Significance of Posttransplant HLA Antibody Monitoring by Solid-Phase Immunoassays in Renal Allograft Recipients

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To my sons Janis & Joris for time taken from them

Zusammenfassung

Die Einführung neuartiger Immunsuppressiva hat in den letzten Jahrzehnten zu herausragenden Fortschritten auf dem Gebiet der Nierentransplantation geführt. Die Funktionsraten in der Frühphase nach Transplantation sind hervorragend. Das Langzeittransplantatüberleben hingegen ist weiterhin unzufriedenstellend. Die Ätiologie des späten Funktionsverlusts ist derzeit unklar. Durch die Weiterentwicklung neuartiger diagnostischer Tests in den letzten Jahren rückte insbesondere die humorale Transplantatabstossung als eine Hauptursache in den Fokus. Gegenstand dieser Dissertation ist die zentrale Fragestellung, inwieweit Antikörper gegen humane Leukozytenantigene (HLA), nachweisbar mittels hochsensitiver Festphasentechnik, als nicht-invasive Biomarker fungieren können, um das Langzeittransplantatüberleben vorherzusagen.

Insgesamt wurden 1075 Nierentransplantatempfänger in die vorliegende unizentrische Querschnittsstudie eingeschlossen und HLA-Antikörper in Serumproben mittels Luminex®-basierter Nachweismethoden detektiert. Der klinische Beobachtungszeitraum betrug im Mittel 8 Jahre nach Antikörperbestimmung. Modifikationen des Luminex-Tests wurden etabliert und validiert, um die Antikörperspezifität als auch Effektorfunktion besser charakterisieren zu können, die ihrerseits potenziell Rückschlüsse auf die Pathogenität zulassen.

Der Nachweis insbesondere donorspezifischer IgG HLA-Antikörper konnte mit einem verringerten Transplantatüberleben assoziiert werden. Es zeigte sich, dass die zusätzliche Bestimmung der Komplementbindungsfähigkeit einen bedeutenderen Einfluss auf die Vorhersage der Transplantatüberlebenswahrscheinlichkeit hatte als die Zuordnung der HLA-Antikörper zu den IgG-Subklassen. Die Ergebnisse des Standard-Luminex®-Tests wurden durch Veränderungen am Testkit (d.h. Nachweis von intakten und denaturierten HLA-Molekülen) und eine veränderte Strategie der Auswertung (Epitopanalyse) verifiziert. Dies ermöglichte eine adäquate Interpretation der Testergebnisse und Identifizierung unspezifischer, klinisch irrelevanter Antikörper. Des Weiteren lieferten die Korrelation mit Biopsieergebnissen und die Erweiterung der Querschnitts- zu einer Längsschnittstudie zusätzliche Hinweise auf einen kausalen Zusammenhang zwischen der Präsenz von HLA-Antikörpern und dem Transplantatfunktionsverlust.

Die Erkenntnisse aus dieser Dissertation führten zur Etablierung eines Schemas zur HLA-Antikörperüberwachung nach Nierentransplantation an allen drei Transplantationszentren der Charité. Damit leistet die HLA-Antikörper-Diagnostik mittels Luminex®-Festphasentechnik einen bedeutenden Beitrag zur adäquaten Risikostratifizierung und Individualisierung der immunsuppressiven Therapie nach Transplantation mit der Zielstellung, das Langzeittransplantatüberleben in der Zukunft signifikant zu steigern.

Abstract

Despite remarkable advances in kidney transplantation over the past decades driven by the introduction of more potent immunosuppressive therapeutics into the clinic, late allograft dysfunction remains the major barrier to long-term survival. The etiology of chronic allograft dysfunction is not well understood. Recently, with the advent of more powerful diagnostic tools humoral rejection processes became a major focus of interest. Therefore, the aim of this thesis was to elucidate the significance of human leukocyte antigen (HLA) antibodies, detected by modern, most sensitive solid-phase immunoassays, as non-invasive biomarkers to predict long-term renal allograft outcome.

In a large single-center study we enrolled serum samples of 1075 kidney transplants that were tested in a cross-sectional manner for the presence of HLA antibodies by Luminex® solid-phase assay. Graft function survival was followed for 8 years after antibody testing. We established and validated modifications to the standard Luminex® assay in order to further delineate antibody specificity as well as effector function as these determinants are anticipated to predict the pathogenicity of antibodies.

HLA antibodies, in particular, those with donor-specificity could be associated with decreased kidney allograft survival. The assessment of the complement-binding ability rather than IgG subclass distribution pattern of HLA antibodies significantly enhanced the predictive value. However, verification of immunoglobulin specificity for intact rather than denatured HLA on detection beads and the delineation of antibody epitopes provided important contributions to evaluate the validity of the standard Luminex® assay and identify unspecific, clinically irrelevant antibodies. Indications for a causal relationship between HLA antibodies and late renal allograft rejection could be deduced from biopsy data of failed transplants and the longitudinal follow-up of posttransplant HLA antibody status.

In conclusion, the findings presented in this thesis have been translated into recommendations for a posttransplant HLA antibody monitoring scheme as a powerful, non-invasive diagnostic tool to detect rejection. The predictive value of antibodies provides further perspectives for patient risk stratification to enable tailoring the individual immunosuppressive therapy and thus significantly improve long-term renal allograft outcome.

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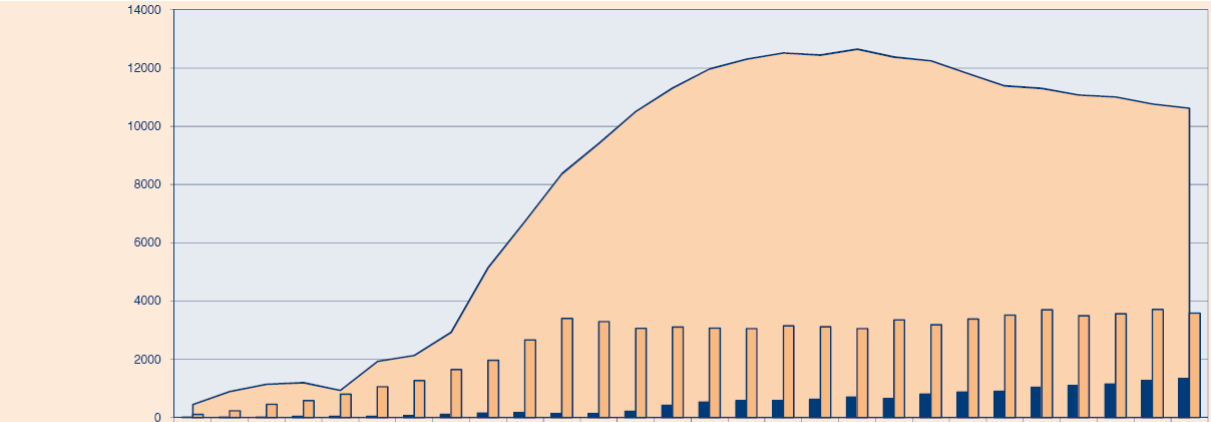
1 Introduction

1.1 The Disparities in Renal Transplantation

When in 2007 reputable press agencies like Reuters and AFP reported breaking news of a forthcoming controversial Dutch television show named “The Big Donor Show”, where three candidates waiting for a renal transplant should compete on a kidney, it provoked a storm of moral disgust among the public worldwide (1). Although the press release turned out to be a publicity campaign it really achieved its goal to draw the public attention towards the problem of donor organ shortage.

Renal transplantation (TX) is still the preferred treatment for patients with chronic kidney disease regardless of the primary cause. As an artificial and temporal replacement for lost kidney function usually dialysis is used to bridge the time gap to TX. Organ replacement provides patients with a higher quality of life (2, 3) and is associated with a lower mortality rate (4). In addition, from an economic point of view TX is preferred over dialysis and provides a relative net saving after a period of 1-2 years (3, 5). In 2011 a total of 1554 kidneys from deceased donors were transplanted in Germany (6). Despite the approached benefits, there are eminent barriers to successful renal TX: (i) the availability of donor organs and in addition to that (ii) the immunobiology of tissue compatibility between donor and recipient.



	1969	1971	1973	1975	1977	1979	1981	1983	1985	1987	1991	1993	199 5	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Kidney waiting list	450	892	1147	1200	934	1929	2135	2928	5150	6740	8376	9418	10510	1 1324	11975	12313	12524	12450	12653	12382	12251	11814	11393	11 308	11082	11010	10768	10622
Living donor transplants	9	5	11	33	33	33	53	108	150	161	129	127	212	411	526	579	569	617	697	646	803	866	901	1032	1091	1150	1262	1339
Deceased donor transplants	102	228	454	583	800	1050	1263	1645	1965	2665	3395	3293	3064	3110	3068	3050	3145	3121	3047	3352	3185	3383	3518	3703	3492	3561	3705	3585

Figure 1. Dynamics of the kidney waiting list. Disparity between the number of transplants and patients on the waiting list from 1969 through 2011 within the organ sharing organization Eurotransplant. Although the number of transplants from deceased and living donors could be increased over time the disparity between the demand for organs and the supply is dramatic. Taken from Eurotransplant annual report 2011 (www.eurotransplant.eu).

The need for expanding the donor pool

In parallel with the enlarging number of patients diagnosed with primary chronic kidney disease or loss of renal transplant function the number of individuals on the kidney waiting list is increasing rapidly. On the contrary, the number of deceased donors remained constant over the past decades (**Figure 1**). In detail, the number of young and healthy deceased organ donors is declining leading to the increased usage of so-called marginal organs from older donors with medical comorbidities, previously precluded donation (7).

As a consequence of the gap between deceased donor organ supply and demand within the organ sharing organization Eurotransplant (i.e., 3585 transplants vs. 10,522 waitlisted patients in 2011) the average waiting time for a renal transplant nowadays hits 6 years. So, it is not surprising that in a recent analysis of the Canadian kidney waiting list the probability of needing a renal transplant exceeds the probability of becoming a deceased organ donor by five to six times depending on age (8).

Alternative means to further expand the donor pool is to increase the number of living donation between familial (e.g. siblings) or emotionally related (e.g. spouse) donor-recipient pairs. The proportion of live donor renal transplants steadily increased over the last decade in Germany up to 22% in 2011. The major benefits of a live donor transplant include the optimization of the recipient due to the elective nature of the procedure, proven low rates of delayed graft function and better long-term outcome (9). In addition, the use of a live donor preserves a deceased donor kidney for another patient on the waiting list.

Steady improvements in patient survival (4) combined with a relative constant renal allograft half-life (10) have led to an increasing number of transplant recipients with failed allografts who face retransplantation (see Eurotransplant Annual Report 2011). Therefore, sustainable management of the limited number of donor organs and efforts to increase long-term renal allograft survival are the major tasks the transplant community is confronted with nowadays.

The immunologic barriers to transplantation

It's been in the early 1960's shortly after the first worldwide renal TX in 1954 by Joseph Murray and colleagues in Boston that the relevance of the ABO blood group antigen compatibility as one major immunological barrier to TX has been recognized (11). Today blood group identity rather than compatibility determines the allocation of deceased donor kidneys. However, attempts have been made to cross the ABO barrier by removing isoagglutinins using immunoadsorption in selected cases of living donations with an overall excellent outcome (12, 13).

In particular, the highly polymorphic nature of the human leukocyte antigens (HLA) encoded by a set of genes situated on the short arm of chromosome 6 forms the basis of the major histocompatibility complex (MHC) in humans. HLA disparities between donor and recipient fundamentally determine the degree of alloreactivity and thus are of great immunological and clinical relevance for transplant outcome.

