/or (myo-) fibroblasts in the MP-PREC cell culture. However, in one cell culture, transcripts for both ACTA and PDGFB could be found (Supplementary Figure 3).

Immunofluorescence staining showed a heterogeneous expression of vWF, VEGFR-2, and PV-1 on MP-PRECs from different donors after CD31 Dynabeads selection and culture for at least 21 days. Variation was observed in the number of positive cells and the intensity of staining. Discrete intracellular structures resembling Weibel-Palade bodies were found, albeit vWF was expressed to various extents between donors. MP-PRECs from some donors expressed VEGFR-2, whereas in cells from another donor VEGFR-2 expression was sparse. Some MP-PRECs isolates also expressed PV-1 and HLA-II, albeit to varying extents (Figure 5B). None of the MP-PREC isolates expressed the lymphatic EC marker podoplanin (*n*=4) (Supplementary Figure 4).
Double staining for vWF and VEGFR-2 also revealed variability between MP-PREC cell cultures (n=3) (Supplementary Figure 5).

Flow cytometry using MP-PRECs from 3 different donors at 7, 9, and 21 days after CD31 Dynabeads selection, showed that CD31 is continuously expressed, along with varying (donor-dependent) expression of Tie-2/TEK, VEGFR-2, and vWF. HUVEC is shown as control (Figure 5C). Variation in expression of EC markers was a consistent observation in all donors examined.

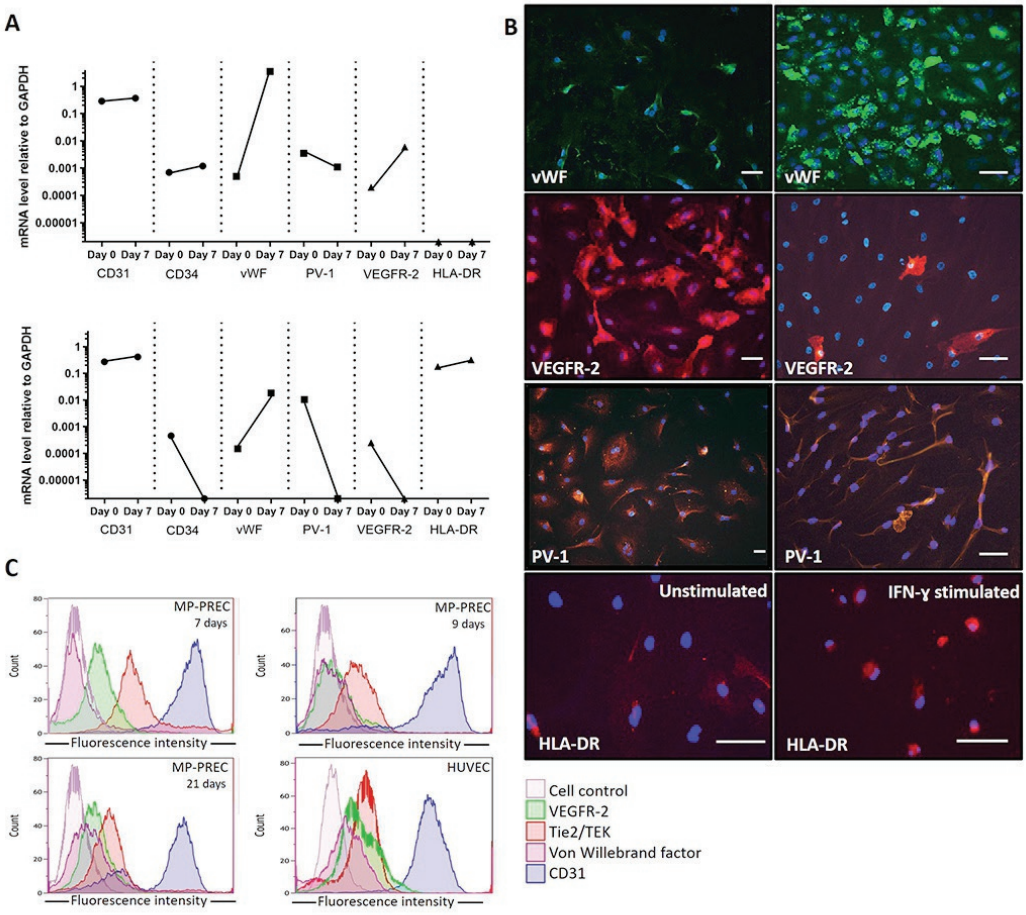

Figure 5. Endothelial cell markers expressed by Machine Perfusion – Primary Renal Endothelial Cells (MP-PRECs) show variation among donors.

(A) Quantitative rt-PCR shows relative mRNA levels of endothelial cell markers; CD31, CD34, von Willebrand factor (vWF), PV-1, VEGFR-2 and of HLA-DR, on MP-PRECs from two different donors directly after CD31 Dynabeads selection (Day 0), and after 7 days of culturing (Day 7).

(B) Immunofluorescence stainings on MP-PRECs from different donors cultured for ~21 days after CD31 beads selection show a heterogeneous expression of vWF, VEGFR-2, PV-1, and HLA-DR on unstimulated and interferon-gamma (IFN-γ) stimulated MP-PRECs. Scale bar represents 100 µm.

(C) Flow cytometry showing expression of VEGFR-2, Tie2/TEK, vWF and CD31 on MP-PRECs from different donors at 7 days after CD31 Dynabeads selection, 9 days after CD31 Dynabeads selection and 21 days after CD31 Dynabeads selection in comparison to Human umbilical vein endothelial cells (HUVEC).

HLA loci on MP-PRECs

Since we developed the MP-PREC isolation technique for the purpose of detection of antibodies directed against antigens expressed by ECs, HLA antigen expression of MP-PRECs was analyzed under interferon-gamma (IFN-γ) stimulated conditions. Expression of pan HLA-I and the HLA-II-DR locus by MP-PRECs increased by 50% and 30% respectively when stimulated with IFN-γ, as assessed by flow cytometry (n=2) (Figure 6A).

Additional immunofluorescence staining on MP-PRECs of different donors (n=4) after 21 days in culture revealed variation in induced expression of HLA-DR between unstimulated cells and their corresponding IFN-γ stimulated cells (Figure 6B and C).

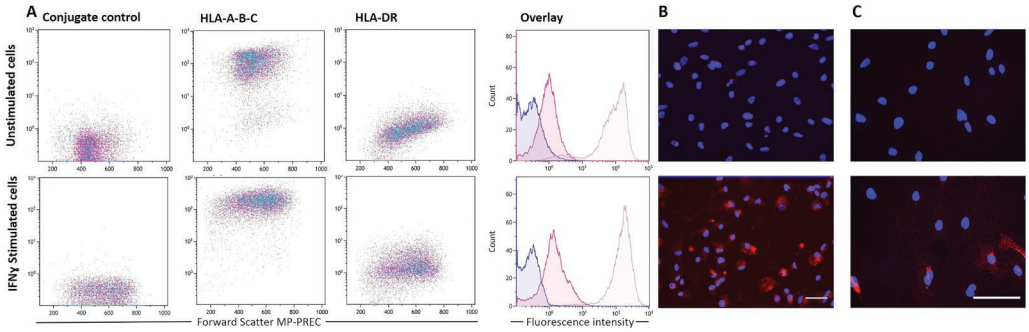

Figure 6. HLA class I and II expression by Machine Perfusion – Primary Renal Endothelial Cells (MP- PRECs).
(A) Representative flow cytometry data showing the expression of HLA class-I and HLA class-II (DR locus) on unstimulated MP-PRECs and IFNγ-stimulated MP-PRECs. Corresponding FACS histograms of unstimulated and stimulated cells are shown in the overlay; HLA class-I (pink), HLA class-II DR (magenta) and the negative control (blue). (B and C) Representative HLA-DR staining on unstimulated MP-PRECs and stimulated MP-PRECs, from two different donors. Scale bar represents 100 µm.

Proof of principle: Complement activation on MP-PRECs by donor specific antibodies

In order to examine the clinical relevance of MP-PRECs, complement activation, measured as activated C3 deposition on the MP-PREC cell membrane, was tested with a custom crossmatch assay using MP-PRECs from 3 different donors. Cross matching with blood group compatible serum (cABO) consistently showed no complement activation (Figure 7A, F and K). Cross-matching with blood group incompatible serum (iABO) (Figure 7B, G and L), monoclonal HLA antibodies (Figure C, H and M) and sera containing HLA class I and/or II antibodies directed against HLA antigens expressed on the MP-PREC cell membrane (Figure 7D and I), resulted in complement C3 activation to varying extents. Serum containing HLA antibodies aspecific for HLA antigens expressed on the MP-PREC cell membrane, did not result in complement activation (Figure 7N).

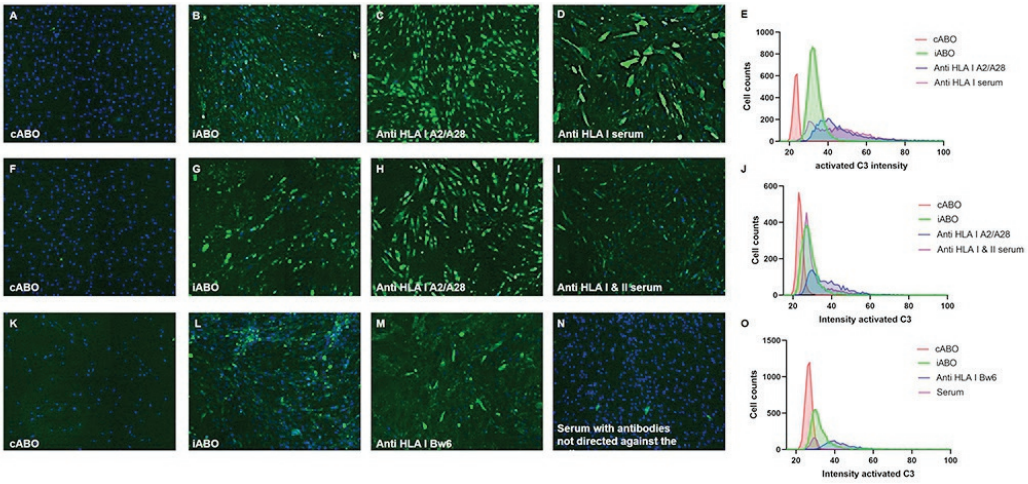

Figure 7. Antibody mediated complement activation as shown by activated C3 staining on Machine Perfusion – Primary Renal Endothelial Cells (MP-PRECs) from 3 different donors.

(A-E) MP-PRECs with blood group A positive and HLA typing A2 A19 A32 B7 B35 Bw6 Cw4 Cw7 DR2 DR15 DR6 DR13 DR51 DR52 DQ1 DQ1 DQ6 DQ6 incubated with blood group compatible AB serum (cABO), blood group incompatible O serum (iABO), a monoclonal human A2/A28 antibody and serum with HLA I & II antibodies (A2, B17, B27, DR52, DR53, DR14, DR15, DR16, DQ5, DQ6, DR12) directed against de HLA typing of the cell.

(F-J) MP-PRECs with blood group A positive and HLA typing A2 A9 A23 B15 B62 B21 B49 Bw4 Bw6 Cw3 Cw7 DR4 DR5 DR11 DR52 DR53 DQ3 DQ3 DQ7 DQ8 DQ6 incubated with cABO, iABO, a monoclonal human A2/A28 antibody, and serum with HLA I & II antibodies (A2, B17, B27, DR52, DR53, DR14, DR15, DR16, DQ5, DQ6, DR12) directed against de HLA typing of the cell.

(K-O) MP-PRECs with blood group A positive and HLA typing A9 A24 A28 A68 B7 B12 B44 Bw4 Bw6 Cw7 DR1 DR2 DR15 DR51 DQ1 DQ1 DQ5 DQ6 incubated with cABO, iABO, a monoclonal human Bw6 antibody, and serum with HLA class II antibodies (DRB4, DR53) not directed against de HLA II typing of the cell. 10x magnification.

(E; J and O) Histograms showing the quantification of the activated C3 staining. Results are expressed as mean fluorescence intensity of the FITC staining in the GFP channel.

Discussion

The lack of availability of primary renal endothelial cells (ECs) from multiple donors remains a major obstacle when studying (patho)physiology of renal ECs and their interindividual variation in reaction to disease processes. Difficulties in obtaining primary cultures of renal EC subsets hampered molecular characterization, transcriptional profiling, and assay development with ECs. We have developed an EC isolation technique, that uses ECs derived from machine perfusates after kidney machine perfusion. We performed isolations after deceased kidney donation, resulting in a bank of primary ECs from multiple donors, featuring general EC characteristics and covering a large spectrum of HLA alleles. These can be used for screening of donor specific antibodies, but also for other applications.

Although ECs in the human body display many common features, they also reveal remarkable morphological and functional heterogeneity.[1,2] The vasculature of the kidney is not only different from that of other organs, it also displays striking intra-organ heterogeneity. Limited accessibility of human kidneys suitable for EC isolations implicates that the majority of research focused on endothelial function in renal diseases, is based on endothelium from diverse origins but generally not the kidney.[7,8,16,24,33,36,41] The presented method in this study has many advantages over existing supply routes and techniques for endothelial cell isolation. First, by taking advantage of the relatively new procedure of organ perfusion between donation and transplantation,[31] a continuous supply of donors is guaranteed. Secondly, the HLA type, blood group, and other characteristics of these cells are already known. Third, the technique is a relatively easy, non-destructive, and robust method to isolate pure populations of ECs from human donor kidneys. Fourth, apart from the CD31 Dynabeads and culture medium, no special reagents or equipment are needed. This, together with the fact that the perfusate is considered as waste material from the transplantation procedure and no additional procedures are required to harvest human kidneys for EC isolation, contributes to the cost effectiveness of this new method. Another major advantage of isolating MP-PRECs is that the cells have an adult tissue origin, as opposed to for example HUVECs, from a fetal origin. Furthermore, the approach outlined may provide a general strategy for isolation and culture of ECs from a variety of organs (provided that the organs are perfused). Finally the method can be used to develop a rich source of renal-specific endothelial cell lines for dedicated purposes.

We also acknowledge several disadvantages of the new method. The isolation technique outlined herein is quite time consuming, and as with all cell culture studies researchers need to be trained. The possible use of FACS was initially investigated for sorting MP-PRECs. Unfortunately, MP- PRECs are vulnerable and relatively large cells, resulting in fragmentation of the cells during the sorting procedure (Data not shown). Also, prolonged culture periods and limited yielded cells are important disadvantages of this study. Nevertheless, implementing this isolation method is worth the investment as it serves the purpose of isolating ECs from a widely available source of donors, along with the possibilities to develop new applications with MP-PRECs. In addition, as discussed later in more detail, the isolated MP-PRECs are heterogeneous in terms of their expression of endothelial cell markers, as shown in Figure 5, and complement activation, as shown in Figure 7. This could lessen their utility in studying kidney rejection or other disease processes involving the renal endothelium because every endothelial cell isolate would require characterization. Primary ECs are in general a superior model for the *in vivo* situation since the cell is not manipulated and not affected by multiple passages, when keeping passages low.[26] In addition, heterogeneity in the response to antibody mediated complement activation, may actually reflect true differences in defence mechanisms to complement mediated attack between individual donors, and thus explain some of the variation of outcome in the presence of DSA. However, the usefulness of primary renal ECs culture could be limited by shifts in their *in vivo* gene expression signature due to the loss of microenvironmental cues,[9] and by early onset of senescence.[42] Therefore, the primary MP-PRECs should be selected based on the gene expression profile in the context of the application of interest and converted into different immortalized cell lines that are then validated thoroughly. The validation process should focus on the expression of endothelial specific markers

during proliferation in cell culture, and multiple donor characteristics like antigen expression by repeated gene analyses and protein staining procedures. Before studying a specific research question, one should evaluate the cellular features and gene expression profile based on the application of interest. Thereafter, following common practice in EC biology, EC lines should be continuously monitored to ascertain their cellular features. Our study is presented as a qualitative methodological paper, as we currently do not have the substantial number of biological replicates of the highly heterogeneous MP-PRECs population that would be necessary for a meaningful analysis.[48] Therefore, we did not present statistical data on differences in EC expression markers between MP-PREC donors and changes over time. Finally, the risk for contamination with other cells should always be taken into account when working with primary cell cultures. We found some contamination by mesangial and/or renal (myo-)fibroblasts in one out of four MP-PREC isolates (Supplementary Figure 3).

The MP-PRECs purification strategy was based on the removal of $CD31^+$ $CD14^+$ monocytes by plastic adherence, positive selection of $CD31^+$ cells using CD31 Dynabeads, and the use of EC specific cell culture medium.[11] MP-PRECs obtained cobblestone morphological EC appearances when viewed with phase contrast microscopy, and there was no evidence of overgrowth by contaminating cells.[18] MP-PRECs from the majority of donors proliferated in cell culture and therefore appear to be a relatively healthy cell population.[35] This raises the following question: why do ECs detach from renal vasculature during kidney perfusion? It is generally known that ischemia/reperfusion injury after kidney donation substantially contributes to a cascade of harmful events leading to inflammation, endothelial dysfunction and cell death.[30,52] *In vivo*, vascular ECs are covered with an endothelial glycocalyx; a gel-like layer consisting of glycoproteins, proteoglycans with bound glycosaminoglycans coating the luminal surface of ECs.[46] This layer is vital for EC function as it participates in vascular permeability, microvascular reactivity, and endothelium interaction with blood constituents. Degradation of the glycocalyx plays an important role in ischemia/reperfusion related endothelial dysfunction; however studies on the effect of kidney machine perfusion after the period of ischemia on the renal endothelial glycocalyx are limited.[45] Preliminary data show that MP-PRECs produce a glycocalyx, as shown with Ulex Europeus-1 positive staining of the MP-PRECs cultured for ~7 days after CD31 bead selection (Data not shown). Further research is needed to assess why the cells detach, and whether this is associated with quality and function of MP-PRECs and kidney allograft outcome.

Phenotypic profiling of MP-PRECs from different donors revealed a remarkable variability among isolates, and within MP-PRECs originating from one donor, although CD31 remained consistently expressed. Variability was apparent for VEGFR-2, vWF, PV-1, and for Tie2/TEK. We showed that MP-PRECs express HLA-I without exception and that HLA-II-DR was either constitutively expressed or could be induced by IFN-γ. The latter is in line with previous studies, showing that renal microvascular ECs are able to express HLA class-II antigens.[34] Our results suggest drift of EC mRNA expression over time, as exemplified by loss of microvascular markers PV-1 and VEGFR-2 and increase of vWF in the first week after CD31 Dynabeads isolation. Tie2/TEK was reduced over time in different isolates as shown with flow cytometry. The reduction of Tie2/TEK could be an indirect effect caused by longer static MP-PREC culture.[21] Together, these alterations in culture composition might indicate EC dedifferentiation over time, or alternatively

overgrowth of MP-PRECs from a specific segment (vWF$^+$ macrovascular EC) over time.[10,37] In addition, MP-PREC immunofluorescent staining for vWF, VEGFR-2, PV-1 and HLA-DR revealed that the distribution of these EC markers within one culture varied for each donor. This likely reflects cultured MP-PRECs obtained from different renal microvasculature structures, having an arterial, glomerular, peritubular or venous origin.[6,39,43,50]

One of the aims of this study was to provide a reliable source of renal ECs to investigate the pathogenicity of HLA antibodies on the surface of the primary target of ABMR.[13] We therefore focused on developing an EC bank from a relatively easily accessible source, by setting up a straightforward isolation technique. Immunological risk stratification still is a major challenge in organ transplantation.[38] Complement fixing HLA antibodies as detected by the complement-dependent-cytotoxicity (CDC) test using donor lymphocytes as target cells will almost certainly lead to hyperacute rejection.[38] However, HLA antibodies only detectable by the more sensitive bead-based Luminex assay are not an absolute barrier to transplantation but are associated with a variably increased risk of chronic antibody mediated damage. To date attempts to accurately predict the individual risk of poor outcomes in patients with DSA detected by the more sensitive bead assays have not been successful.[20] DSA subclass analysis and the assays for complement-binding antibodies have not consistently improved the predictive capacity of current solid phase assays.[28] In addition, non-HLA antibodies are thought to contribute to graft loss in the absence of HLA antibodies.[12,13] However, the nature of these antibodies is still largely unknown, and no widely accepted assays exist to date. Non-HLA antibodies cannot be detected by the current lymphocyte-based CDC. Therefore, the MP-PRECs form an attractive basis for the development of assays to assess the pathogenicity of HLA antibodies, and detect non-HLA antibodies against renal endothelium. Numerous non-HLA antibodies have been reported in the past decade, of which the angiotensin II type-1 receptor (AGTR1) is one of the most established target antigens.[13] Therefore, we have investigated the gene expression of AGTR1 in MP-PRECs from 4 different donors, and established absence of expression in MP-PRECs (Data not shown). This, together with the fact that Leisman *et al.* recently showed that AGTR1 expression is mostly located at the site of mesangial and vascular smooth muscle cells, suggests that the endothelium does not express AGTR1 in the kidney.[23]

As a proof of principle, we have shown in a crossmatch assay using human monoclonal antibodies directed against the HLA typing of the MP-PRECs, but also with patient serum containing donor specific antibodies and ABO incompatible serum, that complement is activated on the cell membrane of MP-PRECs. Complement was activated to varying extents on different MP-PRECs, strengthening the current conception that characteristics of the target cell likely contribute to pathogenicity of donor specific antibodies.[15,25]

We speculate that MP-PRECs could prove invaluable for studying immunological processes in renal allograft rejections and could lead to the development of new and highly required diagnostic tools.[13,47] In addition, after defining strict use,[9] we suggest to use the MP-PREC biobank as a rich source for creating immortalized renal EC lines, which differ in phenotype (e.g. microvascular versus macrovascular; with or without the CD74 GB3 receptor, crucially involved in HUS; blood groups and HLA alleles) and can be manipulated by, e.g., CRISPR/Cas technology.

In conclusion, we developed a technique to isolate and culture renal ECs from machine perfused donor kidneys. The expression of EC markers varies between donors, which could be exploited to generate renal endothelial cell lines. These cells are a unique resource for studying complement (in)dependent cytotoxicity upon binding of donor specific antibodies and for disease monitoring within the general population of patients with renal vasculopathic diseases.

Acknowledgments
We thank H.M. van der Lugt for her support for the preparation of this manuscript, T. Bijma for the assistance in preparing Figure 7, and the endothelial team for their time and assistance in MP-PREC culturing. Part of the work has been performed at the UMCG Imaging and Microscopy Center (UMIC), which is sponsored by NWO grants 40-00506-98-9021.

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Appendix 1

Isolation of Kidney Assist (KA) and LifePort transplant perfusion machine perfusate cells

Perfusate cell isolation was performed using sterile techniques in an enclosed flow cabinet which was cleaned using 70% ethanol.

The KA or Lifeport perfusion machine was collected from storage within 24 hours after kidney perfusion (cold room 4°C) and placed in the enclosed flow cabinet. At the time of cell isolation, the outer part of the KA or Lifeport was no longer sterile and therefore precautions were taken so as not to contaminate the perfusate cells contained inside. Using a 25ml pipette, perfusate fluid was resuspended inside the KA or Lifeport to ensure all cells were removed from surfaces inside the machine. The resuspended fluid was then transferred into two sterile 500mL centrifuge tubes (Corning® SIGMA, Cat#CLS431123). Next, the perfusion machine filter of the Lifeport was unfastened, perfusate fluid retrogradely flushed out of the convoluted filter tubing into the main compartment of the perfusion machine and resuspended. This fluid was then also transferred into the 500mL centrifuge tubes. Once completely emptied, the KA or Lifeport machine was removed and the flow cabinet re-sterilized using 70% ethanol.

Next, the 500mL tubes were centrifuged (acceleration 7, deceleration 7, 4°C, 1500 RPM (300 G)) for 15 minutes. Supernatant was then removed and disposed of leaving a small amount of fluid in the tube. 1/3 of the cells were set aside for long-term storage. With the remaining cells, negative based selection was performed by distributing the cells into uncoated T75 flasks, ensuring that all cells were transferred by rinsing the 500mL flasks with culture medium (M200 culture medium (GIBCO, ref. M200500) supplemented with low growth serum (supplement kit GIBCO, ref. S-003-K)) and adding to the T75 flasks. Additional culture medium was added if necessary to ensure a total volume of 10mL in the flasks. These flasks were then incubated at 37°C for 30-40 minutes. After the incubation period, cells were resuspended within the flasks. Non-adherent cells (containing the endothelial cells among other cell types) were cultured as described in Appendix #2. The 1/3 of cells set aside for storage were centrifuged (1000RPM (200G), 8 minutes, brake 8), supernatant removed leaving cell pellet, freeze medium (M200 cell culture medium with added 10% fetal bovine serum (FBS) and 10% DMSO (Merck, ref. 109678)) added, resuspended with cells, and transferred into labelled ampule for freezing. Ampule containing cells and freeze medium were then placed in Mr. Frosty at -80oC. The following day the ampule was transferred to liquid nitrogen storage.

Appendix 2

All cell cultivation and isolation techniques were performed using sterile techniques in an enclosed flow cabinet which was cleaned using 70% ethanol.

Cell culture of isolated endothelial cells

Kidney transplant machine perfusate cells were isolated from kidney perfusion machines, monocytes/macrophages were removed by plastic based negative selection (See appendix 1) and cultivated as follows. Cells were seeded in two 1% sterile gelatin coated T75 culture flasks in 12mL cell culture medium composed of M200 culture medium (GIBCO, ref. M200500) supplemented with low growth serum (supplement kit GIBCO, ref. S-003-K). The cell culture medium was exchanged every 2-3 days.

Isolation of endothelial cells using CD31 immunomagnetic beads selection

CD31 Immunomagnetic bead selection (Dynabeads® CD31, Invitrogen, ref.11155D, Landsmeer, The Netherlands) of endothelial cells was performed once cultured perfusate cells had reached confluent or almost confluent density.

At forehand, CD31 immunomagnetic beads (400.000 beads/µL) and bead washing buffer, comprising of Dulbecco's Phosphate Buffered Saline (DPBS) (w/o Ca and Mg (LONZA, ref. BE17-512Q)) and 0.1% sterile Bovine Serum Albumine (BSA) solution (preparation: 10% BSA, Sigma, ref. A9647-100G, diluted in DPBS using a 0.2µM filter), were put on ice. Subsequently, Trypsin-EDTA solution (TE) (GIBCO, ref. R-001-100), Trypsin neutralizer solution (TN) (GIBCO, ref. R-002-100) and M200 culture medium (GIBCO, ref. M200500) supplemented with low growth serum (supplement kit GIBCO, ref. S- 003-K) were allowed to heat up to room temperature, and then placed in a water bath at 37°C.

To begin isolation of cells, 2 µl of CD31 immunomagnetic beads were placed in a 1.5mL sterile Eppendorf tube and washed (repeatedly re-suspended) in 1 mL of bead washing buffer. The Eppendorf tube containing the CD31beads-bead wash mix was then place in a magnet for 2 minutes. After 2 minutes, the supernatant was carefully removed, leaving the CD31 beads in the tube. The CD31 beads were then resuspended in 80 µl bead washing buffer to create the bead stock (10.000 beads/ µl). This was then stored on ice.

Next, 1 µl of CD31 bead stock was then added to the T75 flask containing perfusate cells which had previously been in culture, whilst keeping the cells in the medium. If the perfusate cells contained red blood cells, these were removed prior to adding the CD31 beads. The T75 flask containing perfusate cells and CD31 beads were then placed on a slow shaker for 10 minutes. After 10 minutes, cells were examined under a microscope to check if CD31 beads had attached (3-4 beads per cell is optimal). If there was insufficient CD31-bead attachment, the T75 flask was placed back on the slow shaker and the process of checking then mixing was repeated until the CD31 beads had attached.

Once attachment was sufficient, cells were collected as follows. Culture medium was removed and cells washed twice: once with bead washing buffer and subsequently with Phosphate Buffer Saline (PBS), both of which were at room temperature. Trypsin (2mL) was then added to detach the cells from the flask. The flask was gently agitated and 1.5mL of trypsin

instantly removed. This process was done at room temperature. Cells were then checked every 2 minutes under the microscope to assess detachment. Once cells were detached, 2mL of trypsin neutralizer was added and resuspended. The end volume of cells and solution were then transferred to a 4,5 mL facs tube.

To remove the cells with CD31-beads attached from the trypsin-trypsin neutralizer mixture, the 4,5mL facs tube was first placed in a magnet. CD31-bead cells move to the side of the tube by the magnet. After 2 minutes, whilst in the magnet, the CD31-bead negative cells and supernatant were removed carefully using a pipette, making sure not to disturb the CD31-bead positive cells. CD31- bead negative cells and supernatant were placed in a 10mL tube, centrifuged (1000RPM (200G), 8 minutes) into a pellet, supernatant removed and resuspended in culture medium. The procedure of CD31-bead attachment was then repeated on these cells, in order to ensure all CD31-positive cells had the opportunity to become attached. CD31-bead negative cells at this stage were re-seeded in a 1% sterile gelatin coated T75 flask and cultured at 37°C.

Thereafter, the 4,5mL tube with CD31-bead positive cells was removed from the magnet, 2,5 mL of cold bead washing buffer was pipetted down the back side of the tube (cell side), resuspended, placed back into magnet, and after 2 minutes supernatant was carefully transferred into a 15mL tube. This was classified as washing step 1. This was repeated until 5 washing steps were completed, each time placing supernatant into the initial 15mL tube. The appropriate cell culture vessel was chosen according to the estimated quantity of CD31-positive cells (60.000 cells per cm2), coated with 1% sterile gelatin, and culture medium (according to Table 1) was added to the 4,5mL tube and resuspended. Cells and medium were then transferred to the flask/plate and cultured at 37°C.

Table 1. *Quantity of endothelial cell culture medium needed according to cell culture vessel.*

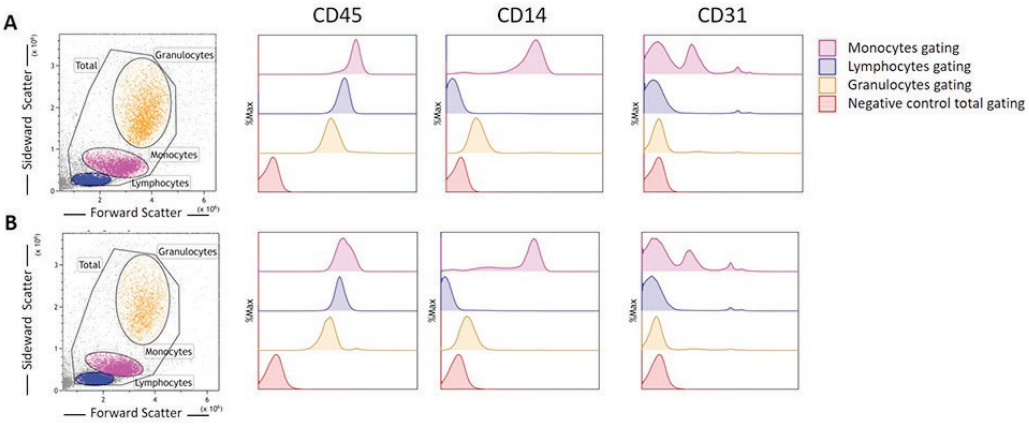
Cell culture vessel	Quantity of culture medium
T25 flask	4,0 mL
6-wells plate	1,5 mL
12-wells plate	0,8 mL
24-wells plate	0,5 mL
48-wells plate	0,3 mL

Finally, the supernatant (containing cells) collected from each wash step in a 15mL tube was centrifuged (1000RPM (200G), 8 minutes) into a pellet, supernatant removed, culture medium (according to Table 1) added, resuspended and plated into a culture flask/plate. These cells were cultured at 37°C.

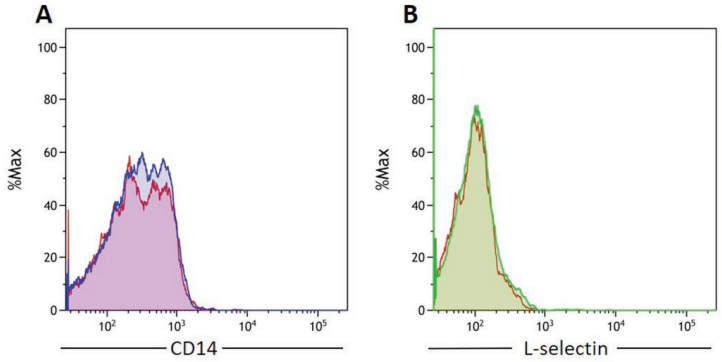
Freezing of confluent cells

Once cells had become confluent, or when cells of interest needed to be stored long-term, they were prepared for freezing as follows. Freeze-medium M200 (M200 cell culture medium with added 10% FBS (general MB) and 10% DMSO (Merck, ref. 109678)) and a Mr. Frosty were pre-cooled. Cells which were to be frozen were first washed twice using PBS. Cells were subsequently dethatched using trypsin. Trypsin was added to the cell containing vesicle, agitate, detachment checked under a microscope, and once detached trypsin neutralizer added (twice the volume of trypsin). Cells and trypsin-trypsin neutralizer supernatant were centrifuged (1000RPM (200G), 8 minutes, brake 8), supernatant removed leaving cell pellet, freeze medium added, resuspended with cells, and transferred into labelled ampule for freezing. Ampule containing cells and freeze medium were then placed in Mr. Frosty at -80°C. The following day the ampule was transferred to liquid nitrogen storage.

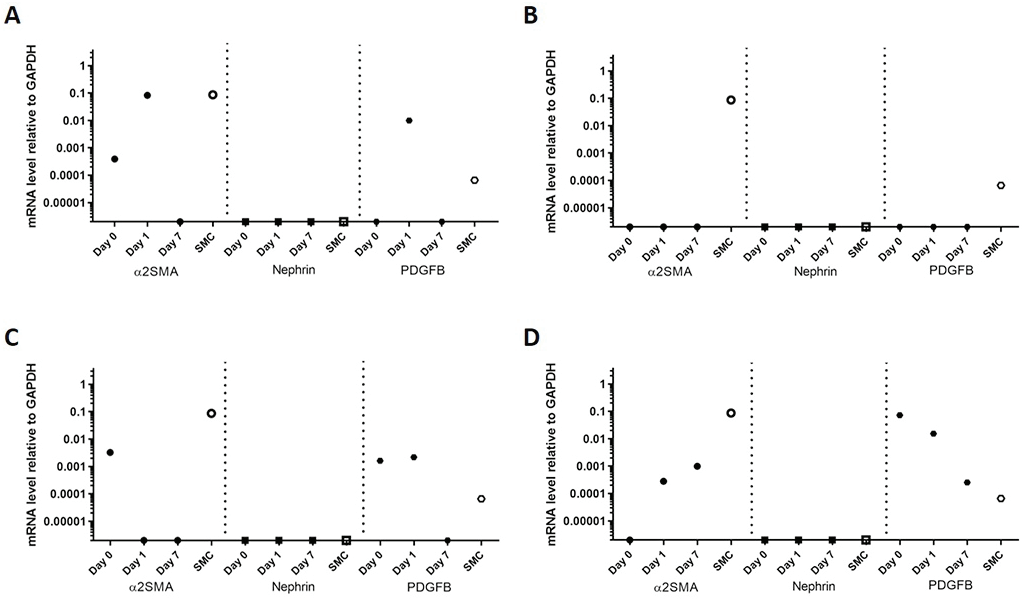
Supplementary figures



Supplementary Figure 1. The presence of CD45+, CD14+ and CD31+ cells in the primary perfusate.
Flow cytometry showing CD45, CD14 and CD31 positive cells in the primary perfusate of 2 different donors (A and B). Cells are gated for the high-density regions (identified as monocytes in purple, lymphocytes in blue, granulocytes in orange). Histograms show the positive cells in the respective regions. The negative control is gated at the total number of cells. https://doi.org/10.6084/m9.figshare.13677019



Supplementary Figure 2. Expression of CD14 and L-selectin on Machine Perfusion-Primary Renal Endothelial Cells (MP-PRECs) cell culture shows no contamination with CD14 and L-selectin positive cells.
CD14 and L-selectin expression was determined by flow cytometry at 28 days after culturing(n=2). Histograms of a representative experiment show that CD14 expression (blue in A) and L-selectin (green in B) are comparable to the isotype control (red in A and B). https://figshare.com/articles/figure/Supplementary_Figure_2/13677067

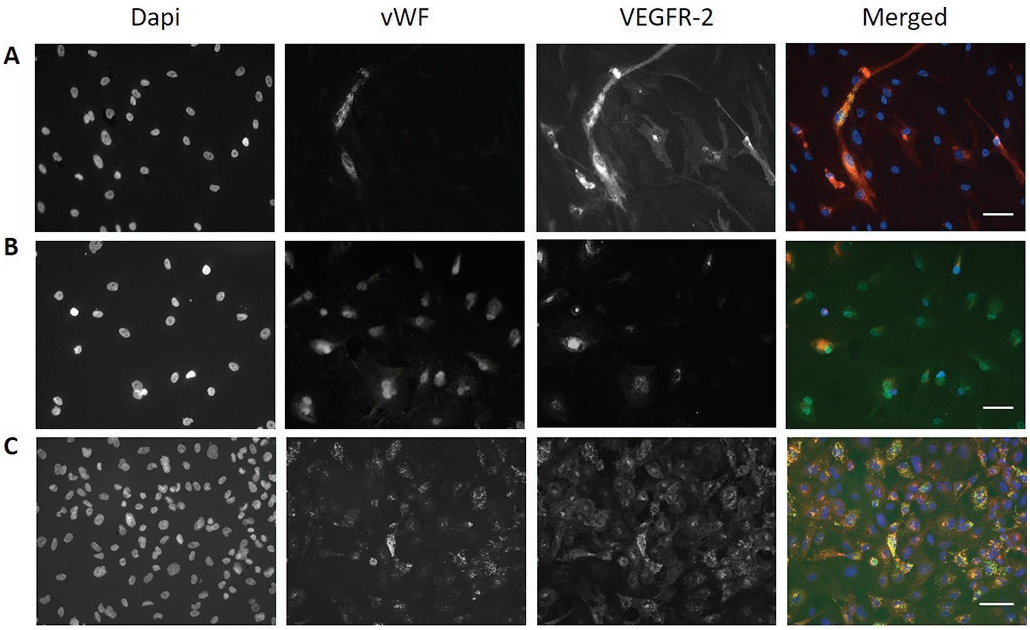


Supplementary Figure 3. mRNA levels of α2SMA, PDGFB and nephrin in the Machine Perfusion- Primary Renal Endothelial Cells (MP-PRECs) cell culture show no contamination with podocytes and occasional contamination with fibroblasts and/or mesangial cells.

Quantitative rt-PCR shows relative mRNA levels of ACTA2 (α2SMA) and PDGFB for (myo-)fibroblasts and/or mesangial cells, and NPHS1 (Nephrin) for podocytes on MP-PRECs from four different donors (A-D), directly after CD31 Dynabeads selection (Day 0), 1 day after CD31 Dynabeads selection, and after 7 days of culturing (Day 7). https://figshare.com/articles/figure/Supplementary_Figure_3/13677220

Supplementary Figure 4. Podoplanin expression by Machine-Perfusion derived Primary Renal Endothelial Cells (MP-PRECs).

A representative staining for podoplanin showing (A) DAPI for nuclear staining; (B) Podoplanin; (C) merge. Scale bar represents 100 µm. https://figshare.com/articles/figure/Supplementary_Figure_4/13677253



Supplementary Figure 5. vWF and VEGFR-2 double staining on MP-PRECs from 3 different donors. *Immunofluorescent double staining of vWF (green) and VEGFR-2 (red) showed variation of staining between MP-PRECs from different donors (A, B and C) cultured for ~21 days after CD31 beads selection. Scale bar represents 100 µm. https://figshare.com/articles/figure/Supplementary_Figure_5/13677298*

Supplementary Table 1. *Percentages of cultures and reached passages.*

Passage	Time (Days)	Percentage of donors (n=102)
P0-P1	18 ± 3.5	75%
P1-P2	10 ± 4.8	75%
P2-P3	8 ± 3.9	43%
P3-P4	8 ± 4.9	32%
P4-P5	12 ± 5.1	21%
P5-P6	11 ± 3.6	14%
P6-P7	9 ± 2.1	7%
P7-P8	9 ± 1.1	4%

Supplementary Table 2. Five-pas leukocyte differentiation. Cellular leukocyte differentiation from 2 different donors.

Cellular differentiation	Count	Percent of total
White blood cells	11.780 [10^6/L]	98%
Mononucleair cells	7.460 [10^6/L]	62%
Polynucleair cells	4.320 [10^6/L]	36%
Red blood cells	0 [10^6/L]	0%
Other	140 [10^6/L]	2%
Total	11.920 [10^6/L]	100%

Cellular differentiation	Count	Percent of total
White blood cells	332 [10^6/L]	98%
Mononucleair cells	253 [10^6/L]	76%
Polynucleair cells	79 [10^6/L]	24%
Red blood cells	0 [10^6/L]	0%
Other	6 [10^6/L]	2%
Total	338 [10^6/L]	100%

CHAPTER 5

Renal endothelial cytotoxicity assay to diagnose and monitor renal transplant recipients for anti-endothelial antibodies

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Frontiers in immunology 2022; DOI: 10.3389/fimmu.2022.845187

Abstract

Tissue specific non-HLA antigens can play crucial roles in allograft immunity and have been shown to trigger humoral responses leading to rejection of HLA-matched kidney allografts. Interest in the role of endothelial specific antigens has grown over the past years, and several case reports have been described in which antibodies reacting with endothelial cells (ECs) are associated with rejection. Such antibodies escape the detection in conventional crossmatch tests, as they do not react with lymphocytes. However, due to the heterogeneity of endothelial cells from different vascular beds, it remains difficult to draw organ specific conclusions from studies describing endothelial cross match assays. We present a case of a 69 year old male patient, whose kidney allograft was rejected hyperacute, in spite of the absence of pre-transplant HLA-specific antibodies. In order to place findings from previous studies in a kidney related context, we performed cross match assays with primary renal endothelial cells. The patient's serum was reactive with primary renal ECs, demonstrated by antibody binding and complement-dependent-cytotoxicity. Antibodies from this patient did not react with lymphocytes, nor were HLA donor-specific-antibodies (DSAs) found. Two years later the patient successfully received a second kidney transplant after treatment with rituximab and plasmapheresis before and after transplantation. We demonstrated that removal of antibodies against non-HLA ECs specific molecules can be monitored using a primary renal EC crossmatch test, possibly contributing to a successful transplantation outcome.