1.2 HLA Compatibility and Transplantation

Organization of the HLA genes

The complex of HLA encoding genes can be distinguished in a class I, II and III region (**Figure 2A**). The class I region is located on the telomeric end of the HLA complex and encodes classical, serologically defined specificities HLA-A, -B, and -C. Class I gene products are constitutively expressed on all nucleated cells with the exception of neural cells, sperm, and trophoblasts. However, the degree of constitutive expression is tissue-dependent and varies between HLA-A, -B, and -C.

The class II region located centromeric to class I comprises several subregions (loci), of which HLA-DRB, -DQB, -DQA, and -DPB have been proven to exhibit clinical relevance with respect to solid organ TX. A hallmark of the HLA-DRB region is that the number of DRB genes is haplotype-dependent. While DRB1 is present in all haplotypes encoding HLA-DR specificities DR1-DR18, additionally expressed DRB genes DRB3, DRB4 and DRB5 are only present on specific haplotypes. Contrary to class I, constitutive class II expression is limited to professional antigen-presenting cells (APC) including B lymphocytes. In addition, expression is inducible by a variety of stimuli on T lymphocytes, monocytes, dendritic and endothelial cells. Although all loci are co-expressed, quantitative differences are observed where HLA-DR expression is generally increased.

The class III region encodes for several immune-related proteins without direct clinical relevance for histocompatibility such as complement factors (e.g. C2, C4A), tumor necrosis factors TNFα and β as well as heat shock protein HSP70.

The structure and function of HLA gene products

HLA class I and II gene products are characterized by a unique domain structure reflecting the biological function of the molecules. Briefly, class I molecules are composed of a polymorphic glycosylated α-chain comprising three extracellular domains (α_1-α_3) which is covalently bound to a monomorphic β-chain, namely, the β_2 microglobulin (β2m). On the contrary, class II molecules are heterodimers composed of a polymorphic α- and β-chain. Both chains contain two extracellular domains (α_1, α_2 and β_1, β_2). The domains α_1, α_2 and α_1,

β_1 of the class I and II molecules, respectively, form a β-pleated sheet platform structure on which two α-helices are located in parallel orientation to form the side walls of the functionally important antigen-binding groove (**Figure 2B**). A further detailed review of the structure of HLA molecules was published by Strominger (14).

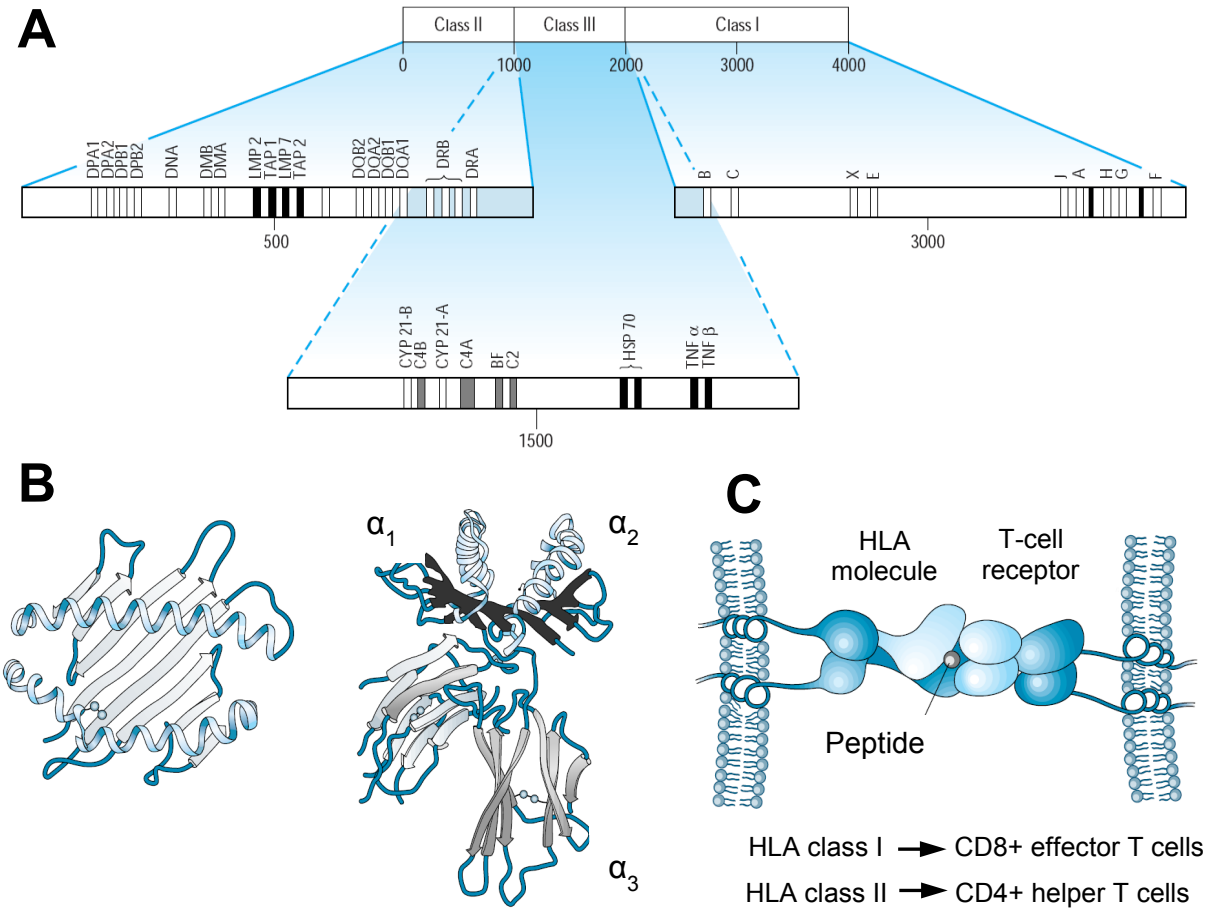


Figure 2. Gene organization, structure and function of HLA. Panel A illustrates the organization of class I, II and III genes of the MHC in humans on the short arm of chromosome 6 (6p21.1-6p21.3). Class I and class II gene products HLA-A, -B, -C and HLA-DRB, -DQB, -DQA, -DPB are of clinical relevance for the histocompatibility between donor and recipient. **Panel B** illustrates the domain structure of a HLA class I molecule (right) comprising three extracellular domains α1-α3 covalently associated with β2m. The peptide binding groove (left) is formed by β-pleated sheets framed by α-helices of the α1 and α2 domain. **Panel C** depicts the central functional role of HLA molecules in the adaptive immune system, namely, antigen presentation. Peptides are presented by class I and II molecules to CD8+ effector and CD4+ helper T lymphocytes, respectively. Figure was adapted from (16).

The peptide binding groove of class I and II molecules can bind peptide fragments of 8-11 and 15-24 amino acids (AA) in length, respectively. Thus, antigen presentation encompasses the primary functional role of HLA molecules in the adaptive immune system whereas class I proteins present antigens to CD8+ effector and class II to CD4+ helper T-cells (**Figure 2C**). The signal is transduced from the APC via HLA towards T lymphocytes and involves the

recognition of the presented antigen. Due to the close contact between HLA and T-cell receptor not only the presented peptide is recognized as being autologous or allogeneic but also the HLA molecule itself (15).

The polymorphism of HLA

Owing to the excellence of HLA to present a diversity of peptide fragments to T lymphocytes there is an extensive allelic polymorphism of HLA genes, especially within the gene segments which form the peptide binding groove.

The HLA typing can be determined by serological or molecular means (detailed in section 2.4). Serologically defined HLA antigens are designated by the name of the respective gene locus and the number of the antigen (e.g. A2, DR13). On the contrary, alleles of HLA genes as assessed by molecular typing, and whose products differ in their AA composition, are designated by the name of the gene locus followed by an asterisk and a 2-digit number of the antigen. Another 2- to 3-digit number designates the respective allele which is separated from the antigen by a colon (e.g. A*02:103, DRB1*13:01). Naming of factors of the HLA system is supervised and regularly published by the WHO Nomenclature Committee. According to the current HLA database as of October 2012 a total of 6725 HLA class I and 1771 class II alleles have been assigned (http://hla.alleles.org/).

Alloreactivity triggered by HLA

The continuing confrontation with the allogeneic graft triggers alloreactivity of the recipient's immune system and makes renal TX without a life-long application of immunosuppressive (IS) drugs almost impossible. The beneficial effect of HLA compatibility in clinical TX demonstrates the dominance of HLA as the major histocompatibility antigens (**Figure 3**).

Although non-immunological factors like waiting time, recipient and donor age as well as duration of organ preservation are playing a role in the decision making of organ allocation from deceased donors, the achievement of HLA compatibility between recipient and donor has traditionally been in focus of organ sharing organizations like Eurotransplant.

Compatibility between donor and recipient is traditionally determined by matching serologically defined HLA antigens. However, the recipient's alloimmune response is triggered by mismatched polymorphic AA residues rather than a whole antigen. Despite the polymorphism there is a substantial homology between HLA alleles resulting in differential immunogenicity of HLA antigen mismatches depending on the recipient's HLA typing exemplarily demonstrated in **Figure 4**.

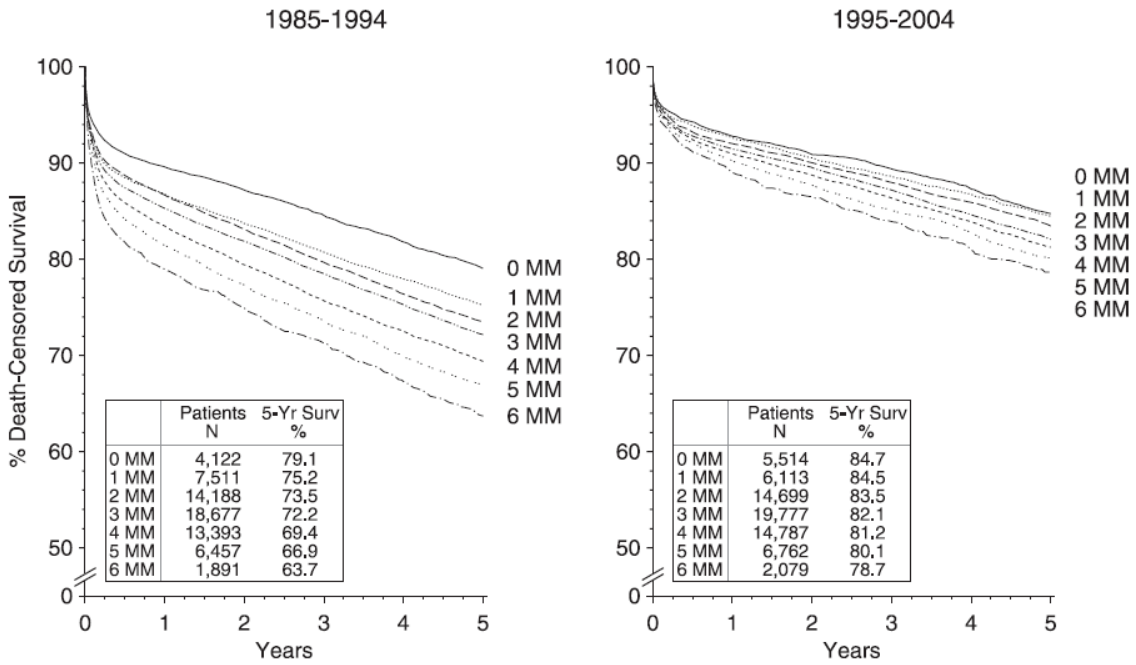


Figure 3. Clinical relevance of HLA compatibility on renal allograft survival. Death-censored 5-year-graft survival rates in relation to the number of HLA-A, -B, -DR mismatches for transplants performed 1985–1994 (left) and 1995–2004 (right). The effect of HLA matching renal allografts has been diminished over time but is nowadays still significantly present. Taken from Opelz et al. (17).