Introduction

The presence of donor-specific HLA antibodies in patients awaiting a renal transplant can either be a contraindication for transplantation or pose an increased risk for antibody mediated rejection (ABMR) and inferior graft survival.[1] However, unexpected ABMR episodes still occur despite thorough pre-transplant screening with the current routinely used techniques. This might be explained by HLA specific memory cells that become activated upon re-exposure to the antigen, or the emergence of de novo antibodies after transplantation. Non-HLA antibodies are also related to graft loss in the absence of HLA antibodies and are generally not detected by routine cross-matching with lymphocytes. In the past years a number of non-HLA antigens were identified in kidney transplantation, including angiotensin type 1 receptor, endothelin type A receptor, collagen-V, K-α1 tubulin, and perlecan.[2,3] Non-HLA anti-endothelial antibody (AECA) concentrations are higher in sera from kidney transplant recipients with acute or chronic rejection compared to stable transplant recipients.[4,5] Screening for non-HLA AECA has not yet been implemented in clinical practice, despite increasing evidence for these antibodies to be involved in rejection. Non-HLA AECA could be detected by endothelial cell (EC) cross matches and the appearance of AECA was associated with an increased risk of allograft rejection.[6–8] However, these studies all have the disadvantage of not utilizing renal EC and therefore underestimating the tremendous heterogeneity of ECs derived from different origins in the human body.[6,8–14] Recently Crespo *et al*. described that a positive endothelial cell crossmatch (ECXM) using aortic endothelial cells did not correlate with the histology of ABMR, neither using pre-transplant serum, nor post-transplant serum. Also, positivity in the EXCM results was found in all investigated patient groups; in patients with normal renal histology, histology of interstitial fibrosis and tubular atrophy and histology of ABMR. This positivity did not associate with any histological signs of endothelial damage e.g., ABMR histology. However, as the authors also clearly stated themselves, the EC cross match was performed with aortic ECs, which may not express the same proteins as renal ECs.[15] Delville *et al*. showed the relevance of a non-HLA AECA cross match test using a renal glomerular endothelial cell line (CiGeNC), confirming specific renal microvascular EC responses and revealing substantial differences in transcriptomic profiles between macrovascular and microvascular ECs.[7]

Cross matching using one cell line does not address the variability of expressed antigens between individuals, which may form the basis for non-HLA antibody formation. In addition, the question how to treat and monitor patients with confirmed anti-non-HLA AECA remains to date unanswered.16 Strategies that are established in the event of HLA antibody-mediated rejection and blood group incompatible transplantation, are based on rapid and effective reduction of antibody titers. We hypothesized that this could also be applicable in the area of non-HLA antibody mediated rejection.[17]

This report presents the case of a patient who developed hyperacute allograft rejection in the absence of HLA-specific antibodies, both before and after rejection occurred. We demonstrate the value of EC based cross matching,[18] and describe the patient's successful re-transplantation, monitored with our EC based cross match assay.

Materials and methods

Human-leukocyte antigen typing and human-leukocyte antibody detection

Human-leukocyte antigen (HLA) typing of a male patient, aged 67, diagnosed with rapidly progressive glomerulonephritis and his donor was performed with sequence-specific oligonucleotide primer (SSOP) technology, analyzed with the IMGT/HLA alleldatabase 3.23. The detailed description of the case is depicted in the section describing the clinical history.

The presence of HLA class I and II Abs in the patients' serum was evaluated using the Life screen the Luxe (LsdL), in accordance with the manufacturer's protocol (Immucor GTI Diagnostics, Inc, Waukesha, USA lot. 3003946-3003920)) and with the Lifecodes Single Antigen Bead (LSA; Immucor Transplant Diagnostics) assay. The routinely lymphocyte based complement dependent cytotoxicity tests were used to test for panel reactive and donor specific antibodies. An overview of HLA antibody detection and endothelial cell crossmatch (ECXM) tests and timepoints is given in Figure 1.

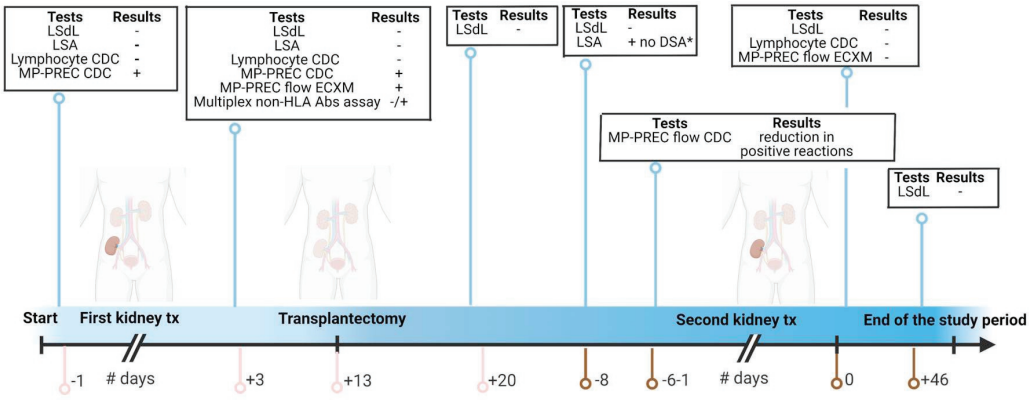

Figure 1. Timepoints of samples obtained and tests performed.

Pink line represents the days related to the first kidney transplantation, brown linerepresents the days related to the second kidney transplantation.

Abbreviations; tx transplantation; LSdL Life Screen Deluxe; LSA luminex single antigen bead assay; CDC complement dependent cytotoxicity; MP-PREC machine perfusion derived primary renal endothelial cells; ECXM endothelial cell crossmatch DSA donor specific antibodies. Figure created with biorender.com.

Staining of CD34, ERG, IgG, IgM, C4d ans C3d in renal biopsies

Pre- and post-implantation biopsies were stained with endothelial specific markers mouse anti-human CD34 (QBEnd/10,Ventana, Mannheim, Germany) and rabbit anti-human ETS-related gene (ERG) (ERP3864, Ventana, Mannheim, Germany) according to the manufacturer's protocol, as part of the standardized pathological evaluation of biopsies. Post implantation biopsies were stained with goat anti-human IgG (760-2680, Ventana, Benchmark automated immunostainer), goat anti-human IgM (760-2682, Ventana, Benchmark automated immunostainer)and rabbit anti-human C4d (clone SP91, Ventana, Benchmark automated immunostainer) also according

to the manufacturer's protocol, as part of the standardized pathological evaluation of biopsies. C3d was staining was performed using an in-house staining. Solutions were prepared using with BSA (A9647, Sigma, St Louis, United States) and PBS (17-512Q, Lonza, Wijchen, The Netherlands) In short, deparafffinization was performed using xylol and alcohol (100%,96%, 70%) and Demi-water in 6 consecutive steps. This was followed by incubation in the dark for 30 min, at 37°C, with 0,4% pepsin from porcine stomach mucosa in 37% 0,1N HCl in demi-water (pH=2.5). Then a blocking step was performed by first adding 0,01% H2O2 (in 1% BSA with PBS) followed by 1% BSA in PBS. Then the polyclonal rabbit antihuman C3d (A0063, Dako, Glostrup, Denmark) at 4°C overnight in a 1:2500 dilution in 1% BSA in PBS was added. This was followed by the first conjugate, HRP polyclonal goat anti-rabbit antibody (Dako, P0448), at room temperature in a 1:100 dilution in 1% BSA in PBS and the second conjugate HRP on polyclonal rabbit anti-goat antibody (Dako, P0449) at room temperature in a 1:100 dilution in 1% BSA in PBS. Then the substrate was added by 0.2 mg/ml 3-Amino9-ethylcarbazole (Sigma 02431MH), in 50mM Acetate buffer and 0.03% H2O2, (pH=5.5) followed by a counterstaining and embedment using hematoxylin 1:2 for 5 seconds and a kaiser's glycerol gelatine embedment (1092420100, Merck, Darmstadt, Germany). An overview of all antibodies used is given in Supplementary table 1.

Endothelial cell-based complement-dependent cytotoxicity test

To provide direct evidence for the presence of potentially pathologic (i.e. cytotoxic) alloantibodies against the MP-PRECs, a cytototic crossmatch was performed combining patient serum and primary renal endothelial cells (ECs). If non-HLA or HLA antibodies are present in patient serum, these antibodies can bind the ECs and form antibody-antigen complexes that activate the complement cascade, leading to complement-mediated cytotoxicity. Terasaki typing trays were prepared as follows: 10 µl patient sera was added to the terasaki wells. As positive control serum containing HLA antibodies directed against HLA-A2 (A2/A28 IgG1 and IgM, provided by Dr. F. Claas, Leiden University Medical Center, The Netherlands) expressed on the endothelial cell was used. As negative control normal human serum (NHS) from healthy volunteers with a compatible blood group and without HLA antibodies was used. Culture machine perfusion derived renal endothelial cells (MP-PRECs) were dissociated using non-enzymatic cell dissociation solution (C5789, Sigma®, Zwijndrecht, The Netherlands). Cells were isolated and characterized as described before and clinical baseline characteristics regarding the donors of the MP-PRECs can be found in our recently published paper describing the isolation and characterization of the MP-PRECs in detail.[18] Experiments were performed with cells cultured up to passage 3-5. The cells were pelleted and resuspended with culture medium (Medium 200 (GIBCO, Cat#M-200-500, Grand Island, NY, USA) containing Low Serum Growth Supplement (LSGS, GIBCO, Cat#S-003-K, Grand Island, NY, USA) for a final concentration of 2000 cells/µl. 1 µl of the cell suspension was added to each terasaki well. Cells were incubated for 30 minutes with sera followed by 60 minutes 5 µl rabbit complement or 60 minutes with sera followed by 120 minutes 5 µl rabbit complement at room temperature. Ethidiumbromide and acridineorange were added to stain for apoptotic and living cells. The reaction was stopped by adding sodium detaat. Results are expressed as percentage of apoptotic cells in relation to the total amount of cells.

A confocal inverted laser microscope (Leica) was used for acquisition of the staining. The fields, systematically digitized throughout the well, were acquired using a computer-based image analysis system. The staining was quantified using built-in specific functions of the software Image J (NIH, Bethesda, MD) and expressed as percentage per well.

Flow cytometry

To explore classical pathway complement activation, MP-PRECs were cultured in a 12-wells-culture plate until they reached confluence and were detached using cell dissociation solution (C5789, Sigma®, Zwijndrecht, The Netherlands), 900 µl/1mL at 37°C, collected in 4.5 mL tubes containing 2 mL cell medium and centrifuged twice at 250 g for 6 min at 20°C. Thereafter, MP-PRECs were incubated with 25% blood group compatible heat inactivated NHS, or 25% blood group compatible heat inactivated serum containing HLA-A2 directed against the antigens on the cell membrane, or 25% of the patient's serum, diluted in M200 medium (M200 culture medium, Ref. No. M200500, Gibco, Bleiswijk, The Netherlands) supplemented with low growth serum (supplement kit, Ref. No. S-003-K, Gibco, Bleiswijk, The Netherlands) for 45 minutes at 37°C. Hereafter, cells were centrifuged twice for 6 min at 250 g at 20°C in culture medium, followed by incubation with 20% NHS as a complement source for 30 minutes at 37°C. After incubation, MP-PRECs were washed twice with ice-cold phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) (FACS buffer) (Sigma®, Zwijndrecht, The Netherlands) at 250 g for 6 min at 4°C. For detection of IgG(G18-145, BD biosciences, San Jose, USA) , IgM(MHM-88, Biolegend, San Diego, CA, USA) and complement activation (activated C3 (HM2168, Hycult, Uden, The Netherlands)), the MP-PRECs were incubated with antibodies depicted in Supplementary table 1, for 30 min on ice. Cells were washed twice with ice cold FACS buffer, centrifuged at 250 g for 6 min at 4°C, and incubated goat anti-mouse FITC (Southern Biotech, Birmingham, USA) for 30 min on ice in the dark. Propidium iodide 1 µg/ml (Molecular Probes, Leiden, The Netherlands) was added just before measuring to be able to exclude apoptotic and necrotic cells. Activated C3 deposition on viable non-apoptotic cells were analyzed in a FACSCaliburTM (FACSCalibur, Becton Dickinson, New Jersey, USA). Results are from at least three independent experiments. The percentage effect was calculated based on the control data (blood group and HLA compatible serum) in mean fluorescence intensity (MFI), % binding is = (MFI test result/MFI control) x 100.

Complement source and handling

Rabbit complement (ThermoFisher scientific (CL3115, Cedarlane, Burlington, Canada) and Normal human serum (NHS) from healthy volunteers having blood group AB were used separately as a complement source. NHS and rabbit complement were stored at -80°, rapidly thawed at 37°C and diluted in M200 medium to a final concentration of 25% for human complement 20% for rabbit complement prior to use. As control NHS was incubated at 56°C for 30 min to inactivate complement proteases.

Multiplex assay

The patients serum from post-operative day 3 was measured using the multiplex assay. The development, technical details and validation of this high-throughput multiplex assay for the detection of non-HLA antibodies is described extensively in the study by Kamburova *et al.*[19]

Statistical analysis

Statistical tests were conducted for complement component measurements. Differences in the percentage effect and percentage apoptosis were analyzed by the unpaired t-test. Computations were performed by SPSS (IBM Statistics Chicago, United States) version 23 and GraphPad software 8.0 (Graphpad Software, San Diego, United States) was used for graphical visualization.

Results

Clinical history

The patient presented in July 2015, aged 67, with rapidly progressive glomerulonephritis, a serum creatinine of 583 µmol/l and an anti-glomerular basement membrane (anti-GBM) antibody titer of >600 IU/mL, without pulmonary involvement. He was diagnosed with anti-GBM glomerulonephritis based on the clinical parameters and a positive anti-GBM titer, but not by kidney biopsy. He was treated with hemodialysis, plasmapheresis, cyclophosphamide and prednisone without improvement of kidney function. During this treatment he received one blood transfusion. The patients' 70 year old brother was considered eligible for kidney donation and in November 2016 the patient received a living related AB0 compatible kidney transplantation. Donor and recipient HLA typing was A2, A68, B62, B44, Bw6, Bw4, Cw1, Cw2, DR4, DR14, DR52, DR53, DQ8,DQ5 and A2, A68, B7, B44, Bw6, Bw4, Cw2, Cw7, DR14, DR15, DR52, DR51, DQ5, DQ6 for donor and recipient respectively, resulting in a 0-1-1 HLA mismatch. The donor and recipient were both tested positive for the Epstein Barr and Cytomegalo viruses. The last anti-GBM titer before transplantation was 14 IU/mL. No panel reactive and donor specific antibodies were detected prior to transplantation in the lymphocyte based CDC test, LSdL andLSA assay.

First post-transplantation clinical course

No relevant pre-existing damage was found in the routine pre-implantation biopsy of the kidney (Figure 2A and B), no surgical complications occurred (cold ischemia time 142 minutes, warm ischemia time 33 minutes) and renal perfusion, as determined by doppler ultrasound, was excellent with immediate diuresis. Serum creatinine values declined from 940 µmol/L to 514 µmol/L after transplantation. Immunosuppressive regimen consisted of mycophenolate mofetil (1000 mg bd) tacrolimus (0.075 mg/kg bd) and prednisone (40 mg iv qd), according to the standard protocol. Within hours after transplantation, the patient developed fever and diuresis dropped from 350 mL/hour to 75 mL/hour.

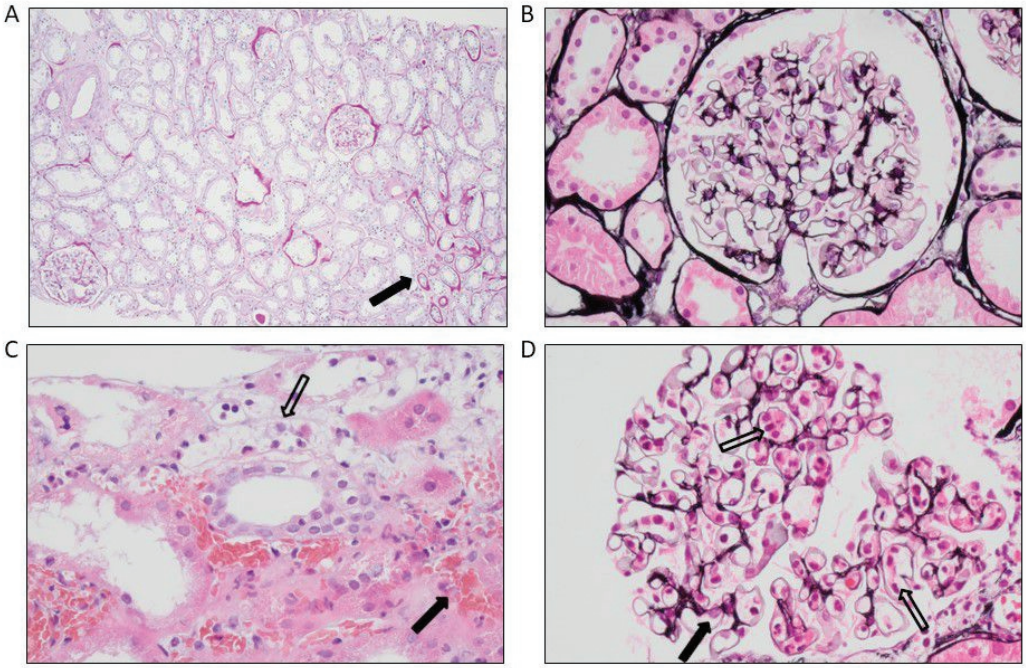

Figure 2. Histology of the pre- and post-implantation biopsies.

A) PAS staining showing no pre-existent damage apart from <5% atrophy (arrow) seen as broadened tubular basement membranes, fibrosis and smaller tubuli in the pre-implantation biopsy.

B) Jones' methenamine Silver staining showing a normal glomerulus in the pre-implantation biopsy.

C) Hematoxyline and eosine staining showing haemorrhagic areas outside the vascular bed (black arrow) and inflammatory cells with destruction of endothelial cells (open arrow) in the post-transplantation biopsy.

D) Jones' methenamine Silver staining showing slightly expanded glomerular capillaries with abundant inflammatory cells (open arrows) and somewhat pronounced endothelial cells (black arrow) in the post-transplantation biopsy.

On post-operative day (POD) 1, nasopharyngeal, urine and blood cultures were performed and prophylactic ceftriaxone was started. Diuresis declined to 25 mL/hour without a response to fluid challenges and cultures returned negative.

On POD 2 diuresis was 430 mL/24 hours accompanied with hematuria. Doppler ultrasound showed diminished perfusion of the kidney, with moderate swelling of the renal parenchyma and no signs of renal vein or artery thrombosis. Thrombocytes declined from $117x10^9$/L to $59x10^9$/L, C-reactive protein values rose to 197 mg/L, serum creatinine values rose to 650 µmol/L and the anti-GBM titer was 10 IU/mL. A Tc-99m MAG3 scintigram revealed poor perfusion of the transplanted kidney, however, during re-exploration surgery no vascular complications were found, though the kidney had a purple discoloration and was swollen and stiff. A biopsy taken during re-exploration revealed granulocytes and lymphocytes in the glomerular capillary loops with occasional small amounts of intraluminal fibrin. Granulocytes were also present in

the peritubular capillaries and associated with detachment and destruction of endothelial cells. There was extensive peritubular hemorrhage. (Figure 2C and D).The tubuli appeared ischemic, with flattening of the epithelial cells. No arterial necrosis was found. Immunoglobulin G (IgG) and immunoglobulin M (IgM) staining by immunofluorescence was barely seen (data not shown) and the C4d staining was negative (Figure 3A).

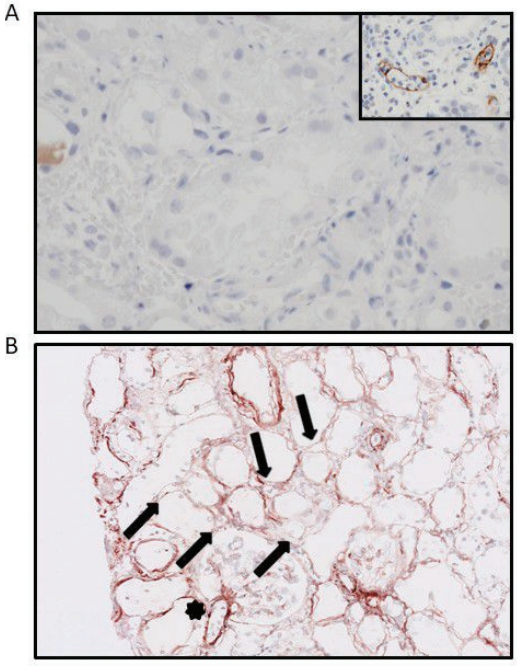

Figure 3. Immunohistochemistry of the hyperacutely rejected allograft.

A) Staining for C4d showing no presence of C4d in the peritubular capillaries. The inset shows a postive control with acute humoral rejection.

B) C3d staining showing variable staining along the peritubular capillaries (arrows) and hilus of the glomerulus (star).

Additional staining for endothelial cell specific markers CD34 and ERG showed less CD34 and ERG positive cells compared to a post transplantation biopsy without rejection (Figure 4A-B). This was most apparent in the peritubular capillaries, indicating pronounced loss of endothelial cells. C3d staining was most pronounced in the peritubular capillaries and the glomerular hilus (Figure 3B). Despite the negative C4d staining, humoral rejection was strongly suspected, hence prednisone, intraveneous immunoglobulin (IVIg) and plasmapheresis was started. On POD 3 no HLA antibodies were found in the LSdL and the LSA assay, nor against A/B/DR loci, nor against C,DQ and DP loci. In addition, serum creatinine rose to 740 umol/L and the patient required dialysis again. During the following days, the patient continued to be treated with plasmapheresis, IVIg and prednisone. Despite this therapy, the kidney function did not improve, and resulted in a transplantectomy on POD 13.

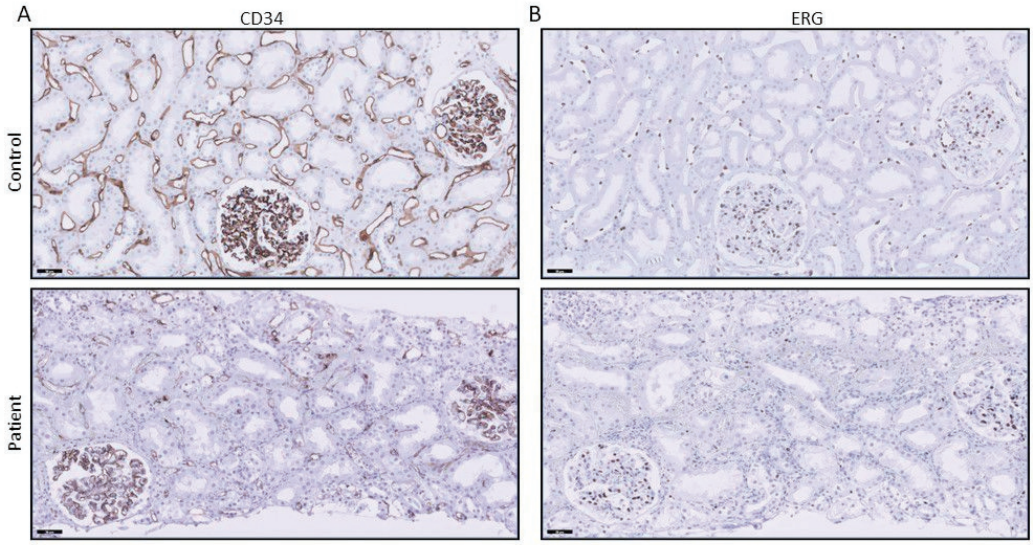

Figure 4. CD34 and ERG stainings show loss of endothelial cells.

A) CD34 staining in the peritubular capillaries of the patient's biopsy is diminished when compared to control tissue (post-transplant biopsy without signs of rejection).

B) ERG staining also shows a loss of endothelial cell nuclei in the peritubular capillaries.

MP-PREC non-HLA tests

Despite the pathological signs of hyperacute ABMR with capillaritis, we could not detect HLA antibodies in patient serum obtained pre-transplant or on POD3. Therefore, non-HLA-mediated rejection was considered. To determine this, we cross matched the patient's serum taken one day prior to transplantation and from POD 3 with human machine perfusion derived primary renal endothelial cells (MP-PRECs), utilizing rabbit complement.

We crossmatched patient serum with MP-PRECs from that were bloodgroup compatible and added rabbit complement as a complement source. Crossmatching with serum taken one day prior to transplantation resulted in clear complement mediated cell death, 72% of all cells appeared to be apoptotic (Figure 5).

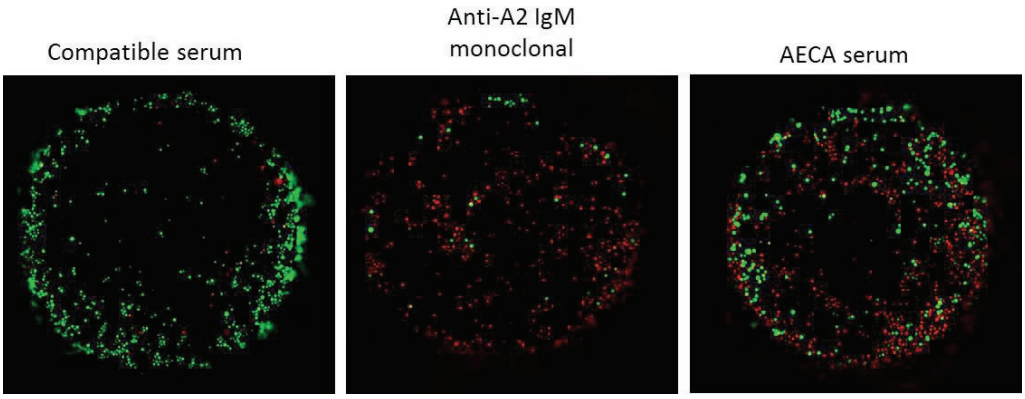

Figure 5. Complement dependent endothelial cytotoxicity using machine perfusion derived primary renal endothelial cells (MP-PRECs) and pre-transplant patient serum.

A representative experiment of a complement dependent endothelial cytotoxicity crossmatch assay (CDC) using MP-PRECs (HLA typing A1 A2 B7 B8 Bw6 Cw7 DR15 DR11 DR51 DR52 DQ6 DQ7 and bloodgroup A, incubated with blood group compatible serum, a monoclonal antibody directed against A2 IgM, and the AECA positive patient serum. Rabbit complement was used as the complement source. Living cells are depicted in green and apoptotic cells in red.

When crossmatching MP-PRECs from 3 different donors with serum taken on POD 3, extensive complement mediated cell death occurred (P=0.002 compared to blood group and HLA compatible serum) (Figure 6). In comparison, control serum from patients with HLA class I directed against HLA antigens expressed on the MP-PRECs caused less complement mediated cell death (P=0.05) when compared to the patient's serum (Figure 6). When compared to human monoclonal IgG and IgM antibodies directed against HLA-A2, a similar extent of complement mediated cell death occurred.

Next, we performed custom endothelial flow cross match tests with MP-PRECs from 3 different donors, using serum from POD 3. IgM, IgG binding (recognizing human immunoglobulin subclasses IgG1, IgG2, IgG3 and IgG4) and complement activation was analyzed using flow cytometry. To better mirror the physiological situation, 20% human serum was utilized as a complement source. Cross matching with the patient's serum resulted in IgG and IgM binding, and activated C3 to varying extents (Figure 6C). Human monoclonal IgG and IgM antibodies directed against HLA-A2 were used as positive cross match test controls, also showing IgG, IgM and complement binding to varying extents. In addition, detection of non-HLA antibodies in the serum from POD 3 was measured using the multiplex assay developed by the PROCARE Consortium,[20] screening for Agrin, APMAP, ARHGDIB, ARHGEF6, AT1R, ETAR, LMNB1, LPLUNC1, PECR PLA2R, PRKCZ, TUBB4B, Vimentin. Only antibodies directed against TUBB4B were found to be present in the patients' serum (absolute mean fluorescence intensity (MFI) 1564.5 and control ratio 2.8). However these antibodies have not been shown to be associated with adverse outcomes after kidney transplantation.

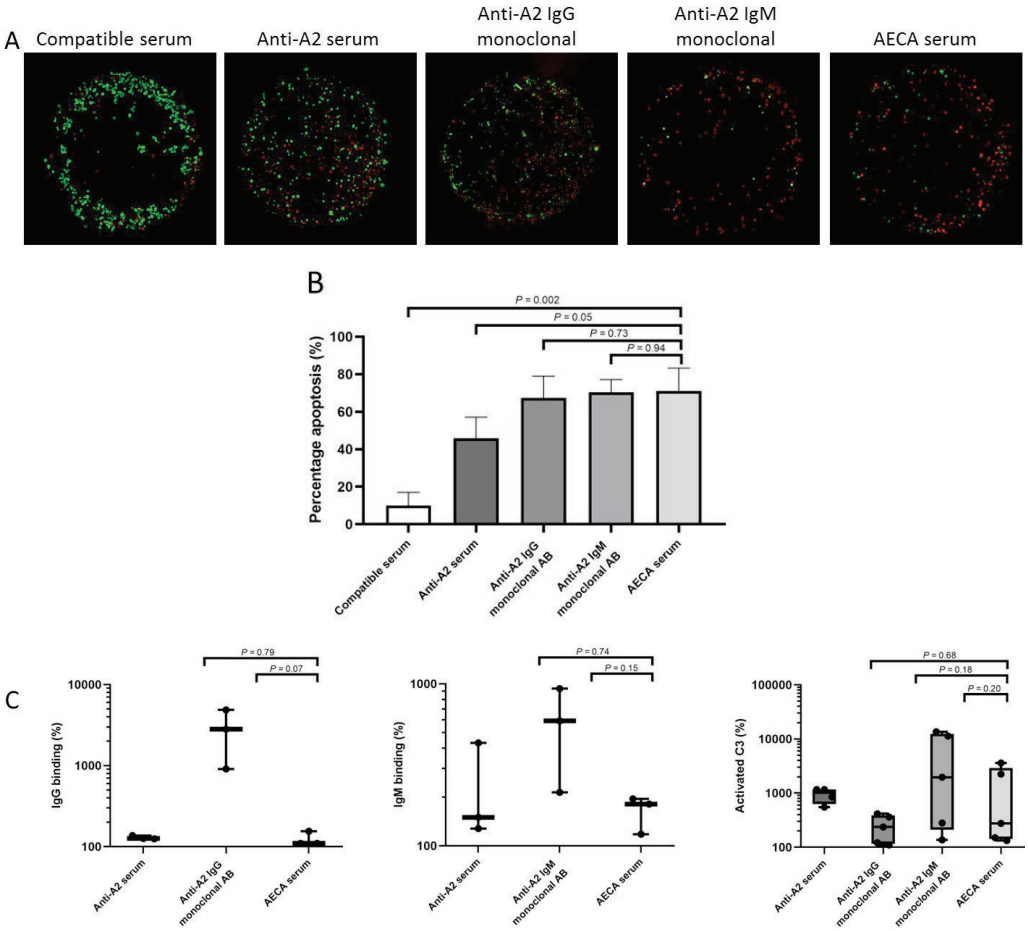

Figure 6. Complement dependent endothelial cytotoxicity and flow crossmatch assays using machine perfusion derived primary renal endothelial cells (MP-PRECs) and post -transplant patient serum.

A) A representative experiment of a complement dependent endothelial cytotoxicity crossmatch assay (CDC) using MP-PRECs (HLA typing A2 B62 B60 Bw6 Bw6 Cw10 DR1 DR4 DR53 DQ7 DQ5 and bloodgroup A, incubated with blood group compatible serum, blood group and HLA incompatible serum (anti-A2 serum), a monoclonal antibody directed against A2 IgG, a monoclonal antibody directed against A2 IgM, and the AECA positive patient serum. Rabbit complement was used as the complement source. Living cells are depicted in green and apoptotic cells in red. B) Quantification of n=3 experiments of apoptotic (red) cells. P values are depicted in the figure.

C) Flow cytometry showing IgG, IgM and activated C3 binding in percentages after incubation of MP-PRECs with blood group and HLA compatible serum, blood group and HLA incompatible serum (anti-A2 serum), a monoclonal antibody directed against A2 IgG, a monoclonal antibody directed against A2 IgM, and the patients' serum having AECA. 20% human serum was used as the complement source. MP-PRECs that were Propidium Iodide negative were included in the analysis, indicating living cells. P values are depicted in the figure. Experiments with a positive result for IgG, IgM and activated C3 binding after incubation with anti-A2 serum were included in the analyzes. Data are normalized to the negative control (Bloodgroup and HLA compatible serum) and are presented on a logarithmic scale. P values depict results using the unpaired t-test. Dots represent individual values.

Second post-transplantation clinical course

Two years later a re-transplantation with a renal allograft from a living unrelated altruistic donor was performed. The donor and recipient both tested positive for the Epstein Barr and Cytomegalo viruses and the donor typing was A1, A2, B7, B60, Bw6, Bw6, Cw10, Cw7, DR13, DR15,DR52, DR51, DQ6, DQ6 resulting in a HLA A/B/DR mismatch of 1-1-0, again blood group compatible and without DSAs. Transplantation was performed after a desensitization procedure consisting of rituximab (750 mg) 9 days before transplantation and 5 sessions of plasmapheresis with exchange of 1 plasma volume per session and albumin 5% substitution. The plasmapheresis regimen was started 6 days before transplantation, followed by 0.1g/kg IVIg (Nanogam) and 1g/kg IVIg after the final session the day before transplantation. The last anti-GBM titer before transplantation was 1 IU/mL. Post transplantation, plasmapheresis was continued for 3 days followed by IVIg 0.1g/kg on the first 2 days and IVIg 1g/kg on the 3rd day. When the patient serum obtained during desensitization treatment was analyzed for the presence of AECA utilizing MP-PRECs, a time-dependent decrease in IgG, IgM, C3, C4d and C5b-9 was observed (Figure 7), suggesting effective removal of non-HLA AECA. No surgical or other post-transplantation complications occurred (cold ischemia time 152 minutes, warm ischemia time 37 minutes) and the patient was discharged with an estimated glomerular filtration rate (eGFR) of 44 mL/min/1.73m^2.21 After two years he had a stable allograft function with an eGFR of 41 mL/min/1.73m^2.

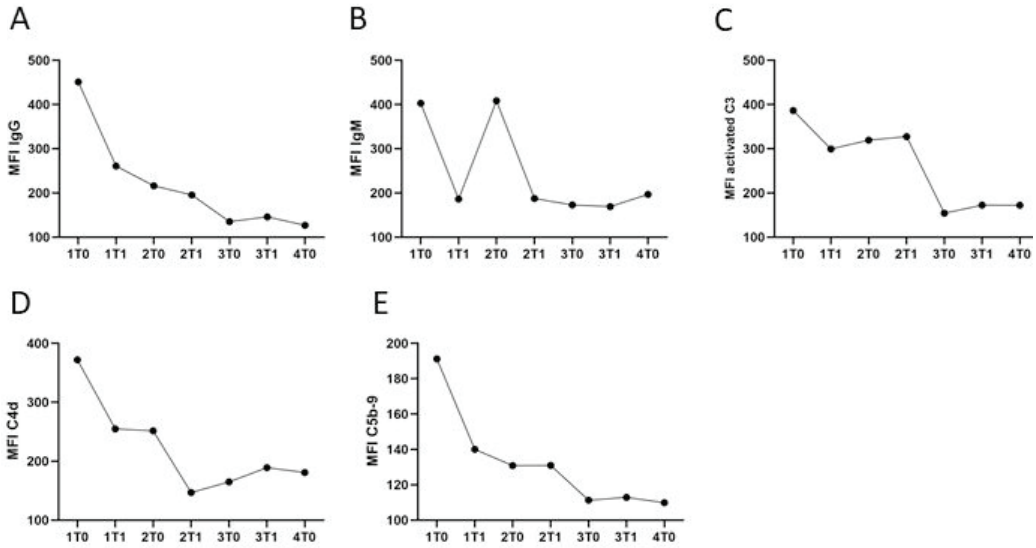

Figure 7. Serum samples collected before and after plasmapheresis show a reduction in binding of IgG, IgM, activated C3, C4d and C5b-9 to MP-PRECs during the course of 4 cycles plasmapheresis.

Serum samples were collected right before every plasmapheresis (1,2,3 and 4) at the timepoints (T) before (0) and after (1) every plasmapheresis.

A) Binding of IgG to MP-PRECs after incubation with the patient's serum collected during the course of plasmapheresis .

B) Binding of IgM to MP-PRECs after incubation with the patient's serum collected during the course of plasmapheresis .

C) Binding of activated C3 to MP-PRECs after incubation with the patient's serum collected during the course of plasmapheresis and 20% NHS as a complement source.

D) Binding of C4d to MP-PRECs after incubation with the patient's serum collected during the course of plasmapheresis and 20% NHS as a complement source.

E) Binding of C5b-9 to MP-PRECs after incubation with the patient's serum collected during the course of plasmapheresis and 20% NHS as a complement source.

Discussion

Although the relevance of non-HLA related humoral rejection is increasingly recognized in solid organ transplantation, its diagnostic identification and monitoring upon treatment has not been sufficiently defined.[22] In this report we describe the accelerated rejection of a blood type compatible, living related donor kidney, where HLA-DSA could not be detected pre- or post-transplantation. A biopsy showed features of C4d-ABMR including extensive hemorrhagic areas and loss of renal vascular endothelial cells. AECAs were identified in crossmatch assays using MP-PRECs from various donors. CDC tests revealed complement dependent cell death in both pre- and post-transplant serum, most likely representing preformed non-HLA antibodies and

not de novo. After desensitization for presumed AECA, a successful second transplantation was performed. Using the MP-PRECs crossmatch assay, we describe monitoring of the effective reduction of complement-mediated renal EC cytotoxicity upon treatment before the second transplantation. Plasmapheresis to treat non-HLA related humoral rejection has been described before.[23] However, to the best of our knowledge, this is the first report describing meaningful diagnostic monitoring of AECA desentization strategy in a kidney transplant recipient.

We showed that MP-PRECs can be utilized instead of lymphocytes in the CDC. However, by using rabbit complement in crossmatch assays, we probably miss the effects of complement regulation that are crucial in the susceptibility to inflammation of endothelial cells.[24] Instead, cross matching using a human complement source showed that the MP-PRECs are heterogeneous in terms of their response to inflammation stimuli.

Histopathological analysis of the patient's biopsy in Figure 2 and 4 emphasizes that renal vascular structures might not be equally involved in rejection processes, as endothelial damage and loss was most prominent in peritubular capillaries. MP-PRECs are primary renal ECs originating from different vascular structures in the kidney (peritubular capillaries, glomerular, macro- and microvascular). Therefore, MP-PREC based CDC is likely representative in an *in vitro* model, as MP-PRECs adequately mirror the immunological situation in its broad vascular heterogeneity and responses to inflammatory stimuli.[10] Our assay with MP-PRECs from various donors makes it possible to prospectively screen for AECA and monitor desensitization before transplantation, as we describe for the second kidney transplantation. It is important to mention that Pereira *et al*. described in 2016 the case of a patient who experienced a hyperacute rejection despite a negative EC crossmatch test, probably due to anti-DQ DSA detected by solid-phase tests based on Luminex technology.[25] However, it has been described that renal microvascular endothelium constitutively expresses DR, without the other class II proteins DQ and DP,[26] minimizing the responsibility of anti-DQ antibodies in acute rejection episodes. Nevertheless, it is important to realize that the MP-PREC crossmatch assay might not detect all possible harmful DSAs, if the antigen is not expressed on the MP-PREC cell membrane. A role for anti-GBM antibodies as a contributing factor to the renal damage seem highly improbable, as the anti-GBM titers remained low during the clinical course post transplantation and the histological pattern in the kidney biopsy was not compatible with anti-GBM disease. Besides, to our knowledge, anti-GBM disease recurrence in a transplant has not been reported to present with fulminant hyperacute rejection as described in this report.[27,28]

The observation of the absence of C4d *in vivo* is similar to the reports about AECA in kidney transplantation described by Jackson *et al*. and Ronda *et al*., who found IgG and IgM binding to respectively endothelial progenitor cells and the cell line EA.hy 926 *in vitro*.[4,22] In a later study, Jackson *et al*. described that patients that were categorized as intermediate or strongly positive for non-HLA Abs measured by ELISA, had higher histological scores of microvascular injury, and non-HLA abs identified through a proteomics approach were able to stimulate expression of adhesion molecules and cytokines, albeit *in vitro*. The effect of antibody mediated damage could also be due to complement independent mechanisms via a mechanism referred to as antibody-dependent cellular cytotoxicity (ADCC). Accumulating evidence suggests that natural killer (NK) cells are important mediators of ADCC in ABMR and it is likely that multiple parallel mechanisms of endothelial activation contribute to the development of ABMR.[29–35]

For example, immune cells like granulocytes could induce injury to the graft endothelium via ADCC.[36,37] The kidney biopsy from the patient described here showed infiltration of granulocytes. It has been described that depending on the FcγR subclass, effector cells like NK cells, macrophages, monocytes and granulocytes can contribute to ADCC.[38–40] On the other hand, neuthrophils are known to act hand in hand with the complement system to defend the host against invading pathogens. Neuthrophils can produce complement factors themselves and this may contribute to local inflammation.[41] We refer to the recently published review by Lebraud *et al.* for a detailed discussion on mechanisms of microvascular damage to the allograft in the absence of HLA antibodies.[36]

We acknowledge several limitations of the study. The usefulness of primary endothelial cells could be limited by early onset of senescence and shifts in the *in vivo* gene expression due to the loss of microenvironmental cues. In addition, the cell isolation can be time-consuming, expensive and the success rates might depend on the experience of the researcher. In order to reproduce this crossmatch assay it is of importance to create immortalized cell lines of the primary ECs derived from the machine perfusate, based on their renal origin and antigen expression. However, cell lines also have their limitations that should not be underestimated, since the process of immortalizing can cause epigenetic changes that alter their phenotypes. Cell lines could change over time due to chromosomal changes during their growth in the lab after generation.[42] Therefore, these cell lines would need thorough validation that focuses on the persistence of expression of endothelium-specific markers.

In addition, as with all laboratory assays, the crossmatch test presented here needs extensive validation, addressing the sensitivity, specificity, intra/inter-assay variability, and the assessment of positive thresholds, and controls. The case we describe is a proof of principle using cultured endothelial cells from different endothelial donors, showing that in all donor-specific endothelial cell antibody-mediated complement activation can be seen, although to a different extent. It has to be noted that validation process might cost a reasonable amount of time, since this heterogeneity in response to non-HLA antibodies may actually reflect true differences in response mechanisms to non-HLA antibodies between individual donors and may explain some of the variation of outcome in the presence of non-HLA antibodies.

Discovery of new targets for non-HLA antibodies is an ongoing proces.[4,19,43–49] Although several non-HLA antibody specificities have been identified, the currently available solid-phase assays are limited to the known non-HLA antigens.[23,50] Only antibodies directed against TUBB4B were found to be present in the serum of the patient described here, however this antibody has never been described to correlated with rejection or graft failure.[20] It has been described that TUBB4B is ubiquitously expressed in patients undergoing chronic hemodialysis, and it might therefore not be surprising that we detect this antibody in the patients serum.[19,51] The pathogenic effect of TUBB4B as the driving force of the hyperacute rejection described here remains questionable, as it seems likely that not all relevant antigens are included in the currently available assays. Differentiation in pathogenic and non significant non-HLA (auto) antibodies remains an important question that is to date unanswered. The advantage of the cell based assay is that it could be used as a screening tool and all pathogenic effects are measured at once. The disadvantage is that the culprit protein remains unkown. Lamarthée *et al.* recently

described the relevance of a tool to identify pathogenic non-HLA antibodies in kidney transplant recipients by utilizing CRISPR/Cas9-engineered HLA-silenced glomerular endothelial cells.[50] Their endothelial crossmatch test identifies non-HLA Abs and strongly predicts graft endothelial injury, independent of HLA-DSAs. The authors suggest that HLA silenced renal endothelial cells from several donors with various genetic backgrounds could provide a wider and more mixed genetic representation, ensuring a more universal cell based assay. This would be important in order to capture more donor specific non-HLA Abs, targeting polymorphic proteins. We think that the MP-PRECs isolation and crossmatch test described in our report could be an attractive way to go. We recognize that it will be important to compare our assay with other EXCMs , such as the XM-One that uses tie2 positive endothelial progenitor cells.[6,52–54] In addition, we did not determine the specificity of the antibody reacting with the MP-PRECs, yet. This identification of the actual antigen may be useful to identify new relevant non-HLA antigen and develop simpler assays.

We hypothesize that our cross match test might add significant value to the characterization of non-HLA antibodies and their detection in terms of standardized diagnostic tests, monitoring therapeutics and for risk stratification prior to transplantation. As organ offers often happen at unexpected moments, a hands on and quick screening assay for anti-endothelial cell antibodies is needed. Also, for HLA sensitized patients a panel of multiple endothelial cell donors might be necessary, if the cells are not made devoid of HLA antigen expression. As it is expensive and time consuming to have endothelial cells in culture without an immediate purpose, a possible suggestion is to grow endothelial cells from multiple donors on cover slips after which the cells could be fixed and stored for future use. We foresee this renal endothelial cell based cross matching assay to be instrumental in further unraveling the pathogenicity of anti-endothelial cell antibodies.

Acknowledgments
We thank H.M. van der Lugt for her support for the preparation of this manuscript and H. Otten for performing the multiplex non-HLA assay.

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Supplementary material

Supplementary table 1. Details on the primary antibodies and conjugates used in staining procedures.

Marker	Antibody	Conjugate
CD34	Ventana bench machine, mouse anti-human CD34 (QBEnd/10,Ventana, Mannheim, Germany)	
ETS-related gene (ERG)	Ventana bench machine, rabbit anti-human ERG (ERP3864, Ventana, Mannheim, Germany)	
IgG	Ventana bench machine, goat anti-human IgG (760-2680, Ventana, Mannheim, Germany)	
IgM	Ventana bench machine, goat anti-human IgM (760-2682, Ventana, Mannheim, Germany)	
C4d	Ventana bench machine, rabbit anti-human C4d (SP91, Ventana, Mannheim, Germany)	
C3d	1:1000 rabbit anti-human C3d, (Dako, A0063, Glostrup, Denmark)	1:10000 anti-human C3d-DIG, (Dako) and 1:8000 polyclonal anti-DIG, (Roche, 11207733910)
IgG	20µl APC conjugated mouse anti-human IgG (G18-145, BD biosciences, San Jose, USA)	
IgM	5 µl PE conjugated mouse anti-human IgM (MHM-88, Biolegend, San Diego, CA, USA)	
Activated C3	1:50 Mouse anti-human activated C3 (HM2168, Hycult, Uden, The Netherlands)	1:100 Goat anti-mouse IgG FITC (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands)
C4d	1:100 Mouse anti-human C4d (12D11, Hycult, Uden, The Netherlands)	1:100 Goat anti-mouse IgG FITC (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands)
Neoantigen C9	1:100 Mouse anti-human neoantigen C9 (HM2264, Hycult, Uden, The Netherlands)	1:100 Goat anti-mouse IgG FITC (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands)

CHAPTER 6

Antigen and cell-based assays for the detection of non-HLA antibodies

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Frontiers in Immunology 2022; DOI: 10.3389/fimmu.2022.864671

Abstract

To date human leukocyte antigens (HLA) have been the major focus in the approach to acute and chronic antibody mediated rejection (AMBR) in solid organ transplantation. However, evidence from the clinic and published studies have shown that non-HLA antibodies; particularly anti-endothelial cells antibodies (AECA), are found in the context of AMBR either, or synergistically in the presence of donor specific anti-HLA antibodies (DSA). Numerous studies have explored the influence of AECA on clinical outcomes, yet the determination of the exact clinical relevance of non-HLA antibodies in organ transplantation is not fully established. This is due to highly heterogenic study designs including differences in testing methods and outcome measures. Efforts to develop reliable and sensitive diagnostic non-HLA antibody tests are continuously made. This is essential, considering the technical difficulties of non-HLA antibody assays and the large variation in reported incidences of antibodies. In addition, it is important to take donor-specificity into account in order to draw clinically relevant conclusions from non-HLA antibody assays. Here, we provide an overview of non-HLA solid-phase and cell-based crossmatch assays for use in solid organ transplantation that are currently available, either in a research setting or commercially.

Introduction

Acute and chronic antibody-mediated rejection (ABMR) are highlighted in studies published in the last decade as an important contributor to organ allograft loss and the lack of long-term survival improvements for transplanted organs. In order to extend the outcomes of the transplanted allografts, a better understanding of the mechanisms of early and late ABMR and the development of protocols to combat and control these processes is needed.[1–4] The interest in the role of donor specific antibodies (DSA) with specificity for antigens other than human leukocyte antigen (HLA) in the contribution of the process of allograft rejection is growing.[5] This has led to the identification of possible non-HLA target antigens and studies into mechanisms of injury.[6–14] However, the understanding of the effect and cause of non-HLA antibodies and the clinical importance of pre-transplant detection remains incomplete, given the fact that non-HLA immunization can both contribute to and arise from allograft injury (Figure 1).[15–20]

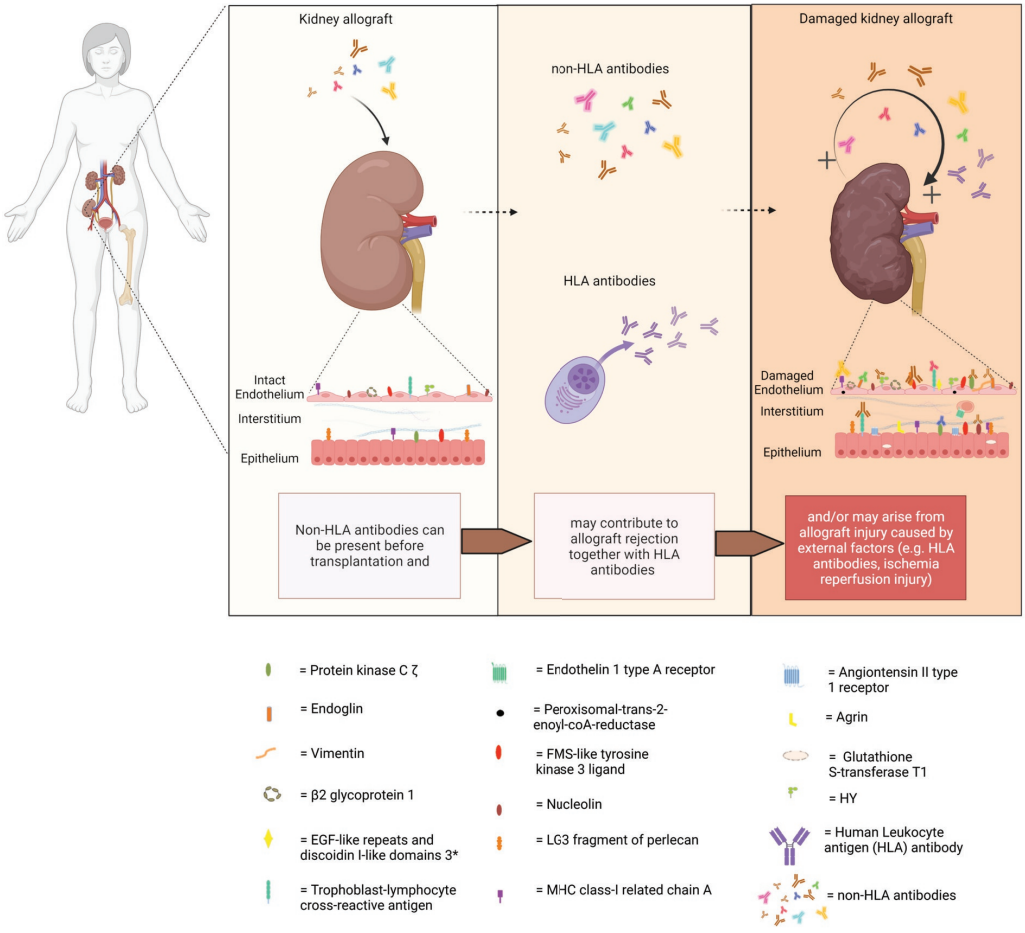

Figure 1. non-HLA immunization can both contribute to and arise from allograft injury.
(non-HLA antibodies depicted in the illustration are examples and no scientific evidence exists that it is this specific antibody that is present at this timepoint). Created with biorender.com

Although several non-HLA antibody specificities have been identified using laboratory-developed assays, large cohort studies into the role of non-HLA antibodies have used commercially available assays.[8,21,22] The most reported non-HLA antibodies are directed against angiotensin II type 1 receptor (AT_1R), MHC class I chain-related antigen A (MICA), tubulin, vimentin, endothelin receptors, collagens and anti-endothelial cell antibodies (AECA), using either commercially available or laboratory-developed assays.[6,7,23] However, despite the large number of published assays, screening for the presence of non-HLA antibodies has still not entered routine clinical practice in transplant medicine. Endothelial crossmatching assays have been used to detect non-HLA antibodies with flow cytometry (e.g., XM-ONE).[24–28] In addition, enzyme-linked immunosorbent assay (ELISA), high-density protein arrays, indirect immunofluorescence, as well as serological analysis of recombinant cDNA expression libraries (SEREX) have also been described.[29–31] Efforts to develop reliable and sensitive diagnostic non-HLA antibody tests capable of detecting new non-HLA antibodies are continuously made. This is essential, considering the technical difficulties of non-HLA antibody assays and the large variation in reported incidences of antibodies. In addition, it is important to take donor-specificity into account in order to draw conclusions from non-HLA antibody assays that have patient related consequences.

In this review, we surveyed the current literature and provided an overview of the recently published non-HLA antibody detection and cell-based crossmatch assays developed for use in solid organ transplantation, either in a research setting or commercially. The articles reviewed in this report were selected based on two criteria; 1) whether the aim of the study was to test a new technology for the detection of non-HLA antibodies and 2) whether study also included a correlation between the identified non-HLA antibodies and rejection episodes (Tables 1-5).

Non-HLA antibodies in kidney transplantation

Cell-based crossmatching assays were among the very first techniques used to detect possible non-HLA antibodies in the serum samples from renal transplant patients.[24,25,28,32–35] In these assays, primary endothelial cells (ECs) are used as targets to be tested against the recipients' sera. These *in vitro* assays are considered to be relatively easy to perform, cost-efficient, and not labor intensive. In addition, it has been suggested that these assays could also detect antibodies against polymorphic antigens such as AT_1R and MICA which might differ between the donors, making their use in the clinical setting more justified.

In kidney transplantation, cell-based crossmatching assays have been used to screen for AECAs. In fact, the use of ECs as targets for crossmatches with fluorochromasia as the reporting technique already exists since the late 1980's.[32] An indirect immunofluorescence procedure was also described by Pontes and colleagues in 2001 where they showed that cultured human umbilical cord vein endothelial cells (HUVECs) could be potentially used to detect non-HLA antibody reactivity in kidney transplant recipients (KTRs). They tested the serum from a single patient who experienced rejection due to antibodies directed against endothelial antigens that were not expressed on platelets (i.e., non-HLA antigens). To ensure that the assay was specific for AECA, they used a platelet pool to remove all pre-existing HLA-class I antibodies, and screened for reactivity against HLA-class I with a complement dependent cytotoxicity (CDC) assay resulting in a negative CDC test.[36]

Ming *et al*. made use of HUVECs for a retrospective crossmatching assay with flow cytometry.[37] The assay was used to test whether the study patient that presented with acute ABMR, who had received kidney transplant without evidence of HLA-DSA, was in fact due to anti-MICA antibodies.[37] High levels of antibodies against MICA were observed using a commercial MICA single antigen Luminex bead assay in both the pre- and post-transplant serum samples, and the donor specificity of these antibodies was confirmed with Sanger sequencing of both the donor and recipient MICA genotype. Finally, endothelial cell-based crossmatching with HUVECs was used to further confirm and characterize the MICA antibodies. The assay demonstrated binding and cytotoxic effects of MICA-DSA in the recipient serum on HUVECs, which indicated the expression of the antigens on the surface of the endothelium and provided evidence that the ABMR experienced by the patient after the first transplantation could have been attributed to MICA-DSA.[37]

Crespo *et al*. recently published a paper in which they described a systematic exploration of pre- and post-kidney transplantation sera for HLA and non-HLA antibodies.[38] 118 kidney transplant recipients were included, based on histological pathology scored as normal histology, interstitial fibrosis and tubular atrophy (IFTA) and ABMR based on Banff'15. The biopsies were either surveillance or clinically indicated biopsies taken after ABO compatible kidney transplantation with a negative CDC crossmatch. HLA antibodies were detected using the Luminex HLA single antigen bead assay and MICA were also detected using Luminex technology. AT_1R-Ab and ETAR-Ab were measured using commercially available ELISAs.[38] They additionally performed EC crossmatch (ECXM) assays, using primary human aortic endothelial cells isolated from aortic rings of explanted donor hearts.39 They found that the combination of pre-transplant HLA-DSA and AT_1R-Abs were strongly associated with ABMR histology. Both pre-transplant DSA and AT_1R-Abs were significantly associated with the development of ABMR. However, none of the patients with HLA-DSA negative ABMR had AT_1R-Abs. In addition, the post-transplant combination of HLA-DSA and AT_1R-Abs did not associate with the development of ABMR. Furthermore, neither pre- or post-transplant MICA- and ETAR-Abs, nor a positive ECXM correlated with ABMR histology, with or without HLA-DSA. Positivity in the ECXM was found in all different patient groups and did not associate with any histological signs of endothelial damage e.g., ABMR histology.

Although the previously mentioned studies made use of primary ECs as targets for the crossmatching test, these cells were not derived from the particular graft donor. An EC-based crossmatching assay with donor derived ECs may provide better risk assessment for the recipients. However, difficulties in obtaining cell cultures of organ specific endothelial subsets have hampered molecular characterization, transcriptional profiling, and assay development with ECs. Nevertheless, isolation of ECs from donors is possible. Although the cell isolation can be time-consuming and expensive, it is a promising technique that will allow us to study donor specific HLA and non-HLA antibody dependent endothelial cytotoxicity.[40] One of the earliest approaches to achieving donor-derived endothelial cell crossmatching test was introduced in 2002 by Vermehren and colleagues[33], which consequently resulted in the development of the commercially available, flow cytometry-based assay XM-ONE®.[25]

The proprietary XM-ONE assay is designed to screen for non-HLA antibodies in a crossmatching test that uses endothelial precursor cells (EPCs) as target cells selected by the

expression of angiopoietin receptor (Tie-2+). The assay was validated in multicenter trial by Breimer *et al*. that used EPCs isolated from donor peripheral blood mononuclear cells (PBMCs) to screen for AECAs in the pre-transplant serum samples of 147 patients.[24] The trial identified AECAs in 35 of the 147 included patients (24%), among which, a significant number (16 of 35; 46%) either experienced rejection (up to 3 months after transplantation), or were at a higher risk of impaired kidney function (as indicated by the increased serum creatinine levels) compared to those without AECAs (13 of 112; 12%).[24]

Several other studies utilized the XM-ONE set-up to screen for the presence of non-HLA antibodies in living donor kidney transplant recipients. These studies describe contrasting results.[27,28] Soyöz *et al*. screened for EPC-reactive IgG and IgM in post-transplant serum from 13 living donor transplant recipients using the XM-ONE kit. In this population, AECAs were not detectable in the serum samples of all patients including the 3 patients who experienced ABMR.[27] In addition, Zitzner *et al*. also investigated the presence of AECAs in the pre-transplant serum samples of 150 living donor kidney transplant recipients and reported a lack of association between the XM-ONE result and biopsy proven rejection or vasculopathy at 1-year post-transplant.[28]

Table 1a. *XM-ONE studies in kidney transplantation.*

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Breimer et al. 2009[24]	Kidney	Patients with pre-transplant sera positive for AECAs had higher risk for rejection or impaired kidney function post-transplant	Pre-transplant serum samples of 147 patients were screened for AECAs	The XM-ONE assay was used to screen for AECAs. EPC-reactive IgG and IgM were detected by flow cytometry	XM-ONE uses EPCs as target cells which lack EC markers such as CD31 and CD34; no target antigens were identified
Soyöz et al. 2020[27]	Kidney	AECAs were not detected in the serum of all patients including the 3 patients who experienced biopsy confirmed rejection	Post-transplant serum samples from 13 living donor KTRs were screened for AECAs	The XM-ONE assay was used to screen for AECAs. EPC-reactive IgG and IgM were detected by flow cytometry	The kidney donors in this study were first degree relatives which might have improved the compliance to AECAs leading to the negative XM-ONE results
Zitzner et al. 2013[28]	Kidney	No association was found between the XM-ONE result and the biopsy proven rejection or vasculopathy at 1-year post-transplant	Pre-transplant serum samples from 150 living donor KTRs were tested for AECAs	The XM-ONE assay was used to screen for AECAs. EPC-reactive IgG and IgM were detected by flow cytometry	A different immunosuppressive protocol (alemtuzumab induction) was used in comparison to other studies
Yu et al. 2020[55]	Kidney	The presence of AT_1R-Abs and AECAs may contribute independently to a worse post-transplant outcome in low-risk, living donor KTRs	Levels of AT_1R-Abs and AECAs were determined in 94 pre-transplant and 29 post-transplant serum samples in living donor KTRs with biopsy proven rejection	AT_1R-Abs levels were assessed with AT_1R ELISA. The presence of AECAs was detected with XM-ONE assay	In most of the patients (65/94), only pre-transplant sera were tested for non-HLA Abs, therefore the post-transplant impact of AT_1R-Abs and AECAs was not reported
Philogene et al. 2017[12]	Kidney	The presence of and AT_1R-Abs may contribute to the microvascular injury observed in ABMR especially in the presence of HLA-DSA	Post-transplant AT_1R-Abs levels were measured in 70 KTRs, and AECAs levels were measured in 35 KTRs who had low to negative HLA-DSA	Commercial ELISA was used to measure AT_1R-Abs, and an XM-ONE assay was used for the AECAs	Pre-transplant serum samples were unavailable for testing; the study included hypertensive patients who underwent ARB treatment at time of graft dysfunction

ABMR, Antibody-mediated rejection; AECAs, anti-endothelial cell antibodies; ARB, angiotensin receptor blocker; AT1R, angiotensin type 1 receptor; EPCs, endothelial precursor cells;
KTR, kidney transplant recipient.
** Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section.*

The XM-ONE assay has several limitations. For instance, the properties of the circulating EPCs might not be reflective of the ECs in the transplanted allograft. It is essential to keep this in mind, especially since these precursor cells lack important general EC markers such as CD[31] and CD[34.33] Additionally, Tie-2+ EPCs express HLA Class I and Class II antigens which can lead to false positivity in the presence of HLA-DSA, and would require the depletion of HLA-DSA from the recipient's serum prior to testing.[33] Furthermore, Alheim and colleagues reported that a large fraction of the cells isolated with the XM-ONE kit were lymphocytes positive for the Tie-2 receptor.[25] This would require further gating for CD3+ CD19+ lymphocytes to exclude the possibility of contamination and interference of Tie-2+ lymphocytes when performing the endothelial crossmatching test. Our group recently published a method of obtaining human renal ECs from machine-perfused donor kidneys. We demonstrated that these cells expressed common EC markers (CD31, CD34, von Willebrand Factor, VEGFR-2, PV-1, and HLA-DR to variable extents). As these ECs are derived from the site of the graft, they could potentially be better candidates as targets for an endothelial crossmatching assay.[40] Delville *et al*. recently demonstrated that non-HLA antibodies that are associated with the histology of ABMR primarily bind in a very specific manner to glomerular endothelial cells (CiGEnC).[41] However, the basal expression of HLA antigens limited the application of CiGEnC for non-HLA antibody detection in patients without circulating anti-HLA antibodies. To tackle this obstacle, the group applied a CRISPR/Cas9 strategy to delete the B2M and CIITA genes, resulting in loss of function and undetectable HLA-ABC and HLA-DR expression.[42] Using these cells, a non-HLA antibody detection immunoassay (NHADIA) was developed. The authors used an unselected cohort of kidney transplant recipients and showed that non-HLA antibodies were increased in patients who underwent a previous kidney transplantation. The pre-transplantation NHADIA value correlated with microvascular inflammation (MVI) in the kidney allograft at 3 and 12 months post transplantation, and was correlated with the risk of the developing ABMR. Interestingly, no correlation between NHADIA results and AT_1R levels was found, indicating that the antibodies detected in the NHADIA results are not AT_1R antibodies. The results from these studies suggest that non-HLA antibodies associated with the histology of ABMR which bind to CiGEnC might recognize a wide diversity of antigens. In addition, these results point directly towards the major limitation of using a single cell line for endothelial cell cross matching assays; as a single cell line does not address the variability of expressed antigens between individuals, which may form the basis for non-HLA antibody formation.

The endothelium has different functions corresponding to the region of the body where it is situated.[43] This results in different antigen expression patterns, and therefore ECs may respond differently to activation.[44,45] The importance of using ECs derived from vessels of the appropriate location has been underlined by several studies. This includes work on AECAs in various small and large vessel diseases and responses of ECs from different organs to inflammation and sepsis.[44–47] Moreover, it was shown that upon binding of HLA class I antibodies on human-aortic, umbilical, and dermal microvasculature ECs, the induction of P-selectin, involved in recruitment of leukocytes, varied between EC types.[48] 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), revealed that EA.hy926 cells have a high similarity with primary ECs, however, they show differences in the expression levels of certain EC markers. They also

express a large number of additional genes mainly related to the cell cycle and EC apoptosis.[48] Circulating EPCs isolated from peripheral blood allow testing for donor specific AECAs. However, it is not clear whether these cells reflect the properties of the endothelial cells present in the graft.[48] Therefore, antibodies reactive against antigen targets on EPCs or HUVECs may not react against antigens expressed on renal microvascular endothelial cells. Li *et al*. underlined that non-HLA antibodies are not exclusively directed against targets on the endothelium, by testing pre- and post-transplant samples of pediatric renal transplant patients for reactivity against over 5000 protein targets selected based on their appearance in the kidney using a ProtoArray. They reported a response to 61% of the targets on average with the highest reactivity against antigens expressed on pelvic epithelial cells and in the renal cortex.[49] In addition, two separate studies by Leisman and Lammerts *et al*. recently showed that AT_1R is possibly not expressed by renal endothelial cells and Delville *et al*. did not find a correlation between AT_1R and non-HLA anti-endothelial cell antibodies, measured with the NHADIA.[40,50,51] Also, the study by Senev *et al*. reported that AT_1R antibodies assessed using the multiplex Luminex assay could not explain the histology of ABMR in the absence of DSA.[22]

In addition to cell-based crossmatching assay, antigen detection methods have also been used to screen for known specific non-HLA antibodies. ELISAs are universally used for convenient and rapid bulk screening of patient serum samples, which is further eased by the possibility of acquiring some of the kits commercially. For instance, currently there are two commercially available ELISA kits that have been developed by Cell Trend (Luckenwalde, Germany) supplied by One Lambda, for detecting anti-AT_1R or anti-ETAR antibodies in kidney transplant patients.[11,12,38,52–54] Unlike conventional ELISAs whereby the proteins of interest are detected by antibodies from a purified or homogenous denatured cell-lysate samples, the commercially available AT_1R and ETAR ELISAs are produced by coating the plate with non-denatured extracts from cells overexpressing the target protein. This ensures that the detected targets (i.e., antibodies against AT_1R and ETAR) are complementary to the receptors in their native, non- denatured form.[53]

Several researchers utilized these commercial ELISAs to investigate the relationship between AT_1R or ETAR and the clinical outcomes in renal transplant recipients.[11,12,54] Philogene *et al*. investigated the presence of AT_1R antibodies with a commercial ELISA in combination with XM-ONE (for AECAs) in the post-transplant sera of kidney transplant recipients who had low or negative HLA-DSA. They observed an association between the development of ABMR and the levels of AT_1R antibodies especially when HLA-DSA were detected.[12] The XM-ONE results revealed that patients whose post-transplant sera were positive for AECAs had increased AT_1R titers compared to those with negative crossmatching test.

Interestingly, 8 out of the 11 patients with positive XM-ONE test developed rejection, one developed transplant glomerulopathy, and 2 had no rejections. Similarly, Pearl *et al*. also reported a strong association between AT_1R and ETAR antibodies, microvascular injury, elevated levels of IL-8, and impaired kidney function upon investigation of AT_1R and ETAR antibody levels in the post-transplant serum samples of kidney transplant recipients using commercial ELISAs.[11]

More recently, Yu and colleagues used the anti-AT_1R antibody ELISA to evaluate their

role in predicting the transplant outcome in low-risk, living donor KTRs.[55] The study included 94 transplant recipients who had negative pre-transplant serum HLA-DSA who underwent ABO compatible, living donor kidney transplantation. In this study, anti-AT_1R antibody titers were measured in 94 pre-transplant serum samples, and 29 post-transplant serum samples in patients experiencing biopsy proven rejection. A significant association was found between increased pre-transplant serum levels of anti-AT_1R antibodies and the risk for developing acute rejection. Interestingly, the authors also investigated the presence of other AECAs in the pre-transplant sera with the XM-ONE assay. They reported that patients with a positive XM-ONE AECA test had poorer kidney function, starting 3 months after transplantation, and continuing to decline up until 20 months (end of the study).[55]

Aside from ELISA, genome wide analyses and protein microarrays have also been described for antigen identification.[56–58] Using genome wide analysis, Reindl-Schwaighofer and colleagues were able to show that genetic mismatches of non-HLA haplotypes coding for transmembrane or secreted proteins were associated with an increased risk of functional graft loss.[58] They genotyped 477 KTRs receiving their first kidney transplant from a deceased donor,, and genetic mismatches between the pairs were measured to identify incompatibilities in both transmembrane and secreted proteins. From this, they were able to identify 16 non-HLA donor-specific peptides mismatches which they then used to construct an array of peptides to screen another group of 25 patients with biopsy rejection for the presence of these mismatches. The study showed that mismatches of non-HLA were associated with worse clinical outcomes independent of HLA.[58]

In protein microarrays, thousands of recombinant proteins are immobilized and arranged on a solid surface and are then probed with florescent labeled sample. Protein microarrays can be used for various applications, including antibody detection.[59]

Jackson *et al*. employed ProteoArray® (a protein microarray technology), in combination with ELISAs, to identify target antigens for AECAs. The sera of 10 KTRs from a discovery cohort experiencing ABMR in the absence of HLA-DSA were used as samples for the protein array assay.56 The assay identified four antigenic targets: endoglin, Fms-like tyrosine kinase-3 ligand (FLT3), EGF-like repeats and discoidin I-like domains 3 (EDIL3), and intercellular adhesion molecule 4, all of which have been shown (*in vitro*) to be capable of endothelial cell activation, and induction of pro-inflammatory cytokines and chemokines. To further validate these findings, additional 150 pre- and post-transplant serum samples from KTRs were tested for antibodies against these antigens with in-house made ELISAs, and positive results were observed in 24% of the tested pre-transplant samples. The presence of antibodies against these antigens was associated with HLA-DSA sensitization, ABMR, and early transplant glomerulopathy.[56]

Li *et al*. utilized the ProteoArray technology to specifically investigate the antibody response against HLA and MICA to determine whether the immunogenic response in the kidney tissue was restricted to certain compartments that express these antigens.[57] Pre- and post-transplant serum samples from 18 pediatric kidney transplant recipients were examined for non-HLA antibody response. The protein microarray assay revealed an increase in the signal for de novo antibodies by an average of 61% in all patients in the post-transplant serum compared to the pre-transplant.

Of note, anti-MICA antibodies were detected in 72% of the patients post-transplant.57 The authors did not test for any correlation between the development of the anti-MICA antibodies and the risk of rejection in the recipients even though previous studies have reported the existence of such correlations.[60,61]

Moreover, a luminex-based non-HLA antibody assay was developed covering 14 possible targets for non-HLA antibodies.[62] Kamburova and colleagues constructed a multiplex Luminex assay with 31 different microspheres consisting of various proteins to screen the serum of kidney transplant recipients for 14 non-HLA antibodies. Various testing conditions were used in order to optimize and validate the highest specific median-fluorescent intensities (MFI) levels that could distinguish between the positive and negative patient sera. The author reported that anti-phospholipase A2 receptor (PLA2R) antibodies showed the highest level of distinction (in terms of MFI vs background signal) followed by anti-Vimentin antibodies. Although the assay did not investigate the presence of commonly described non-HLA targets such as AT_1R and ETAR, the multiplex assay was used in a subsequent study to determine the non-HLA status in the pre-transplant serum samples of 4770 patients in the PROCARE cohort.[8] The analysis revealed that antibodies against rho GDP dissociation inhibitor beta (ARHGDIB) were more clinically relevant in patients receiving a deceased donor kidney transplant compared to those receiving a living donor kidney transplant. Additionally, this non-HLA antibody seemed to be associated with negative clinical outcome on the graft that was independent of the HLA-DSA status. These results were confirmed in a smaller cohort by Senev *et al*.[22] Interestingly, these results of a negative clinical outcome when non-HLA antibodies are present in the pre-transplant sera, were similar to what has been published by other groups for other types of non-HLA antibodies (namely the studies by Soyöz and Zitzner[27,28]). This especially holds true with regards to the presence and clinical relevance of non-HLA antibodies in living donor compared to deceased donor transplantation.

The bottom line is that we now possess several relevant cell-based assays (cytotoxic assays or flow cytometric crossmatch assays) and solid phase assays (Luminex, enzyme-linked immunosorbent assays) that can detect or screen for potentially alloreactive antibodies (Figure

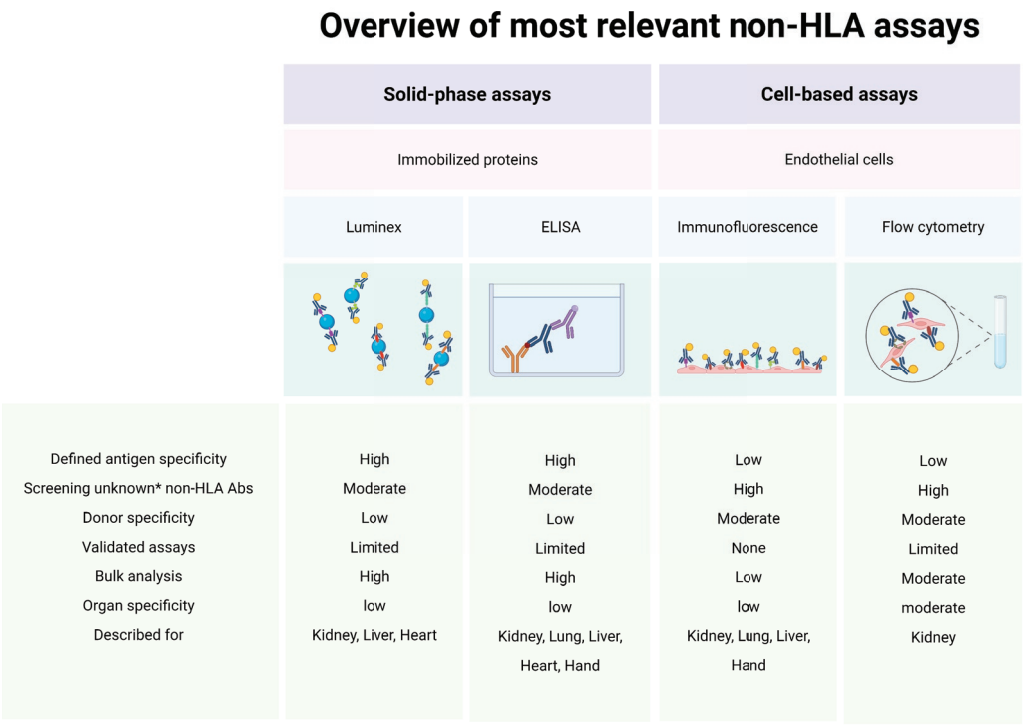

Figure 2. Assays for alloreactive antibodies.

*Abs, antibodies; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen. * new/unknown non-HLA Abs. Created with biorender.com*

Several studies demonstrated that pre-transplant non-HLA antibodies have pathogenic effects on graft survival and may contribute to impaired kidney function post-transplant.[8,10,24,55,58] Others showed that this only holds true when HLA antibodies were also present.[9,11,38,56,57] Moreover, AT_1R antibodies are the most studied non-HLA antibodies despite evidence suggesting that they are not expressed by the ECs involvement in the kidney tissue.[40,50] Importantly, the large variety of non-HLA antibodies used as targets for investigation in the different studies, makes it difficult to compare, or predict the exact role of these antibodies. In addition, although the majority of studies use IgG as readout, many studies report non-HLA antibodies with variable readouts (IgG, IgM, C1q). In the future, it will be important that studies use a comparable and reproducible approach with well-defined study populations and endpoints and thoroughly validate their assays, addressing assay sensitivity and specificity, intra- inter-assay variability, positive thresholds and controls.

Table 1b. non-HLA antibody detection studies in kidney transplantation.

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Pontes et al. 2001[36]	Kidney	Cultured ECs can be used to detect non-HLA Abs with IIF	A single serum sample obtained from a KTR after two rejected grafts and undetectable HLA at baseline	ECXM assay using HUVECs as the target cells. ECs antigens were visualized using IIF with mouse anti-human Ig-TRITC	One post-transplant serum sample from a single patient; non-donor derived ECs for ECXM assay; IIF has lower sensitivity
Ming et al. 2015[37]	Kidney	MICA-DSA in the serum were cytotoxic to ECs expressing MICA-G1 group antigens	A single serum sample positive for MICA-DSAs obtained from a KTR after a previously rejected graft with no HLA mismatch	ECXM assay using HUVECs as the target cells. Mouse anti-human IgG was used to visualize the results by flow cytometry	One post-transplant serum sample from a single patient; non-donor derived ECs for ECXM assay; flow cytometry assays suffer from gating bias
Crespo et al. 2021[38]	Kidney	The combination of pre-transplant HLA-DSA and AT_1R-Abs was strongly associated with the histology of ABMR, the post-transplant combination did not. Nor pre-or post-transplant MICA-Abs, ETAR-Abs or ECXM correlated with ABMR histology, with or without HLA-DSA	19 KTRs with normal histology, 52 KTRs with ABMR histology, and 47 KTRs with IFTA were screened for anti-MICA-Abs, anti-AT_1R-Abs, and anti-ETAR-Abs, and a ECXM was performed in pre-and post-transplant serum	MICA-Abs were detected using commercial Luminex technology. Sandwich ELISAs were used to detect AT_1R- and ETAR-Abs. ECXM assay using primary aortic endothelial cells as the target cells. EC-reactive IgG was detected by flow cytometry	Broad heterogeneity in the inclusion timing and clinical course of the patients. ECXM was not performed with renal ECs. Purification of primary EC isolation and gating strategy is not described resulting in a possible contamination with other cell types
Pearl et al. 2020[11]	Kidney	The presence of ETAR-Abs was significantly associated with AT_1R-Abs. These Abs were found to not be associated with ABMR or HLA-DSA development, however they were significantly associated with microvascular injury, elevated levels of IL-8, and impaired renal function	The relationship between ETAR-Abs and AT_1R-Abs was investigated with regards to biopsy findings, pro-inflammatory cytokine production, and HLA-DSA in a cohort of 67 pediatric KTRs post-transplant	Commercial ELISAs were used to measure AT_1R-Abs and ETAR-Abs in the post-transplant serum samples. A custom magnetic bead kit used to measure the cytokine levels and was visualized with Luminex	Pre-transplant serum samples were not assessed; no ECXMs were performed to assess for other potential AECAs; the use of ATG increased the risk of AT_1R-Abs development
Sorohan et al. 2021[54]	Kidney	No relationship was found between post-transplant AT_1R-Abs and biopsy proven rejection	Pre- and post-transplant serum samples from 56 KTRs were screened for AT_1R-Abs	AT_1R-Abs were measured using a commercial quantitative ELISA	Lack of HLA-DSA in the study population made it difficult to assess the relationship between AT_1R-Abs and HLA-DSA; biopsies were only taken by indication leading to lack of cases with biopsy proven rejection
Reindl-Schwaighofer et al. 2019[58]	Kidney	Genetic mismatches of non-HLA haplotypes coding for transmembrane or secreted proteins were associated with an increased risk of functional graft loss	25 KTRs with biopsy proven rejection were screened for the presence of non-HLA mismatches	Genetic mismatches between 477 pairs of deceased donors-first kidney transplant recipients were measured using a genome-wide analysis	The implementation of this technique as a bulk lab screening method is difficult as it is expensive and time consuming to conduct a genome wide analysis on a routine basis
Jackson et al. 2015[56]	Kidney	ProteoArray identified 4 targets: endoglin, FLT3, EDIL3, and intercellular adhesion molecule 4 which, upon further assessment *in vitro*, were associated with HLA-DSA sensitization, ABMR, and early transplant glomerulopathy	Sera from 10 KTRs from a discovery cohort experiencing ABMR in the absence of HLA-DSA were used to construct the ProteoArray. Additional sera from 150 KTRs was used to validate the ProteoArray results	ProteoArray technology was used to identify antigenic targets for AECAs. The presence of Abs against the detected targets was investigated in pre- and post-transplant serum samples with ELISAs	Many of the patients experiencing rejection post-transplant were also positive for HLA-DSA as indicated by biopsy, therefore the association of ABMR to AECAs in these patients was confounded
Li et al. 2009[57]	Kidney	An increase was detected in the signal for de novo Abs by an average of 61% in all patients in the post-transplant serum compared to the pre-transplant. MICA-Abs were detected in 72% of the patients post-transplant	Pre- and post-transplant serum samples from 18 pediatric KTRs were examined for non-HLA antibody response	ProteoArray technology was used to identify antigenic targets for AECAs. The preferential expression of a particular antibody in the kidney tissue was investigated in 7 micro-dissected kidney compartments	No correlation between the development of the MICA-Abs and the risk of rejection in the recipients was tested
Clotet-Freixas et al. 2021[10]	Kidney	Autoantibodies against Ro/SS-A (52 kDa), CENP-B, and La/SS-B were significantly	Serum samples from 80 KTRs were diagnosed with ABMR,	Protein microarray platform against 134 IgG	The study did not report the impact of these

Sanchez-Zapardiel et al. 2016[9]	Kidney	Preformed MICA-Abs were able to fix and activate the complement system, therefore mediating cell death. Patients with MICA-Abs along with HLA-DSA had worst outcomes	Serum samples from 52 KTRs were tested for MICA-Abs and C1q binding	Both MICA-Abs and C1q binding were analyzed with a Luminex platform	The study did not define whether these anti-MICA antibodies were donor specific
Kamburova et al. 2018-2019[8,62]	Kidney	Antibodies against ARHGDIB were more clinically relevant in deceased donor KTRs compared to living donor KTRs	Pre-transplant serum samples of 4770 KTRs were screened for 14 non-HLA antibodies	A Luminex assay with 31 different microspheres consisting of various proteins was constructed to screen for 14 non-HLA antibodies	The multiplex assay did not investigate the presence of commonly described non-HLA targets such as AT_1R and ETAR
Lamarthée et al. 2021[34]	Kidney	Non-HLA Abs were increased in patients who underwent a previous kidney transplantation	Pre-transplant serum samples from unselected cohort of 389 KTRs	Non-HLA Ab detection immunoassay (NHADIA) using CRISPR/Cas9 deleted B2M and CITTA using CiGEnCs	ECs from a single donor and from a single vascular structure. The study did not measure solid phase non-HLA Abs

ABMR, antibody-mediated rejection; ACR, acute cellular rejection; AECAs, anti-endothelial cell antibodies; ARHGDIB, rho GDP dissociation inhibitor beta; AT1R, angiotensin type 1 receptor; ATG, anti-thymocyte globulin; CiGEnCs, conditionally immortalized glomerular endothelial cells; ECs, endothelial cells; ECXM, endothelial cells crossmatching; ETAR, endothelin type A receptor; IFTA, interstitial fibrosis and tubular atrophy; Ig-TRITC, immunoglobulin-tetramethylrhodamine; IIF, indirect immunofluorescence; IL-8, interleukin 8; KTR, kidney transplant recipient; MICA-DSA, major histocompatibility complex class I chain-related A donor-specific antibody; non-HLA Abs, non-human leukocyte antigen antibodies.

**Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section.*

Non-HLA antibodies in lung transplantation

While several research groups in the field of kidney transplantation have utilized cell-based crossmatching assays to screen for non-HLA targets, this technique is rarely described in lung transplantation research.

Margo and colleagues described an indirect immunofluorescence cell-based crossmatching assay to detect the presence of ACEAs in the serum samples of lung transplant patients.[29] Cytocentrifuged fixed preparations made from human pulmonary microvascular EC cultures were used as the target cells for testing. The study population was tested for panel-reactive antibodies (PRA) and was found to be negative before transplantation and at the time of rejection. The indirect immunofluorescence assay revealed a prominent granular nuclear and cytoplasmic staining pattern indicative of positive reactivity to endothelial cells in 18 of 19 patients.[29] It is important to mention that this study was not explicitly designed to demonstrate the potential role of EC antigens as the antigenic targets, rather, the authors' original goal was to present a report of a series of lung patients who had developed post-transplant septal capillary injury syndrome of a humoral origin as a form of allograft rejection. Furthermore, indirect immunofluorescence is less sensitive compared to newer detection techniques such as flow cytometry which essentially limits the significance of these findings.

In addition to cell-based assays, antigen detection techniques such as the ELISA,[30] and serological analysis of recombinant cDNA expression libraries (SEREX)31 have also been described in the context of lung transplantation.

Reinsmoen *et al*. investigated the presence of antibodies to AT_1R and ETAR in the pre- and post- transplant sera of 162 lung transplant recipients from 3 centers with a commercial sandwich ELISA kit.[30] The serum samples were tested for binding to AT_1R and ETAR at three levels: strong, intermediate, and negative. For AT_1R, the frequencies of bindings were 46%, 37%, and 17% respectively, while ETAR had 26%, 29%, and 45% binding frequencies. Stronger binding frequencies for both AT_1R and ETAR were significantly correlated with increased potential to develop ABMR post-transplant. However, out of the 162 recipients tested, only 5 developed ABMR post-transplant, and these 5 patients were among those who developed de novo DSA.

The SEREX technique is an antigen identification technique that uses cDNA libraries extracted from solid tissue screened against sera from patients to identify gene products (usually expressed by a bacterium such as *Escherichia coli (E.coli)* transfected with the genes of interest), which are then recognized by an IgG antibody.[63] Although mainly used to identify tumor antigens, this technology was applied to lung transplantation to better understand the role of non-HLA antibodies in the pathogenesis of bronchiolitis obliterans syndrome (BOS) in a study by Otten and colleagues.[31]

In this study, RNA from the airway epithelial cells was obtained from 4 donors' tracheae, and was used to construct a complementary DNA (cDNA) library which was then transfected to E.coli. Pre- and post-transplant serum samples from 11 lung recipients were tested for reactivity against the antigens expressed by the transfected bacteria, and the reactivity of the sera was visualized by a goat anti-human IgG. The assay identified six non-HLA targets that were only shared between four of the study patients confirming that the non-HLA profile differs among individuals, and therefore, a larger cDNA library might be needed to cover a wider range of specific non-HLAs that might contribute to the development of BOS in the transplant population.[31] The results of this study, despite the relatively smaller sample size, demonstrated that the SEREX technique was capable of identifying potential non-HLA targets present after lung transplantation, and the authors suggest a potential role of this technique to be implemented for clinical testing.[31]

In conclusion, non-HLA antibodies may contribute to the pathogenesis of lung transplant rejection, but their specific targets have yet to be identified (Figure 2). Even with elaborate technologies utilizing cDNA libraries or validated solid-phase assays (i.e., AT_1R and ETAR ELISAs), the exact contributions of non-HLA to graft dysfunction in lung transplantation is still unknown.

Table 2. non-HLA antibody detection studies in lung transplantation.