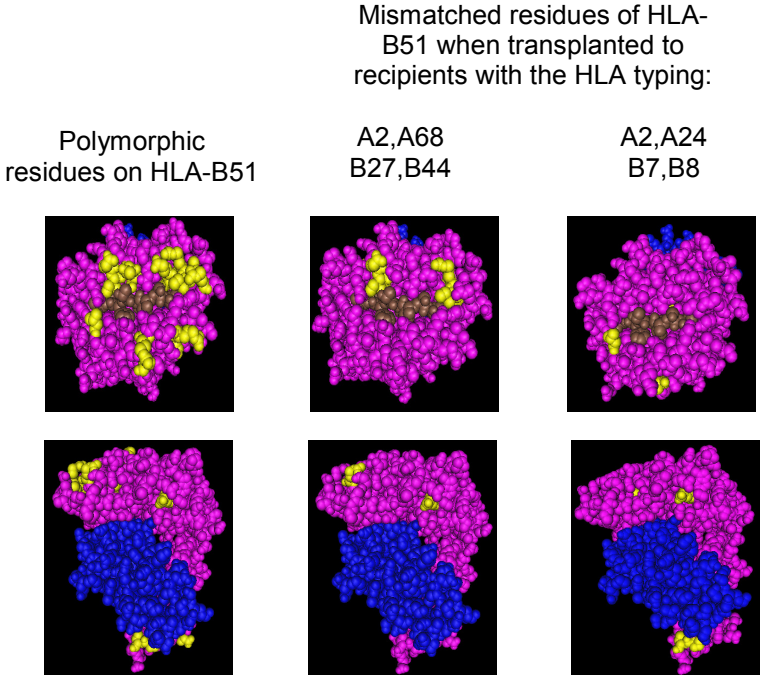


Figure 4. Differential immunogenicity of HLA-B51 dependent on the HLA typing of the recipient. The overall polymorphic residues (yellow) on the α-chain (purple) of HLA-B51 (left) are recognized differentially by the immune systems of two potential recipients with different HLA typings (middle and right). The peptide and β2m are illustrated in brown and blue, respectively.

The computer algorithm HLAMatchmaker developed by R. Duquesnoy facilitates a more adequate matching of HLA based on the AA composition of donor and recipient (18). The algorithm is based on the principle that a patient will not demonstrate alloreactivity against residues present on self-HLA antigens. By comparing the AA composition on the mismatched

HLA antigens with all self-residues, one can define the number of non-self-residues, which might trigger alloreactivity. As a proof of concept it was demonstrated that the number of mismatched residues directly correlates with the probability of pregnant women to develop alloantibodies against the paternal HLA class I alleles of the fetus (19). The HLAMatchmaker algorithm perfectly predicts the humoral but so far failed to predict the cellular immune response to alloantigens (20).

Non-HLA alloantigens

Among the histocompatibility antigens HLA is most important, however, other surface antigens like angiotensin II type-1 receptors (AT_1R), vimentin, collagen V and other still unidentified endothelial antigens have been shown to elicit an alloimmune response in TX (21-24). In particular MHC class I-related chain A (MICA) antigens which are not as polymorphic as HLA but expressed on endothelial cells (not on lymphocytes) have been proven to trigger alloreactivity negatively impacting allograft outcome (25).

1.3 Diagnostic Assessment of Alloreactivity

Measuring the immune response to alloantigens plays an important role in the selection process of a suitable donor for a renal transplant candidate by the assessment of memory alloreactivity. In addition, posttransplant monitoring of the immune response aids in tailoring demand-oriented IS medication and diagnosis of rejection. Based on the type of effector cells primarily involved we can distinguish between cellular and humoral immunity.

1.3.1 Cellular Alloreactivity

The assessment of the cellular alloreactivity elicited primarily by T lymphocytes is not routinely performed in the context of solid organ TX and will be discussed only briefly (26).

The two main assays currently used to monitor lymphocyte-mediated cytoxicity are the ^{51}Cr-release and interferon gamma (IFNγ) ELISpot assay. Both tests are based on the principle of the mixed lymphocyte reaction (MLR), where recipient's T cells are mixed and co-cultured with lymphocytes from the donor. In the presence of alloreactive cytotoxic T lymphocytes ^{51}Cr-labelled target cells are lysed and the release of radioactivity is measured. The ELISpot assay, however, quantifies IFN-γ which is secreted by activated T helper cells.

Although some correlation between cellular alloreactivity and impaired renal allograft outcome could be described, none of these assays is routinely used in the process of donor

selection or posttransplant monitoring mainly due to the high labor costs and lacking standardization (27, 28).

1.3.2 Humoral Alloreactivity

The humoral immune response to alloantigens is mediated by soluble antibodies which are secreted by plasma cells of the B lymphocyte lineage. Naïve B cells can be activated in a T cell-dependent manner by cytokine release of a helper T cell (T_h) recognizing the same antigen, which then will lead to somatic hypermutation, class switch recombination and differentiation into plasma cells or memory B cells.

A detailed discussion on structure and function of antibodies can be found elsewhere (29). However, of special interest is the effector function elicited by antibodies which not only significantly impacts the pathogenicity of the antibody but is also utilized as test principle for the different HLAab detection assays. The major biological antibody functions are: (i) activation of immune effector cells (macrophages, neutrophils) via interactions between antibody and specialized receptors as well as (ii) activation of the complement cascade. Complement activation is initiated by binding of the complement component C1q to an antibody-antigen complex which leads to C1-mediated cleavage of C2, C4 and formation of C3 convertase. Following several intermediate steps then C5 is split to C5b which initiates the assembly of the membrane attack complex together with C6 through C9 finally leading to cell lysis.

The specificity of HLAab can be assessed in a 1x1 crossmatch between recipient serum and donor cells or as an antibody screening assay using a panel of different HLA targets. Based on these targets we can distinguish cell-based from solid-phase immunoassays (SPA).

Cell-based immunoassays

The complement-dependent lymphocytotoxicity (CDC) test, developed by Terasaki and McClelland in the early 1960s is still used in histocompatibility testing (30). Herein, target cells are incubated with test serum, and the addition of rabbit complement results in the lysis of target cells if bound with sufficient complement-fixing antibodies. As readout the proportion of lysed cells is assessed by dye exclusion using ethidium bromide and acridin orange. The CDC principle is used as crossmatch and screening technique. Several modifications of the method have been described including the treatment of sera with dithiothreitol (DTT) to inactivate IgM antibodies, and the introduction of wash steps and an antiglobulin reagent to increase the assay sensitivity (reviewed in (31)).

In 1983 Garavoy et al. introduced a novel crossmatch procedure based on flow cytometry (FC) (reviewed in (32)). FC enabled the detection of different immoglobulin classes and subclasses as well as differentiation of cell targets (e.g. T and B lymphocytes) by staining with fluorophore-labeled monoclonal antibodies to cell-specific surface markers (e.g. CD3 and CD19).

Pretransplant screening of transplant candidates for lymphocytotoxic antibodies and performing a CDC crossmatch using donor lymphocytes as target cells provides a sufficient high level of sensitivity for the prediction of early rejections in renal TX (33). It is therefore still the standard crossmatch technique within Eurotransplant. The FC crossmatch widely accepted in the US has greatly increased the level of sensitivity for predicting antibody-mediated rejection (AMR) (34) but neither test is well standardized or provides an adequate level of specificity (35). The lack of specificity is mostly inherent with the target. Both assays largely depend on vital lymphocytes and are prone to detecting non-HLA specific auto- and alloantibodies (36). This limitation can partly be overcome using CDC by DTT treatment of sera to distinguish between clinically irrelevant IgM from potentially harmful IgG antibodies (37). Interferences may still occur caused by complement fixing therapeutic antibodies, e.g. rituximab which targets CD20+ B lymphocytes.

Solid-phase immunoassays

SPA make use of solubilized HLA molecules bound to a solid matrix that is either a microtiter plate or microbeads. HLA may be purified from platelets, lymphoid cell lines or transfected cells.

Commercially available and most commonly used SPA test kits are based on the enzyme-linked immunosorbent assay (ELISA) or the multiplexed multi-analyte bead assay technique. The ELISA uses colorimetric detection and is usually performed in 96 well microtiter plates.

The bead-based assays utilize microbeads impregnated with fluorescent dyes. In the platform by Luminex® each bead set is impregnated with a unique mixture of two fluorophores which are both excited by a red laser at 635 nm. The emitted light can be detected at wavelengths of 660 nm (red) and 730 nm (infrared) using a dedicated footprint flow cytometer (Luminex®100/200™) (**Figure 5**). By measuring the composition of the emission up to 100 different bead sets with their coated HLA can be individually identified. The detection of HLA-specific antibodies is achieved by using a secondary antibody conjugated with the reporter fluorophore R-phycoerythrin (PE) which is excited by a green laser (532 nm) and detected at 576 nm wavelength. The test principle is depicted in **Figure 6A**.

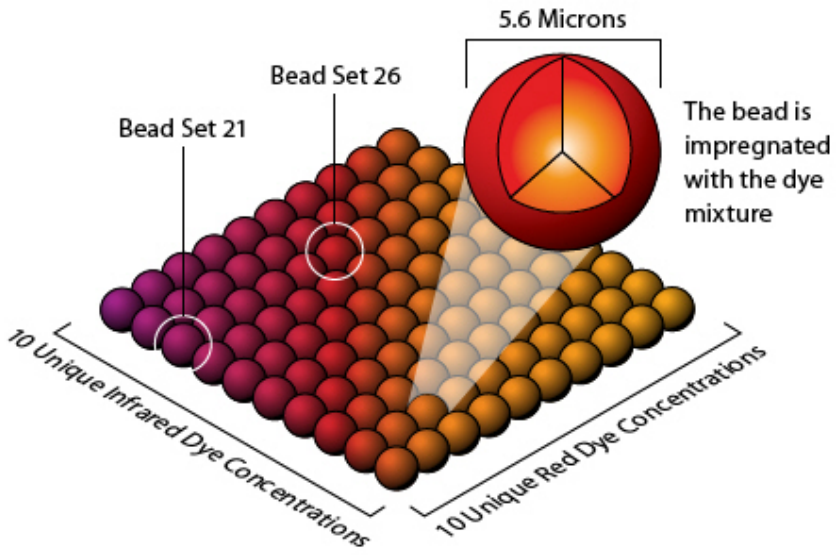


Figure 5. Principle of bead-based SPA by Luminex®. Each Luminex® microbead is impregnated with a unique combination of two fluorophores emitting light in the red and infrared spectrum. The unique fluorescence signals of each bead allows multiplexing using up to 100 beads in one single reaction. Picture taken from Luminex® Inc.

Figure 6B illustrates the three types of panels used for Luminex® beads which can be distinguished based on the composition of their target antigens: (i) pooled antigen panels have bead populations coated with either affinity purified HLA class I (HLA-A, -B, -C) or HLA class II (HLA-DR, -DQ, -DP) antigens which have been pooled from multiple individuals and are used for qualitative HLAab detection; (ii) phenotype beads are coated with either the HLA class I or class II phenotype antigens of a single individual; and (iii) single antigen panels comprise bead populations coated each with a single allelic, recombinant HLA antigen. Screening for HLAab can be achieved best by using pooled antigen panels which are relatively inexpensive and provide information on the presence or absence of antibody to a particular class. Moreover, these panels may be useful for tracking changes in the breadth and/or level of antibody binding in sequential sera. Determination of antibody specificity with highest sensitivity and degree of resolution to the allelic level is achieved by using single antigen beads (SAB).

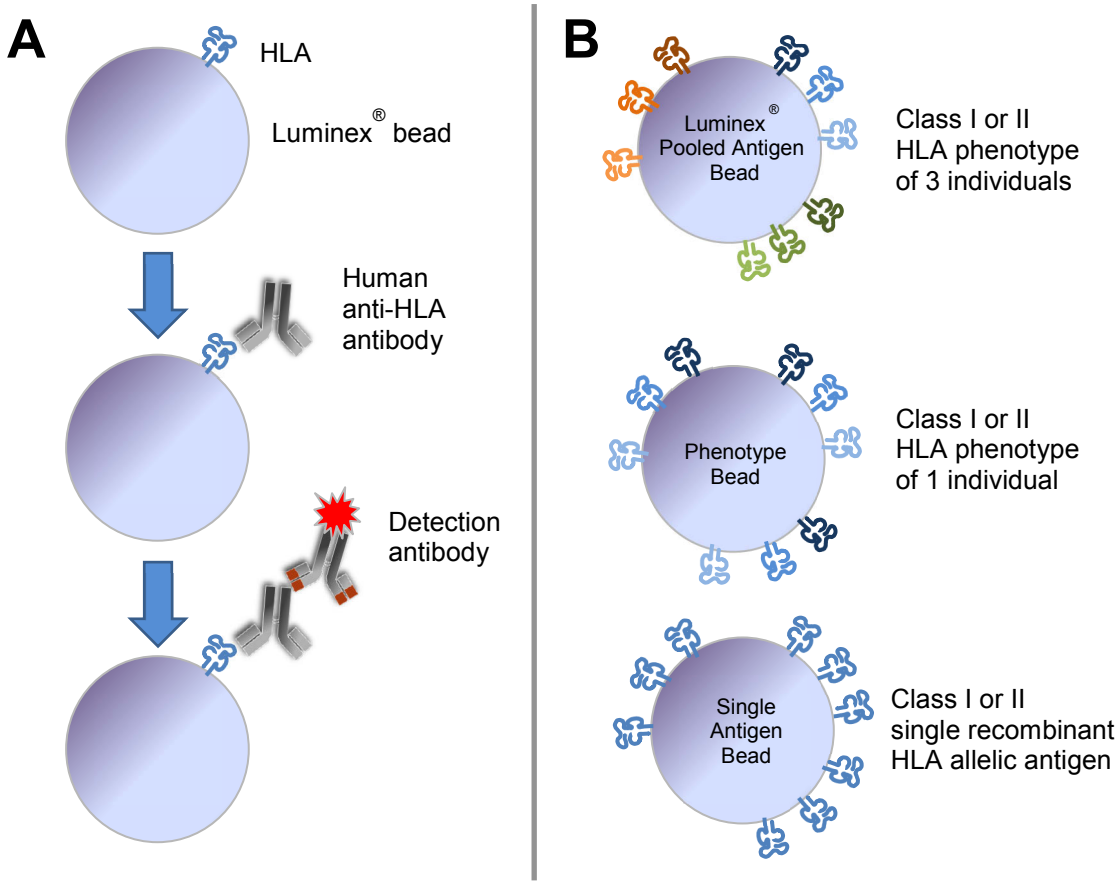


Figure 6. HLAab detection by Luminex® bead array: Principle and panel composition. Panel A illustrates the principle underlying the Luminex® assay. Each specific bead impregnated with two fluorophores is coated with HLA from cell lines, platelets or recombinant HLA. In case the test serum contains antibodies directed against the specific HLA it will bind to the appropriate bead. The binding is then detected by a PE-conjugated secondary antibody specific for human IgG. The combination of the fluorescence signals from each bead indicating the HLA specificity and the secondary reagent indicating bound HLA-specific antibodies is acquired by the Luminex® system using appropriate lasers and detectors. In **panel B** the three different formats of Luminex® beads are illustrated. The Luminex® pooled antigen beads are each coated with class I or II HLA phenotypes (i.e., HLA-A, -B, -C or HLA-DR, -DQ, -DP) from 3-6 different cell lines or platelets. This test format allows the screening for antibodies only. Phenotype or single antigen beads (SAB) are used for specification. SAB are coated with a single recombinant HLA allelic antigen (e.g. A*02:03) and provide the highest specificity and sensitivity in HLAab detection.

Noteworthy, bead-based immunoassays are approved only for qualitative testing by the US Federal Drug Administration FDA and European Commission but several publications showed correlations of the mean fluorescence intensity (MFI) as measured in Luminex® bead arrays with crossmatch results using CDC and FC as well as clinical outcomes (38, 39).

Despite the advantages of the Luminex® technology, there are important limitations such as a prozone-like effect. Similar to the Hook or prozone phenomenon, well known from precipitation immunoassays (40), the serum appears negative or weak when tested neat and becomes more strongly reactive upon dilution, especially in the case of highly sensitized patients (41). Dilution or hypotonic dialysis of the serum as well as the addition of DTT and

treatment with EDTA or heat inactivation were described to overcome this phenomenon (42-44). Furthermore, a technical issue inherent with the manufacturing process of the beads and coating procedure of the HLA molecules on the solid phase matrix are alterations in the tertiary structure of the molecules which could lead to deviant reactions (45). In contrast, SPA in general have the great advantage of allowing the use of a variety of secondary detection antibodies to assess immunoglobulin isotypes or subclasses as well as complement components.

Clinical applications

Depending on the kidney transplant program and its policy to define unacceptable antigens pretransplant, HLAab screening is performed by CDC and/or SPA. To date, posttransplant antibody monitoring is not routinely performed by all transplant centers.

1.4 Mechanisms and Diagnosis of Allograft Rejection

The main immunological reason for graft dysfunction after renal TX is allograft rejection. On one hand, solely based on the time of occurrence different types of rejection can be discriminated: hyperacute (HAR), acute (AR) and chronic rejection (CR). On the other hand, mechanistically we can distinguish between cellular rejection and AMR. Confusingly, HAR, AR and CR have been used in the literature interchangeably to also describe cellular and humoral rejection.

Cellular rejection mechanisms

AR episodes may occur at any time point posttransplant ranging from months to years but is most commonly seen within the first six months following TX. For decades T lymphocytes have been regarded as being the major player in initiation and regulation of AR.

Cellular rejection is thought to result from direct allorecognition of foreign HLA presented to the recipient's alloreactive T cells. According to the passenger leukocyte theory (46) APC of the donor migrate to lymph nodes upon release of pro-inflammatory signals such as TNFα and stimulate alloreactive T lymphocytes. Once activated, these graft-directed T cells differentiate and undergo marked changes in their homing behavior which enables the cells to infiltrate the transplant leading to cell-mediated deterioration of graft cells (47).

Later after TX donor APC are depleted, and the allorecognition is predominated by recipient APC presenting donor HLA in the form of proteasome-processed peptides associated with self-HLA molecules (48). Indirect allorecognition resembles the general process by which most exogenous antigens enter the immune system and are recognized by T cells. Effector

mechanisms primarily responsible for cellular rejection involve cells of the adaptive immune system (CD4+ helper T cells, cytotoxic T lymphocytes and memory T cells) but also natural killer (NK) cells, eosinophils and neutrophils of the innate immune system. In contrast to naïve, memory T lymphocytes already exhibit alloreactivity and are generated via prior exposure to alloantigens (blood transfusions, gravidities, previous transplants) or non-specific exposure to pathogenic antigens due to infections or vaccination (49) that cross-react with alloantigens (50).

The mechanisms by which NK cells promote cellular rejection are not well understood yet. IFN-γ produced by activated NK cells is thought to upregulate HLA expression on endothelial cells, and thus enhances the susceptibility of the graft for allorecognition. In addition, NK cells infiltrating the transplant produce chemokines that subsequently attract inflammatory effector cells (51).

Antibody-mediated rejection mechanisms

AMR also termed humoral rejection was recognized very early in the history of TX as rejection caused by preformed antibodies to ABO blood group or HLA antigens. Briefly, preformed cytotoxic donor-specific antibodies (DSA) cause HAR within minutes to hours after reperfusion of the graft leading to cyanosis due to the activation of the complement system followed by activation of platelets and perfusion blockade (52). The incidence of HAR in recipients with preformed DSA detected by CDC was reported to be as high as 80% (33).

Recent evidence shows that also a substantial proportion of AR are due to circulating HLAab and complement deposition (53). The binding of DSA to the endothelium and subsequent complement activation triggers the recruitment of cellular infiltrates by increased release of pro-inflammatory cytokines, growth factors and upregulation of adhesion molecules leading to platelet coagulation in the capillaries and endothelial cell-necrosis (**Figure 7**).

In addition to alloantibodies, plasma cells itself and CD20+ B cells infiltrating the graft have been correlated with AR (54).

Acute AMR occurring within hours to days posttransplant is resistant to standard therapies and causes rapid graft functional impairment with a poor graft survival prognosis (55). However, in recent years it became apparent that acute AMR may also occur months or years following TX and is referred to “late acute AMR”.

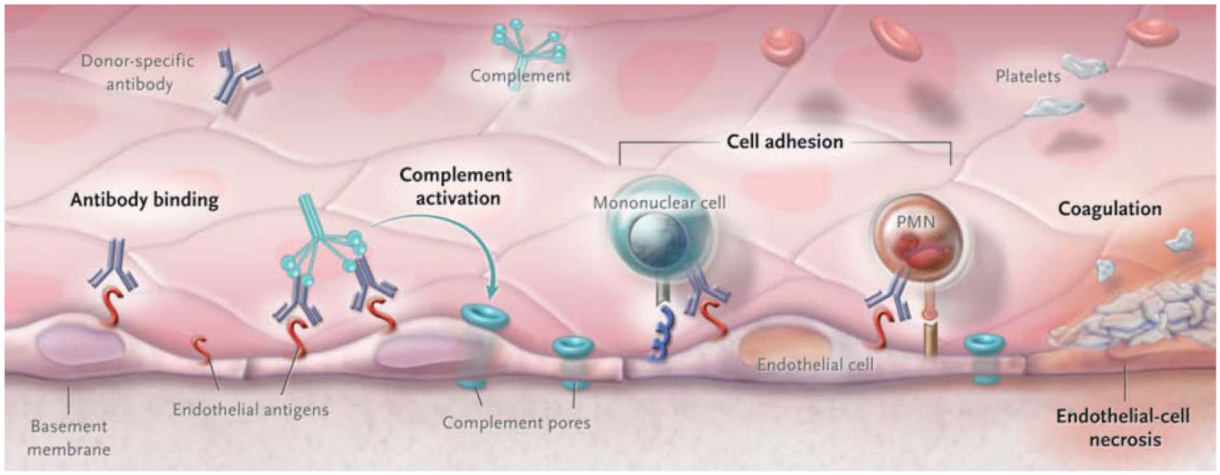


Figure 7. Antibody-mediated injury to allograft endothelium. Donor-specific alloantibodies bind to endothelial antigens (e.g. HLA, MICA) expressed by graft vessel cells. Complement activation and recruitment of mono- and polymorphonuclear cells lead to endothelial-cell necrosis and platelet activation (56).

CR also termed chronic allograft nephropathy (CAN) occurs in later stage posttransplant and confines more than any other entity the long-term survival of renal transplants; however the pathophysiology still remains poorly understood. At the tissue level CR is relatively unspecifically defined as tubulointerstitial, vascular or glomerular fibrosis. As mentioned earlier CR of renal transplants derives from the indirect allorecognition pathway (57). Importantly, HLA disparity between donor and recipient is the primary trigger for the alloimmune response during CR as abrogation of donor HLA expression reduces the immunogenicity of the graft and strongly influences the character and severity of CR in renal transplants (58, 59). It is thought that CR develops as a result of repeated acute or persistent damage from cellular and/or humoral injury to the graft (60). Clearly, a subcategory of AMR is chronic and denotes a slowly progressive but active process of graft deterioration over a long period of time. It could be shown by Cosio et al. (61) that the presence of low level HLAab in the absence of acute AMR leads to transplant glomerulopathy (TG) which is the histological manifestation of chronic AMR.