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Margo et al. 2002[29]	Lung	IIF revealed an increase in the granular nuclear and cytoplasmic staining pattern indicative of positive reactivity to ECs in 18 of 19 patients	Post-transplant sera from 19 LTRs were screened for AECAs	An ECXM assay was employed to assess for AECAs using fixed preparation made from human pulmonary microvascular ECs. The results were visualized with IIF with goat anti-human IgG Abs	IIF is a less specific approach compared to other detection methods such as flow cytometry; only one pre-transplant serum sample was assessed for AECAs; biopsy data showed evidence of prior microbial infections that may have enhanced the alloantigenic response
Reinsmoen et al. 2017[30]	Lung	In LTRs with pre-transplant HLA-DSA, higher frequencies for either AT_1R-Abs or ETAR-Abs correlated with increased potential to develop de novo DSA	The pre- and post-transplant sera of 162 LTRs were tested for anti-AT_1R and ETAR-Abs	Commercially available sandwich ELISAs were used to detect anti-AT_1R and ETAR-Abs	ECXMs were not performed to assess the impact of other AECAs; relatively short follow-up time (3-6months post-transplant samples were tested)
Otten et al. 2006[31]	Lung	The SEREX technique identified 6 potential non-HLA targets that were shared between 4 study patients	Pre- and post-transplant serum samples from 11 LTRs were tested for non-HLAs	SEREX technique was used to test for reactivity against the cDNA library-encoded antigens expressed by the transfected bacteria, and this reactivity was visualized by a goat anti-human IgG	Small sample size; the assay failed to detect gene products in the pre-transplant samples; the assay only detected antigenic targets, but their specific role in rejection was not established

AECAs, anti-endothelial cell antibodies; AT1R, angiotensin type 1 receptor; cDNA, complementary DNA; ECs, endothelial cells; ETAR, endothelin type A receptor; ECXM, endothelial cells crossmatching; IIF, indirect immunofluorescence; LTR, lung transplant recipient; SEREX, serological analysis of recombinant cDNA expression libraries.

**Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section*

Non-HLA antibodies in liver transplantation

Liver transplant recipients rarely experience ABMR even in the presence of antibodies directed against HLA.[64] Although HLA antibodies have been gaining attention for their association with adverse transplantation outcomes, research into non-HLA antibodies in liver transplant patients is still relatively new and data is limited. In fact, most of the studies into the role of non-HLA antibodies focused primarily on the detection of AT_1R and ETAR antibodies.

Ekong and colleagues evaluated the effects of several non-HLA antibodies including and anti- nuclear antibodies, anti-smooth muscles antibodies, anti-liver kidney microsomal antibodies, and AT_1R antibodies on the development of fibrosis in 42 pediatric liver transplant recipients using indirect fluorescence and a commercially available ELISA kit.[65] The results of their analysis revealed that the presence of the aforementioned antibodies had no significant association with fibrosis. That said, the study mainly focused on evaluating whether HLA epitope mismatches were predictors of de novo donor DSA risk and rejection, and less on the role of non-HLA antibodies specifically.[65] Moreover, most of the patients did not have pre-transplant serum samples available for testing for anti-AT_1R antibodies, so the levels of this antibody were not clearly defined in these patients pre-transplant.

Ohe and colleagues also focused on evaluating the effects of anti-AT_1R antibodies in a cohort of 81 pediatric living donor liver transplant recipients using a commercially available ELISA kit.[66] The study concluded that all patients who had anti-AT_1R antibodies in addition to HLA-DSA developed advanced fibrosis compared to those patients who only had one antibody or were double negative for both. Assessment of the AT_1R status especially in patients with confirmed DSA could prove to be useful in predicting the risk for fibrosis. However, even though it was clear that the AT_1R antibodies played an important role in fibrosis in liver transplant recipients, the results only suggested an association between these antibodies and the development of fibrosis, and not a causation. Interestingly, these findings resemble those reported by Crespo in kidney transplantation as discussed above.

In a larger cohort of adult liver transplant patients, O'Leary *et al*. reported that AT_1R or ETAR antibodies (either preformed or *de novo*) were associated with a higher risk of rejection.[64] They analyzed pre- and post-transplant serum samples from 1269 liver transplant recipients for AT_1R or ETAR antibodies using commercially available sandwiched ELISA kits. The results showed that non-HLA antibodies alone did not influence the outcome of the transplant, however, when coupled with an HLA-DSA (particularly of the IgG3 subclass), the synergistic association between these antibodies increased the mortality risk significantly (hazard ratio, 1.66; P=0.02). Additionally, they reported that post-transplant non-HLA antibodies were capable of activating the complement system as seen from the positive C4d staining pattern in the liver tissue.

In addition to AT_1R and ETAR antibodies, C-terminal laminin-like globular domain of Perlecan (LG3) antibodies were also investigated in liver transplantation. Xu and colleagues recently published a study in which they tested the pre-transplant sera of 131 transplant recipients who received a second liver for 33 autoantibodies with a commercially available Luminex antibody panel.[14] Among these 33 antibodies, 15 were significantly higher in 52% of the patients who lost their graft. Specifically, patients with antibodies against LG3 experienced worse secondary graft survival compared to those without this particular antibody (P=0.02). Interestingly, patients with increased AT_1R antibody levels in addition to LG3 were at a higher risk for rejection compared to those with either of these antibodies. Similar association was found between LG3 levels and HLA-DSA, which, once again, suggested a synergistic relationship. Therefore, screening for LG3 (in addition to AT_1R and ETAR antibodies) might be important for liver transplant recipients with or without HLA-DSA as it may significantly help in identifying high risk transplant patients.

In summary, while allograft rejection is relatively rare in liver transplantation due to the highly immunotolerant nature of the organ,[64] research into role of non-HLA antibodies, particularly anti-AT_1R and ETAR antibodies in liver transplant patients is gaining the attention of researchers. However, as it stands, research in this area still relatively new and the reported data is limited (Figure 2).

Table 3. non-HLA antibody detection studies in liver transplantation.

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Ekong et al. 2019[65]	Liver	With regards to non-HLA Abs, no significant association was found between anti-nuclear Abs, anti-smooth muscles Abs, anti-liver kidney microsomal Abs, and AT_1R Abs on the development fibrosis	Serum samples from 42 recipients were assessed for AT_1R-Abs and several other non-HLA Abs	Antinuclear Abs were measured by IIF using an IgG-specific conjugate, anti-smooth muscle, and anti-liver kidney microsome Abs were measured using a semi-quantitative ELISA, and AT_1R-Abs were measured using commercial ELISA	Non-HLA Abs data was missing for a number of patients pre-transplant; exclusion of some patients due to lack of HLA-DSA measurement post-transplant reduced the sample size of the study; the study mostly focused on HLA-DSA and little attention was given to non-HLA Abs
Ohe et al. 2014[66]	Liver	In all patients with increased levels for both HLA-DSA and AT_1R-Abs were found to have advanced fibrosis compared to the other groups positive for either Ab or negative for both	Post-transplant sera from 81 patients were screened for AT_1R-Abs	AT_1R-Abs were detected in the sera using a commercially available ELISA kit	Only patients withdrawn from immunosuppression treatment were included; no assessment of Abs after the reintroduction of the immunosuppression medication; due to limited post-transplant serum samples, the status of preformed or de novo Abs was not established
O'Leary et al. 2017[64]	Liver	Preformed non-HLA Abs alone did not impact the clinical outcomes, however, the synergistic association between these preformed Abs and HLA-DSA increased the mortality risk significantly	Pre- and post-transplant serum samples from 1269 liver transplant recipients were analyzed for anti-AT_1R or ETAR-Abs	Commercially available sandwiched ELISA kits were used to measure the levels of anti-AT_1R or ETAR-Abs	This study was retrospective and single centered; only association between non-HLA Abs and HLA-DSA and development of fibrosis could be established
Xu et al. 2021[14]	Liver	Among all tested autoantibodies, patients with Abs against LG3 experienced worse secondary graft survival compared to those without. The combination of LG3 with AT_1R or HLA-DSA showed higher rejection risk	Pre-transplant sera of 131 transplant recipients who received a second liver were tested for 33 autoantibodies	A commercially available Luminex antibody panel was used to screen for the presence of non-HLA Abs	No post-transplant serum samples were assessed for the changes in the levels of the preformed Abs after transplantation and the development of de novo Abs

AT1R, angiotensin type 1 receptor; ETAR, endothelin type A receptor; IIF, indirect immunofluorescence; LG3, C-terminal laminin-like globular domain of perlecan; non-HLA Abs, nonhuman leukocyte antigen antibodies.
**Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section.*

Non-HLA antibodies in heart transplantation

In heart transplantation, the detection of non-HLA antibodies still remains a major endeavor, especially when it was reported that 40% of patients experiencing biopsy-proven ABMR had no HLA-DSA in blood.[67] Investigation into non-HLA antibodies in this field mainly focuses on the detection of the specific non-HLAs, especially the antigens expressed on the endothelium, utilizing approaches such as ELISAs and Luminex.

Hiemann and colleagues utilized both an AT_1R and an ETAR ELISAs to investigate the impact of anti-AT_1R and anti-ETAR antibodies on the development of ABMR in heart transplant recipients.[68] They prospectively assessed the pre- and post-transplant serum samples from 30 patients for the presence of both anti-AT_1R and anti-ETAR antibodies with commercially available sandwiched ELISA kits. The results showed elevated levels of anti-AT_1R and anti-ETAR antibodies present in patients experiencing both cellular and ABMR compared to the patients with no rejection. Furthermore, increased pre-transplant titers of these antibodies were associated with higher risk for an early onset of micro-vasculopathy, implying negative effects post-transplant.[68]

In addition to using ELISAs for AT_1R and ETAR, Jurcevic *et al* developed an ELISA for detection of anti-vimentin antibodies.[13] Pre- and post-transplant serum samples from 109 cardiac transplant recipients were tested for anti-vimentin antibodies up to 5 years after transplantation, and the antibody titers were correlated to the development of transplant-associated coronary artery disease. The mean titers of anti-vimentin antibodies calculated in the period between 1 to 5 years post-transplant were significantly increased in patients who had developed transplant-associated coronary artery disease compared to those free from the disease. Additionally, the assay also helped establish a predictive test for the development of this disease with 63% sensitivity and 76% specificity based on the mean titer of the antibodies detected in the first two years after the transplant. Therefore, by utilizing this ELISA, anti-vimentin antibodies could potentially be used as biomarkers for identifying patients who have a higher risk for developing transplant-associated coronary artery disease.[13]

Luminex technology for the screening of non-HLA antibodies in heart transplantation has also been described. In their study, Zhang and colleagues employed a multiplex beads panel to profile non-HLA antibodies in heart transplant recipients with treated ABMR.[69] Post-transplant serum samples from 13 patients with treated ABMR and/or ventricular dysfunction, and without HLA-DSA were screened for 32 non-HLA antibodies with a commercially available panel. They were able to show that each tested patient had at least one non-HLA antibody identified, with anti-vimentin antibodies being the most frequent in the patient group with treated ABMR with undetectable HLA-DSA. Additionally, they also examined pre-transplant serum samples for anti-vimentin antibodies, and the analysis revealed that 11 out of the 13 study patients were negative for vimentin pre-transplant, however, in 7 of these patients, anti-vimentin antibodies were detected at the time of ABMR suggesting a de novo development of these antibodies post-transplant.[69]

Butler *et al*. also utilized a commercialized Luminex-based multiplex bead panel for the discovery of non-HLA antigens associated with heart transplant rejection.[70] First, a protein microarray was constructed to identify 366 non-HLA targets from a discovery cohort consisting of 12 heart transplant recipients who had positive endothelial cell crossmatch but no evidence of HLA-DSA at the time of biopsy-proven rejection. A commercial multiplex bead array that included 67 non-HLA targets was then used to screen 546 serum samples from 115 heart transplant recipients for non-HLA antibodies. The array identified 18 non-HLA antibodies associated with rejection, among which, 4 antibodies were not previously described as non-HLA targets. Moreover, the analysis showed that of the 18 identified non-HLA antibodies, 5 predicted rejection, and 4 showed a synergistic effect with HLA-DSA. That said, this study did not include pre-transplant serum testing for these non-HLA antibodies, so the absence of these pre-transplant samples makes it difficult to interpret the relation between rejection and the non-HLA antibodies.

Based on the studies discussed above, a clear direction can be observed with regards to the investigation of non-HLA antibodies in heart transplantation. Research currently focuses on utilizing solid-phase assays (i.e., ELISAs and Luminex) for antibody detection, and while these techniques are practical, and allow for bulk sample processing with high throughput, they fail to consider donor and organ specifies (Figure 2). To our knowledge, only one study described the use of donor-specific human aortic ECs as target cells for a flow cytometry crossmatching

assay.[71] However, it was unclear whether all of the recipients included in the study were paired with their organ donors for the crossmatching test based on the description of the assay in the methodology section. Moreover, reactivity to IgM AECAs was used as the readout which does not allow for comparisons with other crossmatching tests that report IgG. Therefore, to properly appreciate the role of non-HLA in heart transplantation, more organ- and donor-specific assays are needed.

Table 4. *non-HLA antibody detection studies in heart transplantation.*

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Hiemann et al. 2012[68]	Heart	Increased levels of anti-AT_1R and ETAR-Abs were present in patients experiencing both ACR and ABMR compared to the patients with no rejection. Increased pre-transplant titers of these Abs was associated with higher risk for an early onset of micro-vasculopathy	Pre- and post-transplant serum samples from 30 patients were assessed for the presence of both anti-AT_1R and ETAR-Abs	Commercially available ELISAs were used to measure anti-AT_1R and ETAR-Abs levels	Small sample size; large number of patients in this study were on assist device support which represents a high-risk group for sensitization events; no associations were established with regards to pre-transplant HLA-DSA
Jurcevic et al. 2001[13]	Heart	Using the in-house ELISA, a predictive test for the development of CAD was established with 63% sensitivity and 76% specificity based on the mean titer of the vimentin-Abs detected in the first two years after the transplant	Pre- and post-transplant serum samples from 109 patients were assessed for vimentin-Abs	An in-house developed ELISA kit was used to measure anti-vimentin IgM levels in the serum	The majority of the post-transplant serum samples were collected 2 years after transplantation even though the patients were followed for 5 years, therefore the data from the first 2 years only were analyzed with regards to the titers of vimentin-Abs and CAD
Zhang et al. 2011[67]	Heart	The assay showed that each tested patient had at least one non-HLA Ab identified, with vimentin-Abs being the most frequent in this patient group	Post-transplant serum samples from 13 patients with treated ABMR and/or ventricular dysfunction, and without HLA-DSA were screened for 32 non-HLA Abs	The non-HLA Abs were detected using a commercial Luminex kit with fluorescence labeled secondary anti-human IgG	The small sample size made it difficult to establish associations between vimentin-Abs and ABMR in the pre-transplant sera; variations in time between the non-HLA testing and the time the biopsies were taken due to follow up of patients from another center
Butler et al. 2020[70]	Heart	18 non-HLA Abs associated with rejection were identified, among which, 4 Abs were not previously described as non-HLA targets. Within the 18 identified non-HLA Abs, 5 of them predicted rejection, and 4 of showed a synergistic effect with HLA-DSA	546 serum samples from 115 heart transplant recipients were screened for non-HLA antibodies	A commercial multiplex bead array that included 67 non-HLA targets was used. Antibody binding was reported as the MFI of IgG with Luminex	No pre-transplant serum samples were tested; important non-HLA antibodies such as AT_1R could not be included in the Luminex panel

ABMR, antibody-mediated rejection; ACR, acute cellular rejection; AT1R, angiotensin type 1 receptor; CAD, transplant-associated coronary artery disease; ETAR, endothelin type A receptor; MFI, median fluorescence intensity; non-HLA Abs, non-human leukocyte antigen antibodies.

**Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section.*

Non-HLA antibodies in composite tissue transplantation

Most hand transplant recipients rarely experience ABMR, however a few reports have emerged attributing vascular rejection to the presence of anti-AT_1R and other non-HLA antibodies.[72–74] Banasik *et al*. investigated the presence of non-HLA antibodies in the post-transplant serum samples of 5 hand transplant patients.[72] The sera were assayed for non-HLA antibodies including AECAs, anti-AT_1R, and anti-ETAR antibodies. AECAs were detected using the TITERPLANE technique whereby slides coated with HUVECs were incubated with the patient serum, and the reaction of the antigen to IgG, IgA, or IgG was visualized with IIF. Antibodies against AT_1R, and ETAR were detected with commercial ELISAs. Pre-transplant serum samples were also analyzed for the HLA status in all patients using the Luminex technique. Anti-HLA antibodies of class I or II were detected in two patients; albeit these antibodies were not DSA. AECA were present with moderate activity in only one patient, and both anti-AT_1R and Anti-ETAR were found with strong reactivity in another patient who had a bilateral transplant and developed 6 acute rejection episodes. Notably, no association was reported between non-HLA antibodies and HLA and the repeated acute rejection episodes experienced by the patient with the bilateral transplantation was attributed to the presence of anti-AT_1R and anti-ETAR antibodies.[72]

Very recently, Sikorska *et al*. investigated the role of non-HLA antibodies in hand transplant rejection.[74] The post-transplant serum samples from 6 hand transplant recipients were assayed for antibodies against AT_1R, ETAR, protease-activated receptor 1 (PAR-1), and vascular endothelial growth factor A (VECGF-A) using commercial ELISAs. Additionally, the levels of proinflammatory cytokines IL-1, IL-6, and IFNγ were also investigated to evaluate the humoral response post-transplant. The authors reported that repeated episodes of rejection were associated with high levels of anti-AT_1R and ETAR antibodies as well as increased levels of ECs activation markers represented by higher titers of anti-VEGF-A and PAR1 antibodies in 1 out of the 6 included patients. Interestingly, this patient did not develop anti-HLA antibodies. With regards to the proinflammatory markers, no elevations were observed for all the three tested cytokines (IL-1, IL-6, IFNγ). Although these findings are promising, it is difficult to highlight the importance of non-HLA in composite tissue transplantation making it difficult to draw firm conclusions at this stage, especially since both of the abovementioned studies base their conclusions on the observation from one single patients.

Table 5. *non-HLA antibody detection studies in composite tissue transplantation.*

Reference	Organ	Overall conclusion	Sample details*	Type of assay*	Key limitations
Banasik et al. 2014[72]	Hand	The repeated occurrence of rejection episodes was associated with high levels of anti-AT_1R and ETAR-Abs in one patient with bilateral hand transplantation	Post-transplant serum samples from 6 patients were assayed for AECAs, and anti-AT_1R and ETAR-Abs	AECAs were detected with the TITERPLANE technique using HUVECs, and the reaction of the antigen to IgG, IgA, or IgG was visualized with IIF. Antibodies against AT_1R, and ETAR were detected with the commercial ELISAs	The study only included 5 patients; only post-transplant serum samples were assessed for non-HLA Abs
Sikorska et al. 2022[74]	Hand	Repeated episodes of rejection were associated with high levels of anti-AT_1R and ETAR Abs, as well as increased levels of ECs activation in 1/6 included patients. No elevations in proinflammatory cytokines (IL-1, IL-6, IFNγ) were observed	Post-transplant sera from 6 hand transplant recipients were assayed for anti-AT_1R, ETAR, PAR-1, and VECGF-A Abs. Proinflammatory cytokines (IL-1, IL-6, IFNγ) were also assayed to evaluate the humoral response post-transplant	Anti-AT_1R, ETAR, PAR-1, and VECGF-A Abs using commercial ELISAs. Proinflammatory cytokines (IL-1, IL-6, IFNγ) were also assayed with ELISAs	Small sample size of 6 patients; no pre-transplant samples were analyzed; unclear how the ELISA protocols were conducted or whether the kits were made in-house or acquired commercially

AECAs, anti-endothelial cells antibodies; AT1R, angiotensin type 1 receptor; ECs, endothelial cells; ETAR, endothelin type A receptor; IIF, indirect immunofluorescence; IFNg, interferon gamma; IL-1, interleukin 1; IL-6, interleukin 6; non-HLA Abs, non-human leukocyte antigen antibodies; PAR-1, protease-activated receptor 1; VECGF-A, vascular endothelial growth factor A.

**Sample details as well as the type of assay pertain only to the non-HLA detection method described in the article and not the entire methodology section.*

Discussion

The relevance and importance of non-HLA antibodies in transplantation in addition to HLA-antibodies is increasingly being acknowledged. In the past decade, several non-HLA detection and screening assays have been developed, resulting in the identification of multiple non-HLA antibodies.

Summary points

- The significance of non-HLA antibodies in organ transplantation is not fully understood yet
- High heterogeneity between study designs results in considerable variability in the reported findings regarding the role of non-HLA antibodies in transplant outcomes
- The clinical relevance of non-HLA antibodies is dependent on the tissue expression of the target antigens that might differ between organs
- As it stands, non-HLA mainly have detrimental effects on transplant outcomes in combination with HLA-DSA
- Donor specific target cells derived from the particular graft site may provide a better risk assessment on the transplant outcome
- Laboratory guidelines to harmonize and validate non-HLA antibody investigation are required

Non-HLA antibodies are associated with a wide range of autoimmune diseases, but can also be produced as antibodies after transplantation, possibly due to increased antigen exposure in the context of tissue dammage.[75–78] It has also been shown that the process of allosensitization to minor histocompatibility non-HLA antigens after previous kidney transplantation affects long-term graft outcomes.[58,79] Most of the potential non-HLA target antigens are ubiquitously expressed throughout the body, resulting in the possible involvement of non-HLA antibodies in all organ transplantations. However, non-HLA antibodies have most extensively been studied in kidney transplantation. Within this line of research, the focus was mostly on the presence of anti-AT_1R antibodies in serum of transplant patients and the effect of several non-HLA antibodies on the allogenic endothelium.[75,80] It has been proposed that anti-AT_1R and anti-HLA antibodies have a synergistic role in mediating kidney allograft rejection through the induction of overexpression of HLA molecules after binding to the ECs.[81] AECAs can be detected with flow cytometry, ELISA, indirect immunofluorescence, and high-density protein array as described in this review, and most assays use either EPCs isolated from peripheral blood or HUVECs as an antigen substrate.[82–85] A commercial kit for EC crossmatching is available, and it uses magnetic coated beads against angiopoietin receptor Tie-2 to isolate EPCs (XM-ONE). However, as discussed in the section describing EC based assays, the current detection methods have several significant limitations.

Many of the assays discussed in this review have reported non-HLA antibodies with variable readouts (IgG, IgM, C1q) which might, in turn, make it difficult to compare and correlate the results obtained from different assays and studies. The lack of uniform read-outs, cut-offs, and the potential confounders by the presence of HLA antibodies (or other non-HLA antibodies) results in studies describing conflicting relations between non-HLA and rejection and/or graft survival, as described in this review. Moreover, it is yet to be resolved whether non-HLA antibodies mediate graft injury themselves, or whether they are produced due to processes following tissue damage. Up until now, it seems that non-HLA mainly have detrimental effects in combination with HLA-DSA. Finally, an ideal antigen identification technique requires considerable degree of sensitivity and specificity. It must be easily reproducible and can be used to screen samples in bulk (i.e., high throughput). Although the techniques discussed in this report fulfil some of these requirements, an ideal antigen detection technique is yet to be developed.

All in all, the identification of new non-HLA antibodies on endothelial cells and other cell types involved in the transplanted organ and their relevance in transplantation require a rigorous step-by-step scientific process, combining relevant experimental models with clinical investigations in adequately phenotyped, preferably prospective cohorts. The data on the involvement of non-HLA in other solid organ transplantations than the kidney is scarce and might need more attention. The use of organ donor-derived cells allows for detection of specific antibody responses against polymorphic proteins that are mismatched between the donor and the recipient. The development of a library of organ specific endothelial cell lines that are devoid of endogenous HLA expression would be necessary. This organ specific non-HLA endothelial cell crossmatch assay could eventually help identify different non-HLA risk profiles for rejection and impaired graft survival, and should be applied in a systematic fashion in all types of solid organ transplantations.

In conclusion, several *in vitro* non-HLA assays have been developed for the screening of non-HLA antibodies pre- and/or post allograft transplantation, mainly in kidney transplantation. Although some of the published studies have common characteristics between the studied cohorts, there is a considerable clinical variability among the patient groups. There are currently barely any laboratory guidelines for the investigation of patients with non-HLA antibodies, resulting in a considerable variability on the accuracy of investigation and heterogeneric study populations that are compared with each other. Additionally, the reported clinical significance for organ transplantation of the non-HLA antibody is variable and is very much dependent on tissue expression of its target antigen, its relationship with HLA-DSA, as well as the inflammatory context in which it developed. Therefore, not all non-HLA antibodies have pathological relevance. To be able to fully elucidate the clinical relevance of non-HLA antibodies, harmonization and validation of existing non-HLA assays is necessary, in addition to a rigorous step-by-step scientific process to identify and test for new and relevant non-HLA antibodies.

Acknowledgments

The PROCARE 2.0 consortium is an inter-university collaboration in the Netherlands that is formed to study the prediction of kidney graft survival by immune profiling and the clinical application in personalized medicine. Principal investigators are S. Heidt (Department of immunohematology and Blood Transfusion, LUMC Leiden), S.P. Berger (Department of Nephrology, UMCG Groningen), J.S. Sanders (Department of Nephrology, UMCG Groningen), B.G.Hepkema (Transplantation immunology, department of Laboratory Medicine), M.Reinders (Department of Nephrology, LUMC Leiden) D.L. Roelen (Department of immunohematology and Blood Transfusion, LUMC Leiden), H.G. Otten (Department of laboratory of Translational Immunology, UMC Utrecht), F.H.J. Claas (Department of immunohematology and Blood Transfusion, LUMC Leiden). We thank H. M. van der Lugt for the support for the preparation of this manuscript.

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CHAPTER 7

Weak expression of terminal complement in active antibody-mediated rejection of the kidney

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Frontiers in Immunology 2022; DOI: 0.3389/fimmu.2022.845301

Abstract

Background

The role of the complement system in antibody-mediated rejection (ABMR) is insufficiently understood. We aimed to investigate the role of local and systemic complement activation in active (aABMR). We quantified complement activation markers, C3, C3d, and C5b-9 in plasma of aABMR, and acute T-cell mediated rejection (aTCMR), and non-rejection kidney transplant recipients. Intra-renal complement markers were analyzed as C4d, C3d, C5b-9, and CD59 deposition. We examined *in vitro* complement activation and CD59 expression on renal endothelial cells upon incubation with human leukocyte antigen antibodies.

Methods

We included 50 kidney transplant recipients, who we histopathologically classified as aABMR (n=17), aTCMR (n=18), and non-rejection patients (n=15).

Results

Complement activation in plasma did not differ across groups. C3d and C4d deposition were discriminative for aABMR diagnosis. Particularly, C3d deposition was stronger in glomerular (P<0,01), and peritubular capillaries (P<0,05) comparing aABMR to aTCMR rejection and non-rejection biopsies. In contrast to C3d, C5b-9 was only mildly expressed across all groups. For C5b-9, no significant difference between aABMR and non-rejection biopsies regarding peritubular and glomerular C5b-9 deposition was evident. We replicated these findings *in vitro* using renal endothelial cells and found complement pathway activation with C4d and C3d, but without terminal C5b-9 deposition.

Complement regulator CD59 was variably present in biopsies and constitutively expressed on renal endothelial cells *in vitro*.

Conclusion

Our results indicate that terminal complement might only play a minor role in late aABMR, possibly indicating the need to re-evaluate the applicability of terminal complement inhibitors as treatment for aABMR.

Introduction

Antibody-mediated rejection (ABMR) is a major reason for impaired function and reduced longevity of the kidney allograft, accounting for about 60% of chronic graft failure in kidney transplant recipients (KTR).[1-5] Understanding the underlying pathoimmunological processes associated with ABMR is crucial for accurate diagnosis and implementation of effective treatment strategies. Former Banff classifications identified ABMR based on the presence of donor-specific HLA-antibodies (DSA), C4d complement deposition, microvascular inflammation, and/or transplant glomerulopathy in renal specimens.[6]

From a traditional perspective, ABMR pathomechanism was thought to be based on the activation of the classical complement pathway, initiated by HLA-Antibodies (HLA-Abs), subsequent binding of C1q, C3-convertase formation, and final cellular cytotoxic damage through C5b-9.[2,7-11] C4d, a split product of the classical and lectin pathway, is relatively stable and binds covalently to renal capillaries. Therefore, C4d was introduced and is still utilized as a diagnostic marker for complement pathway activation.[8,12]

However, recent Banff classifications recognize ABMR subtypes, including C4d-negative ABMR and C4d-positive ABMR without DSA, thereby questioning the universal pathogenic role of the classical complement pathway in ABMR).[4,13-15] These Banff updates indicate that ABMR encompasses a more heterogeneous group, which is possibly linked to diverse pathomechanisms. Moreover, studies evaluating the efficacy of terminal complement inhibitors as a potential treatment for ABMR, showed that terminal complement inhibition was insufficient in preventing ABMR occurrence in the long term.[13,16,17]

A possible reason for the lack of C4d deposition in the biopsies of some ABMR patients and the insufficient efficacy of complement inhibitors might be that the classical complement pathway does not serve as a universal pathogenetic model for ABMR. Coherently, a recent study by Mezőet al. (2019) showed that complement factors in plasma failed to predict ABMR outcome.[18] A more nuanced view on the role of the complement system in ABMR is necessary to precisely define the role of the complement system in its development. However, evaluation of the full complement orchestra with analysis of the consecutive steps of the classical complement pathway activation in ABMR is still missing. We aimed to analyze the role of the classical complement pathway in ABMR, specifically active ABMR, by quantification of representative complement activation products, both in plasma, in kidney transplant biopsies, and upon HLA-Ab binding to renal endothelial cells *in vitro*.

Materials and methods

Study Population and Sample Collection

The study population encompassed a total of 227 KTR of whom blood samples for direct complement measurement were taken and who had either an indication or protocol kidney biopsy at the University Medical Center Groningen (UMCG), the Netherlands, between 2010 and 2020. KTR were recruited with informed consent in the *Transplantlines biobank cohort* study, following the Declarations of Helsinki and Istanbul, with NCT02811835 as ClinicalTrials.gov identifier. The research was approved by the UMCG institutional review board (METc 2008/186).[19]

KTRs were retrospectively included and classified as aABMR, acute T-cell mediated rejection (aTCMR), and non-rejection (NR) patients. All NR patients had a protocol biopsy taken without signs of rejection. Inclusion and exclusion criteria are depicted in Figure 1. 50 KTR were included, of whom 17 were classified as aABMR, 18 as aTCMR patients, and 15 as NR patients. As included patients were diagnosed based on different versions of the Banff guideline depending on the year of consultation, standardized re-evaluation of the diagnosis by the most recent Banff'19 guideline was performed. Re-evaluation according to Banff'19 classification considered presence of HLA-Abs, signs of microvascular inflammation in the biopsy, and C4d deposition.[15] In case antibody screening in serum revealed no HLA-Abs in pre-categorized cases of aABMR, we only included these HLA-Abs-negative patients as aABMR patients, if otherwise clear histopathological signs of microvascular inflammation were present. Coherently, C4d-negative patients were only included as aABMR patients, if histopathological microvascular inflammation was unambiguously identified and serum samples were positive for either HLA-Abs class I or II or both.[15]

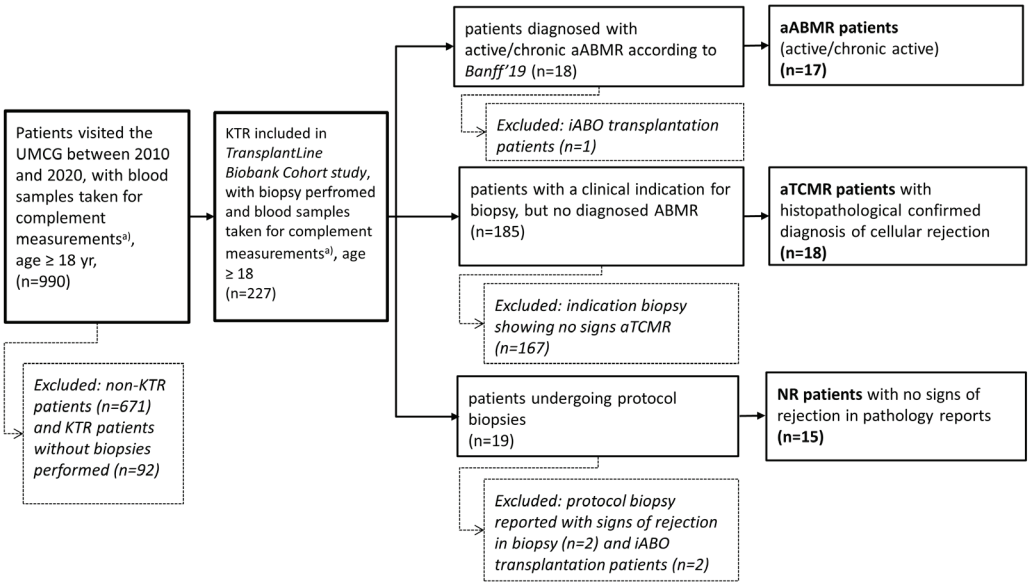

Figure 1. Patient Selection with Inclusion and Exclusion Criteria.

Flow chart representing selection of patients based on exclusion criteria. Total amount of patients excluded and included are depicted as (n). Exclusion of patients is presented in box with dotted lines. KTR, Kidney Transplant Recipients; UMCG, University Medical Center; yr, years; non-KTR, patients without a renal transplant; aABMR, active antibody-mediated rejection; iABO, blood group incompatible; aTCMR, acute T-cell mediated rejection; NR, non-rejection. a) with blood samples directly placed on ice.

Plasma samples taken closest to the date of biopsy were immediately stored at minus 80°C and only thawn directly prior to complement measurements. Clinical data regarding patient demographics, underlying renal disease, transplantation, immunological status, and kidney function around the time of sampling were derived from the hospital electronic patient file (Epic Systems Cooperation, EPIC, Wisconsin, United States). Kidney function was assessed by estimating glomerular filtration rate (eGFR) by applying the Chronic Kidney Disease Epidemiology Collaboration equation.[20] Protein excretion of ≥ 0.5 g per day was defined as proteinuria. Renal biopsy samples were taken based on clinical indication or in the context of protocol biopsies, fixed in formalin, embedded, and stored in paraffin.

Human-Leukocyte Antibody Diagnostics and Complement Quantifications Detection of Donor-Specific Antibodies

The presence of HLA-Abs class I and II n serum was evaluated prior to anti-rejection therapy using the Life screen the Luxe (LsdL), in accordance with the manufacturer's protocol(Immucor Transplant Diagnostics, Norcross, Georgia). Serum samples of aABMR and aTCMR patients, which were tested positive in LsdL class I and/or II were subsequently tested with Lifecodes Single Antigen Bead (LSA; Immucor Transplant Diagnostics) class I and/or II assay respectively. In light of the HLA-donor type, LSA test results were evaluated regarding donor-specificity and mean fluorescence intensity (MFI) by B.G.H. and R.G.L. (Department of Transplant Immunology, Groningen).

Quantification of Systemic C3, C3d, and C5b-9

C3 quantification in patients' plasma was performed using radial immunodiffusion assay (RID) measurement according to Mancini *et al*.[21] C3d and soluble C5b-9 were quantified by enzyme-linked immunosorbent assay (ELISA).[22,23] C3d measurements were preceded by Polyethylene Glycol precipitation for fractionation of C3d.[22] For accurate quantification, standard curves were derived from a serial dilution with a standard sample kindly provided by the University of Leiden for C3d; and by serial dilution of zymosan-activated normal human serum in PTB [PBS (Phosphate Buffered Saline) with 0.05% Tween and 1% Bovin Serum Albumin (BSA)] of defined concentration for C5b-9. The C3d/C3 ratio was determined by dividing the C3d values in ng/ mL by the C3 concentration in ng/mL. Details on antibodies and conjugates used for quantification of respective complement factors are provided in Table 1.

Table 1. Antibodies for ELISA on plasma samples.

Target	Primary antibody	Secondary antibody	Tertiary antibody	substrate	Stop solution	Sample diluent (solution)
C5b-9	Monoclonal mouse anti-human C5b-9, (Dako, MO777) (1:1000)	Polyclonal goat anti-human C5, (Quidel, Ca92121) (1:1000)	Polyclonal mouse anti-goat HRP, (Jackson, 205-035-108) (1:5000)	TMB (Sigma, T0440)	1M H2S04	PTB (PBS with 0.05% Tween, 1% BSA) and 0,01M EDTA (1:3)
C3d	Polyclonal rabbit anti-human C3d, (Dako, A0063) (1:1000)	Polyclonal anti-human C3d-DIG, (Dako) (1:10000)	Polyclonal anti-DIG, (Roche, 11207733910) (1:8000)	ABTS (Sigma, A1888)	N/A	PTB (PBS with 0.05% Tween, 1% BSA) and 0,01M EDTA (1:100)

Antibodies for ELISA analysis on kidney biopsy samples from aABMR, aTCMR, and NR patients. Applied dilutions are given below each antibody used. TMB, 3,3',5,5'-tetramethylbenzidine, N/A, not applicable; ABTS, 2,2'-Azino- bis(3-ethylbenzthiazoline-6-sulfonic acid); PBS, Phosphate Buffered Solution.

Staining of C4d, C3d, C5b-9 and CD59 in Renal Biopsies

Paraffin-embedded renal specimens were stained with rabbit anti-human C4d (clone SP91, Ventana, Benchmark automated immunostainer) according to the manufacturer's protocol, as part of the standardized pathological evaluation of biopsies. C3d, C5b-9, and CD59 staining were performed using in-house stainings. Details on C4d, C3d, C5b-9, and CD59 staining in renal biopsies are given in Table 2. Solutions were prepared with BSA (A9647, Sigma, St Louis, United States) and PBS (17-512Q, Lonza, Wijchen, The Netherlands) with specific dilutions provided in Table 2. Between every incubation step, slides were washed three times with PBS (17-512Q, Lonza, Wijchen, The Netherlands). A renal pathologist (M. vd H.) blindly scored the presence of peritubular capillary C4d according to the recent Banff`19 classification standard (15). For inter-group statistical analysis, semiquantitative scores of the pathologist were categorized as 'C4d positive' or 'C4d negative'. Slides stained for C3d and C5b-9 were digitalized by the Department of Pathology & Medical Biology of the UMCG, using a Hamamatsu slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). Stained slides were blindly graded by M. vdH. in the Aperio ImageScope software (Leica Biosystems, Nussloch, Germany). Complement factors C3d and C5b-9 were scored with a semiquantitative scoring system previously described by Bobka *et al*. (2018) as a method of analyzing complement deposition in biopsies.[24] Glomerular and peritubular semiquantitative scores ranged from 0 to 4, indicating lack of any deposit, weak (≤25%), moderate (≤50%), substantial (≤75%), and intense deposition.

Table 2. *Complement Factor Staining in Biopsies from Kidney Transplant Recipients..*

Antigen	Fixation and Embedment	Deparaffinization	Antigen retrieval	Blocking steps	Antibody	Conjugate 1	Conjugate 2	Substrate	Counterstaining and embedment	Control sample
C3d	Paraffin-embedded tissue	Xylol and Alcohol (100%,96%, 70%) and Demi-water in 6 consecutive steps	Incubation in dark for 30 min, 37°C with 0,4% pepsin from porcine stomach mucosa in 37% 0,1N HCl in demi-water (pH=2.5)	First, 0,01% H2O2 (in 1% BSA with PBS) Second, blocking step with 1% BSA in PBS	Polyclonal rabbit anti-human C3d (A0063, Dako) at 4°C overnight 1:2500 dilution in 1% BSA in PBS	HRP on polyclonal goat anti-rabbit antibody (Dako, P0448) at room temperature 1:100 in 1% BSA in PBS	HRP on polyclonal rabbit anti-goat antibody (Dako, P0449) at room temperature 1:100 dilution in 1% BSA in PBS	0.2 mg/ml 3-Amino-9-ethylcarbazole (Sigma 02431MH), in 50mM Acetate buffer and 0.03%H2O2, (pH=5.5)	Hematoxylin 1:2 for 5 seconds Kaiser's glycerol gelatine embedment (1092420100, Merck, Darmstadt, Germany)	Kidney biopsy of a patient with systemic lupus erythematosus; Pre-transplantation biopsy of non-heart-beating and living donor
C5b-9	Paraffin-embedded tissue	Xylol and Alcohol (100%,96%, 70%) and Demi-water in 6 consecutive steps	Incubation for 30 min in dark at room temperature with 0,1% protease	0,01% H202 in PBS	Monoclonal mouse anti-human C5b-9 (Quidel, A239) at 4°C overnight 1:10000 dilution in 1% BSA in PBS	HRP on polyclonal rabbit anti-mouse antibody (Dako, P0260) at room temperature 1:100 dilution in 1% BSA in PBS+1%NHS	HRP on polyclonal goat anti-rabbit antibody (Dako, P0448) at room temperature 1:100 dilution in 1% BSA in PBS+1%NHS	0.2 mg/ml 3-Amino-9-ethylcarbazole (Sigma 02431MH) in 50mM and 0.03%H2O2, (pH=5.5)	Hematoxylin 1:2 for 5 seconds Kaiser's glycerol gelatine embedment (1092420100, Merck, Darmstadt, Germany)	Pre-transplantation biopsy of non-heart beating donor
CD59	Paraffin-embedded tissue	Xylol and Alcohol (100%,96%, 70%) and Demi-water in 6 consecutive steps	Heat-induced epitope retrieval with Pascal pressure-cooker (DakoCytomation, Glostrup, Denmark) at 115°C for 7 min in 0.2N HCl in Demi-water	0,3% H202 in PBS	Monoclonal mouse anti-human CD59 (Hycult, HM2120) at room temperature for 60 minutes 1:50 dilution in 1% BSA in PBS	HRP on polyclonal rabbit anti-mouse antibody (Dako, P0260) at room temperature 1:100 dilution in 1% BSA in PBS	HRP on polyclonal goat anti-rabbit antibody (Dako, P0448) at room temperature 1:10 dilution in 1% BSA in PBS	0.5 mg/ml 3,3'-Diaminobenzidine (Merck, D5637) incubation for 20 min, in dark at room temperature in PBS + 0,3% H202	Periodic Acid-Schiff counterstaining for 5 and 15 min respectively embedment in Permount™ Mounting Medium (FisherScientific, Loughborough, United Kingdom) after dehydration	Pre-transplantation biopsy of non-heart beating and living donor kidney

HCl, Hydrochloric acid; BSA, bovine serum albumin; HRP, Horseradish peroxidase; NHS, N-Hydroxysuccinimide; PBS, Phosphate Buffered Solution.

Antibody-Mediated Complement Factor Activations in a Renal Endothelial Cell Model

To evaluate whether HLA-Abs can activate the complement cascade up to C5b-9 on renal endothelial cells, *in vitro* experiments using conditionally immortalized glomerular endothelial cells (CiGEnCs) were conducted.[25] In addition, the presence of terminal complement regulator CD59 was analyzed. Details on handling and characterization of the CiGenCs cell-line are provided in the Supplementary Material 2A. Depending on the incubation condition of the complement activation assay, cells were incubated with either human monoclonal HLA-antibodies (A2/A28 IgG1, provided by Dr. F. Claas, Leiden University Medical Center, The Netherlands), or 25% human serum containing HLA-A2 antibodies directed against HLA antigens expressed on the CiGEnC cell membrane, or a pan-HLA-class I antibody (clone W6/32). 25% heat-inactivated blood group compatible serum was used as a control. The following washing steps are described in Supplementary Material 2B. For detection of activated C3, C4d, C5b-9, and CD59, cells were incubated with primary and secondary antibodies, as depicted in Table 3. To exclude apoptotic and necrotic cells, propidium iodide 1 mg/ml (Molecular Probes, Leiden, The Netherlands) was added just before measuring. Activated C3, C4d, and neoantigen C9 deposition on viable non-apoptotic cells were analyzed in a FACSCalibur™ (FACSCalibur, Becton Dickinson, New Jersey, USA). Results were derived from three independent experiments.

Table 3. Antibodies for flow cytometric analysis on CiGEnCs.

Target	Activated C3	C4d	C5b-9	CD59
Primary antibody	Monoclonal Mouse anti-human activated C3 antibody, which recognizes C3b, iC3b, and C3c fragments (Clone bH6, HM2168S, Hycult biotech, Uden, The Netherlands) 1:50	Mouse anti-human C4d antibody (Clone 12D11, HM2229 20UG, Hycult biotech, Uden, The Netherlands) 1:100	Mouse anti-human neoantigen-C9 antibody (HM2264, Hycult biotech, Uden, The Netherlands) 1:100	Mouse anti-human CD59 (HM2120, Hycult biotech, Uden, The Netherlands) 1:100
Secondary antibody	Goat anti-mouse FITC-labelled antibody (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands) 1:100	Goat anti-mouse FITC-labelled antibody (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands) 1:100	Goat anti-mouse FITC-labelled antibody (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands) 1:100	Goat anti-mouse FITC-labelled antibody (Cat. No. 1031-02, SouthernBioTech, Uden, The Netherlands) 1:100

Incubation with primary antibodies was performed on ice for 30 min. Incubation with secondary antibodies was conducted in dark for an additional time of 30 min.

Statistics

Statistical tests were conducted for the baseline characteristics and complement component measurements. Normality was examined by the Kolmogorov Smirnov test for each variable. Categorical parameters are represented as n (%). For continuous data, normally distributed variables are expressed as mean ± standard deviation (SD) and skewed data as median and interquartile range (IQR). All three groups were analyzed by Kruskal-Wallis for non-parametric variables and by one-way ANOVA for parametric variables. Analyses between subgroups were additionally performed for complement measures in serum and biopsy, comparing aABMR with aTCMR patients, and aABMR with NR patients. These two-group analyses were conducted by Mann-Whitney U test for non-parametric and by independent unpaired t-test for parametric data. Correlations between plasma and local complement factors were derived from Spearman's correlation analyses. Computations were performed by SPSS (IBM SPSS Statistics, Chicago, United States) version 23, and GraphPad software 8.0 (GraphPad Software, San Diego, United States) was used for graphical visualization.

Results

Study Population

Among 50 KTR, 17 were identified as aABMR patients according to the Banff '19 classification, 18 as aTCMR, and 15 as NR (Figure 1) (2, 4). Mean age at biopsy was 46.8 ± 12.5 years in the ABMR group, 48.1 ± 16.8 years in the aTCMR group, and 51.1 ± 15.3 years in the NR group. No significant differences were found in demographic characteristics between all groups (Table 4). We included ABMR patients based on biopsy specimens with clear histopathological evidence for ongoing rejection, and only considered cases of active or chronic active ABMR, following the Banff'19 criteria.[15] Thus, KTR with chronic, inactive ABMR were excluded (Figure 1 and Table 5). Within the aABMR group, 3 patients showed acute active ABMR, with the diagnosis made within the first 30 days after transplantation (Table 5). Based on inclusion criteria, all transplants were ABO-compatible.

Table 4. Baseline characteristics of aABMR, aTCMR, and NR patients.

Variable	aABMR* *n=17*	aTCMR* *n=18*	NR *n=15*	*p*
Recipient				
Age at time of transplantation in (yr, mean ± SD)	43,9 ± 13,9	47,5 ± 17,7	49,9 ± 15,6	0,47
Type of transplantation				**0,04**
Living Related (n, %)	3 (17,6)	3 (16,7)	6 (40)	
Living Unrelated (n, %)	7 (41,2)	2 (11,1)	5 (33,3)	
Donation after Brain Death (n, %)	6 (35,5)	9 (50)	3 (20)	
Donation after Cardiac Death (n, %)	1 (5,9)	4 (22,2)	1 (6,7)	
Gender (n, %)				0,07
Female sex	4 (23,5)	11 (61,1)	5 (33,3)	
Primary renal disease (n, %)				0,73
Diabetic nephropathy	0 (0)	2 (11,1)	0 (0)	
IgA nephropathy	4 (23,5)	1 (5,6)	5 (33,3)	
Glomerulonephritis	0 (0)	2 (11,1)	0 (0)	
Polycystic kidney disease	3 (17,6)	5 (27,8)	1 (6,7)	
Pyelonephritis	0 (0)	0 (0)	0 (0)	
Hypertensive nephropathy	3 (17,6)	3 (16,7)	1 (6,7)	
MPO-vasculitis	0 (0)	0 (0)	2 (13,3)	
Unknown	2 (11,8)	0 (0)	3 (20)	
Other	5 (29,4)	5 (27,8)	3 (20)	
Donor				
Gender (n, %)				0,68
Female sex	6 (35,3)	7 (38,9)	8 (53,3)	
missing	4 (23,5)	1 (5,6)	1 (6,7)	
Age at time of donation (yr, median ± IQR)	47 ± 20	54 ± 13)	54,5 ± 20	0,09
Transplant and Transplantation characteristics				
HLA mismatches (n, %)				
mismatches of HLA-AB				0,13
0	0 (0)	4 (22,2)	1 (6,7)	
1	2 (11,8)	4 (21,1)	2 (13,3)	
2	9 (52,9)	6 (33,3)	7 (46,7)	
3	4 (23,5)	2 (11,1)	2 (13,3)	
4	1 (5,9)	1 (5,6)	1 (6,7)	
missing	1 (5,9)	1 (5,6)	2 (13,3)	
mismatches of HLA- DR				0,32
0	4 (23,5)	6 (33,3)	3 (20)	
1	7 (41,2)	10 (55,6)	6 (40)	
2	4 (23,5)	1 (5,6)	4 (26,7)	
missing	2 (11,8)	1 (5,6)	2 (13,3)	
CMV serological status (n, %)				0,08
Donor-/recipient-	7 (41,2)	1 (5,6)	2 (13,3)	
Donor+/recipient-	5 (29,2)	6 (33,3)	4 (26,7)	
Donor-/recipient+	1 (5,9)	5 (27,3)	3 (20)	
Donor+/recipient+	4 (23,5)	6 (33,3)	6 (40)	

Biopsy				
Time since transplantation (yr, mean ± std)	4,4 ± 3,4	1 ± 1,4	0,8 ± 0,3	**<0,01**
Age at biopsy (yr, mean ± SD)	46,8 ± 12,5	48,1 ± 16,8	51,1 ± 15,3	0,71
Rationale for undergoing biopsy (n, %)				**<0,01**
Delayed graft function	1 (5,9)	4 (22,2)	0 (0)	
elevated creatinine level	4 (23,5)	4 (22,2)	0 (0)	
proteinuria (n, %)	8 (47,1)	1 (5,6)	0 (0)	
elevated creatinine and proteinuria	0 (0)	3 (16,7)	0 (0)	
cyst formation	0 (0)	1 (5,6)	0 (0)	
BK viremia	0 (0)	1 (5,6)	0 (0)	
transplantectomy	2 (11,8)	0 (0)	0 (0)	
general kidney function decline	2 (11,8)	4 (22,2)	0 (0)	
protocol	0 (0)	0 (0)	15 (100)	
C4d in biopsy (n, %)				**<0,01**
positive	16 (94,1)	1 (5,6)	0 (0)	
negative	1 (5,9)	17 (94,4)	15 (100)	
SV40 in biopsy (n, %)				0,32
positive	0 (0)	1 (5,6)	1 (6,7)	
negative	15 (88,2)	17 (94,4)	13 (86,7)	
missing	2 (11,8)	0 (0)	1 (6,7)	
Laboratory measurements				
LsdL class I (n, %)				**<0,01**
positive	11 (64,7)	1 (5,6)	0 (0)	
negative	6 (35,5)	13 (72,2)	3 (20)	
missing	0 (0)	4 (22,2)	12 (80)	
LsdL class II positive (n, %)				
positive	14 (82,4)	3 (16,7)	0 (0)	**<0,01**
negative	3 (17,6)	11 (61,1)	3 (20)	
missing	0 (0)	4 (22,2)	12 (80)	
LsdL class I and I (n, %)				**<0,01**
LsdL class I and II both negative	1 (5,9)	11 (61,1)	3 (20)	
At least one of LsdL class I or class II positive	16 (94,1)	3 (16,7)	0 (0)	
missing	0 (0)	4 (22,2)	12 (80)	
eGFR (ml/min/1.73m, median ± IQR)	26 ± 19	17,5 ± 12,1	49 ± 18	**<0,01**
Creatinine Clearance (ml/min, median ± IQR)	34 ± 37	23 ± 15	70 ± 21	**<0,01**
Protein in 24-hour Urine Samples (g/24 h, median ±IQR)	1,5 ± 1,9	0,3 ± 1,0	0,2 ± 0,1	**<0,01**
Proteinuria (>0.5g/24h, n, %)				**<0,01**
yes	13 (76,5)	7 (38,9)	0 (0)	
no	1 (5,9)	10 (55,6)	12 (80)	
missing	3 (17,6)	1 (5,6)	3 (20)	

*Categorical and dichotomous variables are presented as absolute numbers and respective percentages. Continuous, non-parametric variables are presented as median and with interquartile range. Continuous, parametric data is provided as mean and standard deviation. Categorical data, as well as parametric continuous variables, are compared between groups by ANOVA, continuous non-parametric by Kruskal-Wallis test. Statistical significance is defined as P < 0,05. aABMR, active antibody- mediated rejection; aTCMR, acute T-cell mediated rejection; NR, non-rejection; yrs, years; aHUS, atypical hemolytic syndrome; MPO, Myeloperoxidase; IQR, interquartile range; HLA, human leukocyte antigen; CMV, Cytomegalovirus; SV40, simian virus 40; IFTA, Interstitial Fibrosis and Tubular Atrophy; n.a., not applicable; LsdL, Life screen de Luxe; LSA, Lifecodes Single Antigen; DSA, donor-specific antibodies; eGFR, Estimated Glomerular Filtration Rate. *According to Banff'19.*

Presence of HLA-Antibodies

LsdL-tests were performed on serum samples, which were taken at the same timepoints as plasma samples for aforementioned complement measurements. LsdL-tests for HLA-Abs class I and class II in serum were more commonly positive in aABMR patients when compared to TCMR and NR patients (P<0,01 respectively) (Table 4). Specifically, LsdL tests showed that 52,9% of ABMR patients were positive for HLA-Abs class I and 82,4% positive for HLA class II (Table 4).

HLA-Abs were identified as donor-specific in 70,6% of tested aABMR patients and in 16,7% of tested aTCMR patients. Respective mean MFIs of HLA class I of 13187,5 ± 12421 for aABMR and of HLA class II of 129991 ± 11711. For aTCMR with DSA class II, mean MFI was 90000± 9539. DSA-negative aABMR patients showed otherwise clear histopathological evidence for aABMR diagnosis according to the Banff'19 classification (Tables 4, 5).

Table 5. *Detailed immunological, histopathological and clinical information of individual patients included.*

P.		LsdL class I (+/-/np)	LsdL class II (+/-/np)	DSA class I	MFI	DSA class II	MFI	DSA: p.e./dn	Days between biopsy/ diagnosis and trans-plantation	Biopsy type	C4d	C3d glom	C3d ptc	C5b-9 glom	C5b-9 ptc
1	aABMR	+	+	B62	6000			dn	751	InB	C4d+	3	0	1	1
2	aABMR	+	+	A23	16000	DR53 DQ2	40000	dn	3095	InB	C4d+	3	1	1	0
3	aABMR	-	+			DQ2 DQ5	9000		440	InB	C4d+	2	0	1	1
4	aABMR	-	+			DQ2	3000	dn	1485	InB	C4d-	ms	0	1	0
5	aABMR	-	+			DQ6	10000	dn	3453	InB	C4d+	3	1	1	1
6	aABMR	+	+	B38, B51	40000	DR8 DR13 DR52	12000	dn	5	InB	C4d+	3	3	1	0
7	aABMR	-	+						5625	InB	C4d+	1	1	1	3
8	aABMR	+	-	B62	10000			dn	2256	InB	C4d+	3	1	1	0
9	aABMR	-	+	DQ1	20000			dn	3016	InB	C4d+	ms	ms	ms	ms
10	aABMR	+	-	A2	3000			dn	1006	InB	C4d+	3	1	1	0
11	aABMR	-	+			DQ6 DQ8 DR52	27900	dn	1499	InB	C4d+	3	1	1	1
12	aABMR	-	-						3297	InB	C4d+	ms	1	ms	1
13	aABMR	+	+			DR12 DR52 DQ9	14500	dn	7	InB	C4d+	3	1	ms	0
14	aABMR	+	+	A24,Cw9,Cw10	8000	DR13 DR52 DQ8	3500	pe	20	InB	C4d+	3	0	1	0
15	aABMR	+	+			DQ2	16000		969	InB	C4d+	1	1	1	1
16	aABMR	+	+	A24	2500	DR13 DR52	6000	pe	141	InB	C4d+	3	1	1	0
17	aABMR	+	+			DQ6	1000	dn	531	InB	C4d+	2	3	1	2
18	aTCMR	+	+						6	InB	C4d-	1	0	0	0
19	aTCMR	-	-						1921	InB	C4d+	1	0	ms	ms
20	aTCMR	-	-						154	InB	C4d-	1	0	0	0
21	aTCMR	-	-						883	InB	C4d-	1	0	1	0
22	aTCMR	-	-						305	InB	C4d-	ms	ms	ms	ms
23	aTCMR	+	+						451	InB	C4d-	1	1	ms	1
24	aTCMR	-	-						18	InB	C4d-	ms	ms	ms	ms

25	aTCMR	-	-						1111	InB	C4d-	3	0	1	0
26	aTCMR	-	-						15	InB	C4d-	1	0	1	1
27	aTCMR	-	-						77	InB	C4d-	1	1	0	0
28	aTCMR	-	-						55	InB	C4d-	0	1	0	2
29	aTCMR	-	-						142	InB	C4d-	3	1	0	0
30	aTCMR	+	+			DQ7	20000	dn	531	InB	C4d-	1	0	1	0
31	aTCMR	np	np						192	InB	C4d-	0	1	0	1
32	aTCMR	-	+			DR13	3000	p.e	585	InB	C4d-	1	0	0	0
33	aTCMR	-	+			DQ2 DR53	4000	dn	117	InB	C4d-	2	1	0	1
34	aTCMR	-	-						11	InB	C4d-	2	0	1	0
35	aTCMR	-	-						18	InB	C4d-	0	0	ms	0
36	NR	np	np						387	PB	C4d-	1	1	1	0
37	NR	np	np						166	PB	C4d-	2	0	1	1
38	NR	np	np						357	PB	C4d-	0	0	1	0
39	NR	np	np						380	PB	C4d-	ms	ms	1	0
40	NR	-	-						372	PB	C4d-	0	0	0	0
41	NR	np	np						379	PB	C4d-	ms	ms	1	0
42	NR	np	np						392	PB	C4d-	1	0	1	0
43	NR	-	-						358	PB	C4d-	1	1	1	0
44	NR	-	-						388	PB	C4d-	1	0	0	0
45	NR	np	np						180	PB	C4d-	ms	0	ms	1
46	NR	np	np						169	PB	C4d-	1	0	1	1
47	NR	np	np						379	PB	C4d-	ms	ms	1	0
48	NR	np	np						193	PB	C4d-	ms	1	ms	0
49	NR	np	np						190	PB	C4d-	ms	ms	ms	ms
50	NR	np	np						178	PB	C4d-	1	0	ms	0

Histopathological diagnosis and Banff scores are based on Banff'19 classification. Anti-HLA-Abs are screened for in serum with LifeScreen de Luxe (LsdL). C4d deposition are displayed dichotomously in this table, indicating presence (+) or absence (-) in biopsy specimen. Complement factors C3d and C5b-9 were scored semiquantitatively. C3d and C5b-9 scores range from 0 to 4, indicating lack of any deposit, weak deposition (≤25%), moderate deposition (≤50%), substantial deposition (≤75%), and intense deposition (>75%). 25 p., patient; DSA, human-leukocyte antigen donor-specific antibody; MFI, mean fluorescence intensity; p.e., pre-exisiting; dn, deNovo; ABMR, Antibody-mediated rejection; aTCMR, acute T-cell mediated rejection; NR, non-rejection; IndB, biopsy due to clinical indication; protocol biopsy; +, positive test; -, negative test; np, test was not performed; ms, missing sample.

Systemic Complement Activation

Quantification of plasma complement activation markers C3d, and C5b-9 did not show significant differences across groups. C3d/C3 ratio was evaluated as an indication for complement catabolism (26). Both C3 and C3d/C3 did not differ when comparing aABMR with aTCMR (*P*=0,7; *P*=0,4) and when comparing aABMR with NR (*P*=0,1; *P*=0,4) (Figure 2). Similarly, plasma C5b-9 did not differ when comparing ABMR with aTCMR (*P*=0,5) and with NR KTR (*P*=0,9). Plasma levels of C3d and C5b-9 complement factors, as well as C3d/C3 ratio, did not correlate with the intensity of complement deposition in renal biopsies of ABMR patients.

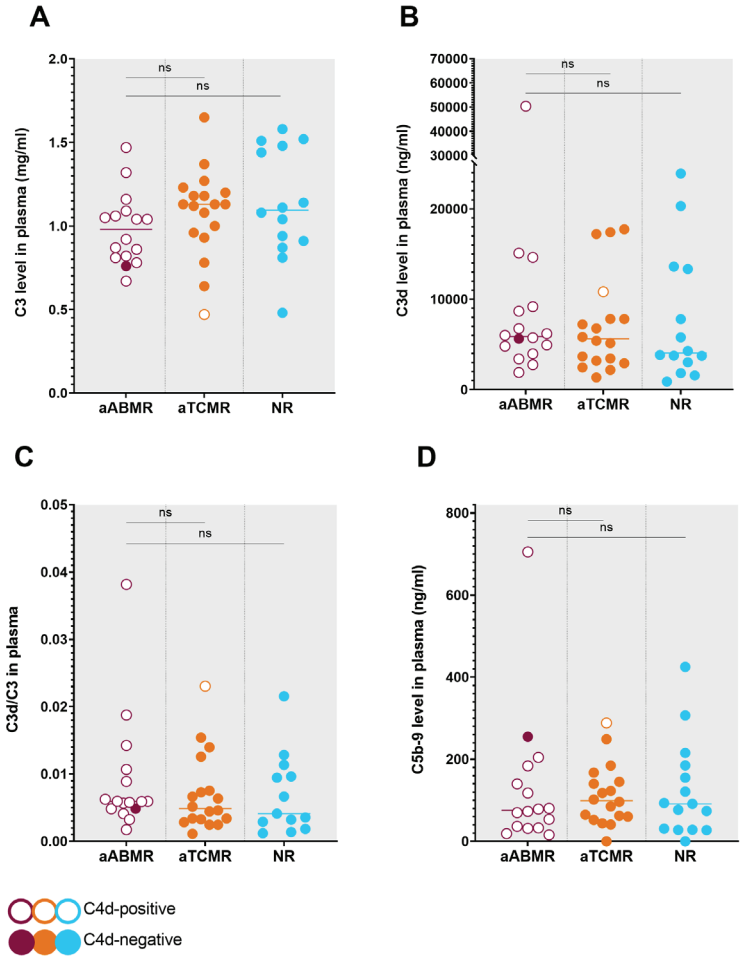

Figure 2. Complement Levels in Plasma of aABMR, aTCMR, and NR patients.

Plasma levels were measured for C3 (A), C3d (B), and C5b-9 (D). Ratios were calculated from respective C3 and C3d levels (C), indicating systemic complement consumption. Circles represent individual patients with open circles indicating C4d-positive patients and filled circles symbolizing C4d-negative patients. P values depict results from Mann-Whitney U statistical analysis. Statistical significance is defined as P < 0.05. aABMR, active antibody-mediated Rejection; aTCMR, acute T-cell mediated rejection; NR, non-rejection; ns, not significant.

Local Complement Deposition in Renal Biopsies

Biopsy samples were chosen with the closest temporal proximity to plasma samples, with a mean time difference of 3,9 ± 44 days. In a number of cases, complement deposition could not be determined due to missing biopsy material or due to missing glomeruli in available specimens (Tables 3, 5).

C4d, as the standard clinicopathological marker, was scored according to Banff'19.[2,15] C4d deposition was clearly linked to ABMR diagnosis, with 16 of 17 C4d-positive patients identified as aABMR patients (Tables 3, 5).

The median score for glomerular C3d deposition in the aABMR group was 3, and in the aTCMR and NR group 1. Glomerular C3d deposition was significantly higher in the aABMR patients compared to aTCMR patients (P<0,01) and NR (P<0,01) (Figure 3A). The median score for C3d in the peritubular capillaries in the AMR group was 1, and in the aTCMR and NR group 0. Expression of C3d in peritubular capillaries was higher in aABMR compared to aTCMR (P=0,04) and NR patients (P=0,03) (Figure 3B). C3d expression appeared more intense in glomerular than in peritubular structures across all three groups (Figure 3).

Median scores for glomerular C5b-9 showed mild intensity (1) for aABMR and NR patients and negativity (0) for aTCMR patients. While glomerular C5b-9 deposition was not significantly increased in aABMR compared to NR patients (P=0,5), glomerular C5b-9 staining was stronger in aABMR compared to aTCMR patients (P<0,01)(Figure 3C). Peritubular capillary deposition of C5b-9 was similar across groups with median semiquantitative scores of 0 in all groups (Figure 3D).

Figure 4 exemplifies the expression pattern in a C4d-positive aABMR patient, who presented with strong C3d deposition in the glomerulus, but only mild C3d in peritubular capillaries and scarce glomerular and no peritubular C5b-9. In comparison, in aTCMR and NR patients, C3d and C5b-9 were absent or only mildly expressed (Figures 5, 6).

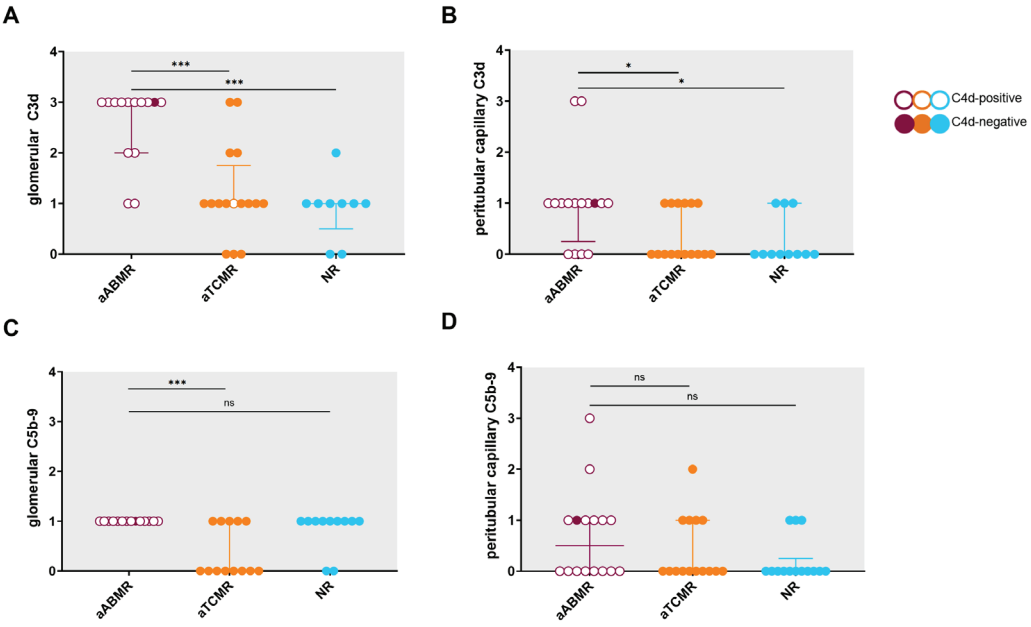

Figure 3. Semiquantitative scores for C3d and C5b-9 deposition in renal biopsy specimens of aABMR, aTCMR, and NR patients.

*Semiquantitative scores range from 0 to 4, with higher scores indicating increased positivity in biopsy. Groups are compared regarding glomerular C3d deposition (A), peritubular capillary C3d (B), glomerular C5b-9 (C), and peritubular capillary C5b-9 (D) deposition. Data represent staining results of individual patients. Error bars represent median with interquartile range. Unfilled circles symbolize biopsies of C4d-positive patients, filled circles C4d-negative patients. P-values are derived from Mann-Whitney U tests with statistical significance defined as *P < 0.05, **P < 0.001; aABMR, active antibody-mediated rejection; aTCMR, acute T-cell mediated rejection; NR, non-rejection; ns, not significant.*

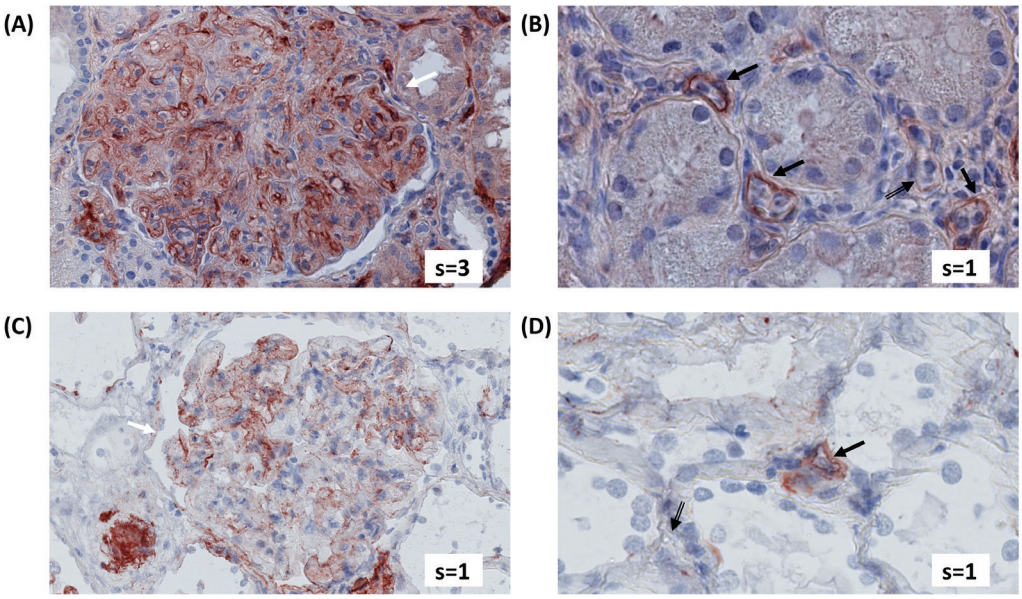

Figure 4. Renal biopsy staining for complement factors in a KTR diagnosed with C4d-positive, aABMR.
Selected section of biopsy slide stained for C3d in glomeruli (A) and in peritubular capillaries (B). Selected section of biopsy slide stained for C5b-9 in glomeruli (C) and in peritubular capillaries (D). White arrows indicate glomeruli and black arrows indicate peritubular capillaries. Double-compound arrows indicate C5b-9 negative peritubular capillaries (D). Semi- quantitative scores (0-4) are indicated in the lower right corner of each picture. KTR, kidney transplant recipients; aABMR, active antibody- mediated rejection; s, semi-quantitative score.

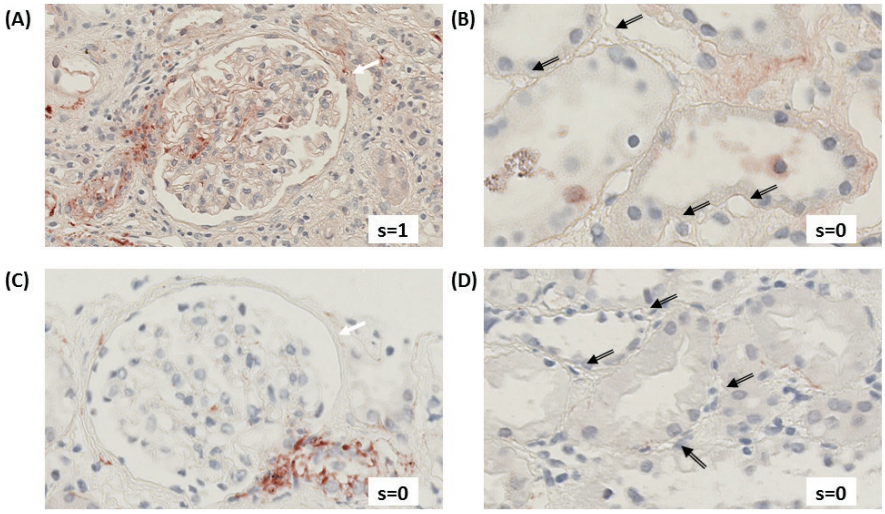

Figure 5. Renal biopsy staining for complement factors in a KTR diagnosed with aTCMR.

Selected section of biopsy slide stained for C3d in glomeruli (A) and in peritubular capillaries (B). Selected section of biopsy slide stained for C5b-9 in glomeruli (C) and in peritubular capillaries (D). White arrows indicate glomeruli and black arrows indicate peritubular capillaries. Semi-quantitative scores (0-4) are indicated in the lower right corner of each picture. KTR, kidney transplant recipients; aTCMR, acute T-cell- mediated rejection; s, semi-quantitative score.

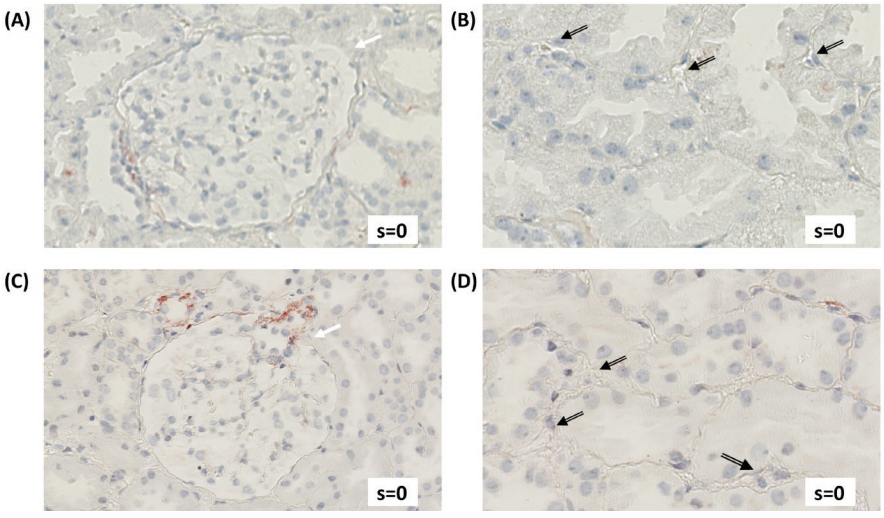

Figure 6. Renal biopsy staining for complement factors in a KTR without indication for undergoing biopsy and without signs of rejection in biopsy (NR patient).

Selected section of biopsy slide stained for C3d in glomeruli (A) and in peritubular capillaries (B). Selected section of biopsy slide stained for C5b-9 in glomeruli (C) and in peritubular capillaries (D). White arrows indicate glomeruli and black arrows indicate peritubular capillaries. Semi-quantitative scores (0-4) are indicated in the lower right corner of each picture. KTR, kidney transplant recipients; s, semi-quantitative score.

Complement regulator CD59 in human renal tissue derived from a pre-transplantation biopsy is depicted in Figures 7A, B. CD59 was clearly expressed in both glomeruli and peritubular capillaries, with stronger deposition in peritubular structures (Figure 7B). CD59 staining in 5 aABMR patients showed a tendency of lower CD59 expression (Figures 7C, D) as compared to pre-transplantation control biopsy (Figures 7A, B).

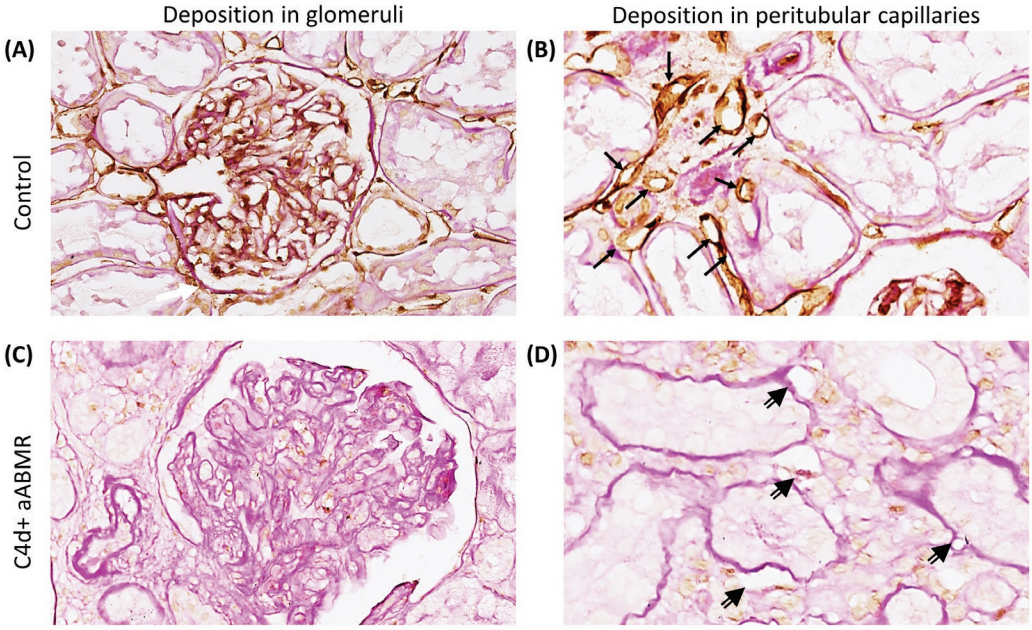

Figure 7. CD59 staining in vivo .

Renal biopsy staining for complement regulator CD59 in glomeruli (A, C) and peritubular capillaries (B, D). Staining was performed on human kidney before transplantation as control (A, B), on biopsy specimen from a C4d-positive (C4d+) aABMR (C, D). Black arrows point to CD59- positive peritubular capillaries, double-compound arrows to CD59-negative peritubular capillaries. aABMR, active antibody-mediated rejection.

Complement Deposition on CiGEnCs

Quantification of complement factors on CiGEnCs showed strong expression of C3d and C4d, but only slight or no deposition of C5b-9 after incubation with HLA-Abs. This expression pattern varied across the different incubation conditions but revealed a general trend of strong C3d and C4d with minor or absent C5b-9 expression. Notably, incubation with IgG HLA-A2 antibodies resulted in expression of complement factors C3d and C4d without simultaneous C5b-9 deposition on the cell surface. In the pan-HLA antibody incubation condition, C5b-9 expression was highest when compared to the other incubation conditions (Figure 8A). Complement regulator, CD59, was constitutively expressed on CiGEnCs, independent of the incubation condition (Figure 8B).

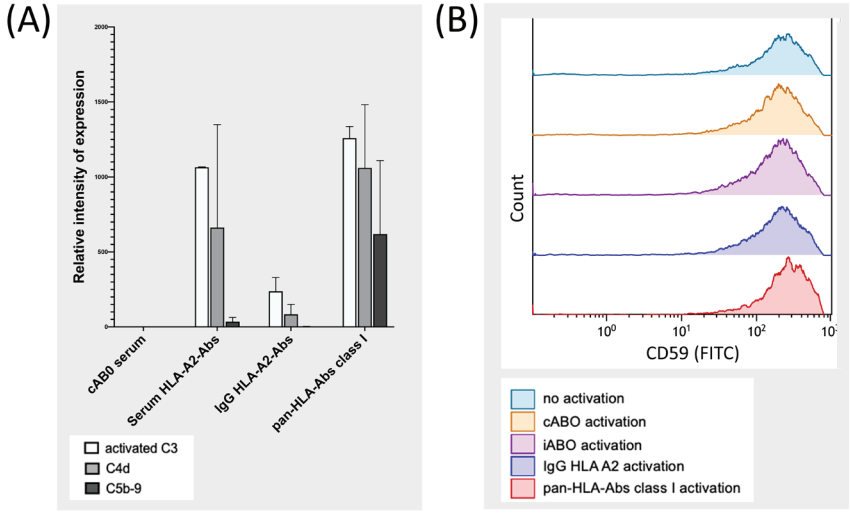

Figure 8. Complement system activation on conditionally immortalized glomerular endothelial cells in vitro in flow cytometric analysis.

Complement factors C3 (activated), C4d, and C5b-9 on conditionally immortalized glomerular endothelial cells in vitro in flow cytometric analysis are depicted in (A) with the four different incubation conditions plotted on the y-axis. Deposition of complement regulator, CD59, was measured in flow-cytometry under five different incubation conditions (B). cABO, ABO-compatible; HLA, Human leukocyte Antigen; Abs, antibodies; IgG, Immunoglobulin G; FITC, Fluorescein isothiocyanate; iABO, ABO-incompatible.

Discussion

In this study, we show robust proximal complement activation in aABMR renal biopsies compared to controls, without or only mild, concomitant C5b-9 deposition. The absence of co-expression of proximal and terminal complement factors might imply that terminal complement is less involved in aABMR pathogenesis. In this line of reasoning, the minor role of terminal complement could explain the disappointing efficacy of terminal complement inhibition as treatment for aABMR, particularly for late, aABMR.[27-30]

Earliest research on immunohistopathology of ABMR by Feucht *et al*. revealed similar results. Next to their primary findings on the role of C4d deposition and its diagnostic value, Feucht *et al*. demonstrated the absence of C5b-9 deposits in C4d-positive ABMR patients.[8]

Terminal complement regulation could account for the absence of C5b-9 deposits in aABMR patients, who did otherwise stain positive for C3d and C4d (Figure 4).[10,31] Other authors indicated that complement regulation and degradation by CD59 could explain why C5b-9 deposition is volatile, discounting C5b-9 as a diagnostic marker in ABMR.[10,32,33] Stronger protection against terminal complement by CD59 compared to the proximal regulators CD46 and CD55 might further explain why C3d and C4d but not C5b-9 was pronounced in aABMR.[34]

We confirmed this assumed influence of CD59 *in vitro*, as it was continuously expressed on CiGEnCs, independent of the antibody stimulation condition. Similarly, we revealed clear endothelial CD59 expression *in vivo*, in pre-transplantation kidney biopsies (Figures 7A, B). Interaction of CD59 and C5b-9 might cause internalization and/or degradation of the CD59/C9 complex, possibly explaining the absence of both C5b-9 and CD59 in ABMR biopsies. Particularly, Cai *et al*. showed that binding to the complement-binding side of CD59 can cause internalization of the complex in cancer cells.[35] Despite these findings, literature on CD59 actions, degradation, and recycling processes is scarce. Thus, interpretations of CD59 expression in our aABMR samples remain speculative. Regarding the sustained CD59 positivity after complement activation *in vitro*, we assume that down-modulation by internalization of the CD59/C9 complex takes more time than the 45-minute time frame used in the *in vitro* complement activation experiments. In short, while the kidney endothelium seems to be highly protected against C5b-9 activation, this protection does not prevent aABMR. The absence of systemic and local C5b-9 fundamentally questions the relevance of terminal complement in aABMR pathogenesis.[11,31]

A broad body of evidence proved the efficacy of terminal complement inhibition in complement-mediated diseases like atypical hemolytic uremic syndrome or paroxysmal sleep hemoglobinuria.[36,37] In contrast, studies on the efficacy of terminal complement inhibition for ABMR presented with more equivocal results.[27-30,38,39] Particularly, these studies were unable to show universal and enduring effectiveness of the terminal complement inhibitor, eculizumab.[27-30] Interestingly, two case studies demonstrated substantial effectiveness of eculizumab in a pre-sensitized child[39] and a young adult,[38] who showed clear deposition of immunoglobulins, C4d, and C5b-9 in renal biopsies. Complement deposits fully resolved within days after eculizumab administration in both case studies. Case studies in which eculizumab was effective as ABMR treatment, showed C5b-9 positivity, stressing the importance of C5b-9 involvement for eculizumab treatment.[38,39] Thus, we do not deny the potential relevance of identifying C5b-9-positive ABMR as an immunophenotypic subtype,[40] although we showed that only a minority of our ABMR patients were C5b-9-positive. Our *in vitro* results provide some indication for why C5b-9 deposition occurs in particular ABMR subgroups. Specifically, they suggest that a particularly high level of antibody-antigen interaction on the endothelial surface, as in the pan-HLA incubation condition, might be able to overwhelm any local acute rejection, partially because early acute ABMR is a rare event in recipients without pre-transplant HLA sensitization. Therefore, our findings are of particular relevance for the majority of ABMR cases in the clinical setting.[41]

In C5b-9 negative aABMR, complement cascade split products like C4a, C3dg, iC3b, and C5a arising proximal to ultimate C5b-9 assembly might be sufficient to cause ABMR by binding to respective receptors on endothelium, creating a pro-inflammatory environment, or recruiting other immunogenic agents.[33,44-46]

Besides the complement system, alternative immunopathogenic pathways could be involved in aABMR, accounting for aABMR in the absence of C5b-9 in biopsies. A broad body of evidence emphasizes complement-independent mechanisms in ABMR pathogenesis, possibly mediated via Natural Killer cells.[47-51] Others emphasized that DSA-binding could directly cause complement-independent endothelial cell activation.[52-55]

To evaluate systemic evidence of complement activation in aABMR, complement factors in plasma were quantified, and no differences were found regarding C3, C3d, and C5b-9 levels between groups. This is in line with recently published findings revealing that ABMR and TCMR with circulating DSAs could not be distinguished by plasma complement factors[18]

Notably, aABMR patients showed increased expression of C3d in glomerular and peritubular structures compared to aTCMR and NR patients. C4d and C3d are considered stable split products that deposit on vascular structures after cleavage of C4 and C3.[11,31] Pronounced C3d deposition in C4d-positive aABMR patients suggests that HLA-Abs cause classical pathway activation at least up to the level of C3 convertase in aABMR.

Other studies indicate that renal, C3d, and not, C4d deposition, could serve as a prognostic factor for graft functioning and survival in KTR with acute rejection.[56,57]

Our study has several strengths. First, we provide a sophisticated level of comparison for complement activation by including a well-defined, biopsy-proven cohort of aABMR, NR, and aTCMR patients. The aABMR group encompasses a relatively homogenous and clinically relevant group, with the majority of ABMR patients showing active or chronic active ABMR, and classical C4d-and DSA-positivity. Second, we provide a holistic picture of complement system activation, with factor quantification on local and systemic levels. This integrative complement analysis is crucial, considering that local and systemic complement system activation did not mirror each other. Third, we strengthened our findings *in vivo* by reproducing similar complement deposition patterns on glomerular, endothelial cells *in vitro*.

Some limitations of our study have to be noted. Our study was based on a small, single-center cohort, with some patients missing biopsy material. Future research should be performed on a larger group of ABMR patients to reflect and address the immunopathogenic heterogeneity of ABMR subgroups. Notably, we mainly included late, chronic active and active ABMR patients, whereas patients with chronic inactive ABMR, early ABMR and DSA-/C4d-negative ABMR are missing or underrepresented in our study. This underrepresentation limits any implications for these particular ABMR subgroups. Chronic active and active ABMR might differ in terms of the underlying complement-related pathogenesis, particularly as chronic active ABMR was more commonly C4d-negative.[58] The possible influence of chronicity on complement activation cannot fully be addressed by our study due to the small sample size. However, as 16 of 17 included aABMR patients were C4d-positive, we do not expect that the chronicity component had a significant effect on complement analyses in our study. In addition, the Banff classification has its limitations and may not always represent the true underlying pathophysiology. For example, one patient classified as aTCMR on the basis of tubulitis and interstitial inflammation. This patient showed C4d-positivity in biopsy, but no other histological evidence for ABMR to fulfil the diagnostic criteria for aABMR. Within the boundaries of the Banff classification, the origin of C4d-deposition in this patient remains unexplained and a potential influence of undetected (non-)HLA-Abs cannot fully be excluded. With regard to the CD59 staining, it should be noted that it was only performed for 5 aABMR patients and preliminary results need to be validated in a larger aABMR population, as well as in aTCMR patients.