As shown, immunologic but also several non-immunologic risk factors for CR have been identified including drug toxicity, ischemia and senescence which are reviewed by Pratschke et al. (62).

It became obvious that antibodies play an important role in the process of allograft rejection, and that AMR may occur in any stage after TX. The incidence of AMR in unselected populations is as low as 5-7% (63) but reaches up to 40% in high risk patients who underwent

desensitization protocols prior to TX (reviewed in (64)). The true incidence is probably underestimated due to undetected subclinical AMR.

Diagnosis of rejection

Symptoms of clinical rejection include an unexplained decrease in allograft function or in the initial period after TX insufficient improvements usually accompanied by relatively unspecific symptoms like fever and hypertension. Today diagnosis of rejection mainly relies on two pillars: (i) assessment of renal function by measuring the serum creatinine and/or urinary protein level, and (ii) histological examination of the allograft.

Creatinine, a waste product of the creatine phosphatase pathway in muscle, is produced at a relatively constant rate depending on muscle mass and filtered out of the body by the glomerulus. Noteworthy, if serum creatinine (SCr) level doubles, the allograft nephron mass is known to have been dropped by half already. Based on the serum creatine concentration the glomerular filtration rate (GFR), a better predictor for renal function by adjusting for age, gender and ethnicity, can be estimated using various empiric equations (65, 66) which all have their limitations in the assessment of allograft function (67). Proteinuria is a condition in which urine contains an abnormal amount of protein and may indicate impaired renal function.

As mentioned earlier the second pillar in the diagnosis of rejection is the histological examination by allograft needle core biopsy (i.e., indication biopsy). Protocol biopsy performed according to a predefined schedule was introduced recently by several transplant centers to rule out subclinical rejections (i.e., without indication of allograft function impairment) but is controversially discussed regarding the justification to biopsy a well-functioning graft (69). The histological differentiation between the types and severity (grade) of rejections was first standardized in 1991 based on a consensus meeting of clinicians, pathologists, surgeons and immunologists which took place in Banff, Canada (historically reviewed by Solez (70)). This Banff classification was initially established for renal TX and then extended to liver, pancreas and composite tissue grafts. It makes use of semi-quantitative lesion scorings from 0 to 3+ in six diagnostic categories, and is regularly updated based on the most recent research results in the field of renal transplant pathology. Details can be found in the Banff meeting report of 2009 which mainly focused on the humoral rejection of renal allografts (71).

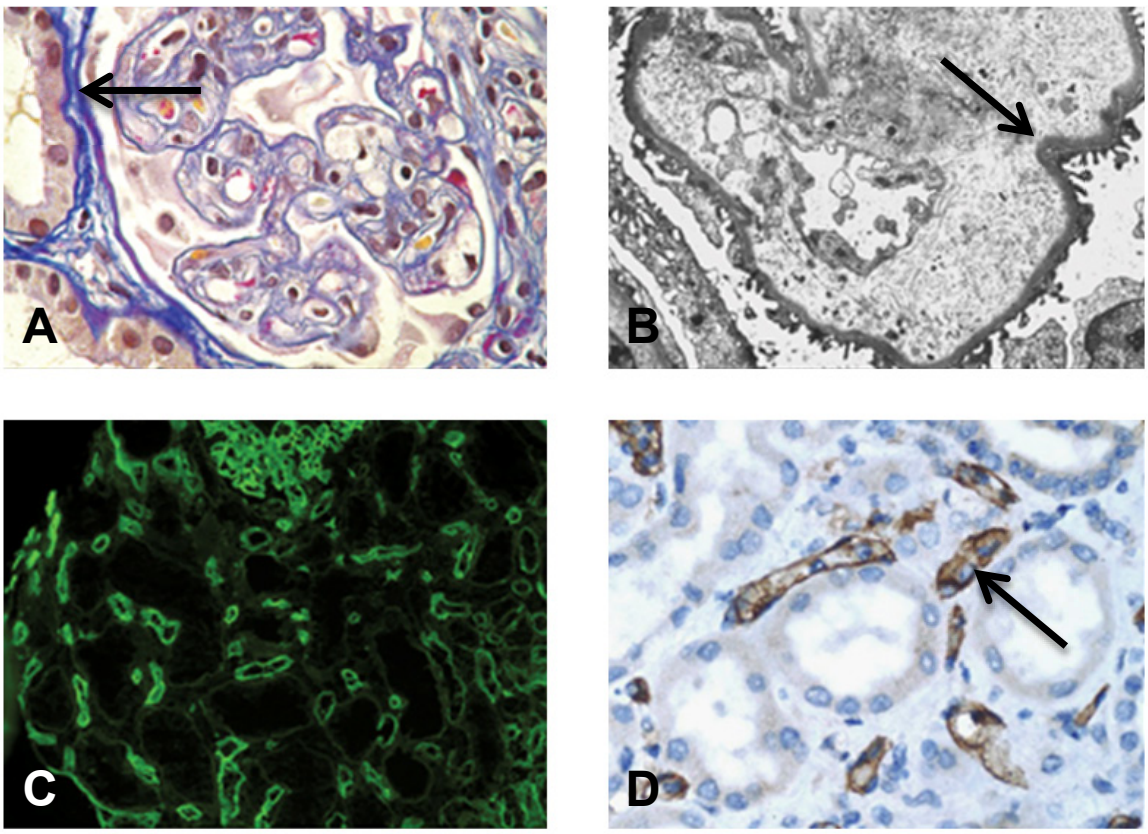


Figure 8. Histology of TG and C4d staining as the characteristic findings in AMR. (A) The glomerulus shows global duplication (arrow) of capillary walls (trichrome stain, 160x). **(B)** Activated capillary endothelial cells with marked widening of the *lamina rara interna* (arrow) representing the early phase of basement membrane remodeling in TG (electron microscopy, 5000x). **(C)** Indirect immunofluorescence detection of strong, linear C4d depositions (green fluorescence) along the walls of peritubular capillaries (PTC) on frozen sections (100x). **(D)** Immunhistochemic staining for C4d (arrow) along PTC on formalin-fixed and paraffin-embedded tissue sections (200x). Pictures taken from (68).

To date, the biopsy is regarded as the gold standard for detection and classification of transplant rejection. However, staining for the complement component C4d shows exemplarily the limitations of renal biopsies. Detection of C4d on PTC is a prerequisite feature for the proper diagnosis of AMR according to the Banff classification (**Figure 8**). Beside significant technical issues accompanied with the C4d staining procedure (72), there is growing evidence that AMR may occur in the absence of detectable C4d on renal biopsies first shown by Sis et al. (73) and extensively reviewed by Cohen et al. (74).

More recently, with the advent of the modern, sensitive SPA-based HLAab detection assays antibodies were recognized by the Banff committee as an additional pillar in the diagnosis of rejection - in particular AMR, where detection of circulating DSA is one key feature of the diagnostic criteria.

1.5 Objectives of the Thesis

Despite the remarkable success in treating AR, which could have been achieved over the past two decades due to more potent IS regimens targeting the cellular alloimmune response, it remains that many renal transplants undergo vascular injury typical for CR. The result has been that long-term graft survival has not notably improved as compared to twenty years ago (10).

We hypothesize, supported by most recent findings (75), that late renal allograft rejection is mainly driven by antibody-mediated processes which have been fairly underestimated in the past due to insufficient diagnostic tools. Therefore, we wanted to evidence the potential of HLAab detected by modern, most sensitive SPA as the predictive, non-invasive biomarker for long-term renal allograft survival. In this thesis we put a special focus on modifications of the standard Luminex® SPA to further improve the determination of HLAab pathogenicity by assessment of the IgG subclass and complement-fixing ability. Furthermore, we aim at the establishment of methods to facilitate the determination of HLAab specificity on the epitope level of highly complex allosera which should provide more evidence for a causal relationship between DSA and late allograft rejection. Finally, we intend to elaborate recommendations on an effective posttransplant HLAab monitoring scheme, which should enable posttransplant risk stratification and thus may guide tailoring the individual IS strategy to facilitate uncomplicated long-term allograft survival.

2 Materials and Methods

2.1 Patient Population

A total of 1179 consecutive kidney transplant recipients from the three out-patient clinics Charité Mitte, Charité Virchow-Klinikum and dialysis center "Am Reichstag", who gave their informed consent, were considered for enrollment in this analysis. 26 patients had to be suspended from the study, who showed an impaired graft function indicated by an elevated baseline SCr level >3 mg/dL at the time of enrollment. In addition, 17 and 57 patients were excluded as they were transplanted before 1980 and sample collection was performed before the 90th postoperative day, respectively, while another 4 patients had to be excluded with a follow-up of less than 100 days (illustrated in **Figure 9**). The cohort of the remaining 1075 patients were all transplanted between 1980 and 2004 at the KfH Friedrichshain (1980-1990) and the Charité – Universitätsmedizin Berlin (1990-2004), and showed stable graft function. As the contribution of HLAab and MICAab to late renal allograft loss is discussed controversially in the literature (76, 77) sera of these patients were monitored in a cross-sectional manner for the presence of these antibodies using Luminex® SPA. Patient's sera were tested once at a median of 4.2 years posttransplant with an interquartile range (IQR) of 9.8 to 1.8 years. Graft function as indicated by urinary protein secretion, SCr and the estimated GFR (eGFR) was monitored for a period of 6.2 years (IQR = 3.9 to 8.5 years) after testing. The calculation of eGFR was based on the simplified Modification of Diet in Renal Disease equation (67, 78).

Despite limited availability of specimens a subset of 788 patients' sera could be further analyzed, in which the HLAab were additionally characterized according to their effector function (i.e., IgG subclass and complement-binding capability) and specificity against HLA heavy chain (HC) molecules.

In addition, starting from the cross-sectional study for 531 patients the study design could be further extended to a longitudinal design, in which patients were monitored for the development of HLAab over time.

Patient's records (e.g. SCr, urinary protein levels and medication) were followed with help of the electronic patient record file TBase (79-81). Additional sources of information comprised the Eurotransplant ENIS database and the former TIS database (Deutsche Stiftung Organtransplantation). Relevant information were gathered from all sources by considering the federal Data Protection Act and joined to a HLA-Research SQL database established by M. Niemann (bachelor thesis: Niemann M. Datenintegration in einer medizinischen Laborumgebung zur Vorbereitung auf Frequent Itemset Mining. 2010).

The independent ethics committee of the Charité reviewed and approved the study with the reference number 161/2002.