In conclusion, local C3d, and C4d are predominant hallmarks in the majority of aABMR patients, indicating upstream complement cascade activation of the classical pathway, which is not reflected by systemic complement activation. Terminal complement regulation, especially in peritubular capillaries, might explain this absence of C5b-9 despite C3d and C4d deposits. Thus, cytotoxicity is probably not mediated through C5b-9 in a significant proportion of aABMR patients. Together with our findings *in vitro*, our results provide sufficient justification to hypothesize that proximal complement factors or Fc-receptor mediated pathways could be more efficient pharmacological targets than terminal complement treatment.

Acknowledgments

Dr. T. F. Claas from the University, Leiden (Groningen, the Netherlands) for kindly providing the HLA-antibodies for *in vitro* experiments; H. Burgerhoff for his advice regarding analyses and graphical display (Figure 3) and A.W. Gomez for his initial help in designing the database and requesting samples.

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Supplementary material and results

Supplementary Material 1. *Individual Banff scores for aABMR and aTCMR patients.*

	Group	n(g)	gs	g	mm	cg	i	t	i-IFTA	ah	v	cv	ptc	C4d	SV40	Histopathological diagnosis and Banff classification score
1	aABMR	12	1	0	0	0	1	1	3	3	0	1	2	3	0	active ABMR
2	aABMR	0		0	1	3	0	0	3	1	1	0	0	3	0	chronic active ABMR
3	aABMR	16	0	3	1	1	1	1	1	0	0	2	1	3	0	active ABMR
4	aABMR	15	2	1	1	2	0	0	0	2	0	1	2	0	0	chronic active ABMR
5	aABMR	13	2	0	1	3	0	0	1	1	0	0	0	3	0	chronic active ABMR
6	aABMR	9	0	0	0	0	0	0	0	0	0	0	0	3	0	active ABMR
7	aABMR	0		0	1	3	0	0	3	3	1	3	0	3	0	chronic active ABMR
8	aABMR	7	0	0	1	3	1	1	2	1	0	2	3	3	0	chronic active ABMR
9	aABMR	10	2	0	1	2	0	0	3	3	0	3	0	3	0	chronic active ABMR
10	aABMR	19	2	1	1	3	2	2	2	1	2	0	1	3	0	chronic active ABMR, Banff IIB, IA
11	aABMR	25	4	1	1	3	3	2	0	0	0	3	1	3	0	active ABMR, Banff IA
12	aABMR	0					0	0	0	0	1	0	1	3	0	chronic active ABMR
13	aABMR	9	0	0	0	0	1	0	0	0	1	0	0	3	0	active ABMR, Banff IIA
14	aABMR	12	0	0	0	0	1	0	0	0	0	0	0	3	0	active ABMR
15	aABMR	10	1	0	1	0	1	1	1	1	0	0	2	2	0	active ABMR
16	aABMR	7	0	0	1	3	0	0	0	0	0	0	0	3	0	chronic active ABMR
17	aABMR	6	0	0	1	0	1	1	1	1			0	3	0	chronic active ABMR**
18	aTCMR	8	1	0	0	0	1	1	0	0	1	0	1	0	0	acute TCMR, Banff IIA
19	aTCMR	8	0	0	0	0	3	3	1	0	0	0	0	3	0	acute TCMR, Banff IB
20	aTCMR	4	1	0	0	0	3	2	1	1	0	0	0	0	0	acute TCMR, Banff IA
21	aTCMR	12	0	0	0	0	2	3	0	0	0	1	0	0	0	acute TCMR, Banff IB
22	aTCMR	7	0	0	0	0	3	3	1	1	0	1	0	0	0	acute TCMR, Banff IB
23	aTCMR	10	1	0	1	0	2	2	2	1	0	3	0	0	0	acute TCMR, Banff IA
24	aTCMR	9	2	0	0	0	0	0	1	0	2	0	0	0	0	acute TCMR, Banff IIB*
25	aTCMR	16	1	0	0	0	3	3	1	0	0	1	0	0	0	acute TCMR, Banff IB
26	aTCMR	7	3	0	0	0	3	2	3	0			0	0	0	acute TCMR, Banff IA**
27	aTCMR	18	1	0	0	0	2	2	0	0	0	0	0	0	0	acute TCMR, Banff IA
28	aTCMR	5	0	0	0	0	1	2	1	0	1	1	1	0	0	acute TCMR, Banff IA
29	aTCMR	10	1	0	0	0	2	3	1	3			0	0	0	acute TCMR, Banff IB**
30	aTCMR	6	0	0	0	0	3	3	0	0	0	0	0	0	0	acute TCMR, Banff IB
31	aTCMR	5	1	0	0	0	3	2	0	0	0	3	0	0	0	acute TCMR, Banff IA
32	aTCMR	11	1	0	0	0	2	2	1	0	0	0	0	0	0	acute TCMR, Banff IA
33	aTCMR	11	0	0	0	0	3	3	0	0	0	1	0	0	0	acute TCMR, Banff IB
34	aTCMR	4	0	0	0	0	1	0	0	0	2	0	0	0	0	acute TCMR, Banff IIB
35	aTCMR	7	0	0	0	0	1	1	0	0	1	0	1	0	0	acute TCMR, Banff IIA

*Individual scores were determined according to the Banff'19 guideline, evaluating glomerulosclerosis (gs), glomerulitis (g), mesangial matrix expansion (mm), glomerular basement membrane double contours (cg), interstitial inflammation (i), tubulitis (t), inflammation in the area of IFTA (i-IFTA), arteriolar hyalinosis (ah), intimal arteritis (v), vascular fibrous intimal thickening (cv), peritubular capillaritis (ptc), C4d and SV40. SV40, Simian virus; IFTA, interstitial fibrosis and tubular atrophy; n, number; a, arteries; TMA, thrombotic microangiopathy; aTCMR, acute T-cell aTCMR patients were not available for Banff-reclassification. ** no arteries present in the biopsy.*

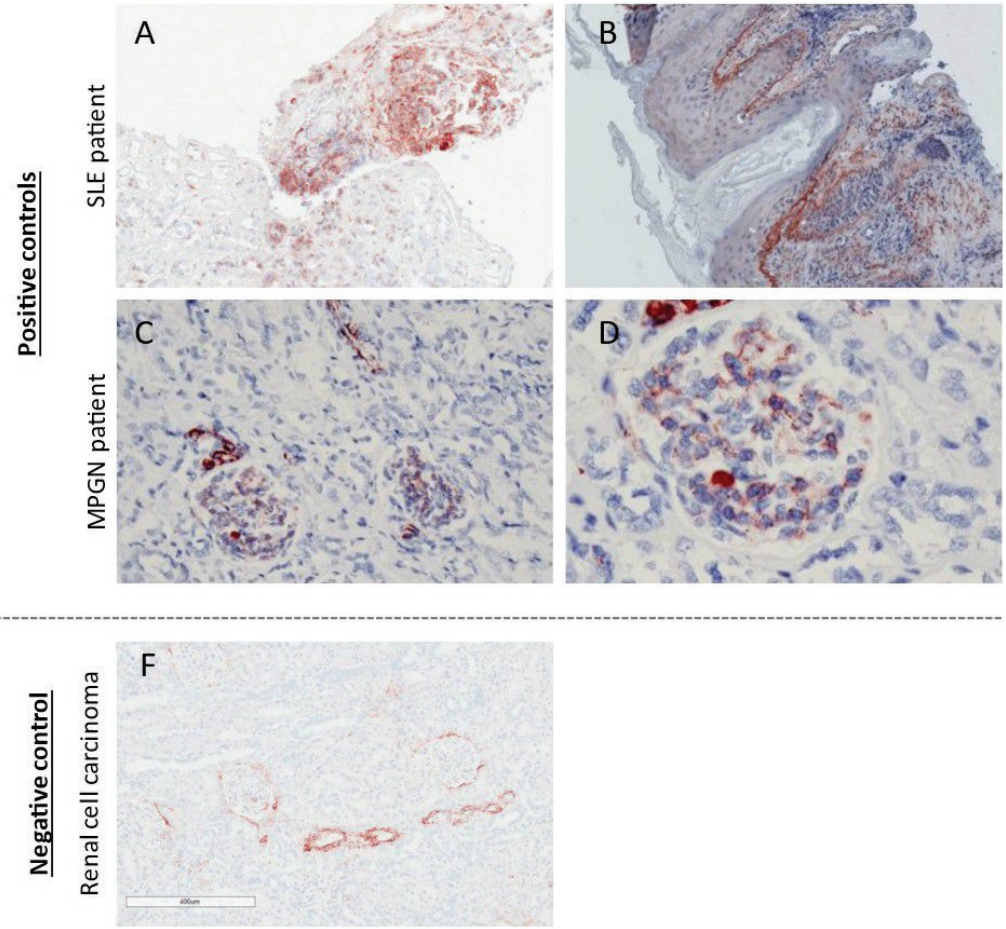
Supplementary Material 2. *In Vitro* Study with Conditionally Immortalized Glomerular Endothelial Cells

Supplementary Material 2.1. Cell Culture Handling

HLA and blood group typing of the CiGEnCs was determined using polymerase chain reaction (PCR) for sequence-specific oligonucleotide probes (SSOP) (Immunocor, Atlanta, USA) and polymerase chain reaction (6645, BAG health care, Lich, Germany), according to the manufacturer's protocol. Cells were cultured in a 6-wells-culture plate in EGM2- MV medium (EGM-2 endothelial med bullet kit, CC-3202, Lonza, 500 mL without VEGF supplemented) until they reached confluence and detached with cell dissociation solution (C5789, Sigma®, Zwijndrecht, The Netherlands) 900µl/1mL at 37°C. This cell suspension was collected in 4.5 mL tubes containing 2mL cell medium (EGM-2 endothelial med bullet kit, CC-3202, Lonza, 500 mL without VEGF supplemented) and centrifuged twice at 250 g for 6 min at 20°C.

Supplementary Material 2.2. Washing steps after HLA-incubation steps, as well as after incubation with FACS-labelling antibodies

Hereafter, cells were centrifuged twice for 6 min at 250 g at 20°C in culture medium, followed by incubation with 20% normal human serum as a complement source for 45 min at 37°C. After incubation, cells were washed once with 2 mL 20°C PBS/1% bovine serum albumin (BSA) (Fluorescence-automated cell sorting buffer) (Number, Sigma®, Zwijndrecht, The Netherlands) at 250 g for 6 min at 4°C and once with 2 mL 4°C FACS buffer at 250 g without a break for 6 min at 4°C. Before incubation with secondary labeling antibodies, two washing steps with ice-cold FACS buffer (Number, Sigma®, Zwijndrecht, The Netherlands) at 250 g without a break for 6 min at 4°C were performed.



Supplementary Material 3. Positive and Negative Controls for the in-house C5b-9 staining.

Positive controls for the used C5b-9 in-house staining protocol as described in Table 2. The following tissues were used as positive controls with evident C5b-9 expression in A. renal biopsy tissue of a patient with diagnosed systemic lupus erythematosus, B. skin tissue of a patient with diagnosed systemic lupus erythematosus and C. renal biopsy tissue of a patient with membranous proliferative glomerulonephritis. As negative control for the in-house C5b/9 staining, renal cell carcinoma tissue was used and is depicted in 3.F. SLE, systemic lupus erythematosus; MPGN, mesangial proliferative glomerulonephritis.

time difference between blood and biopsy in days	number of aABMR patients (n=17)	Percentage (%)
-7,00	1	5,9
-1,00	3	17,6
,00	8	47,1
1,00	3	17,6
122,00	1	5,9
missing	1	5,9

Supplemental Material 4. *Time difference between date of blood and biopsy sampling for aABMR patients. Date of blood sampling included the serological samples for anti-HLA-Abs diagnostics and plasma samples for complement measurements. Time difference is provided in days. n, number.*

CHAPTER 8

General discussion and future perspectives

Discussion

In the Eurotransplant region, ten-years-death-censored graft survival is around 75-80% for patients receiving a kidney from a living donor and 65-70% for patients receiving a kidney from a deceased donor.[1] As the complement system plays an important role in the cause of acute and chronic kidney allograft failure, we explored in this thesis the pathomechanisms of the complement system in kidney transplant recipients, focusing on both systemic and renal cellular complement activation. We showed the importance of novel complement assays to determine the complement profile, screen for the optimum choice of complement inhibition and monitoring patients upon treatment.

PROTEINURIA-DRIVEN, ALTERNATIVE PATHWAY ACTIVATION IN KIDNEY TRANSPLANT RECIPIENTS

Urinary complement markers and kidney transplantation

In Chapter 2 of this thesis, we have shown the presence of properdin and soluble (s)C5b-9 in the urine of kidney transplant recipients (KTR), and hypothesized that this could indicate involvement of the alternative complement pathway (AP) in chronic allograft failure. In clinical practice, surveillance of allograft health after transplantation relies on invasive biopsy procedures that enable pathologic assessment of graft failure and rejection.[2–6] In addition, current noninvasive methods to assess graft function and rejection are primarily based on serum creatinine and urinary protein measurements.[7] These markers are unspecific for rejection and therefore numerous urinary and serum biomarkers have been evaluated as potential alternative rejection biomarkers.[8–12] Both clinical and experimental work showed a critical contribution of the alternative pathway (AP) in the pathogenesis of several renal diseases. Literature has shown that properdin is an important mediator in proteinuria induced tubular epithelial damage.[13,14] We thus hypothesized that properdin and/or the membrane attack complex; C5b-9, are present in the urine of kidney transplant patients with proteinuria,[15,16] and may have an important role in the proinflammatory and pro-fibrotic effects that directly contribute to chronic tubulo-interstitial damage and graft loss. Next to showing the presence of properdin and sC5b-9 in the urine of KTR, we furthermore assessed the potential of urinary properdin and sC5b-9 as novel biomarkers for chronic allograft failure. Indeed, we could show that graft survival is reduced in kidney transplant patients in whom properdin and sC5b-9 are detectable in urine. This association was independent of proteinuria and kidney function. Also in patients without overt proteinuria, we showed that properdin, sC5b-9 or both properdin and sC5b-9, were associated with worse graft survival. Interestingly, the prevalence of both death censored and overall graft failure were significantly higher in the patients with the combination of both properdin and sC5b-9 present in the urine. More importantly, not only the presence of properdin and sC5b-9 was significantly associated with graft failure, but both factors were robustly associated with graft failure when analyzed as continuous parameters, pointing toward a dose-dependent effect. It could be possible that locally produced properdin or filtered properdin with other small complement components cause intratubular complement activation, that leads to progressive transplant failure even in the absence of manifest proteinuria.17 It is generally believed that small amounts of proteinuria,

defined as proteinuria <0.5g/24h, are harmless in KTR.[18,19] According to the American Society of Transplantation guidelines, it is the persistent proteinuria of >0.5g/24h for at least 3-6 months that is considered significant. However low-grade proteinuria, that is often referred to as 'subclinical proteinuria', might be less harmless than originally described.[20] Also within the low-grade proteinuria KTR group, it has already been shown that with every 0.1g/24h increase in proteinuria the risk for graft loss rises with 25%.[21] Our observation that adjustment for proteinuria, defined as >0.5 g/24h did not alter the prospective association of properdin and sC5b-9 measurements, supports our hypothesis that AP complement activation could be an important driving force of chronic graft failure. Urinary properdin and sC5b-9 might be novel noninvasive biomarkers that could add important supplemental information for treatable complement mediated injury processes when compared to proteinuria alone.

Alternative pathway complement activation on proximal tubular epithelial cells

In recent years a debate has emerged on whether properdin, as the single positive regulator of the AP, also functions as a pattern recognition molecule by direct binding to specific surfaces and thereby forming an initiation site of AP activation. Epithelial cells in the renal tubules, that barely express complement regulatory proteins, may be particularly susceptible for properdin pattern recognition, and this could be followed by complement activation during proteinuria after kidney transplantation, leading to allograft failure. Our group revealed that, under proteinuric conditions, properdin binds to heparan sulphate proteoglycans on proximal tubular epithelial cells (PTECs). These studies also revealed that the properdin binding to HSPGs occurs via the heparan sulphate glycosaminoglycan side chains and that the binding of properdin to PTECs was significantly different from binding to endothelial cells.[22,23] However, Harboe *et al*. showed that properdin binding to endothelial cells is dependent on the initial binding of C3b.[24] We thus questioned whether prior deposition of C3b is also necessary for properdin binding to PTEC, as this would essentially direct therapeutic approaches targeting complement activation in the tubules.

In **Chapter 3** we investigated the mechanism of properdin mediated complement activation on the tubular epithelium. We identified and characterized molecular structures on PTECs that are able to interact with properdin. We demonstrated that both serum-derived and recombinant properdin bind to PTECs and showed that binding of properdin to PTECs is independent of prior C3b deposition. Syndecan-1 is one of the most important heparan sulfate proteoglycans, and by utilizing syndecan-1 knockout PTECs, we showed that properdin binding largely depends on syndecan-1. This binding can be dose-dependently inhibited by Salp20 *in vitro*, but not with the C3 inhibitor Compstatin, showing direct binding of properdin to the tubular surface. On the contrary, subsequent C3 activation via the AP was effectively blocked by Compstatin and also Salp20. Heparin(oids) and Salp20 could also block the binding of properdin to C3b. Salp20 mediated blocking of both the binding of C3b and HSPGs to properdin suggests that C3b, HSPGs and Salp20 share the same binding epitope on properdin. However, others have shown that the epitopes for sulfated glycoconjugates and C3b on properdin are positioned very close to each other, but are not the same. These studies showed that C3b and HSPG binding is dependent on trombospondin type I repeats (TSR 4 & 5) in the properdin monomer, but that trypsinisation of properdin resulted in a cleaved TSR5 domain, abolishing C3b binding while still allowing sulfated

glycoconjugates to bind.[25,26] In addition, van den Bos *et al*. recently showed that C3b binding to properdin occurs mainly via the TSR5 domain.[27] Yet, Salp20 blocks both C3b and HSPGs binding to properdin. All in all, data suggests that the binding site for C3b and glycosaminoglycans on properdin is probably different, although very close, insinuating glycosaminoglycan binding to properdin via TSR4 and C3b via TSR5.
AP activation is involved in many proteinuric renal diseases, as described in the introduction of this thesis. The dual effects of Salp20 could be a useful way to effectively block alternative pathway activation in proteinuric diseases. The applicability of Salp20 has already been demonstrated in pre-clinical setting by reducing AP activation and subsequent injury in elastase induced aortic aneurysms and Ovalbumine-induced asthma.[28] When aiming at going towards an ultimate introduction of Salp20 in clinical practice, the immunogenicity of Salp20 has to be addressed after which the overall clinical safety and efficacy of this strong inhibitor should be tested in future trials.

ANTIBODY-MEDIATED COMPLEMENT ACTIVATION IN KIDNEY TRANSPLANT RECIPIENTS

Primary renal endothelial cells to study antibody-mediated complement activation

As endothelial cells (ECs) are the main players in vascular homeostasis and inflammation, and are aligned in the inner layer of the blood vessel, they are also a direct alloimmune target in kidney transplantation. In order to be able to study ECs from the vasculature of the kidney, we developed in a renal EC isolation technique. In **Chapter 4** we showed that primary renal microvascular and glomerular EC can realistically be derived from numerous donors with a wide variety of blood group and human leukocyte antigen (HLA)-types. We developed a straightforward procedure to isolate machine perfusion derived primary renal endothelial cells (MP-PRECs) from the perfusion fluid of human donor kidneys by a combination of negative selection of monocytes/macrophages, positive selection by CD31 Dynabeads and propagation in endothelial specific culture medium. At the site of the endothelium, the immunogenic reactions are mediated by both innate and adaptive immune mechanisms. HLA antibodies can recognize and bind to endothelial cells and subsequently activate the classical route of the complement cascade. As an additional result of complement activation by these donor specific antibodies (DSAs), the cellular immune response is stimulated by directly activating antigen-presenting cells, including monocytes and T-cells.[29–31] The recognition of the detrimental role of HLA antibodies was one of the most important advances in transplantation medicine.[32,33] The alloimmune response mediated by HLA antibodies plays a key role in the failure of kidney allografts.[33] However, there is a wide spectrum of graft injury related to these antibodies, ranging from florid rejection to no recognizable damage.[34,35] These variations in pathogenic potential of anti-HLA DSAs highlight the need to carefully characterize the pathogenicity of DSAs and their interaction with the donor endothelium after transplantation in a nuanced manner. A major advantage of isolating MP-PREC is that the HLA typing and blood group of the EC donor is known, saving time and costs before cross-matching can start. CD31 Dynabeads were used to select the endothelial cells from the perfusate, as CD31 is known to be a general endothelial cell marker.[36] For further endothelial phenotyping, also Tie2/TEK and CD34 were selected as general endothelium-resticted markers.[36,37] vWF was selected as a macrovascular

marker, VEGFR-2 as a glomerular and peritubular microvascular marker, PV-1 as a peritubular microvascular marker, and podoplanin was selected as a lymphatic endothelial marker.[38–42] The MP-PRECs were positive for endothelial cell markers from these different vascular compartments of the kidney; they were positive for CD31, von Willebrand Factor, CD34, VEGFR-2, Tie2/TEK, and PV-1, all to variable extents. HLA class I was constitutively expressed, and HLA class II either constitutively expressed or could be induced by IFN-γ. These differences in EC markers between different donors could be due to alterations in culture composition, indicating endothelial cell dedifferentiation over time or, alternatively, overgrowth of MP-PRECs from a specific segment over time.43,44 We also found that the distribution of vWF, VEGFR-2, PV-1, and HLA-DR within one culture varied for each donor. This likely reflects cultured MP-PRECs obtained from different renal microvasculature structures, having an arterial, glomerular, peritubular, or venous origin.[36,39–41] The MP-PREC biobank could therefore be a rich source for creating different immortalized renal endothelial cells, which differ in phenotype. However, it is important to realize that the usefulness of the MP-PRECs could be limited by shifts in their *in vivo* gene expression signature. This could be due to the loss of micro-environmental cues and by the early onset of senescence.[45,46] In the future, we should select the MP-PRECs based on their gene expression profile, in the context of the application of interest. In line with other modified cell lines, the immortalized cell lines should then be validated thoroughly.[47]

As explained in the introduction of this thesis, due to the lack of proper and reliable sources of renal EC, the ability to properly investigate (antibody-mediated) rejection at the site of the endothelium is still limited.[48–51] Consequently, antibody-mediated rejection (ABMR) remains a diagnostic and therapeutic challenge. In **Chapter 4** we additionally showed potential diagnostic value of a renal endothelial biobank in a kidney specific endothelial cross match test. This allows us to study the pathogenetic role of various types of DSAs (anti-HLA I, anti-HLA II, and anti-endothelial antibodies on complement mediated endothelial cell injury and/or death. We postulate that the MP-PRECs form an attractive basis for the development of assays to assess the pathogenicity of HLA antibodies and detect non-HLA antibodies against the renal endothelium.

HLA and non-HLA antibodies in kidney transplantation

Approximately 30% of the patients on the waiting list for a kidney transplant in the Netherlands are sensitized against alloantigens. Allogenic sensitization is defined as a cumulative panel reactive antibodies (PRA) percentage >0%.[52] Sensitizing events leading to the formation of antibodies against HLA are pregnancies, blood transfusions, and previous transplantations.[53] Serum of patients on the waiting list is regularly screened for antibodies by the HLA antibody screening assays and with the classic complement dependent cytotoxicity (CDC) cross match. Sensitized transplant candidates may experience prolonged waiting times,[54] as the presence of these pre-transplant HLA antibodies leads to exclusion of donors with the antigens to which the antibodies are directed. In addition to this, patients can form antibodies against targets other than HLA.[55] In **Chapter 5** we utilized the primary renal endothelial cells (MP-PRECs) described in **Chapter 4** to perform EC crossmatch studies. In **Chapter 5**, we describe the accelerated rejection of a blood group compatible, living related donor kidney, in the absence of DSA pre- or post-

transplantation. The biopsy taken during the rejection episode showed features of C4d negative ABMR including extensive hemorrhagic areas and loss of renal vascular ECs. Anti-endothelial cell antibodies (AECAs) were identified in crossmatch assays using MP-PRECs from various donors. Two years later, after desensitization for presumed AECA with plasmapheresis and rituximab, a successful second transplantation was performed. The success of the desensitization procedure could be monitored with our MP-PREC cross-match assay. The results suggest that the patient had antibodies against ECs and that effective reduction of complement mediated renal endothelial cytotoxicity upon treatment before transplantation can be monitored. Multiple cases of acute allograft dysfunction with histological signs of ABMR but without (detectable) circulating DSAs have been described.[56–58] This phenomenon has partly been addressed in a cohort study of 935 transplantations.[59] In this cohort, 208 patients who underwent a biopsy for a specific indication or patients who underwent protocol biopsies met the histological criteria of ABMR the according to Banff 2015 and 2017 classification.[4,5] However, it was found that 59% of the patients had no detectable DSA at time of biopsy. Unlike for HLA-antibodies, it not known yet if there is a distinct relation with (classical) sensitizing events and non-HLA-antibody formation.[60] Also, these antibodies are also known to occur in healthy individuals.[61,62] It could very well be that due to graft injury induced by ischemia reperfusion injury and DSA binding to the donor endothelium or chronic inflammation results in the exposure of cryptic antigens and formation of neoantigens.[63,64] This suggests that, as sensitizing mechanism, non-HLA antibodies can be considered to arise de novo early post-transplantation or following acute rejection, or as autoantibodies that bind self-antigens.[63,65] As the allograft vascular endothelium represents the first contact surface between the recipient's immune system and the donor organ, a significant proportion of non-HLA antibodies that are associated with allograft rejection are directed against antigens expressed by endothelial cells. However, also antigens expressed on the underlying basement membrane and the extracellular matrix of the endothelial cells that may only be expressed under certain circumstances related to allograft injury, may be targeted.[60,66]

Current immunological risk stratification for patients awaiting a kidney transplant is based on the detection of circulating HLA antibodies reacting with lymphocytes. Although lymphocytes have HLA antigen expression in common with the graft, they have a differential repertoire of cell surface antigens as compared to endothelial cells. A well-known example is the absence of blood group antigens on lymphocytes while these are present on endothelial cells. Unfortunately, despite the aforementioned increasing evidence for these antibodies to be involved in rejection, screening for non-HLA anti-endothelial antibodies (AECA) has not yet been implemented in clinical practice. Our report illustrates the need for further studies evaluating in depth the mechanisms leading to hyperacute, acute and late rejection caused by AECAs.

In **Chapter 6** we review the literature and report current non-HLA antibodies assays and provide an overview of non-HLA crossmatch assays developed for use in solid organ transplantation, either in a research setting or commercially. The term non-HLA antibody in transplantation covers auto-reactive and allo-reactive antibodies, specific for targets other than HLA. However as said, the nature of these antibodies is still largely unknown, and to date no

widely accepted assays exist. Immune triggers that lead to non-HLA antibody formation and pathogenicity are complex and poorly understood.[57,60,67] The ability of non-HLA antibodies to mediate allograft injury may depend upon their affinity and strength (titer), target specificity, density of the target antigen, and synergy with donor-specific HLA antibodies. The discovery of new targets against which non-HLA antibodies develop, is ongoing.[68–70] Although several non-HLA antibody specificities have been identified, the current available assays are limited by identifying only the known non-HLA antibodies.[71,72] It seems likely that not all relevant antigens are included in the current available assays, underlining the need for solid *in vitro* crossmatch assays that aid in studying these differences.[72] Efforts to develop reliable and sensitive diagnostic non-HLA antibody tests are continuously made. This is essential, considering the technical difficulties of non-HLA antibody assays and the large variation in reported incidences of antibodies, resulting in highly heterogenic study outcomes. However, despite the heterogenic study designs and partly conflicting results, the clinical relevance of non-HLA on graft survival should not be neglected. In order to be able to fully elucidate on the clinical relevance of non-HLA antibodies, future studies should harmonize and validate the existing non-HLA assays, in addition to a rigorous step-by-step scientific process to identify and test for new and relevant non-HLA antibodies.

Profiling systemic and renal complement activation in patients with renal transplantation and antibody-mediated rejection

Despite the fact that the histopathological heterogeneity of ABMR is increasingly acknowledged and reflected in diagnostic standards, pathomechanistic models to comprehensively address this heterogeneity are missing.[2,73] To be more precise, the role of complement activation in the development of ABMR is insufficiently understood. In **Chapter 7** we analyzed systemic and local complement activation in kidney transplant recipients with ABMR, cellular rejection, and without rejection. Compared to the patients experiencing cellular rejection or without rejection, plasma complement activation was not increased in ABMR patients. On a local level, only C4d and C3d, but not C5b-9 depositions, were more pronounced in the biopsies from patients with ABMR. In the *in vitro* renal EC assay similar to the one described in **Chapter 4** and **Chapter 5**, we replicated these findings and showed classical complement pathway activation by C4d and C3d deposition, but minor C5b-9 deposition. The kidney endothelium appeared to be effectively protected against C5b-9 activation, although this protection does not seem to prevent ABMR. In order to be able to investigate the prognostic value of local C3d for graft failure in ABMR, it would be interesting to expand the number of biopsies and to extend the follow up period. Others have already indicated that C3d might be valuable in KTR with rejection though this has not been thoroughly studied to date.[74] Initial analysis on our small ABMR cohort is promising, showing peritubular C3d deposition linked to graft failure.

The presence of complement regulatory proteins like CD46, CD55 or CD59 on the endothelial cell membrane might account for the absence of C5b-9 in ABMR patients.[75] To test this hypothesis, we investigated the presence of CD59 in the biopsies of ABMR patients and deposition of CD59 on endothelial cells *in vitro*. We found a constitutive expression of CD59 *in vivo* and in pre-transplant biopsies *in vitro* on renal ECs. Surprisingly, post-transplantation no CD59 was found in ABMR biopsies. This finding needs further exploration, but could possibly be due to

internalization of CD59/C9 complexes.[76] Earliest research on immunohistopathology of ABMR by Feucht *et al*. in 1993 revealed that proximal depositions of complement activation products C4d and C3d were present in a kidney biopsy with ABMR, yet no convincingly terminal complement activation was found. Based on the findings by Feucht *et al*. and confirmations by following research, C4d was established as a robust and sensitive marker for ABMR. Guidelines started to be developed stating that C4d needed to be present for diagnosis of ABMR. In addition to that, the C5 complement inhibitor eculizumab, that proved to be effective in complement driven diseases like aHUS, started to be investigated in trials with patients with for ABMR.[77–79] Some case studies describe impressive therapeutic effectiveness in ABMR with eculizumab.[80–82] However, in recent years more studies describe cases in which patients are highly suspected of having ABMR based on the morphology of the rejected transplant, but no C4d staining is present. The diagnosis C4d-negative ABMR emerged and also the most recent Banff diagnostic criteria was adapted. In addition to that, Nishi *et al*. reported the absence of co-deposition of C4d and C5b-9 in peritubular capillaries in kidney transplant recipients with acute rejection.[83] Moreover, studies showing disappointing results with treatment with eculizumab started to emerge.[84,85] These facts place a question mark behind the universal role of complement in ABMR. The results from **Chapter 7** indicate that terminal complement pathway activation is probably not central in the pathogenesis of ABMR. Future studies should, therefore, examine whether complement regulation indeed plays an important role in the lack of involvement of C5b-9 during in ABMR. A possible explanation that needs further exploration would be the development of accommodation, an adaptation of the graft resulting in resistance against the acute pathogenic effects of DSAs and the fixation of complement,[86,87] that is primarily recognized in AB0-incompatible transplantation. Biopsies from AB0-incompatible transplants regularly show C4d deposition without other signs of rejection, suggesting regulatory mechanisms to prevent terminal complement activation.[87] In AB0-incompatible transplantation, the endothelium appears to become resistant to blood group antibody-induced complement-mediated lysis by upregulating its expression of cytoprotective proteins, including complement regulatory proteins CD55 and CD59.[88,89] Our data on CD59 *in vivo* and on glomerular endothelial cells *in vitro* might point to a similar involvement of complement regulation in ABMR. Other processes that potentially limit host EC injury by complement, could be clearance of complement by ECs, (with or without the antibody)[90] or exocytosis/endocytosis, as is described for C5b-9 on neutrophils.[91]

In addition, to the accommodation theory, our data presented in **Chapter 7** could also suggest that complement independent mechanisms might account for the heterogeneous picture in ABMR, and thus explain why patients with ABMR do not always benefit from complement inhibition.[72,77,78,92,93] Complement-independent pathomechanisms via Fc-receptors (FcR) could be involved in ABMR; a mechanism referred to as antibody-dependent cellular cytotoxicity (ADCC). Accumulating evidence suggests that natural killer (NK) cells are important mediators of ADCC in ABMR. Experimental models as well as analysis of clinical biopsies have shown that NK cells are increased in ABMR and that their activation status is increased when patients have DSA.31,[94–97] In addition, NK cell depleted mice show a reduced incidence of acute ABMR, however these mice still develop chronic ABMR.98,99 Taken together, multiple parallel mechanisms of EC activation could contribute to the development of acute ABMR and chronic ABMR.

Future perspectives

In this thesis we focused on the potential mechanism of tubular complement activation in the proteinuric setting and we showed the potential of heparins and Salp20 in inhibiting properdin binding to proximal tubular epithelial cells, which might result in reducing alternative pathway mediated renal tubular injury. Since the reduction of proteinuria is a major therapeutic goal in reducing the risk for progression of renal injury, it is very interesting that Salp20 can inhibit both the binding of C3b and HSPG to properdin. This bimodal inhibiting effect should be further explored for developing an AP-blocking agent for proteinuric patients. The focus should be on constructing non-immunogenic analogues and on determining the exact properdin-binding epitope. The latter could be tested *in vitro* and in experimental (animal) models for their AP-inhibiting potential. With regard to heparins, it is interesting to study whether non-anticoagulant heparins or related glycomimetics can inhibit complement activation on tubular cells and reduce injury , as for instance in experimental proteinuria models.

The results presented in this thesis support the interpretation that C5b-9 plays a relatively minor role in most cases of ABMR compared to proximal complement components, consistent with the failure of the C5 inhibitor Eculizumab to prevent and resolve ABMR. We also presented data suggesting that CD59 expression on renal endothelial cells may play an important role in limiting C5b-9 formation in ABMR. After AB0-incompatible transplantation, endothelial cells appear to become more resistant against antibody-induced complement-mediated cell lysis through upregulation in the expression of cytoprotective proteins like complement regulatory proteins CD55 and CD59. These findings support the concept that the capacity of donor organ cells to regulate complement can influence the susceptibility to antibody-mediated and complement-dependent allograft injury.

Whilst there are differences in the characteristics of HLA- and AB0-antibodies, and desensitization therapy in HLA-incompatible transplantation seems to be less effective when compared to AB0-incompatible transplantation, the hypothesis of the occurrence of some form of accommodation in the context of HLA-DSAs explaining the variance of damage associated with circulating HLA atibodies is intriguing. As we also developed a robust method to isolate primary renal endothelial cells, it will be interesting to perform experiments utilizing these renal endothelial cells, in which we expose endothelial cells to AB0i and HLAi serum and measure complement regulation and damage markers.

In addition to complement regulation, ADCC as a complement-independent mechanism might be an important mediator in the development of ABMR without the presence of C5b-9. Apparently complement activation is not detrimental in all cases and it is important to define the conditions for complement-dependent and complement-independent antibody-mediated rejection. Quality and quantity of involved antibodies might be pivotal in this context. From cross matching comparison studies it appears that it is both the nature of the heterogenous mixtures of antibodies and the particular set of antigens on the target cell that mainly determines the effectiveness of antibody-antigen binding and thus further immune system activation. The renal

endothelial cell bank (Chapter 6), covering the majority of known and probably also unknown HLA and non-HLA polymorphisms, could help us in unraveling the pathogenic effects of various antibodies and their regulation on a cellular level. In the near future, further development of the MP-PREC crossmatch assay into routinely applicable assays is necessary. In order to achieve this, the primary cells need to be immortalized and characterized in depth. Additionally, the MP-PRECs might also facilitate the investigation of a broad array of non-HLA polymorphisms. As patients may have a mixture of HLA and non HLA antibodies immortalized primary ECs need to be made devoid of endogenous HLA expression.

The EC crossmatch test could add important value to the recent immunological risk markers and could, after thorough validation, also clinically be relevant to predict or diagnose rejection. Integration of the more traditional risk markers like (pe-) transplant antibody profile, recipient age and HLA mismatches with other, newer, risk markers may result in a more personalized and complete risk profile. This risk profile can then be used for more precise donor-recipient matching, posttransplant surveillance and assess personalized immunosuppression adequacy (Figure 1). In addition, the MP-PRECs might aid in further *in vitro* studies to understand the mechanisms leading to hyperacute, acute and late rejection caused by HLA and non-HLA antibodies on an endothelial level.

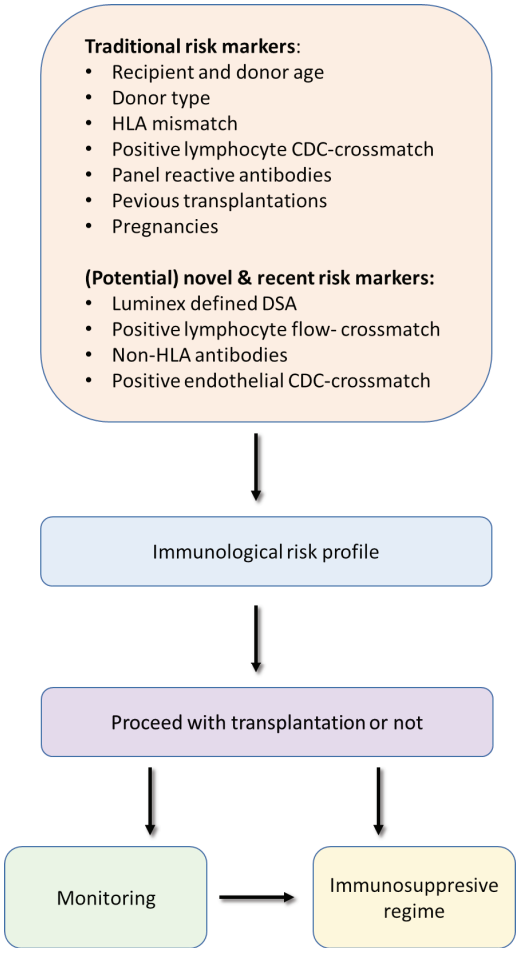


Figure 1. Immunological risk assessment.

The observations described in this thesis that machine perfusion of donor kidneys leads to detachment of ECs are intruiging. Machine perfusion is a relatively new technique, that is now routinely being used in kidney transplantation in The Netherlands. However, especially in the case of ECs, much is unknown about the impact of machine perfusion. It will be interesting to study whether endothelium in DCD (donation after circulatory death) donors is affected differently compared to donation after brain death (DBD) kidneys. Mainly the effects of brain death on endothelium and complement activation have been described, resulting in endothelial dysfunction. In contrast, the effects of DCD are much less clear. Future studies might correlate transplantation outcomes to observed changes in endothelial phenotype during and after machine perfusion of the various donor kidneys, but also to the number and phenotype of the endothelial cells that have detached from the renal vasculature.

The introduction of highly effective complement treatments in complement mediated diseases like aHUS has demonstrated that treatments targeting complement are feasible and have an enormous potential. This means that complement oriented research has to bridge the gap between bench and bedside in order to develop a rational approach to complement targeted treatment. Hopefully, the approaches described in this thesis will eventually aid in reducing the incidence of rejection, improve graft survival and the development of complement diagnostics.

COMBAT

This thesis was conducted in the context of the COMBAT Consortium, COMplement: Basic mechanisms, Assay development and Translation. The consortium was funded by the Dutch Kidney Foundation and led by prof. dr. C. van Kooten. It brought researchers together from the Universities of Leiden, Utrecht, Nijmegen and Groningen, resulting in an intensive interuniversity collaboration. The overall aim was to investigate the complement related mechanisms in kidney diseases and kidney transplant rejection and to define novel complement biomarkers. The consortium also aimed to develop a complement diagnostic toolbox, which could be used in complement-mediated renal diseases and kidney transplant rejection and would lead to the optimum choice, use and monitoring of complement inhibition. The consortium contained three work packages, WP1-basic mechanisms, WP2-assay development and WP3-translation and started in 2015 and ended in 2020.

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CHAPTER 9

Nederlandse samenvatting / Dutch summary
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Nederlandse samenvatting, discussie en vooruitzichten

Patiënten met eindstadium nierfalen kunnen een niertransplantatie ondergaan om niet afhankelijk te zijn van dialyse. De belangrijkste oorzaak van transplantaatfalen is afstoting. In de Eurotransplant-regio (Oostenrijk, België, Kroatië, Duitsland, Hongarije, Luxemburg, Nederland en Slovenië) ligt de tienjaarsoverleving rond de 75-80% voor patiënten met een nier van een levende donor en rond de 65-70% voor de patiënten met een nier van een overleden donor. Bij afstoting wordt de donornier door het afweersysteem van de patiënt herkend als lichaamsvreemd, waarna een afweerreactie tegen de nier op gang komt. Daarbij spelen donorspecifieke T-lymfocyten en/of donorspecifieke antilichamen een rol. Laatstgenoemde antilichamen kunnen weefseleiwitten (HLA-antigenen) op de cellen van de donor herkennen, maar ook een scala aan andere donorspecifieke eiwitten (non-HLA-antigenen). Binding van antilichamen aan de donornier kan leiden tot activatie van het complementsysteem. Dit is een afweermechanisme van het lichaam tegen lichaamsvreemde cellen zoals bacteriën, maar ook tegen de niet-eigen cellen van een donornier. Daarnaast kan complementactivatie in getransplanteerde nieren ook op een andere manier kan verlopen, namelijk via eiwitlekkage in de urine. Bij dit proces raakt de filterfunctie van de nier verstoord en lekken de filterorgaantjes in de nieren eiwitten uit het bloed. Die eiwitten komen vervolgens in de urine terecht. Tussen deze eiwitten bevinden zich ook complementeiwitten, die tubuluscellen van de nier kunnen beschadigen. Ook kunnen complementfactoren in beschadigde nieren uitgescheiden worden via de urine. Omdat het complementsysteem een belangrijke rol speelt bij acuut en chronisch transplantaatfalen, hebben we in dit proefschrift de pathologische mechanismen van complementactivatie onderzocht bij patiënten die een niertransplantatie hadden ondergaan. Daarbij hebben we gefocust op zowel systemische activatie in de circulatie, als op lokale cellulaire complementactivatie in de getransplanteerde nier en in de urine. We hebben het belang laten zien van nieuwe complementtesten, die complementactivatie kunnen aantonen, kunnen helpen in de optimale keuze van complementremming en kunnen bijdragen in het monitoren van patiënten gedurende een behandeling.

Samenvatting en Discussie

Hoofdstuk 1 vormt de inleiding van dit proefschrift en geeft een beschrijving van het complementsysteem. Het complementsysteem is een onderdeel van het aangeboren immuunsysteem en bestaat uit verschillende eiwitten, die elkaar via een kettingreactie kunnen activeren. Het complementsysteem is belangrijk voor de verdediging tegen invasieve micro-organismen en voor het opruimen van veranderde gastheercellen en lichaamsvreemde objecten. Het complementsysteem kan razendsnel reageren op gevaren, omdat het niet getraind hoeft te worden om geactiveerd te worden. Dit in tegenstelling tot het verworven immuunsysteem, dat wel getraind moet worden om in actie te kunnen komen. De activatie van complement kan plaatsvinden door herkenning van 'gevaarsignalen' maar ook door continue, langzame, aspecifieke activatie. Het complementsysteem surveilleert namelijk continu het lichaam op binnengedrongen gevaren. De activatie van het complementsysteem kan resulteren in het direct doden van micro-organismen en veranderde en/of vreemde cellen, het markeren van vreemde lichaamsobjecten of micro-organismen voor opruiming door het immuunsysteem en in het opstarten van een ontstekingsreactie.

Het complementsysteem is al meer dan 100 jaren geleden ontdekt en kan worden geactiveerd via drie verschillende routes. De eerste route, de klassieke route verloopt door binding van C1q aan antigeen-immunoglobulinen-complexen, en daarnaast bijvoorbeeld door bepaalde virussen, gramnegatieve bacteriën of door apoptotische (dode) cellen. De componenten van de klassieke route zijn genummerd van C1 tot C9, waarbij het terminale deel van de keten, C5b-9, het gemeenschappelijke membraan-aanval-complex is van deze drie routes. C1q-binding leidt tot de activatie van de serine-proteasen C1r en C1s. C2 en C4 worden daarna gesplitst door het C1q,r2s2-complex, waardoor het C3-convertase gevormd wordt. Dit wordt ook wel C4bC2a genoemd. De tweede route, de lectineroute wordt met name geactiveerd door suikergroepen op het oppervlak van micro-organismen zoals virussen, schimmels, bacteriën en parasieten. Deze route wordt geïnitieerd door een aantal patroonherkennings-receptoren, die macromoleculaire complexen vormen met mannosebindend lectine serine proteasen 1,2 en 3 (MASP 1, 2 en 3). Na activatie splijten deze C2 en C4 en vormen het C3-convertase. Mannosebindend lectine en verschillende leden van de ficoline- en collectine-families zijn de belangrijkste lectine-route geassocieerde patroonherkennings-receptoren. De derde route, de alternatieve route wordt geactiveerd door oppervlakten van bacteriën, virussen, schimmels, IgA-bevattende immuuncomplexen en tumorcellen, waardoor spontaan gehydrolyseerd C3 wordt gestabiliseerd als complex met factor B. Dit leidt vervolgens tot de vorming van het alternatieve C3-convertase onder invloed van factor D en properdine.

PROTEÏNURIE-GEDREVEN, ALTERNATIEVE COMPLEMENTACTIVATIE IN NIERTRANSPLANTATIEPATIËNTEN

Niertransplantatie en complementmarkers in de urine

In **hoofdstuk 2** van dit proefschrift hebben we de aanwezigheid van properdine en C5b-9 in de urine van niertransplantatiepatiënten gemeten. We hebben de hypothese onderzocht dat dit zou kunnen wijzen op betrokkenheid van de alternatieve route van het complementsysteem in het ontstaan van chronische nierschade. Klinisch wordt de conditie van het niertransplantaat beoordeeld door het nemen van biopten, die het mogelijk maken om eventuele pathologie van transplantaatfalen en afstoting te beoordelen. Daarnaast worden niet-invasieve methoden toegepast, namelijk de bepaling van creatinine in het serum en het meten van eiwit in de urine. Deze markers zijn echter niet specifiek voor afstoting en daarom zijn er in studie verband veel urine- en bloed(serum)-biomarkers onderzocht als mogelijke alternatieve biomarkers voor rejectie. Zowel in klinische als experimentele studies is beschreven dat de alternatieve route van het complementsysteem een belangrijke rol speelt in de pathogenese van verschillende nierziekten. Ook is in de literatuur beschreven dat properdine een belangrijke mediator is in door proteïnurie geïnduceerde tubulaire epitheelschade. Vandaar dat wij de hypothese wilden toetsen dat properdine en het membraan-aanval-complex C5b-9 aanwezig zouden kunnen zijn in de urine van transplantatiepatiënten met proteïnurie, en dat ze daarmee een belangrijke rol kunnen spelen en direct kunnen bijdragen aan chronische tubulo-interstitiële schade en het verliezen van het niertransplantaat. Naast dat we in dit hoofdstuk hebben laten zien dat properdine en oplosbaar C5b-9 inderdaad aanwezig kunnen zijn in de urine van niertransplantatiepatiënten, hebben we onderzocht of properdine en C5b-9 in de urine nieuwe biomarkers zouden kunnen zijn voor het ontstaan van chronisch transplantaatfalen. En inderdaad konden we laten zien dat de levensduur van het transplantaat verminderd is bij niertransplantatiepatiënten bij wie properdine en C5b-9 te meten is in de urine. Deze associatie was onafhankelijk van de nierfunctie en de hoeveelheid eiwit in de urine. Ook bij patiënten met duidelijke proteïnurie konden we laten zien dat properdine en/of C5b-9 geassocieerd waren met een slechtere overleving van het niertransplantaat. Belangrijk was dat niet alleen de aanwezigheid van properdine en/of C5b-9 significant geassocieerd was met het falen van het transplantaat, maar ook dat beide factoren sterk geassocieerd waren met het falen van het transplantaat, wanneer ze geanalyseerd werden als continue parameters. Dit laatste wijst op een dosis-afhankelijk effect. Het onderzoek geeft aan dat lokaal geproduceerde properdine en/of het gefilterde properdine activatie van complement kan veroorzaken op een tubulair niveau. Dit kan vervolgens leiden tot de progressie van nierschade, zelfs in de afwezigheid van proteïnurie. Over het algemeen wordt gedacht dat kleine hoeveelheden proteïnurie, gedefinieerd als proteïnurie < 0.5g/24h, niet schadelijk zijn voor niertransplantatie ontvangers. Volgens de richtlijnen van de Amerikaanse Transplantatie Vereniging veroorzaakt juist de blijvende aanwezigheid van >0.5g proteïnurie/24h gedurende minstens 3-6 maanden schadelijke effecten. Echter, de kleine hoeveelheid proteïnurie, waar vaak naar gerefereerd wordt als 'subklinische proteinurie', zou toch schadelijker kunnen zijn dan aanvankelijk beschreven werd. Ook is al beschreven dat binnen de groep patiënten met een kleine hoeveelheid proteïnurie, elke 0.1g/24h verhoging in proteïnurie het risico op het verliezen

van het transplantaat met 25% verhoogt. Onze observatie dat de correctie voor proteïnurie, de prospectieve associatie met properdine en C5b-9 niet beïnvloedde, ondersteunt onze hypothese dat tubulaire activatie van de alternatieve route van het complementsysteem een belangrijke aanjager is van chronisch transplantaatfalen. Properdine en C5b-9 in de urine zouden wellicht nieuwe, niet-invasieve biomarkers kunnen zijn. In vergelijking met het meten van proteïnurie alleen zouden properdine en C5b-9 in de urine belangrijke aanvullende informatie kunnen geven voor behandelbare door complement gemedieerde schade processen.

Alternatieve complement route activatie op proximale tubulaire epitheelcellen
In de afgelopen jaren is een debat ontstaan over de vraag of properdine, als de enige positieve regulator van de alternatieve route van complement, ook kan fungeren als patroonherkenningsmolecuul. Dit zou kunnen door directe binding aan specifieke oppervlaktes, waardoor dit het begin zou kunnen zijn van alternatieve routeactivatie. Epitheelcellen in de tubuli van de nier, die nauwelijks complementregulerende eiwitten tot expressie brengen, zouden extra gevoelig kunnen zijn voor patroonherkenning door properdine. Als er proteïnurie is na niertransplantatie, zou dit gevolgd kunnen worden door properdine-gemedieerde alternatieve complementactivatie, wat vervolgens zou kunnen leiden tot transplantaatfalen. Onze onderzoeksgroep heeft ontdekt dat, bij proteïnurie, properdine bindt aan heparan sulfaat proteoglycanen (HSPGs) op proximale tubulaire epitheelcellen (PTECs). Ook hebben we laten zien dat de binding van properdine aan HSPGs gebeurt via de heparan sulfaat glycosaminoglycaan zijketens en dat de binding van properdine aan PTECs significant hoger was dan aan endotheelcellen. Aan de andere kant heeft de groep van Harboe en collega' s laten zien dat binding van properdine aan endotheelcellen afhankelijk is van de initiële binding van C3b. We vroegen ons daarom af of C3b- depositie ook nodig is voor properdinebinding aan PTECs, omdat dit uiteindelijk de richting geeft aan de therapeutische benaderingen gericht op het voorkomen van complementactivatie in de tubulus.

In **hoofdstuk 3** is het onderzoek naar het mechanisme van properdine gemedieerde complementactivatie op gekweekte PTECs beschreven. Daarbij hebben we moleculaire structuren op PTECs geïdentificeerd en gekarakteriseerd, die interacties kunnen aangaan met properdine. We hebben laten zien dat zowel properdine uit serum als recombinant properdine bindt aan PTECs. Tevens hebben we laten zien dat binding van properdine aan PTECs onafhankelijk is van eerdere depositie van C3b op PTECs. Syndecan-1 is een van de meest belangrijke epitheliale heparan sulfaat proteoglycanen, en door het gebruik van syndecan-1 knockout PTECs, hebben we laten zien dat properdine binding grotendeels afhankelijk is van syndecan-1. Deze binding kan dosisafhankelijk geremd worden door de properdine remmer Salp20 *in vitro*, maar niet door de C3-remmer Compstatin, wat de directe binding van properdine aan het tubulaire oppervlakte laat zien, onafhankelijk van C3b. Verdere activatie van de alternatieve route werd effectief geremd door Compstatin en door Salp20. Heparin(oids) en Salp20 konden ook de binding van properdine aan C3b blokkeren. Salp20 remt dus zowel de binding van C3b als de binding van HSPGs aan properdine, suggererend dat C3b, HSPGs en Salp20 dezelfde bindings-epitoop hebben op properdine. Aan de andere kant hebben andere onderzoekers laten zien dat de epitopen voor gesulfateerde glucoconjugaten en C3b op properdine erg dicht bij elkaar

liggen, maar niet hetzelfde zijn. Deze studies laten zien dat C3b- en HSPG-binding afhankelijk zijn van de trombospindine type I repeats (TSR 4 & 5) in het properdine monomeer, maar dat trypsinisatie van properdine resulteerde in een gekliefd TSR 5-domein, wat de C3b-binding teniet deed, terwijl de gesulfateerde glycoconjugaten nog steeds konden binden. Daarnaast hebben Van den Bos et al. recentelijk laten zien dat C3b-binding aan properdine voornamelijk gebeurt via het TSR 5-domein. Toch blokkeert Salp20 zowel C3b- als HSPG-binding aan properdine. Alles tezamen suggereren deze data dat de plek van de binding van C3b en glycosaminoglycanen op properdine waarschijnlijk verschillend is, maar wel dicht bij elkaar liggen. Dat wijst erop dat glycosaminoglycaanbinding aan properdine loopt via TSR4 en dat C3b- binding loopt via TSR5.

Alternatieve routeactivatie van het complementsysteem is betrokken bij veel verschillende nierziekten met proteïnurie, zoals beschreven is in de introductie van dit proefschrift. Het tweedelige effect van Salp20 zou een bruikbare manier zijn om effectief alternatieve routeactivatie te kunnen blokkeren in nierziekten met proteïnurie. De toepasbaarheid van Salp20 is al aangetoond in pre-klinische studies, door alternatieve routeactivatie te verminderen en vervolgens ook de schade in elastase geïnduceerde aorta aneurysma's en ovalbumine - geïnduceerde astma. Als we ons richten op het uiteindelijke introduceren van Salp20 in de kliniek, moet de immunogeniciteit van Salp20 (analogen) ongedaan worden gemaakt, waarna we de klinische veiligheid en effectiviteit van deze sterke properdine remmer kunnen testen in toekomstige klinische trials.

ANTILICHAAM-GEMEDIEERDE COMPLEMENTACTIVATIE IN NIERTRANSPLANTATIEPATIENTEN

De isolatie van nier-endotheelcellen om antilichaam-gemedieerde complementactivatie te bestuderen

Omdat endotheelcellen (ECs), als belangrijkste spelers van vasculaire homeostase en inductie van ontsteking, de binnenste laag van een bloedvat vormen, zijn ze ook een direct immunologisch doelwit na niertransplantatie. Om de rol van ECs uit de nier te kunnen bestuderen, hebben we een techniek ontwikkeld om menselijke ECs te isoleren uit donornieren. In **hoofdstuk 4** hebben we laten zien dat primaire ECs verkregen konden worden uit nieren van verschillende donoren, met een grote variëteit in bloedgroep weefsel antigeen (HLA)-types. We hebben een relatief eenvoudige procedure ontwikkeld om endotheelcellen van mensen te isoleren uit machine perfusievloeistof van geperfundeerde donornieren; machine-perfusion derived primary renal endothelial cells (kort MP-PRECs). Dit deden we door een combinatie van negatieve selectie van monocyten/macrofagen, positieve selectie door CD31 Dynabeads en groei in endotheelspecifiek kweekmedium. Op het niveau van het endotheel worden de immunologische reacties gemedieerd zowel door aangeboren als verworven immuunmechanismen. Humaan leukocyt antigeen (HLA) antistoffen kunnen ECs herkennen en eraan binden en vervolgens de klassieke route van de complementcascade activeren. Naast complementactivatie door deze donorspecifieke antistoffen (DSAs), wordt door direct activerende antigeen-presenterende cellen de cellulaire T-cel gemedieerde immuunrespons gestimuleerd. De ontdekking van de nadelige rol van HLA-antilichamen was een van de meest belangrijke ontdekkingen in de

transplantatiegeneeskunde. De allo-immuun-response gemedieerd door HLA-antistoffen speelt een belangrijke rol in het falen van niertransplantaten. Echter, er is een breed spectrum van transplantaatschade gerelateerd aan deze antilichamen, variërend van een duidelijke afstoting tot afwezigheid van herkenbare schade. Deze variaties in pathogeniciteit van anti-HLA DSAs onderstrepen de noodzaak om de interactie van verschillende DSA's met het donorendotheel zorgvuldig te karakteriseren. Hiervoor gebruikten wij endotheel-specifieke fenotypische markers. Allereerst CD31 omdat het bekend is dat CD31 een algemene endotheelcelmarker is. Ook tie2/TEK en CD34 werden geselecteerd voor endotheel fenotypering. Von Willebrand factor (vWF) werd geselecteerd als macrovasculaire marker, vasculaire endotheel groei factor receptor-2 (VEGFR-2) werd geselecteerd als glomerulaire en peritubulaire microvasculaire marker, PV-1 als peritubulaire microvasculaire marker en podoplanin werd geselecteerd als lymfatische endotheelmarker. We vonden dat de MP-PRECs verschillend waren voor endotheelcelmarkers van deze verschillende compartimenten van de nier. Ze waren allemaal positief voor CD31. In variabele hoeveelheden waren ze ook positief voor Von Willebrand factor, CD34, VEGFR-2, Tie2/TEK and PV-1. HLA klasse I kwam continue tot expressie, en HLA klasse II kwam ook continue tot expressie of kon geïnduceerd worden door interferon-γ. Deze verschillen in endotheelmarkers tussen verschillende donoren, maar ook EC-variatie over de tijd, kan wijzen op EC-kweken met variabele hoeveelheid ECs afkomstig uit verschillende compartimenten van de nier. Daarnaast kan het ook wijzen op EC-de-differentiatie over de tijd, of als alternatief, overgroei van MP-PRECs afkomstig uit een bepaald vasculair segment van de donornier. De MP-PREC biobank kan een rijke bron zijn voor het creëren van verschillende geïmmortaliseerde nierendotheel cellijnen, welke verschillen van fenotype. In de toekomst moeten we cellijnen, afkomstig van de MP-PRECs, selecteren op basis van hun gen-expressie profiel, en in de context van de applicatie waarvoor we ze willen gebruiken.

Zoals beschreven in de introductie van dit proefschrift, is wegens het gebrek aan passende en betrouwbare bronnen voor nier-ECs, de mogelijkheid om (antilichaam-gemedieerde) afstoting op het niveau van het endotheel grondig te onderzoeken nog steeds beperkt. De consequentie is dat antilichaam gemedieerde rejectie nog steeds een diagnostische en therapeutische uitdaging blijft. In **hoofdstuk 4** hebben we de mogelijke diagnostische waarde van een nier-endotheelbank beschreven, in een nierspecifieke endotheel-crossmatch-test. Dit gaf ons de mogelijkheid om de pathogene rol van verschillende types DSAs (anti-HLA klasse I, anti-HLA klasse II, en anti-endotheelcel antilichamen) op complement gemedieerde endotheelcelschade en/of dood te bepalen. MP-PRECs zouden een aantrekkelijke basis kunnen zijn voor het ontwikkelen van assays om de pathogeniciteit van HLA-antilichamen te kunnen beoordelen en voor het detecteren van non-HLA-antistoffen tegen nier-endotheel.

HLA- en non-HLA-antilichamen in niertransplantatie

Ongeveer 30% van de patiënten op de wachtlijst voor een niertransplantatie in Nederland blijkt geïmmuniseerd te zijn tegen allo-antigenen. Allogene immunisatie is gedefinieerd als een cumulatieve panel reactieve antilichamen (PRA) percentage van >0%. Zwangerschappen, bloedtransfusies en eerdere transplantaties kunnen allemaal leiden tot immunisatie. De sera van patiënten op de wachtlijst worden regelmatig gescreend voor antilichamen met HLA-antilichaam screeningassays en met de klassieke complementafhankelijke crossmatchtest (CDC-test). Geïmmuniseerde patiënten kunnen noodgedwongen langer op de wachtlijst staan, omdat de aanwezigheid van pre-transplantatie HLA-antistoffen leidt tot de exclusie van donoren met de antigenen, waartegen de DSA-antilichamen gericht zijn. Daarnaast kunnen patiënten antilichamen maken tegen andere doelwitten dan HLA. In **hoofdstuk 5** hebben we de primaire nier-endotheelcellen, zoals beschreven in **hoofdstuk 4**, gebruikt voor EC-crossmatchstudies. In **hoofdstuk 5** hebben we de versnelde afstoting beschreven van een bloedgroepcompatibele nierdonatie, van een levende en gerelateerde donor, in de afwezigheid van DSA pre- of post-transplantatie. Het biopt genomen in de periode van de afstoting liet het beeld van C4d negatieve antilichaam gemedieerde afstoting zien, met uitgebreide hemorragische gebieden en verlies van nier endotheel. Anti-endotheelcel antilichamen (AECAs) werden geïdentificeerd met crossmatchtesten, waarbij we MP- PRECs van verschillende donoren hebben gebruikt. Twee jaar later werd een succesvolle tweede niertransplantatie uitgevoerd, na desensitisatie voor veronderstelde AECA met plasmaferese en rituximab. We konden de effectieve vermindering van de AECAs door deze behandeling voorafgaand aan de tweede niertransplantatie nauwkeurig monitoren met onze MP-PREC crossmatchtest. Dit resultaat toont aan dat de patiënt antistoffen had tegen donor-endotheel en dat effectieve verlaging van complement gemedieerde nierendotheel cytotoxiciteit tijdens behandeling voor de transplantatie gemonitord kan worden. Meerdere casussen van acuut transplantaatfalen met histologische tekenen van antilichaamgemedieerde afstoting, maar zonder (detecteerbare) circulerende DSAs, zijn beschreven in de literatuur. Dit fenomeen is ook beschreven in een cohortstudie van 935 transplantaties. In dit cohort voldeden 208 patiënten aan de histologische criteria van ABMR volgens de Banff 2015 of 2017. Allen hadden een protocollair biopt gehad of een biopt voor een specifieke indicatie. Desondanks had 59% van deze patiënten geen detecteerbare HLA-specifieke DSAs op het moment van het biopt. In tegenstelling tot HLA-antistoffen, is het voor non-HLA-antistoffen nog niet bekend of er een duidelijke relatie is met (klassieke) immuniserende gebeurtenissen en non-HLA-antilichaam formatie. Daarnaast is bekend dat deze antistoffen kunnen voorkomen in gezonde individuen. Het zou heel goed kunnen dat transplantaatschade geïnduceerd door ischemie reperfusie schade, DSA-binding aan het donor-endotheel of chronische inflammatie resulteert in de blootstelling van cryptische antigenen en formatie van neo-antigenen. Dit suggereert dat non-HLA-antilichamen zowel de novo kunnen ontstaan, bijvoorbeeld als gevolg van acute rejectie, of reeds als autoantilichamen die (ook) eigen antigenen binden aanwezig zijn. Omdat het vasculaire endotheel van het transplantaat het eerste 'contact oppervlak' is tussen het immuunsysteem van de ontvanger en het donororgaan, is een heel groot deel van de non-HLA-antistoffen, gericht tegen antigenen die tot expressie komen op ECs. Echter, ook antigenen die voorkomen in het onderliggende basaal membraan en de extracellulaire matrix van de ECs, die alleen tot expressie komen tijdens transplantaatschade, kunnen fungeren als doel.

Inschatting van het huidige immunologische risicoprofiel van patiënten die op de wachtlijst staan voor niertransplantatie, is gebaseerd op de detectie van circulerende HLA-antilichamen die reageren met lymfocyten. Hoewel lymfocyten het HLA-antigeen expressieprofiel delen met dat van het transplantaat, hebben ze een verschillend repertoire van cel oppervlakte antigenen in vergelijking met endotheelcellen. Een bekend voorbeeld is de afwezigheid van bloedgroep antigenen op lymfocyten terwijl deze wel aanwezig zijn op endotheelcellen. Helaas, ondanks het eerder genoemde stijgende bewijs van betrokkenheid van deze AECAs in afstoting, is screening voor deze antistoffen nog niet geïmplementeerd in de kliniek. Onze studie laat zien dat er een noodzaak is voor meer studies die grondig de mechanismen evalueren die leiden tot hyperacute, acute en late afstoting veroorzaakt door AECAs.

In **hoofdstuk 6** geven we een overzicht van de literatuur en rapporteren we de huidige non-HLA-antistof testen en geven we een overzicht van non-HLA-crossmatchtesten die ontwikkeld zijn voor orgaantransplantatie, zowel in de onderzoeksfase als ook commercieel beschikbaar. De term non-HLA-antistof in transplantatie dekt alle auto-reactieve en allo-reactieve antilichamen, specifiek voor andere targets dan HLA. Echter zoals hierboven beschreven is het ontstaan van deze antistoffen nog steeds onduidelijk, en er bestaan ook nog geen algemeen geaccepteerde testen. Immuun triggers die leiden tot de aanmaak van non-HLA-antistof formatie en pathogeniciteit ervan zijn complexe en nog niet goed begrepen processen. De mogelijkheid van non-HLA-antistoffen om transplantaat schade te mediëren kan afhangen van de affiniteit en sterkte (titer), specificiteit voor het doel, dichtheid van het doel antigeen, en ook de synergie met HLA DSAs. De ontdekking van nieuwe antigenen waartegen non-HLA-antistoffen kunnen ontstaan is een continue proces. Ook al zijn er verschillende non-HLA-antistof specificiteiten geïdentificeerd, zijn de huidige beschikbare testen gelimiteerd door het identificeren van uitsluitend non-HLA-antistoffen die bekend zijn. Het is hoogstwaarschijnlijk dat nog niet alle relevante antigenen geïncludeerd zijn in de huidige beschikbare testen, wat de noodzaak onderstreept voor goede *in vitro* crossmatch testen die helpen in het bestuderen van deze verschillen. Pogingen om betrouwbare en sensitieve diagnostische non-HLA-testen te ontwikkelen gaan nog steeds door. Om de klinische relevantie van non-HLA-antistoffen volledig te kunnen beoordelen moeten toekomstige studies geharmoniseerd worden en bestaande non-HLA-testen gevalideerd, dit naast een rigoureus stap-voor-stap wetenschappelijk proces om nieuwe en relevante non-HLA-antistoffen te identificeren en te testen.

Systemische en lokale complementactivatie in patiënten met een niertransplantatie en antistof gemedieerde afstoting

Ondanks het feit dat de histopathologische heterogeniteit van antilichaam gemedieerde afstoting steeds meer herkend wordt en geïntegreerd is in diagnostische standaarden, ontbreken de pathomechanistische modellen om deze heterogeniteit te onderzoeken nog steeds. Om preciezer te zijn, de rol van complementactivatie in de ontwikkeling van antilichaam gemedieerde rejectie is nog steeds niet compleet begrepen. In hoofdstuk 7 van dit proefschrift analyseren we systemische en lokale complementactivatie in niertransplantatiepatiënten met antilichaam gemedieerde afstoting, met cellulaire afstoting en zonder afstoting. In vergelijking met patiënten

met cellulaire afstoting of zonder afstoting, is plasma-complementactivatie niet verhoogd in patiënten met antilichaam gemedieerde afstoting. In de nier echter waren C4d en C3d, maar niet C5b-9 depositie, aanwezig in de biopsieën van patiënten met antilichaam gemedieerde afstoting. In de *in vitro* nier-endotheelcel crossmatch beschreven in **hoofdstuk 4** en **hoofdstuk 5**, konden we deze bevindingen repliceren en klassieke route complementactivatie laten zien door C4d- en C3d-depositie, maar eveneens met weinig C5b-9-depositie. Het nier-endotheel lijkt effectief beschermd te zijn tegen C5b-9-activatie, ondanks dat dit niet beschermend lijkt te zijn voor de ontwikkeling van antilichaam gemedieerde afstoting. Om de prognostische waarde van lokaal C3d voor transplantaatfalen in antilichaam gemedieerde rejectie goed te kunnen onderzoeken, zou het interessant zijn om het aantal nierbiopsieën te vergroten en de follow-up-periode te verlengen. Anderen hebben eveneens laten zien dat C3d waardevol zou kunnen zijn in niertransplantatie ontvangers met afstoting, al is dit tot nu toe nog niet heel grondig onderzocht. Eerste analyses in ons kleine cohort van patiënten met antilichaam gemedieerde afstoting lijkt veelbelovend, en laat zien dat peritubulaire C3d-depositie associeert met transplantaatfalen.

De aanwezigheid van complementregulerende eiwitten zoals CD46, CD55 of CD59 op de endotheelcelmembraan zou de afwezigheid van C5b-9 in patiënten met antilichaam gemedieerde rejectie kunnen verklaren. Om deze hypothese goed te kunnen testen, hebben we de aanwezigheid van CD59 in de biopsieën van de patiënten met antilichaam gemedieerde rejectie en de depositie van CD59 op endotheelcellen *in vitro* onderzocht. Het was verassend dat we na transplantatie geen CD59 konden vinden in de biopsieën van patiënten met antilichaam gemedieerde afstoting. Dit moet verder uitgezocht worden, maar het zou kunnen dat CD59/C9-complexen geïnternaliseerd worden. Onderzoek in 1993 door Feucht. et al. heeft al laten zien dat depositie van complementactivatie producten C4d en C3d aanwezig was in een nierbiopt van patiënten met antilichaam gemedieerde rejectie. Ook deze onderzoekers konden toen geen overtuigende depositie van terminaal complement vinden in de biopten. Op basis van dit onderzoek van Feucht et al. en vervolgonderzoek dat deze bevindingen bevestigde, werd C4d vastgesteld als een robuuste en gevoelige marker voor antilichaam gemedieerde afstoting. Richtlijnen werden opgesteld die vermelden dat C4d aanwezig moest zijn voor de diagnose van antilichaamgemedieerde afstoting. Daarnaast werd begonnen met het onderzoeken van de effectiviteit van het gebruik van de anti-C5 complementremmer eculizumab in klinische trials van niertransplantatiepatiënten met antilichaam gemedieerde afstoting. Eculizumab bleek namelijk zeer effectief te zijn in complement gemedieerde ziekten zoals aHUS. Sommige casestudies beschreven een indrukwekkende therapeutische effectiviteit van eculizumab in patiënten met antilichaam gemedieerde afstoting. Desondanks worden steeds meer casussen beschreven in studies waarin patiënten met een sterke verdenking op het hebben van antilichaam gemedieerde rejectie, geen aanwezigheid laten zien van C4d in het biopt. Hierdoor ontstond de diagnose C4d-negatieve antilichaam gemedieerde afstoting en ook de meest recente Banff diagnostische criteria werden aangepast. Daarnaast hebben Nishi et al. eveneens de afwezigheid beschreven van co-depositie van C4d en C5b-9 in de peritubulaire capillairen in niertransplantatie-ontvangers met acute rejectie. Ook worden steeds meer studies beschreven die teleurstellende resultaten laten zien met behandeling met eculizumab. Deze feiten plaatsen allemaal een vraagteken

achter de rol van terminaal complement in antilichaam gemedieerde afstoting. De resultaten van **hoofdstuk 7** laten zien dat terminale complementactivatie waarschijnlijk geen centrale rol speelt in de pathogenese van antilichaamgemedieerde afstoting. Toekomstige studies zullen daarom moeten onderzoeken of complementregulatie inderdaad een belangrijke rol speelt in de afwezigheid van C5b-9 tijdens antilichaam gemedieerde afstoting. Een mogelijke verklaring die verder uitgezocht moet worden, is de ontwikkeling van accommodatie, een aanpassingsvermogen van het transplantaat endotheel dat resulteert in ongevoeligheid voor de acute effecten van donorspecifieke antistoffen en de fixatie van complement. Dit is een proces dat voornamelijk herkend wordt in bloedgroepincompatibele transplantaties. Biopsieën van bloedgroepincompatibele transplantaten laten geregeld C4d-depositie zien zonder enige tekenen van afstoting. Dat suggereert dat er regulerende mechanismen zijn die preventief terminale complementactivatie kunnen afremmen, mogelijk door de verhoogde expressie van cytoprotectieve eiwitten, inclusief complement regulerende eiwitten CD55 en CD59. Onze data over CD59 *in vivo* en over glomerulaire endotheelcellen *in vitro* zouden op een vergelijkbare betrokkenheid van complementregulatie in antilichaam gemedieerde afstoting kunnen wijzen. Andere processen die mogelijk endotheel schade door complement kunnen voorkomen, zouden het klaren van complement door endotheelcellen kunnen zijn (met of zonder de antistoffen), of exocytose/endocytose, zoals beschreven is voor C5b-9 op neutrofielen.

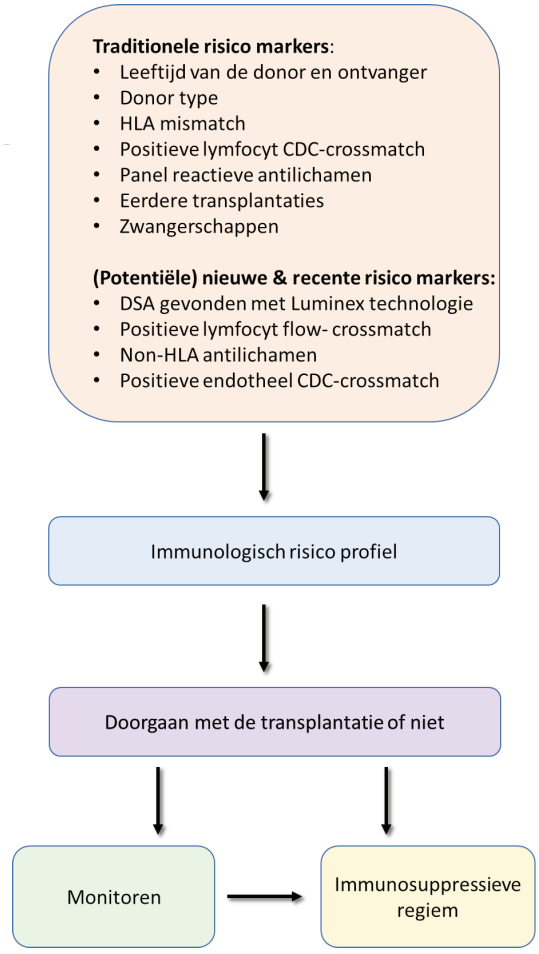
Naast de accommodatietheorie, kunnen onze data zoals gepresenteerd in **hoofdstuk 7**, ook een complement onafhankelijk mechanisme suggereren, dat zo verantwoordelijk is voor het heterogene beeld tijdens antilichaam gemedieerde afstoting, en daarmee tevens verklaren waarom patiënten met antilichaam gemedieerde afstoting niet altijd voordeel hebben van complementremmers. Complement onafhankelijke pathomechanismen via Fc-receptoren (FcR) zouden een rol kunnen spelen in antilichaam gemedieerde afstoting, een mechanisme dat antilichaam afhankelijke cellulaire cytotocixiteit (ADCC) wordt genoemd. Er ontstaat steeds meer bewijs dat natural-killercellen (NK-cellen) belangrijke cellen zijn in ADCC tijdens antilichaam gemedieerde afstoting. Experimentele modellen en analyses van nierbiopsieën hebben laten zien dat NK-cellen verhoogd zijn tijdens antilichaam gemedieerde afstoting, en dat hun activatiestatus verhoogd is, wanneer patiënten donor specifieke antistoffen hebben. Daarnaast is beschreven dat muizen zonder NK-cellen een verminderde incidentie hebben van acute antilichaam gemedieerde afstoting, ook al kunnen deze muizen nog steeds wel chronische antilichaam gemedieerde afstoting ontwikkelen. De conclusie is dus te zijn dat er meerdere, parallelle mechanismen van endotheelcelactivatie kunnen bestaan (complementafhankelijk en complementonafhankelijk) die belangrijke bijdragen leveren in de ontwikkeling van acute en chronische antilichaam gemedieerde afstoting.

Toekomstperspectieven

In het eerste deel van dit proefschrift focussen we op het potentiële mechanisme van tubulaire complement activatie en hebben we laten zien dat heparines en Salp20 de potentie hebben tot het inhiberen van properdine binding aan proximale tubulaire epitheelcellen, wat zou kunnen leiden tot een reductie van alternatieve route gemedieerde tubulaire schade. Omdat de reductie van proteïnurie een groot therapeutisch doel is in het reduceren van het risico op progressie van nierschade, is het erg interessant dat Salp20 zowel de binding van properdine aan C3b als aan HSPG kan remmen. Dit tweeledig remmende effect zou verder uitgezocht moeten worden om een product te kunnen ontwikkelen voor patiënten met proteïnurie, dat de alternatieve route blokkeert. De focus zou moeten liggen op de constructie van niet-immunogene Salp20-analogen en op het bepalen van het exacte properdine-bindings epitoop. De alternatieve route remmende capaciteiten van Salp20, of een klein Salp20-fragment met daarin zowel de HSPG- als C3b-binding-site, zouden zowel *in vitro* als in experimentele diermodellen getest moeten worden. Met betrekking tot de heparines is het interessant om te onderzoeken of niet-anticoagulante heparines of gerelateerde glycomimetica complementactivatie kunnen remmen op tubulaire cellen en hiermee de schade kunnen reduceren, bijvoorbeeld in experimentele proteïnurische modellen.

De resultaten gepresenteerd in dit proefschrift ondersteunen de interpretatie dat C5b-9 een relatieve kleine rol speelt in de meeste gevallen van antilichaam gemedieerde afstoting in vergelijking met meer proximale complementcomponenten, wat het falen van de complement C5-remmer eculizumab in het behandelen van antilichaam gemedieerde afstoting zou kunnen verklaren. In dit proefschrift hebben we ook data laten zien, die suggereren dat CD59-expressie op nier-endotheelcellen een belangrijke rol lijkt te spelen in de beperkte formatie van C5b-9 tijdens antilichaam gemedieerde afstoting. Na bloedgroep incompatibele transplantatie lijken endotheelcellen meer resistent te worden tegen antilichaam geïnduceerde complement gemedieerde lysis door opregulatie van de expressie van cytoprotectieve eiwitten, zoals complement regulerende eiwitten CD55 en CD59. Deze bevindingen ondersteunen het concept dat de capaciteit van de donor-orgaancellen om complement te reguleren, de vatbaarheid voor antilichaam gemedieerde en complement afhankelijke transplantaat falen beïnvloeden. Terwijl er verschillen zijn in de karakteristieken van HLA- en bloedgroep-antistoffen, en desensitisatie therapie in HLA-incompatibele transplantatie minder effectief lijkt te zijn in vergelijking met bloedgroep-incompatibele transplantatie, is de hypothese dat er wel een vorm van accommodatie plaatsvindt in de context van HLA-DSA, uiterst interessant. Dit zou de variatie in schade, die geassocieerd wordt met circulerende HLA-antistoffen kunnen verklaren. Omdat we nu een robuuste methode ontwikkeld hebben om primaire endotheelcellen te isoleren, zou het uiterst interessant zijn om experimenten uit te voeren, waarbij we deze gekweekte nier-endotheelcellen gebruiken, en ze blootstellen aan bloedgroep- en HLA-incompatibel serum en dan de complementregulatie en schademarkers meten.

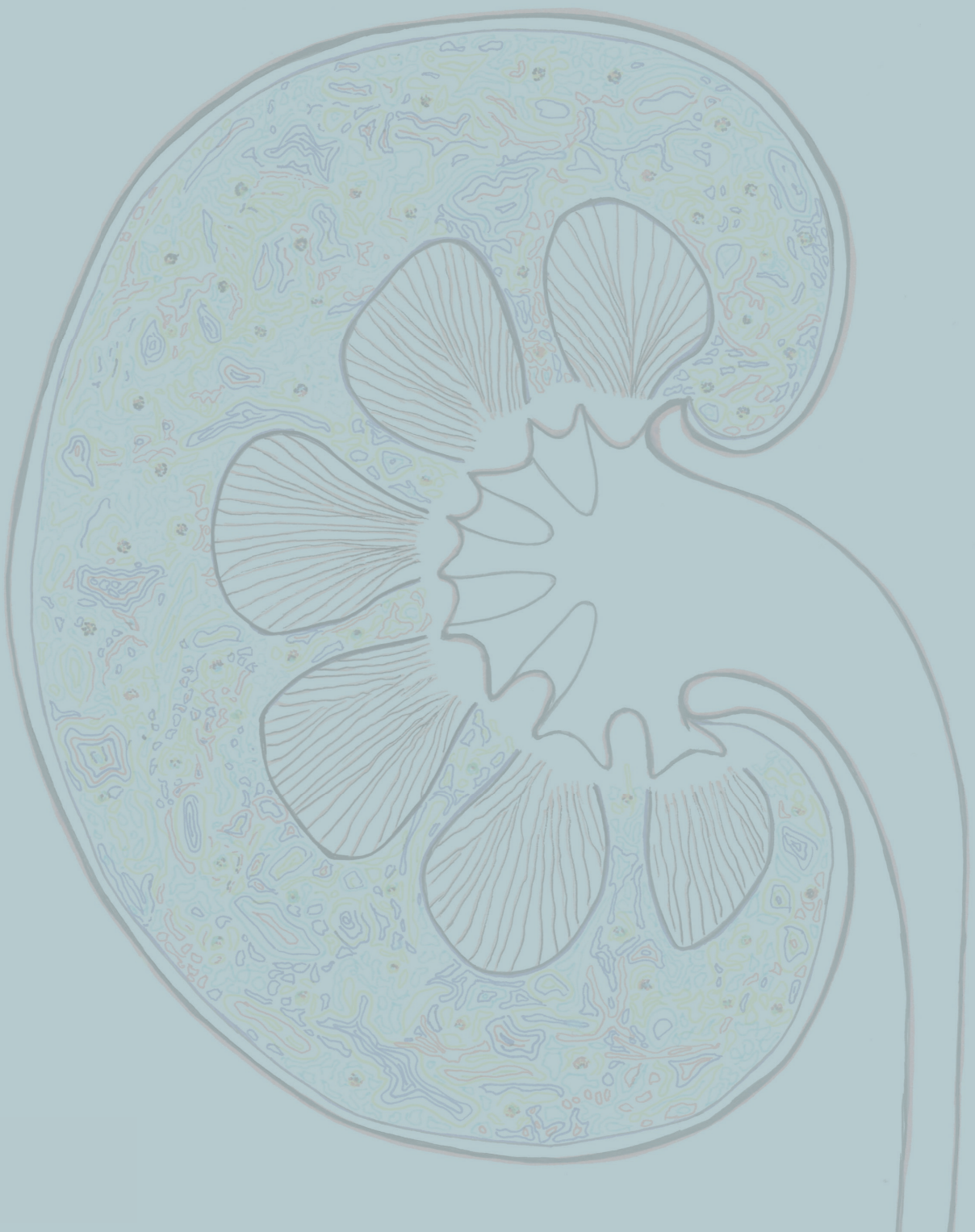
Naast complementregulatie kan ADCC als complement onafhankelijk mechanisme een belangrijke mediator zijn in het ontwikkelen van antilichaam gemedieerde afstoting zonder de aanwezigheid van C5b-9. Blijkbaar is complement activatie niet bepalend in alle casussen en is het belangrijk om de condities te definiëren voor complement afhankelijke en complement onafhankelijke antilichaam gemedieerde afstoting. De kwaliteit en kwantiteit van de betrokken antistoffen zou doorslaggevend kunnen zijn in deze context. Uit vergelijkende crossmatching studies blijkt dat het zowel de natuur van de heterogene mix van antistoffen, als de specifieke set van antigenen op het endotheel is, dat de effectiviteit van de antistof-antigeen-binding bepaalt en daarmee verdere immuunsysteem activatie. De nier-endotheelcel-bank zoals beschreven in hoofdstuk 6, welke de meerderheid van de HLA-en non-HLA-polymorfismen behelst, zou ons kunnen helpen in het ontrafelen van de pathogene effecten van verschillende antilichamen en hun regulatie op een cellulair niveau. In de nabije toekomst is de verdere ontwikkeling van de MP-PREC crossmatchassay in een routineassay belangrijk. Om dit te bereiken, moeten de primaire cellen eerst geïmmortaliseerd worden en grondig gekarakteriseerd. Daarnaast zijn de MP-PRECs ook bruikbaar voor het faciliteren van het onderzoeken naar de grote hoeveelheid non-HLA-polymorfismen. Omdat patiënten een mix kunnen hebben van HLA- en non-HLA-antistoffen, zouden we ook HLA-expressie moeten elimineren op een deel van de endotheelcellen. De endotheel-crossmatchtest zou een belangrijke toevoeging kunnen zijn aan het immunologische risicoprofiel van ontvangers en zou, na grondige validatie, ook klinisch relevant kunnen zijn om afstoting te voorspellen. De integratie van de meer traditionele risicomarkers zoals (pre-) transplantatie antilichaam profiel, de leeftijd van de ontvanger, HLA-mismatches met andere, nieuwere risicomarkers, zou kunnen resulteren in een meer gepersonaliseerd en completer risicoprofiel. Dit risicoprofiel kan dan gebruikt worden voor een nauwkeuriger donor-ontvanger matching, post-transplantatie monitoring en om gepersonaliseerde immunosuppressiva adequaat in te stellen (figuur 1). Daarnaast zouden de MP-PRECs wellicht verder kunnen helpen in in-vitro-studies om het mechanisme dat leidt tot hyperacute, acute en late rejectie veroorzaakt door HLA- en non-HLA-antistoffen, beter te begrijpen op het niveau van de endotheelcel.



Figuur 1. Immunologische risico beoordeling.