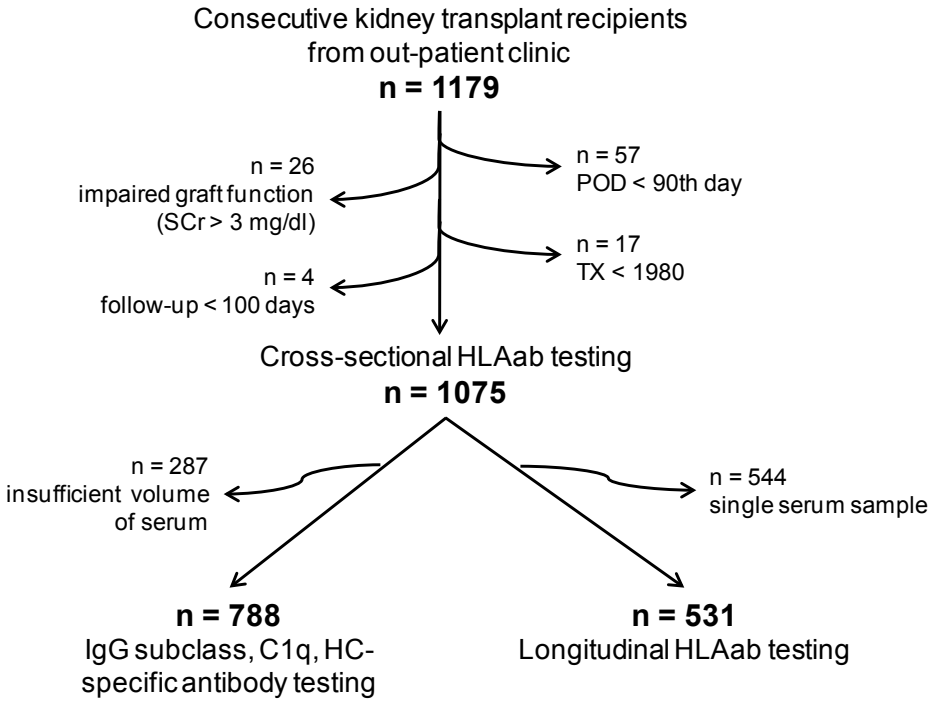


Figure 9. Composition of patient cohorts. Overall 1075 patients, who were transplanted between 1980 and 2004 showing stable graft function were enrolled in the cross-sectional HLAab study. Further assessment of HLAab effector functions and specificity could be accomplished for a subgroup of 788 patients. The extension of the cross-sectional study design to a longitudinal design was feasible for another subgroup of 531 patients with several specimens.

2.2 Materials

2.2.1 Buffers and Reagents

Bovine serum albumin (BSA), 30%	PAA Laboratories
Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
Sodium citrate buffer (0.6% w/v sodium citrate)	
6.0 g sodium citrate dihydrate	Sigma-Aldrich
ad 1000 mL distilled water	Millipore
DTT inactivation buffer	
480 mg cystine	Sigma-Aldrich
ad 100 mL Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
colored by phenol red	Sigma-Aldrich

DTT (1 M stock solution)	
1.543 g 1,4-Dithiothreitol	Sigma-Aldrich
ad 10 mL Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
Elution buffer (EB)	
1.37 g citric acid	Sigma-Aldrich
468 mg disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich
1 % (v/v) BSA	PAA Laboratories
ad 50 mL distilled water	Millipore
Beads blocking solution (P2BSA, 2% BSA)	
1.0 mL 30% BSA	PAA Laboratories
14 mL Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
Beads blocking solution (P0.1BSA, 0.1% v/v BSA)	
50 µL 30% BSA	PAA Laboratories
ad 15 mL Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
Beads storage solution (0.1% v/v BSA, 0.1% w/v NaN_3)	
50 µL 30% BSA	PAA Laboratories
150 µL 10% sodium azide stock solution	
14.8 mL Dulbecco's PBS w/o Ca^{2+}, Mg^{2+}, pH 7.4	PAA Laboratories
Sodium azide, 10% w/v stock solution	
10 g sodium azide (NaN_3)	Sigma-Aldrich
ad 100 mL distilled water	Millipore
1M TRIS-HCl pH 9.5	
12.1 g TRIS base	Sigma-Aldrich
ad 100 mL distilled water	Millipore
adjust pH with HCl	
Ficoll-Hypaque®, density 1.077 g/mL	Biochrom
FluoroQuench®	OneLambda
Human C1q protein	Quidel
ImmunoPure IgG Elution Buffer	Pierce
LABScreen® Negative Control Serum	OneLambda
Luminex® sheath fluid	Luminex Corp.
Lymphostabil® (w/o Mg^{2+}, Ca^{2+})	Biotest
Mineral oil	Biotest
Rabbit complement	Biotest

RPMI 1640	Biochrom
Sodium azide (NaN_3)	Sigma-Aldrich

2.2.2 Antibodies

Anti-human HLA-A2/B17, clone SN230G6	Leiden University (LUMC)
Anti-human HLA-B12/B13/B40/B21/B41, clone ROU9A6	LUMC
Anti-human HLA-B51/B35, clone HDG 8D9	LUMC
Anti-human HLA-B60/B7/B55/B18, clone ZEL4F11	LUMC
Chimeric mouse/human IgG1, clone F3.3	provided by M. Dechant
Chimeric mouse/human IgG2, clone F3.3	provided by M. Dechant
Chimeric mouse/human IgG3, clone F3.3	provided by M. Dechant
Chimeric mouse/human IgG4, clone F3.3	provided by M. Dechant
Mouse anti-human C1q, clone 3R9/2	AbD Serotec
Mouse anti-human HLA-A/B/C, clone HC10	provided by OneLambda
Mouse anti-human HLA-A/B/C, clone W6/32	BioLegend
Mouse anti-human HLA-DR (CDC B cell control)	Biotest
Mouse anti-human HLA-DR/DP, clone MEM-136	provided by OneLambda
Mouse anti-human HLA-DR/DQ, clone F2	provided by OneLambda
Mouse anti-human HLA-DR/DQ, clone FJ	provided by OneLambda
Mouse anti-human IgG1 ($Fc\gamma_1$), clone HP6069	Merck
Mouse anti-human IgG2 ($Fc\gamma_2$), clone HP6002	Southern Biotech
Mouse anti-human IgG3 (hinge), clone HP6050	Southern Biotech
Mouse anti-human IgG4 ($Fc\gamma_4$), clone HP6025	Southern Biotech
Mouse anti-human IgM (CDC IgM positive control)	provided by Eurotransplant
Mouse anti-human β2-microglobulin, clone 2M2	BioLegend
PE mouse anti-human IgG1 (hinge), clone 4E3	Southern Biotech
PE mouse IgG1 κ isotype control, clone MOPC-21	BD Pharmingen
PE polyclonal goat anti-human pan-IgG (Fcγ) (LS-AB2)	OneLambda
PE polyclonal goat anti-mouse IgG (H+L), hum adsorbed	Southern Biotech
Polyclonal rabbit anti-human C4d	Biomedica

2.2.3 Commercial Test Kits

C1qScreen	OneLambda

Dynabeads® CD19	Invitrogen Dynal
Dynabeads® CD3	Invitrogen Dynal
EZ1 DNA Blood 350 µL Kit	Qiagen
LABScreen® Mixed	OneLambda
LABScreen® Single Antigen	OneLambda
LAT1288	OneLambda
LATM10x5	OneLambda
Lympho-Kwik® (LK50-MN)	OneLambda
Lymphoscreen ABC 60	Biotest
Lymphoscreen DR 30x2	Biotest
Lymphotype HLA-ABC 144 Italia	Biotest
Lymphotype HLA-ABC 72	Biotest
Lymphotype HLA-DR/DQ 72	Biotest
Olerup SSP HLA-A low	Olerup
Olerup SSP HLA-B low	Olerup
Olerup SSP HLA-C low	Olerup
Olerup SSP HLA-DQ low	Olerup
Olerup SSP HLA-DR low	Olerup
Olerup SSP HLA-DRB1*03	Olerup
RELI SSO Strip Detection Reagent Kit	Invitrogen Dynal
RELI SSO Typing Trays	Invitrogen Dynal
RELI SSO-HLA-A	Invitrogen Dynal
RELI SSO-HLA-B	Invitrogen Dynal
RELI SSO-HLA-Cw	Invitrogen Dynal
RELI SSO-HLA-DQB	Invitrogen Dynal
RELI SSO-HLA-DRB	Invitrogen Dynal

2.3 HLA Antibody Analysis

2.3.1 Pretransplant Antibody Detection and Crossmatching

Pretransplant HLAab status was assessed using CDC and ELISA. In the era before 1997 screening for class I HLAab was performed quarterly and after each immunizing event (i.e., transfusion, gravidity) by CDC with and without DTT. DTT was used to discriminate between IgG and IgM. It reduces disulfide bonds of the pentamer IgM molecule and abolishes the complement-binding capability. DTT-sensitive reactions are attributed to IgM. Class II

HLAab were detected with a panel of B lymphocytes from patients suffering from chronic lymphoid leukemia after adsorption of class I HLAab using a pool of platelets from HLA-typed individuals. HLAab screening by ELISA (82) was introduced to the laboratory in 1997, and was then used in addition to CDC. Based on HLAab screening unacceptable HLA-A, -B, -DR mismatches to be excluded for donor selection were defined. Preformed lymphocytotoxic DSA leading to hyperacute or accelerated acute AMR were excluded by T- and B-cell CDC crossmatches (CDC-XM) using current and historical serum samples.

2.3.1.1 Complement-Dependent Lymphocytotoxicity Antibody Detection (CDC)

For the detection of cytotoxic HLAab commercially available and in-house cell trays were used. Lymphoscreen® ABC60 and Lymphoscreen® DR30x2 were used for the detection and specification of class I and class II HLAab, respectively. Each cell tray of the Lymphoscreen® ABC 60 assay contains a deep-frozen panel of 56 HLA-A, -B and -C-typed lymphocytes for screening of one serum sample. Each Lymphoscreen® DR30x2 plate contains two identical panels of 26 HLA-DR and -DQ-typed B lymphocytes for the screening of two serum samples. The in-house panel used until 2005 comprised 50 HLA-A, -B and -C-typed peripheral blood lymphocytes of healthy blood donors. The panels were compiled reflecting the frequency of HLA antigens in the normal German population (83). Cell isolation was performed using Ficoll-Hypaque® density gradient (see 2.3.1.2)

Cell trays were thawed for 2-5 min at 37°C and 20 µL RPMI-1640 (22-25°C) was added per well. After 15 min of incubation at room temperature (RT) the medium was aspirated and 3 µL mineral oil was added to each well to prevent evaporation. If applicable, DTT was added to a final concentration of 5 mM and incubated for 30 min at RT. Next, 2 µL serum with and without DTT were added to each well of the cell tray and incubated for another 30 min at RT. Rabbit complement was then added (5 µL/well) and trays were incubated for 70 min at RT. Trays supplemented with DTT were preincubated with 1 µL DTT inactivation buffer per well for 5 min before complement was added. Finally, cells were stained and fixed by adding 3 µL FluoroQuench® AO/EB to each well. Trays were analyzed using a fluorescence microscope Leitz DM IL. The percentage of lyzed cells was converted to Terasaki scores (see **Table 1**).

Table 1. Terasaki scores. Translation of the percent lyzed cells as determined in the CDC to Terasaki scores.

percent lysed cells	Terasaki score
0-10%	1 (negative)
11-20%	2 (equivocal positive)
21-50%	4 (weak positive)
51-80%	6 (positive)
81-100%	8 (strong positive)
n/a	0 (not interpretable)

2.3.1.2 Isolation of T, B and Whole Lymphocytes

T, B and whole lymphocytes for histocompatibility testing are usually isolated from peripheral blood or spleen.

a) Isolation from peripheral blood

Citrated or heparinized peripheral donor blood was diluted 1:2 with PBS and each 20 mL diluted blood was then underlayered with 20 mL Ficoll-Hypaque®. Tubes were centrifuged at 1150 *g* for 15 min without brake, and peripheral mononuclear cells (PBMC) at the interface between erythrocytes and plasma were carefully aspirated. Next, the PBMC suspension was split into two parts. Part A (1/3) for the isolation of whole PBMC was washed with PBS at 2700 *g* for 2 min and each 100 µL of cell sediment was incubated with 800 µL of freshly thawed Lympho-Kwik® reagent. The suspension was incubated at 37°C for 15 min, mixed well, and overlayered with 200 µL PBS. After centrifugation at 2700 *g* for 2 min without brake the supernatant containing dead cells and granulocytes was carefully aspirated and discarded. The cell pellet was washed twice using 1 mL PBS at 2700 *g* for 1 min and finally resuspended in the appropriate volume Lymphostabil® to a cell concentration of 2000-4000/µL.

Part B (2/3) for the isolation of T and B cells was washed with PBS at 2700 *g* for 2 min and resuspended in 5 mL (2-4°C) 0.6% sodium citrate buffer. Next, 80 µL Dynabeads® CD19 was added and incubated for 20 min with gentle rotation at RT. The tube was placed on a magnet (Dynal MPC™; Invitrogen Dynal, Oslo, Norway) for 2 min, supernatant was aspirated for downstream T cell isolation, and the bead-bound B cells were washed three times with chilled PBS on the magnet. The aspirated supernatant on its part was then incubated with 100 µL

Dynabeads® CD3 for 20 min with gentle rotation at RT. Next, tube was placed on a magnet, the supernatant was discarded, and cells were washed three times as described above. Finally, the isolated T and B cells were resuspended in an appropriate volume of Lymphostabil®, and cell density was adjusted to 2000-4000/µL.

b) Isolation from spleen

Spleen was cut into 2x2 cm, and gently crushed in 20 mL PBS using a syringe piston. The crude suspension was then filtered through several layers of mull in a funnel and subsequently rinsed with additional 40 mL PBS. The cell suspension was split into two tubes with 30 mL each, and underlayered with 20 mL Ficoll-Hypaque®. The suspension was centrifuged at 1150 *g* for 15 min without brake, and the cell layer was carefully aspirated. The isolated cells were processed downstream as described above to isolate whole lymphocytes, T and B cells.

2.3.1.3 Complement-Dependent Lymphocytotoxicity Crossmatch (CDC-XM)

The pretransplant CDC-XM is used to identify preformed antibodies in recipient serum reactive with donor lymphocytes, which represent a contraindication for TX (84). Cell populations were isolated as described in section 2.3.1.2.

In case of a living donor a CDC-XM was performed using the recipient's current serum and the donor's PBMC as well as B cells with and without DTT. In case of a deceased donor crossmatch was performed using the recipient's current pretransplant and historical serum. Sera were incubated with whole lymphocytes with and without DTT as well as T and B cells both without DTT.

Where appropriate, isolated cell suspensions were centrifuged at 2500 *g* for 30 sec, supernatant was discarded and the cells resuspended in the original volume of 0.01 M DTT. Next, 1 µL cells and 1 µL serum were added to the appropriate wells of a 72-well Terasaki cell tray (Greiner Bio-One, Frickenhausen, Germany) and incubated for 30 min at RT. Cells pretreated with DTT were incubated with serum on a separate tray at 37°C for 30 min. Rabbit complement was then added (5 µL/well), and trays were incubated for 70 min at RT. Trays supplemented with DTT were preincubated with 1 µL DTT inactivation buffer per well for 5 min before complement was added. Cells were then stained and fixed by adding 3 µL FluoroQuench® AO/EB to each well, and analyzed using a fluorescence microscope. A positive crossmatch was reported at a Terasaki score ≥ 2 (see **Table 1**). For the correct interpretation of crossmatch results four different controls were used on each cell tray: (a) negative control serum, (b) pooled serum from highly immunized patients with IgG

antibodies, (c) IgM anti-human monoclonal antibody, and (d) mouse anti-human HLA-DR antibody as HLA class II positive control.

2.3.1.4 Enzyme-Linked Immunosorbent Assay (ELISA)

The commercially available ELISA-based Lambda Antigen Tray (LAT^{TM}) allows the detection of IgG antibodies to HLA class I or class II antigens in human serum. For the qualitative detection of HLAab the LATM10x5 was used, which provides a specific mix of class I and class II HLA antigens per well of a Terasaki tray. The determination of HLAab specificity was accomplished by using the LAT1288 providing a panel of the most common 56 class I and 32 class II HLA antigens. Both tests were performed using the same protocol. In detail, the appropriate volume of patient serum was diluted 1:2 and 1:3 for LATM and LAT1288, respectively, using antibody diluent, and 10 µL of the diluted serum was added to the wells. After incubating the trays for 60 min at RT the sera were removed by hand flicking the trays. Next, 20 µL washing buffer was added to each well, aspirated using a vacuum pump with a 12-needle comb and repeated once. After removal of washing buffer 10 µL of prediluted alkaline phosphatase-conjugated anti-human IgG was added to each well and incubated for 40 min at RT. Trays were washed twice before 10 µL of the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate was dispensed to each well. After incubation for 12 min at 37°C in the dark, 5 µL stop reagent was added. Reagents equilibrated within 15 min and trays were then analyzed using the ELISA reader ELX 800 (BioTek Instruments, Winooski, VT, USA) in conjunction with the LAT Software V1.7 (OneLambda).

The software calculated individual cutoff values for each serum sample based on the provided positive control serum and the non-specific background of the test serum tested in the wells carrying no antigens (i.e., NAC well). HLAab specificity was determined by analyzing the reaction patterns. The percentage panel reactive antibody (PRA) was calculated according to the following equation:

$$\%PRA = \frac{\#\ of\ \ positive\ \ HLA\ \ wells}{n} \cdot 100$$

n = panel size (i.e., n = 56 for class I and n = 32 for class II)

%PRA was rounded to whole numbers

2.3.2 Posttransplant Antibody Detection

2.3.2.1 Pan-IgG HLA Antibody Detection

The screening for pan-IgG HLA class I, class II and MICAab was performed using LABScreen® Mixed antigen beads (i.e., pooled antigen beads). Antibody specificity was assessed by LABScreen® SAB. The assays were performed according to an in-house procedure. Test sera were filtered using a 96-well AcroPrep® filter plate with 0.2 µm pore-sized Bio-Inert® membrane and 3.0 µm pore-sized glass fiber prefilter (Pall, Port Washington, NY, USA) at 1400 *g* for 5 min. Filtered serum (20 µL) was transferred into a polypropylene 96-well V-bottom microplate (Greiner Bio-One) and incubated with 3 µL LABScreen® beads for 30 min at RT in the dark while gently agitating at 200/min. LABScreen® washing buffer (LSWB) was added (150 µL) and centrifuged at 1400 *g* for 5 min. LSWB was removed by flicking the plate. The pelleted beads were resuspended in another 200 µL LSWB and transferred to a 96-well AcroPrep® filter plate with 1.2 µm pore-sized Supor® membrane (Pall). LSWB was aspirated using a plate vacuum manifold (Pall) not exceeding 13.3 kPa vacuum pressure. The washing procedure was repeated three times. Secondary PE-conjugated anti-human pan-IgG (100 µL of a 1:100 dilution) was added to each well and incubated for 40 min at RT in the dark with gentle agitation at 200/min. The supernatant was aspirated and beads were washed three times. Finally, beads were resuspended in 80 µL PBS and acquired on a Luminex100™ system (Luminex Corp., Austin, TX, USA).

As negative controls for each batch the commercially available LABScreen® Negative Control Serum (LSNC) or an in-house validated negative control serum from a non-immunized, healthy, male blood donor with blood group AB was used. The adjusted in-house cutoff definition for the LABScreen® Mixed (i.e., MFI ratio > 3) was based on 48 HLAab negative serum samples from patients waiting for a transplant, which have been proven HLAab negative by LABScreen® SAB. HLAab specificities using LABScreen® SAB were considered positive exceeding a normalized MFI value of 500.

2.3.2.2 HLA Antibody Detection of IgG Subclasses

The standard pan-IgG HLAab detection assay (see 2.3.2.1) was modified by replacing the generic secondary pan-IgG by monoclonal antibodies specific for IgG1-4 subclasses. In detail, 20 µL of patient serum and 3 µL of LABScreen® beads were incubated for 30 min at RT with gentle agitation at 200/min. Next, 150 µL LSWB was added and the plate was centrifuged at 1,400 *g* for 5 min. After discarding the supernatant, three more washing steps were performed on AcroPrep® filter plate with 1.2 µm pore-sized Supor® membrane (Pall) using 200 µl LSWB. Then, 100 µL of appropriately diluted IgG1-4 subclass secondary antibody

(concentration: anti-IgG_1=5 µg/mL; anti-IgG_2=2.5 µg/mL; anti-IgG_3=5 µg/mL; anti-IgG_4=5 µg/mL) was added and incubated for 30 min at RT in the dark with gentle agitation. After two washing steps, beads were resuspended in 100 µl PE-conjugated polyclonal goat anti-mouse IgG antibody (1:500 dilution) and incubated for 30 min at RT in the dark with gentle agitation. The beads were washed three times, finally resuspended in 80 µl PBS, and acquired on a Luminex®100™. Positive results were defined by a MFI-based cutoff which was generated for each IgG subclass and each test run by using a negative control serum from a healthy, non-sensitized and HLA-antibody negative male blood donor. The cutoff was calculated the following:

$Cutoff\ MFI = 3 \cdot mean\ MFI_{NC}$.

2.3.2.3 Detection of Complement C1q-Fixing HLA Antibodies

The following protocol describes our newly established sandwich C1q assay (sC1qA) which was adopted from the procedure published by Chin et al. (85). The appropriate volume of serum was transferred to a PCR tube and heated in a GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) at 56°C for 30 min, then cooled down to 4°C and filtered using a 96-well AcroPrep® filter plate with 0.2 µm pore-sized Bio-Inert® membrane and 3.0 µm pore-sized glass fiber prefilter (Pall). Heat-inactivated serum was then spiked with purified human complement component C1q to a final concentration of 200 µg/mL and 5 µL incubated with 3 µL LABScreen® beads for 20 min in the dark. Monoclonal mouse anti-human C1q antibody was added to the sample (8 µL of 1:20 dilution) and incubated for 20 min at RT with gentle agitation. Beads were washed twice with 150 µL LSWB, centrifuged at 1400 *g* for 5 min and resuspended in 100 µL PE-conjugated polyclonal goat anti-mouse IgG antibody (1:500). After 30 min incubation at RT in the dark, beads were washed twice with 150 µL LSWB and finally resuspended in 80 µL PBS. The assay was acquired on a Luminex100™. MFI values exceeding 20 and sample-to-control ratio of 2 were considered positive.

2.3.2.4 Denaturation of HLA Class I Antigen Beads by Mild Acid Treatment

Untreated class I LABScreen® pooled antigen or SAB, which contain both intact and β2m-free HC on the surface, were used to detect intact antigen and/or β2m-free HC-specific antibodies. The presence of both intact and β2m-free HC of class I antigens were confirmed by using a murine monoclonal antibody W6/32 which only binds to β2m-associated HC (86), and murine monoclonal antibody HC10 which probes β2m-free HC (87). Antibody-positive sera were further tested with elution buffer (EB)-treated LABScreen® beads which have only

β2m-free HC on the surface, to detect HC-specific antibodies. LABScreen® beads were spun down at 10,000 *g* for 2 min, supernatant was carefully aspirated and the remaining bead pellet was incubated with five times the original volume of EB. Beads were incubated for 20 min with gentle agitation (200/min) at RT in the dark, spun down at 10,000 *g* for 2 min and washed with 1 mL of LSWB. This washing step was repeated with 1 mL of a 2% BSA solution in PBS (P2BSA). Subsequently, 1 mL of P2BSA was added and beads were blocked for at least 1 h at RT or preferably overnight at 2-4°C. Beads were centrifuged and resuspended in 90% of the original volume storage buffer to compensate for bead loss and dilution effects during the denaturation process. Removal of β2m from HC after treatment with EB was confirmed by negative staining with W6/32 and β2m-specific antibody but positive staining with HC10. The test was performed as described in section 2.3.2.1 with two modifications: (a) beads were incubated with monoclonal antibody instead of serum at dilutions of 1:200, 1:500 and 1:320 for W6/32, 2M2 and HC10, respectively, and (b) a polyclonal PE-conjugated goat anti-mouse IgG antibody (100 µL of a 1:500 dilution) was used as secondary reagent. Positive results were defined by a MFI-based cutoff using a negative control serum from a healthy, non-sensitized and HLA-antibody negative male blood donor. The cutoff was calculated the following:

$Cutoff\ MFI = 3 \cdot mean\ MFI_{NC}$.