De observaties beschreven in dit proefschrift dat machine perfusie van donornieren leidt tot loslating van endotheelcellen, is uiterst interessant. Machine perfusie is een relatief nieuwe techniek, die nu routinematig gebruikt wordt bij niertransplantatieprocedures in Nederland. Desondanks is er, vooral in de context van endotheelcellen, nog weinig bekend over de impact van machine perfusie. Het is interessant om te bestuderen of het endotheel van DCD (donation after circulatory death) donoren op een andere manier aangetast wordt dan het endotheel van DBD (donation after brain death) donoren. Beschreven zijn voornamelijk de effecten van hersendood en complementactivatie op endotheel, wat resulteert in endotheeldysfunctie. De effecten van DCD op endotheel zijn echter nog veel onduidelijker. Toekomstige studies zouden de transplantatie uitkomsten kunnen correleren aan geobserveerde veranderingen in het endotheel fenotype tijdens en na machine perfusie van verschillende donornieren, maar ook aan het aantal en fenotype van de endotheelcellen die zijn los gespoeld uit de donornier.

Effectieve complement behandelingen in complement gemedieerde ziekte zoals aHUS hebben laten zien dat behandelingen gericht op complement mogelijk zijn en een groot potentieel hebben. Dit betekent dat complement georiënteerd onderzoek de brug moet slaan tussen de labtafel en de kliniek om een rationele benadering te kunnen ontwikkelen om complement gerichte behandelingen te kunnen opstarten, maar ook om vermindering van complementactivatie te kunnen monitoren tijdens behandeling. Hopelijk gaan de benaderingen beschreven in dit proefschrift uiteindelijk helpen bij het verminderen van de incidentie van afstoting, het verbeteren van transplantaat overleven en het ontwikkelen van aanvullende complement diagnostische methoden.



Dankwoord
Toen ik klaar was met mijn proefschrift keek ik voldaan terug op een periode gevuld met wetenschap. De laatste zes jaren stond mijn leven niet alleen in het teken van de wetenschap. Het werd ook beheerst door het tragische verlies van mijn innig geliefde moeder Hella én de geboorte van onze zoon Philippe, een nieuw leven. De totstandkoming van dit proefschrift was voor mij een leerzame ervaring, die ik voor altijd bij me zal dragen. Natuurlijk was alles wat in dit boek beschreven is niet mogelijk als velen het werk niet ondersteunden, en daar wil ik iedereen uiteraard hartelijk voor bedanken. Ik begin dat ik het schrijven van mijn dankwoord wel lastig vond. Er zijn vele 'major life events' geweest in de tijd dat ik werkte aan dit proefschrift. De meest ingrijpende was dat mijn moeder veel te jong is overleden en een tijdje later hebben we hebben kleine Philippe erbij gekregen. Ik wil vooral iedereen gewoon bedanken voor het er zijn als het nodig was. We moeten elkaar er tenslotte doorheen helpen, door het gedoetje wat 'het leven' heet.

Om te beginnen wil ik alle transplantatie-patiënten, donoren en hun families bedanken. Zonder hen had mijn proefschrift niet tot stand kunnen komen.

Professor P. Heeringa, professor M.E.J. Reinders en professor C.A. Stegeman wil ik bedanken voor hun bereidheid zitting te nemen in de beoordelingscommissie van mijn proefschrift.

Als startende promovendus kon ik niet veel beginnen zonder begeleiders. Mijn promotoren Stefan Berger en Jaap van den Born, ben ik dankbaar voor jullie betrokkenheid, vertrouwen en support. Jullie zijn beide op jullie eigen manier van onschatbare waarde geweest in de totstandkoming van dit proefschrift en ik heb veel bewondering voor het feit dat jullie mij mijn eigen gang lieten gaan, maar toch ook bijstuurden en corrigeerden op de momenten als het moest. Jullie waren kritisch waar nodig en jullie benoemden regelmatig dat ik af en toe best wel tevreden mocht zijn met mijn behaalde resultaten. Het aantal geluksmomentjes verandert als je van kliniek (co-schappen) naar onderzoek gaat, maar jullie leerden me er gewoon naar op zoek te gaan en het te benoemen.

Stefan, bedankt voor de mogelijkheid om onderzoek te doen bij de afdeling nefrologie en de wekelijkse meetings, de adviezen, de verhalen, de voorstellen en de discussies. Ik heb er bewondering voor dat je snel tot de kern van een zaak kunt komen en tevens heb je een ijzersterk geheugen. Ik heb ongelooflijk veel van je geleerd als wetenschapper, als arts en als mens. Ook buiten het ziekenhuis hebben we al een flink aantal anekdotes verzameld en ik ga er vanuit dat we nog vele nieuwe verhalen gaan verzamelen.

Jaap, je grote enthousiasme voor ons onderzoek werkte aanstekelijk. In gesprekken voelde je keer op keer goed aan waar ik behoefte aan had, en benoemde in no-time mijn hulpvraag. Als ik tegen problemen aan liep kon ik altijd bij je terecht en door jouw grote relativerende vermogen en pragmatische advies kon ik na een gesprek met jou weer vol moed verder.

Voor mij waren jullie samen het perfecte promotie team. Jullie waren tevens op een warm en ondersteunende manier betrokken bij het ziekteproces van mijn moeder en jullie voelden goed aan wanneer er op de stop-knop gedrukt moest worden. Toen ik weer terug kwam, had ik het gevoel dat jullie mij goed in de gaten bleven houden, ik wil jullie bedanken voor jullie betrokkenheid. Ik denk dat we het goed gedaan hebben zo.

Mijn eerste stapjes in de transplantatie geneeskunde zette ik al in mijn eerste jaar van de studie geneeskunde. In de eerste week vroeg mijn moeder mij; 'bestaat er ook zoiets als een studentenmail?' We gingen samen op zoek. Toevallig ging het eerste mailtje wat wij openden over de vacature van het Prometheus Nierteam die spoedig zou vrijkomen. De vacature omschrijving sprak me erg aan. Goede vriend Paul Bos was daar de voorzitter van, en hij wist mij nog meer te enthousiasmeren voor het buitengewoon interessante vakgebied waarbinnen dit team opereerde; de transplantatie geneeskunde. En zo gebeurde het dat ik aan het begin van mijn studie geneeskunde, onder supervisie van prof. Dr. Henri Leuvenink samen met Ernst Buiter of Cees Brugman mee ging naar multi-orgaan donatie-procedures en orgaantransplantaties. Het gebeurde regelmatig dat we met de zwaailichten aan naar een ander ziekenhuis gingen, soms met het vliegtuig of de helikopter. Al met al was het een spannende ervaring. Het zaadje voor onderzoek in de transplantatiegeneeskunde werd hier geplant. Van de transplantatie chirurgen Sijbrand Hofker, Christina Krikke, Robert Pol of Vincent Nieuwenhuijs kregen wij biopten van organen die werden opgeslagen in vriezers in het UMCG, voor verder wetenschappelijk onderzoek. Hechte vriendschappen ontstonden in deze tijd; via Prometheus leerde ik Marije en Michele kennen, tevens mijn paranimfen op de dag van mijn promotie.

Iedere complement promovendus in Groningen is steevast grote dank verschuldigd aan prof. Dr. Moh Daha. Vóór de COVID-19 pandemie reisde je elke maand af naar Groningen en voerde je integere begeleidende gesprekken met de promovendi. Dankzij jouw jarenlange ervaring en enthousiasme voor het complement-onderzoek is jouw kundige input van onschatbare waarde. Je vertelde me ooit dat het een natuurlijk proces is dat je ook een beetje vrienden wordt met de mensen met wie je nauw samenwerkt. Ik denk dat dit ontzettend waar is en goed gelukt! Ik hoop dat je het niet erg vindt dat ik je in deze 'post-COVID-19 periode' in plaats van een keer per maand, iedere week een uitnodiging stuur voor de online werkbespreking.

De andere emeritus professor aan wie ik grote dank verschuldigd ben is Willem van Son. Met jou liep ik voor de eerste keer mee op de poli transplantatienefrologie. Je liet me me de weegschaal zien waarmee je de immunologische respons na een niertransplantatie altijd uitlegde. Ook had je het over HLA en complement. Als eerstejaars geneeskunde student vond ik het maar ingewikkeld. Gedurende mijn verdere ontwikkeling ben je altijd erg betrokken geweest. We woonden vlakbij elkaar en kwamen elkaar regelmatig tegen. Geregeld lunchten we in café Huis de Beurs, waar ik van je leerde dat je nooit teleurgesteld wordt als je gewoon een tosti bestelt voor de lunch. Of we kwamen elkaar zomaar tegen op de stoep voor de deur van mijn studentenhuis terwijl ik met mijn ouders een te groot matras door een te klein raam probeerde te krijgen. Je sprong een gat in de lucht toen je hoorde dat ik transplantatie immunoloog wilde worden. Ontzettend bedankt voor alles Willem!

Leden van het COMBAT Consortium, bedankt voor de samenwerkingen en de interessante en nuttige bijeenkomsten. Cees van Kooten, als drijvende kracht van het consortium, ben je een groot voorbeeld. Jouw betrokkenheid bij alle projecten heb ik als prettig ervaren. Bedankt dat je plaatsneemt in mijn Corona. Mieke, Marloes en Ramon, het was fijn om samen met jullie de COMBAT PhDers te vormen. Ik denk dat we erg trots kunnen zijn op wat we hebben bereikt en we komen elkaar vast weer tegen in het complement-wereldje.

Professor Ingrid Molema, bedankt voor je engagement en innemendheid. Ik heb ontzettend veel geleerd van jouw ervaring, je scherpe blik en je schrijfvaardigheden. Eveneens ben ik erg blij dat we elkaar hebben leren kennen en voel ik me vereerd dat je in mijn Corona plaatsneemt.

Henri Leuvenink, bedankt voor alle kansen die je me hebt gegeven. Voor mij ben je altijd op de achtergrond aanwezig en ik kan à la minute bij je terecht voor de meest uiteenlopende vragen en adviezen. Jouw analytische blik en scherpe humor werken voor mij heel relativerend. Dank voor alles.

Robert en Maaike Pol, ik vind onze jaarlijkse oud & nieuw traditie erg leuk. Ik zal wel nog wat beter oefenen op de nieuwjaarsrolletjes! Daarnaast, Robert, ik hoop dat we interessante projecten gaan oppakken in de toekomst. Maaike, hartelijk dank voor je betrokkenheid bij en goede zorgen voor Philippe.

Ik wil graag de hele experimentele-nefrologie-club bedanken voor de leuke sfeer op het lab. Wendy en Anita, wat moest ik als experimentele nefrologie PhD student zonder jullie? Jullie stonden altijd voor me klaar, jullie dachten mee over ingewikkelde vraagstukken en sprongen bij als ik de dag weer te vol gepland had. Ditmer, wat hebben we veel gelachen. Jouw Friese nuchtere blik leidt tot goede en amusante gesprekken. Nu we allebei een zoontje hebben zal er wel wat minder gefietst worden, al kunnen de kindjes natuurlijk ook gewoon mee met de fietsweekendjes in Tecklenburg of de Ardennen. Pragyi, you always seem to be happy and smiling. You give all the people around you tons of energy! Firas, Dania and Tobias, I am a huge fan of your research projects. Thanks for having me involved and I am looking forward to the future! Tina, Mariana en Felix, mede-complement-PhDers van het eerste uur. Dank voor alle hulp, adviezen en brainstorm sessies. Marloes en Bram, ook al deden we geen gezamenlijke projecten, we bespraken de verschillende technieken op het lab met elkaar, wie welke antilichamen had, we gaven elkaar tips over hoe je bepaalde zaken snel geregeld kreeg, en hielpen elkaar op die manier goed verder.

Ook wil ik graag de studenten, die ik begeleid heb tijdens mijn onderzoek, hartelijk danken. Het was zeer nuttig voor mijn onderzoeksprojecten en essentieel voor de endotheelcel isolaties en kweken; meerdere 'endotheelteams' zijn geboren tijdens mijn PhD periode. Jessy, Marjolein, Jeanine, Lisanne, Mohammed, Franzi, Nienke, Harriet, Laura en Cor, bedankt voor alles en heel veel succes met jullie verdere carrière. Gesa, ooit begonnen als studente die fanatiek meehielp met de endotheel kweken, nu ben je zelf gestart aan een MD/PhD traject. We zijn echte vriendinnen

geworden en hebben aan één blik al genoeg. Samen verdwalen op de fiets in Milaan, het ABMR project in goede banen leiden, werken tot in de late uurtjes, het was voor mij allemaal wat minder zwaar omdat jij ook mee deed. Dank dat ik je heb mogen leren kennen! We gaan van jouw projecten ook iets prachtigs maken.

Henk Moorlag, Klaas Sjollema, Theo Bijma, Peter Zwiers, Geert Mesander en Johan Teunis, ook al behoorden we niet tot dezelfde onderzoeksgroep, jullie hebben me geregeld uit de brand geholpen. Bijna à la minute de HUVEC cellen klaar, nú helpen met de compensatie en erg mooie immunofluorescentie foto's die gelijk publicatie waardig zijn. Jullie zijn stuk voor stuk waardevolle toppers.

Ook alle andere nefrologie PhDers wil ik natuurlijk graag bedanken. Te beginnen met mijn kamergenoten op de brug. Antonio, mijn buurman. Erg toevallig kwamen we vaak in dezelfde kledingkleuren naar het ziekenhuis. We zouden samen een mooie reis gaan maken in Amerika na het congres in Seattle, maar ik ben erg blij dat jij er voor koos om mee terug te vliegen naar Nederland toen het noodlot in mijn familie toesloeg. Bedankt voor alle adviezen met betrekking tot statistiek, grapjes en de tijd die we met elkaar door hebben gebracht. Let our journey continue! Maarten, ook mijn kamergenoot op de brug. Dank voor de vele goede en nuttige (onderzoeks) gesprekken. Ik heb veel aan je gehad toen ik weer startte na het overlijden van mijn moeder. De gespreksonderwerpen zijn inmiddels een beetje veranderd nu Jan-Jaap en Philippe ook vrienden zijn geworden op de opvang, desalniettemin waardeer ik ze uiteraard enorm. Karin, wat ben je een sterke dame. Je maakt bewuste keuzes en staat echt voor je gezin klaar. Je rondt je PhD vast ook snel af, daarna zullen we elkaar hopelijk weer wat vaker zien. Stanley, held. Wij op vrijdag middag 'mhe, nee, morgen ga ik niet werken hoor.' Op zaterdag; 'hé, jij ook hier?'.

Rianne, het is moeilijk om geen vriendinnen met je te worden. Uren hebben we samen doorgebracht op de brug, en op een gegeven moment zijn we ook de zaterdagochtenden samen gaan typen. Joëlle kwam daar ook gezellig bij en we hebben flink wat meters gemaakt in deze periode. We zien elkaar gelukkig nog geregeld en ik kijk uit naar ons weekendje weg, na mijn verdediging, samen met Maryse, Jip en Yvonne. Maryse, al boksend hebben we elkaar door de COVID-19 lockdowns heen geholpen, in deze tijd waren onze gesprekjes op de fiets heldere lichtpunten. Kom maar snel weer terug naar Groningen en dan gaan we weer verder. Yvonne, onze korte groeten tijdens het werk waarbij je altijd lekker ondeugend kijkt bezorgden me altijd een grote glimlach. Jip, je nam mijn plek in op de brug toen ik er een aantal maanden niet was. Toen ik terug kwam werden we gelijk vriendinnen. Je fietst in fietsbroeken die de naam 'Hella' dragen, en ik waardeer het enorm dat je ook bezig bent met mijn moeder (los van dat de broeken ook gewoon heel lekker zitten). Inmiddels fiets je mee in het tempo van mijn vader en ga je komende zomer voor de tweede keer met ons naar de Pichounetta in Zuid-Frankrijk. Het voelt alsof ik je al mijn hele leven ken!

Ook de nefrologie PhDers die al lang klaar zijn of in het zusterhuis werken, wil ik graag bedanken. Allereerst omdat ze weinig klagen over het feit dat ze boven de kinderopvang moeten werken, waardoor (onder andere door Philippe) soms de nodige afleiding ontstaat als de kindjes enthousiast buiten spelen. De Lianne's, Messi en Kienie. Fijn dat we elkaar nog geregeld zien in het zusterhuis en ik wens jullie veel succes met jullie projecten. Anna-Sophie, het was een leuk avontuur om samen op congres te gaan, je bent lief en zorgzaam. Samen met Amber werken jullie erg hard en ik vind het attent dat jullie altijd een gesprekje aanknopen met de mensen die het nefro-gedeelte van het zusterhuis binnen komen.

Gouden nefrologie koppels Suzanne en Marco en Bart en Marieke. Ik noem jullie nu allemaal even in één adem, maar uiteraard waardeer ik jullie individuele persoonlijkheden stuk voor stuk enorm. We hebben allemaal inmiddels een huis gekocht en ik kom jullie paleisjes graag snel bewonderen. Yusof, we hebben allebei op het Praedinius gezeten en dat zorgt voor een klik. Dank voor al je adviezen. Silke, Tim en Daan, de nieuwe brug bewoners; veel succes met de projecten en ik kom snel mijn bureau opruimen... Audrey en Simke, ook al kennen we elkaar niet heel erg goed, we hebben toch cruciale zaken met elkaar gedeeld. Dank daarvoor!

Lieve vriendinnen en vrienden. Krista en Jildau, mijn vriendinnen van het eerste uur. We kunnen altijd bij elkaar terecht. Anouk, nos divertimos mucho en Almelo y Nicaragua. Creo que deberíamos volver a bailar salsa y espero que hagamos hermosas nuevas aventuras. Laura, mijn mede-krullenbol en enorme wijn liefhebber. Leonie, houdt een oogje in het zeil in Zuidwolde én fijne gesprekspartner met betrekking tot vele promotie perikelen. Wat heb jij toch een fijne 'vibe' om je heen. Welmoed en Mathijs, waardevolle nieuwe vrienden én de ouders van Philippe z'n vriend Bodi. Arden en Martijn, samen met To vorm(d)en we de Il Flatto eetclub, inmiddels uitgebreid met aanhang plus kinderen. Elk jaar in Domburg komt er een jonge deelnemer bij. Sara-Lisa, Veera en Femke, het voelt met jullie alsof we bij elkaar op de basisschool hebben gezeten. Fareeda, Youri, Inge en Martijn, mijn lieve collega's van het Barrel Wijnlokaal. Joline, we blijven elkaar in alle fases van ons leven weer tegenkomen. Lotus en Martin, erg leuk en fijn dat jullie ook op de Pichounetta zijn geweest, hopelijk gaan we snel weer eens samen een tochtje maken op de racefiets. Anne, je bent meer dan welkom bij de club! Marieke, ik ben erg blij dat jij de vrouw van Michele bent en wij de peetouders zijn van Federico. Stan en Tamar, los van alle leuke borrelavonden, jullie wil ik danken voor het snelle zoeken van een vlucht uit Seattle naar Nederland. Jop, soms spreken we elkaar een hele periode niet, maar ik vind het altijd leuk om je weer te zien. Cynthia en Florine, lieve dames voor al uw acute baby vragen, hihi. Sanne, zo verweven in mijn leven. Mijn moeder herkende je zelfs van achteren op een foto toen ze niet eens wist dat we elkaar zouden treffen in Londen. Lieke, Corine en Marly, we hebben elkaar door de studie geneeskunde heen geholpen en nu bevinden we ons verspreid over het hele land. Kortom, met ieder van jullie heb ik al zo veel beleefd. Pagina's vol kan ik schrijven over hoe blij ik ben dat jullie in mijn leven zijn. Bedankt voor alle steun en vertrouwen die jullie me hebben gegeven. Ook dank voor het geduld dat jullie hebben, zoals jullie weten ben ik door het afronden van mijn proefschrift, de opleiding die ik volg en de zorg voor mijn gezin niet altijd bereikbaar geweest. Ik kijk uit naar de avonturen die we nog allemaal gaan beleven.

Rosie and Zoe, I met you both at the Oxford Hockey Club, that I joined together with Michiel Voskuil in October 2012. You both really helped to make me feel at home in England. And because of our friendship it still feels like a second home. I am really happy that we manage to see each other almost every year, with the end-to-end cycle route in 2017 as a cherry on top. We're already discussing a second long distance cycle trip, hopefully with a little more sun and a little less rain.

My other good friend and colleague from my period in Oxford is Maria Kaisar. Talking to you gives me a shot of energy and I am happy that we run into each other at transplant congresses and philosophize about joint projects. I am honored that you are willing to take place in my defense committee.

Lieve Angelique en Jaap, we hebben elkaar op een hele grappige manier leren kennen, de klik was er gelijk! Toen Guido en ik van Maastricht naar Groningen gingen fietsen een jaar geleden, hebben we een tussenstop gemaakt bij jullie in Sittard. Het was zo gezellig dat we de etappe van die dag maar hebben gelaten voor wat het was. Ik ben erg blij dat jullie ook aanwezig zijn op deze bijzondere promotie dag en bedankt voor jullie betrokkenheid, de berichtjes en fijne gesprekken. We gaan vrolijk verder!

Momenteel bevind ik me dagelijks op de laboratoria transplantatie immunologie en medische immunologie. Mijn fijne werkplek, waar ik me als een vis in het water voel. Ook daar zette ik mijn eerste stapjes al in mijn eerste studiejaar geneeskunde, waar ik onder strikte begeleiding van Richard mijn HLA typering ging uitvoeren. Tijdens mijn promotietraject kwam ik weer geregeld op het transplantatie immunologie lab en meerdere artikelen in dit proefschrift zouden niet tot stand gekomen zijn zonder de experimenten uitgevoerd samen met Magdalena en de samenwerking met Bouke. Caroline, Bart-Jan, Annechien, Michaël, Laura, Bouke en Door, bedankt dat ik jullie team mocht komen versterken. Dank voor alle uren onderwijs die ik al heb genoten en voor jullie steun in deze laatste fase van mijn promotie. Lieve, Els, Ilby, Bram, Lisette, Sylvana, Stan, Magdalena, Diana, Theo, Niels, Richard, Ruben, Suzanne, Grietje, Wineke, Ineke, Marijke, Sevgim Jurjen en Hans van de transplantatie en Johan, Bessel, Brigitte, Marcel, Marieke, Janneke, Inez, Greet, Betty, Maaike, Grytsje, Gezina, Ibolya en Hessel medische immunologie. Ik fiets iedere dag met plezier naar mijn werk en dat komt mede door jullie. We benaderen elkaar laagdrempelig en er wordt gewerkt op hoog niveau. Toch is er altijd ruimte voor een grapje en een gesprekje. Ik ben trots op het werk wat we doen, we zijn een mooie club met elkaar!

Isidor en Kees, wat een fantastische collega's zijn jullie. Het feit dat collega's in de aangrenzende kamer ons appen met de vraag of het gelach wat zachter kan, zegt denk ik wel genoeg over de reden dat ik het geen straf vind als ik weer een stage aan 'de overkant' heb.

Lieve schoonfamilies, rotsvast aanwezig in goede en slechte tijden. Lieve Mech, wat fijn dat Guido jou als vriendin gekozen heeft. Je was eigenlijk nog maar net in onze familie toen Hella ziek werd, gelukkig had je daarvoor al meerdere vakanties en Bourgondische avonden meegemaakt. We proberen allemaal de draad weer goed op te pakken en we zorgen goed voor elkaar. Veel dank hiervoor. Je bent ook een fantastische tante voor Philippe. Als wij ons als gezin soms even moeten haasten vertraagt dat de boel geregeld enorm; want Philippe moet eerst even bellen met Mechteld, met de randomreader van de bank als telefoon.

Lieve Martha, Peter, Mirjam, Nils en Mylo. Als er een ding is wat ik zeker van jullie geleerd heb, is dat eten verbindt. Ik vind het fantastisch om te zien dat er met 'een goed genoeg gerecht' geen genoegen wordt genomen; wat we elkaar serveren moet perfect zijn. En zo is het maar net. Mirjam en Nils, di mama en di papa, super leuk dat Mylo erbij gekomen is. Ik kan niet wachten op weekendjes weg met onze mannetjes. Wijn drinken met jullie is erg gezellig, jammer dat jullie in Amsterdam wonen, maar dan rijden we maar wat vaker op en neer. Martha en Peter; mijn lieve schoonouders. Dank dat jullie altijd voor ons klaar staan. Martha, voor alle weekenden die je al op Philippe hebt gepast, gelukkig vind je het hartstikke leuk en snijdt het mes aan twee kanten. Ook apprecieer ik het dat je naar me luistert als ik soms gestresst op bel. Samen met jou, Mirjam en Micha zijn we na mijn afstuderen een maand naar de Molukken geweest. Ik vond het erg speciaal om te verblijven in het familiehuis op Ambon en helemaal ondergedompeld te worden in jullie oorspronkelijke cultuur. Zelfs na twee nachtjes op een prachtig onbewoond eiland konden we het nog goed met elkaar vinden, terima kasih! Peter, we zijn van 10 seconden loop afstand verhuisd naar 10 minuten met de bus. We moesten even een weg vinden om elkaar weer wekelijks te kunnen zien, maar dat lukt gelukkig. Dank voor de heerlijke gerechten die we samen gegeten hebben, het altijd luisterende oor, de spoed-oppas momenten, en voor de tip dat magnum aardbei ook gewoon fruit eten is.

Ook wil ik Abies en Nagi even noemen. Mijn bonus nichtjes. Het is altijd erg lachen met jullie, ik vind jullie prachtige mensen.

De vrienden van mijn ouders zijn de afgelopen periode ook nog meer betrokken geraakt met de levens van Henk, Guido en mij. Lieve Marjan en Fons, Eddy en Toos, Terry en Nico, Ger en Annemiek en nichtje van Hella, Marja samen met Gerard. Dank voor jullie tips en trics, relativerende gesprekken en geëngageerdheid. Geregeld checken jullie even in hoe het bij ons gaat en dan kan ik me weer even lekker 'kind' voelen. Marja, dank voor het onderwerpen van de kaft van mijn proefschrift. Je hebt het ontzettend goed gedaan en ik ben er erg trots op! Nico, ik was blij verrast toen je mailde dat jij als rector magnificus de voorzitter van de Corona zal zijn tijdens verdediging; ik vind het erg speciaal.

Gosse, Jacqueline en Eloise. In 't Rookshuys kom ik altijd enorm tot rust, ook al ging ik er vroeger heen voor een rondje marathon studeren. Onder strikt bewind van Gosse haalde ik goede cijfers op mijn tentamens, ik mocht pas schieten op een leeg flesje water als ik minstens een 8 had op mijn oefententamen. Dank voor de goede gesprekken en avondjes wijn drinken in de tuin.

Siska, mijn lieve betrokken tante. Op avonden met de familie houd je me altijd goed in de gaten en neem je me dingen uit handen als ik teveel hooi op mijn vork heb genomen. Ik ben erg blij dat je er bent.

Jolanda en Robert, levensgenieters. Jol, wat heb je een boel meegemaakt in je leven. Je hebt het omgedacht naar alles uit het leven halen en samen met Robert maak je mooie reizen in de zelf omgebouwde camper. Jullie genieten ook van Philippe en hadden al kaplaarsjes voor hem nog voordat hij geboren was. Jullie zijn altijd present als Guido en ik iets te vieren hebben, dank voor jullie participatie. Nu mijn weekenden wat minder vol zijn zien we elkaar hopelijk weer vaker!

Mijn paranimfen, Marije, Michele en To. Mijn bonus zusje en bonus broertjes, dank dat jullie mij bijstaan op deze dag. Wat ons denk ik het meest verbindt is dat we 'bewust' leven. Lieve Marije, niet te bevatten dat het noodlot al vroeg in ons leven heeft toegeslagen in onze stabiele gezinnen. We steunen elkaar door dik en dun, we kunnen altijd bij elkaar terecht en we accepteren elkaars feedback moeiteloos. Als we samen op vakantie gaan, plannen we dagen in dat we even in de 'rust-stand' kunnen komen en filosoferen we over onze levens. Allebei hebben we veel ambitie en zin in het leven. De laatste jaarwisseling van Hella waren jij en To er, we lachten en kletsten met elkaar en jullie bleven slapen. Dat betekent buitengewoon veel voor me. Lieve To, we gaan bijna ons dertigjarig jubileum in? Toen we klein waren maakten we samen heksensoep voor onze ouders, dit werd gevolgd door gezamenlijk op vioolles, dezelfde scholen, dezelfde sport, dezelfde studie en vele vakanties. Ook speelde je viool op mijn moeders begrafenis, wat zij graag wilde en ik onvoorstelbaar knap van je vind. Dank voor de jarenlange hechte vriendschap, je bent er altijd voor mij en je bent altijd bereikbaar voor me. Binnenkort word je papa, een nieuwe fase in je leven. Ik verheug me op de komende 100 jaren waarin we nog vele fasen met elkaar gaan meemaken. Lieve Michele, ik ben erg dankbaar voor onze waardevolle vriendschap. Geleid door onze Zuid-Europese temperamentjes kunnen wij uren te praten over gebeurtenissen binnen en buiten het ziekenhuis en kunnen we elkaars handelen en functioneren tot op het bot analyseren. Los van dat ik er veel van leer, voel ik me erg gehoord en gezien door jou. Ik vond het een eer de ceremoniemeester te zijn op jullie bruiloft en ik vind het ontzettend fijn dat jij en Micha het zo goed met elkaar kunnen vinden, evenals Marieke en ik, en Federico en Philippe. Mathilde leert goed haar 'mannetje' te staan, later gaan de kindjes vast met z'n allen op boevenpad. Ik kijk er naar uit dat we eens met onze beide gezinnen naar Italië gaan.

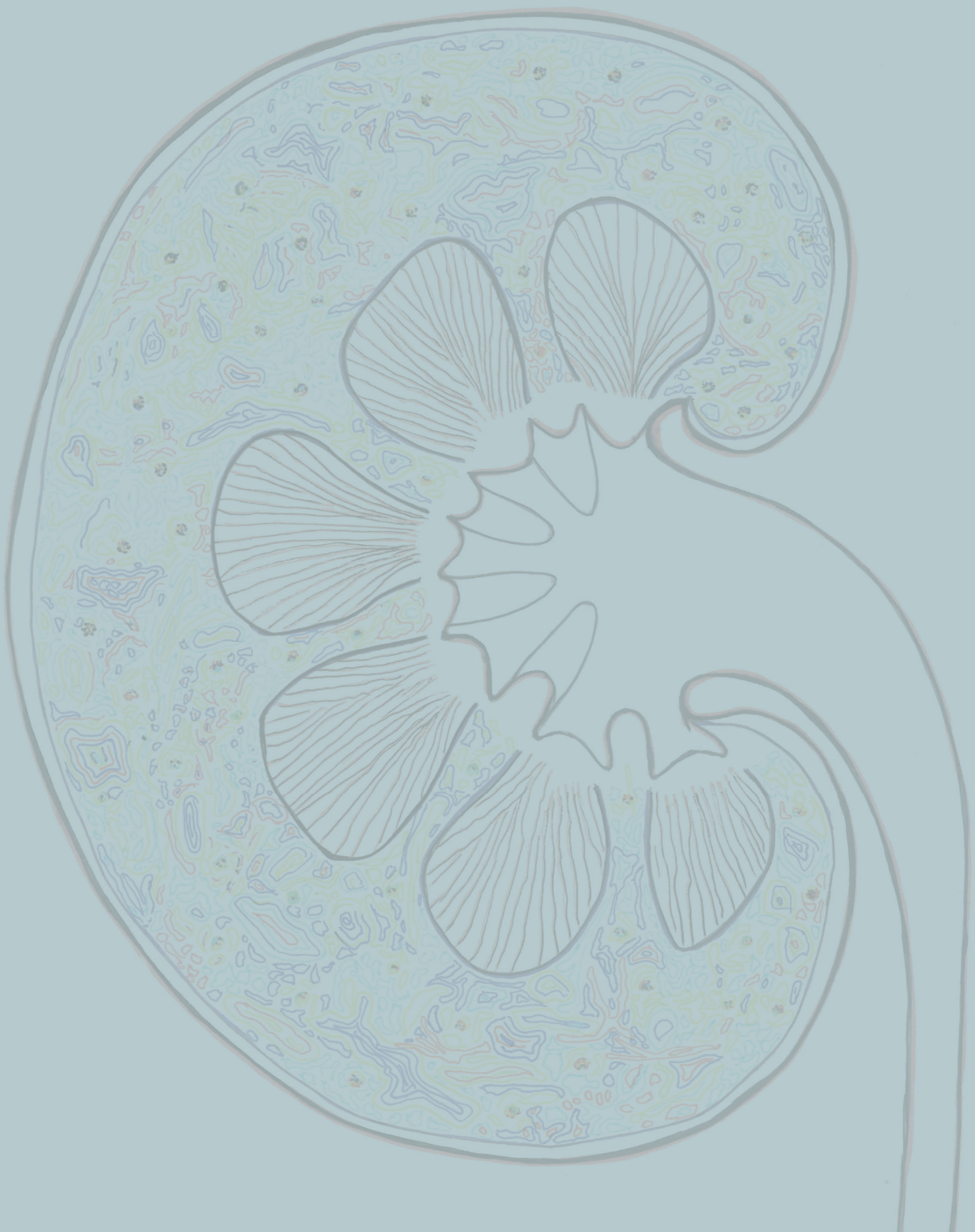
Lief thuisfront, papa en Guido. Henk, dank dat je er zo voor ons bent. We hebben het niet erg makkelijk gehad de afgelopen jaren. Relativerend noemde je mijn proefschrift een werkstuk, waar ik eerst een beetje pissig om werd maar nu vind ik het eigenlijk gewoon heel erg grappig en misschien ook wel een beetje waar; allemaal kleine werkstukjes gebundeld in een boek. De laatste periode aten we samen vele avonden in het ziekenhuis, waarna ik nog even een paar uurtjes door kon werken. Voor ons beiden uiterst waardevol, even één op één kletsen. Enkele keren omzeilden we de Corona regels en smokkelden we je in doktersjas het personeelsrestaurant in; die witte jas misstaat je niet! Ik ben erg trots op hoe je je leven zonder mama nu vormgeeft. Daarnaast ben ik super blij dat we nu zo dicht bij elkaar wonen, we eten geregeld met z'n allen en eigenlijk hebben we met beide huishoudens nu drie mooie auto's die we regelmatig onderling uitwisselen. Bij jou ligt de lekkere wijn altijd koud en we proberen de speciale momenten die we mee maken weer samen te vieren. Ik ben zuinig op je en kijk soms met argus ogen toe hoe je samen met Hopie vele kilometers wandelt of fietst, erg knap. Je bent een voorbeeld voor Guido en mij!

Lieve Guido, mijn kleine broertje. We kennen elkaar door en door, hebben aan één blik genoeg om te weten hoe het met de ander gaat. Lekker jezelf zijn bij elkaar, een beetje ruzie maken, samen een team vormen, met Henk en Hella als voorbeeld kunnen we de wereld wel aan. Ik vind het knap hoe je je leven vormgeeft en goed naar je eigen gevoel luistert. Samen met Mechteld ben je verhuisd naar Zwolle om met een baan in Nijmegen te beginnen, een baan die volgens mij erg goed bij je past. Gelukkig kom je in je mooie Volvo C30 vaak heen en weer naar Groningen gereden en zien we elkaar daardoor bijna wekelijks. Ik ben erg blij dat jij mijn broertje bent en vind je een belangrijk voorbeeld figuur voor kleine Philippe! We hebben al heel wat sportieve avonturen samen beleefd en ik kijk uit naar onze wandeltocht in de week na mijn verdediging.

Lieve Micha en lieve papa van Philippe. Degene die de 'last van de PhD afronden' het meest mét mij ervaren heeft. Bijna 10 jaar zijn we nu in elkaars leven. Toen ik je bezig zag op de festivals waar je onder hoge druk soms meer dan dertig mensen moest aansturen, wist ik gelijk dat je een prachtig mens bent. Je had een luisterend oor voor iedereen en loste moeiteloos en met veel geduld kleine ruzietjes op tussen mensen. Ik verbaas me keer op keer over jouw fotografische geheugen; je kijkt één keer op de kaart en rijdt hup zonder verdere navigatie naar de meest ingewikkelde locaties. Als jong gezin moe(s)ten we snel en efficiënt leren plannen en organiseren. Nu konden we dat ieder voor zichzelf al uitstekend, samen zijn we een groeiend team. Wat hebben we al ongelooflijk veel meegemaakt met zijn tweetjes. Rollercoasters aan gebeurtenissen, rollercoasters aan emoties. We staan er gewoon nog steeds, hand in hand. Ik ben gigantisch trots op jou, op ons. Natuurlijk op ons geweldige kind, Philippe. Lieve, lieve, lieve kleine Philippe, wat ben je een heerlijk mannetje. Uiterst wijs en vol met positieve energie. Micha en ik zijn buitengewoon blij dat je ons hebt uitgekozen om jouw ouders te zijn! We halen zo veel plezier uit dit leven samen met jou.

Maar het meest van iedereen wil ik mijn moeder bedanken. Voor de gesprekken over alle mogelijke onderwerpen die ik 28 jaren lang met je heb gevoerd, waarin je me het zelfvertrouwen en de discipline hebt gegeven die ik onder andere nodig had om dit proefschrift af te ronden. Ik ben ontzettend blij dat jij mijn moeder bent. 'Een prachtige en inspirerende vrouw' is zoals mensen je onder andere omschrijven. Je wordt door velen onvoorstelbaar gemist. Ik weet dat je trots op me bent, maar wat had ik je er graag bij gehad, al was het maar om 10 seconden in je stralende ogen te kijken.

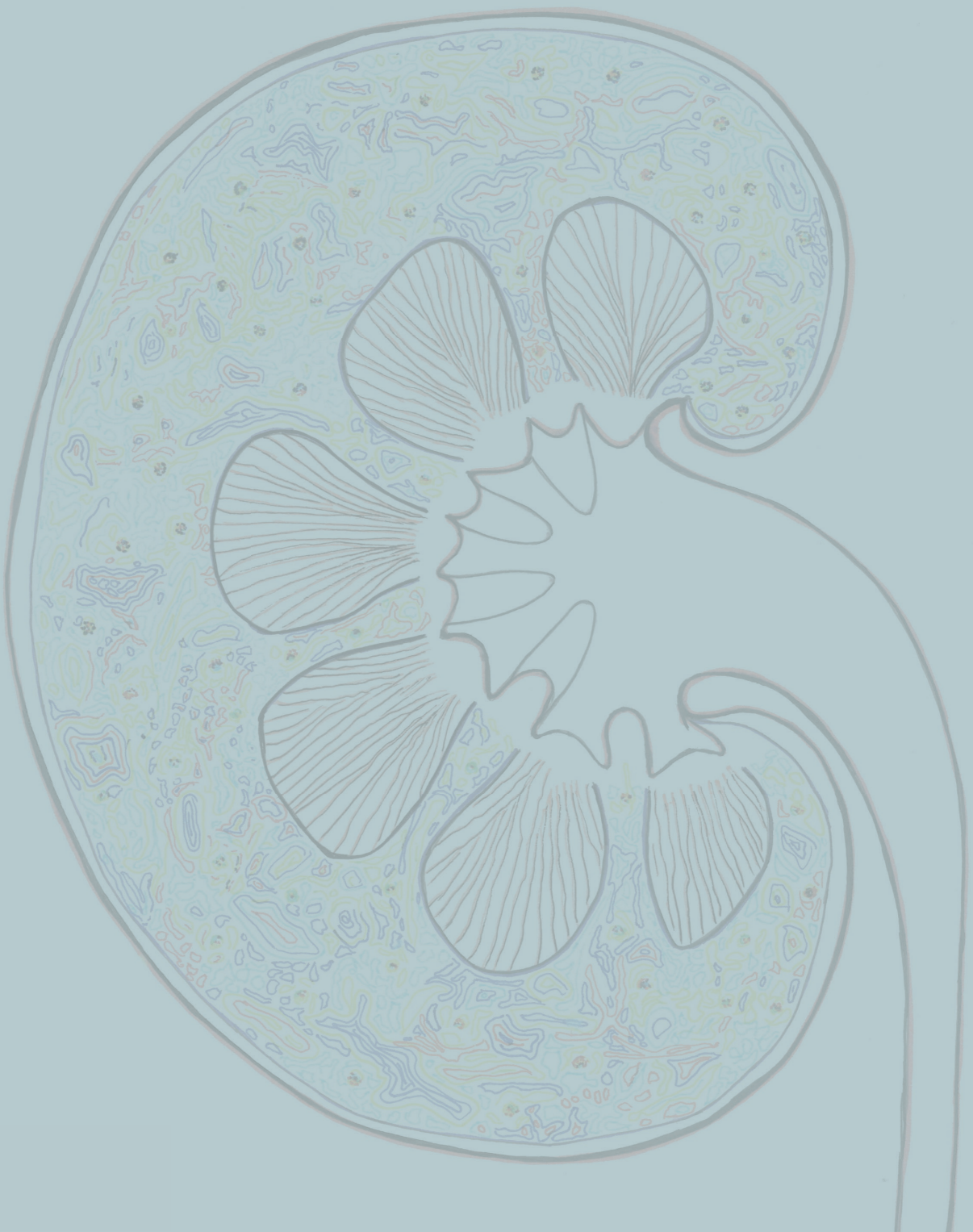
Mijn dankwoord schrijvende realiseer ik me hoeveel mensen mij op hun eigen wijze vanuit hun eigen discipline en hoedanigheid hebben bijgestaan om deze grote opdracht tot een goed einde te kunnen brengen.



About the author

Rosa Lammerts werd op 11 juli 1989 geboren in Zuidwolde, Groningen. Ze groeide op in Groningen met haar ouders en jongere broer Guido. Zij behaalde haar Gymnasium diploma aan het Praedinius Gymnasium in Groningen in 2008. Ze begon met haar studie Geneeskunde aan de Rijksuniversiteit Groningen en ging werken bij het Prometheus nier(transplantatie)team. Na het behalen van de bachelor Geneeskunde deed zij haar stage wetenschap gedurende 8 maanden bij de groep van professor Rutger Ploeg in Oxford en zij was betrokken bij het opzetten van het QUality in Organ Donation team UK. Hierna volgde zij haar co-schappen in het UMC Groningen en de Ziekenhuis Groep Twente in Almelo. In deze periode heeft Rosa tevens gewerkt bij het Wijnlokaal Barrel, in Groningen. Ook volgde zij een paar maanden een co-schap in San Carlos in Nicaragua. Haar oudste co-schap deed zij op de afdeling chirurgie en de intensive care in het Isala in Zwolle. Na het behalen van haar artsenbul startte ze op 1 mei 2016 met haar promotie traject in het UMC Groningen op de afdeling nefrologie met als promotoren professor Stefan Berger en dr. Jaap van den Born. Haar promotie traject werd voor 8 maanden onderbroken om intensief voor haar moeder te zorgen, die in juni 2018 uitgezaaide endometrium kanker bleek te hebben waaraan ze op 7 januari 2019 overleed. In maart 2019 hervatte zij het wetenschappelijke onderzoek weer. De inhoud van dit proefschrift heeft geleid tot meerdere presentaties en posters op internationale en nationale congressen. Ook heeft het geleid tot huidige samenwerkingen met onderzoekers van het UMC Utrecht en de Universiteit van Hannover. Rosa houdt erg van reizen en wielrennen en heeft in 2017 de 'End-to-end cycle route' gefietst samen met twee Engelse vrienden en haar jongere broer Guido. Dit is een fietstocht, die start in John o' Groats, het noordelijkste puntje van Schotland, en eindigt in Land's end, het zuidelijkste puntje van Engeland. Op 13 november 2019 is zij de trotse moeder geworden van Philippe Hendrik Joachim Müller.

In juni 2021 is Rosa begonnen met de opleiding tot laboratoriumspecialist medische immunologie in het UMC Groningen met als opleiders dr. Caroline Roozendaal en dr. Bouke Hepkema.



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