2.3.2.5 Heat Treatment for Denaturation of HLA and MICA Antigen Beads

LABScreen® beads were centrifuged at 10,000 *g* for 2 min and washed once with PBS containing 0.1% BSA (P0.1BSA). Beads were incubated in an appropriate volume of P0.1BSA at 90°C for 5 min and cooled down to 4°C in a Gene Amp® PCR System 9700. Subsequently, beads were washed with P0.1BSA three times, spun down at 10,000 *g* for 2 min and resuspended in 90% of the original volume of P0.1BSA buffer to compensate for bead loss and dilution effects which occurred during the denaturation process. Alloantibodies recognizing heat-denatured and non heat-treated HLA and MICA antigens and their ability to fix complement were identified using heated and unheated antigen beads by the Luminex® assays described earlier (see 2.3.2.1 and 2.3.2.3). The cutoff for determination of positive results was calculated as described above (see 2.3.2.4)

2.4 HLA Typing

Donor and patient HLA typing was performed using both serological and molecular typing methods. Kidney donors and recipients were typed twice. Recipient typing for HLA-A,-B,-C, -DR,-DQ was determined first by molecular means using sequence-specific oligonucleotides

(SSO). Verification typing was performed for HLA class I and II using the serological and SSO method, respectively. Living donors were typed analogous to recipients. Deceased donors from our donor region (Germany Northeast) were typed in our laboratory for HLA-A, -B,-C,-DR,-DQ by molecular means using sequence-specific primers (SSP) and serological methods. However, deceased donors from outside our donor region were typed by the respective HLA laboratory of that region using SSP, sequencing and/or serological methods for HLA-A,-B,-DR as required by the Eurotransplant guidelines. Verification typing was performed in our laboratory by SSO.

2.4.1 Serological HLA Typing

Serological typing of HLA class I and II antigens was performed using PBMC and B cells, respectively. Cells were isolated as described in section 2.3.1.2. The commercially available serological HLA typing kits Lymphotype HLA-ABC 144 Italia, Lymphotype HLA-ABC 72 and Lymphotype HLA-DR/DQ 72 were used to type for class I and class II antigens, respectively. Tests were performed according to the manufacturer's protocol. Briefly, plates were thawed and 1 µL of cell suspension was added to each well. HLA-ABC and HLA-DR/DQ typing plates were incubated for 30 min and 60 min at RT, respectively. Rabbit complement was added (5 µL/well) and plates were incubated for 70 min at RT. The reaction was stopped, and cells were fixed using FluoroQuench® AO/EB. Trays were analyzed using a fluorescence microscope and the typing was analyzed using Lambda Scan® Software 5.7 (OneLambda).

2.4.2 Molecular HLA Typing

DNA extraction from whole blood was accomplished by using the EZ1 instrument (Qiagen, Hilden, Germany) and the EZ1 DNA Blood 350 µL Kit. DNA was extracted from 350 µL peripheral blood samples and eluted in 200 µL. Molecular HLA typing was performed by PCR-SSP or SSO where appropriate (see 2.4).

a) PCR-SSO

The PCR-SSO technique allows low resolution HLA typing and is based on three major sequential processes: (1) PCR HLA loci amplification using biotinylated primers, (2) hybridization of the amplicons to an array of immobilized SSO probes, and (3) colorimetric detection of probe-bound amplicons. The automated AutoRELI™ 48 instrument for

hybridization and detection and RELI^{TM} SSO HLA typing kits were used according to the manufacturer.

b) PCR-SSP

The PCR-SSP methodology for HLA, which was originally described by Olerup et al. (88), is based on the principle that matched oligonucleotide primers are more efficiently used in the PCR reaction using DNA polymerase without proof-reading properties than unmatched primers. Primers are designed to match with single HLA alleles (high resolution) or allele groups (low resolution). Amplicons are then detected using gel electrophoresis. The primarily used PCR-SSP kits for donor typing were Olerup SSP HLA-A low, -B low, -DR low, -DRB1*03 and DQ low. All tests were performed according to the manufacturer.

2.5 Histopathology

Currently, the gold standard for assessing pathological changes in the renal allograft is histopathology including light microscopic and immunohistochemical staining procedures.

A total of 175 clinically indicated renal biopsies were evaluated taken from the total of 364 patients who experienced graft failure during follow-up. On average these biopsies were taken on day 77 before allograft dysfunction was diagnosed. Clinical indications in the late phase posttransplant included a successively rising SCr level of ≥ 0.3 mg/dL above baseline, which was persistent and of unclear etiology, as well as elevated proteinuria ≥ 1 g per day. Ultrasound-assisted percutaneous renal allograft biopsies were performed using a 16 gauge needle.

Paraffin sections were prepared and graded according to a modified Banff classification based on current Banff criteria (89). Immunohistochemical detection of the complement cleavage product C4d was performed on formalin-fixed, paraffin-embedded sections using a polyclonal anti-C4d antibody. Details are described elsewhere (90). Biopsies were considered C4d positive in case of diffuse or focal endothelial C4d deposition of peritubular or glomerular capillaries. AMR was defined according to the original Banff criteria in the presence of TG and/or PTC basement membrane multilayering and/or interstitial fibrosis/tubular atrophy (IF/TA) and/or fibrous intimal thickening in coincidence with C4d deposition and the presence of DSA. In cases of DSA without or undetermined C4d deposits but with signs of morphologic capillary changes the diagnosis “suggestive of AMR” (sAMR) was made according to Banff. Modifications to the Banff classification included the addition of another entity to sAMR in which capillary changes and C4d deposits but non-donor-specific HLAab (NDSA) were detectable. In addition, a new classification termed probable AMR (pAMR)

was defined based on capillary changes with C4d deposits but no HLAab or capillary changes and NDSA without C4d.

2.6 Statistics

The cumulative probability of death-censored graft and patient survival was estimated according to Kaplan and Meier, while the statistical comparison of curves was performed by means of the log-rank test. The prognostic relevance of covariates (univariate and multivariate) was assessed by means of the Cox proportional hazards model. In a stepwise backward multivariate analysis data were adjusted for the potential confounders: year of TX, recipient age and gender, number of previous transplants, HLAab status pretransplant, donor organ source and eGFR calculated by the abbreviated MDRD equation at time of testing (78). Logistic linear regression models were employed in a stepwise backward manner to elucidate the contribution of potentially explanatory variables (gender, preimmunization, retransplantation and HLA-A,-B,-DR mismatch) in predicting the development of HLAab, MICAab and DSA.

Normally distributed continuous data were summarized as mean and standard deviation (SD); others as median and IQR. The 95% confidence interval (95% CI) was calculated where appropriate. Chi-square and Fisher's exact test were used where appropriate in the analysis of categorical data; the unpaired t-test, Mann-Whitney U or Kruskal-Wallis test for continuous data. Two-sided *P* values less than 0.05 were considered significant. The starting point for the follow-up of patients was the time of antibody testing. The combined end point for allograft failure comprised the need for dialysis, retransplantation, return to the kidney waiting list, and an impaired graft function indicated by an eGFR level < 30 mL/min/1.73 m^2. In the analysis of graft function survival data were censored for death with functioning graft (n = 108, 10%) and loss of follow-up (n = 60, 5.6%) at their last recorded visit. Statistical analysis was performed using Stata 11 (StataCorp, College Station, TX, USA).

2.7 HLA Epitope Analysis

2.7.1 Adsorption and Elution Technique

A total of 9 kidney transplant recipients who underwent nephrectomy (Nx) after chronic allograft dysfunction were selected from the total cohort. Three sera taken at different time points per patient were tested by LABScreen® SAB: (i) pretransplant, (ii) before graft dysfunction, and (iii) after Nx. Sera after Nx were additionally adsorbed by an appropriate recombinant single HLA allele-expressing cell line (kindly provided by OneLambda) derived

from the LCL712.2 cell line. The respective cell line used for adsorption of the antibody was selected based on the known serological specificity of each sample. In detail, 40 µL of serum (diluted 1:3 in PBS) was mixed with 3 to 5 × 10^6 cells and incubated for 30 min at RT. Cells were then centrifuged at 2700 *g* for 2 min, washed twice with 200 µL PBS at 2700 *g* for 2 min. The adsorbed antibody was eluted by adding 60 µL of ImmunoPure IgG Elution Buffer and incubated for 10 min at RT. Cells and eluates were separated by centrifugation (2700 *g*, 2 min). The eluates were transferred to another tube and neutralized by 3 µL of 1 M TRIS-HCl pH 9.5. Most eluates were from an initial adsorption and elution (A&E) step. However, in some cases the initial eluate was adsorbed by another recombinant cell line. All eluates were tested with LABScreen® SAB (see section 2.3.2.1).

2.7.2 Raw Data Processing

Data generated by the Luminex®100™ were analyzed manually. Median fluorescence values were obtained from the output (*.csv) file and adjusted for background signal using the formula:

adjusted MFI = (sample #N bead – sample NC bead)
– (negative control #N bead – negative control NC bead)

The adjusted reaction values were then normalized by multiplying each value by a corresponding normalization factor derived from the results of the monoclonal antibody W6/32 with the same beads. Normalization factors were calculated by dividing the average value of all W6/32 reactions by the adjusted fluorescence value for each bead. The data were then visualized with Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). All normalized reactions that were above zero were considered as potential positive reactions.

2.7.3 HLA Epitope Prediction

AA sequences of the HLA alleles were retrieved from the ImMunoGeneTics (IMGT) web site (91). An epitope search program (kindly provided by N. Sasaki) was used to identify distinguishing AA that are exclusively shared by the positive antigens at particular sequence positions based on the positive HLA antigens for each eluted antibody. The program searched for one, two or three common unique AA positions. All potential epitopes generated were subjected to a validation/selection process based on two major criteria: (i) positions had to be exposed on the surface of the HLA molecule and accessible to an antibody, and (ii) positions had to be within the antibody binding span estimated to a maximum of 880 $Å^2$ (92).

Approximate distances between two AA were calculated using the Cn3D Viewer software (93) and the three-dimensional structure of an HLA-A*02:01 (94), HLA-B*27:05 (95), and HLA-Cw3 (96). AA that were exclusively unique to a group of antigens reacting with an eluted alloantibody and fulfilled the criteria mentioned above were considered a distinguishing characteristic of the functional epitope.

3 Results

In the following chapter we first describe the development of methods which were essential to answer the objectives of this thesis. Then we focus in a cross-sectional analysis on the prevalence, specificities and effector functions of HLAab detected posttransplant before we elucidate the impact on late allograft survival. Later, supportive data are presented from a longitudinal study and by the analysis of the etiology of observed allograft failures.

3.1 Development of Methods

Current HLAab detection assays are conceived of antigens coated to a planar (plate) or globular (bead) solid phase tray. HLA-specific antibodies bind to the corresponding well or bead and are subsequently detected by anti-human-IgG-specific reporter antibody. Broad consensus exists that to date Luminex® bead assays are considered the most sensitive method. However, one of the primary concerns arises from suspicious false-positive reactions as exhibited by the reactivity of sera from non-allo-immunized healthy males (45). False-positive reactions could be attributed to partly denatured antigens present on the beads. In an attempt to assess the incidence and clinical relevance of antibodies directed against denatured vs. native antigens among our cohort of patients two mechanistically different methods were developed to completely denature HLA on the beads by heat and mild acid treatment (3.1.1). Antibodies directed against native antigens are supposed to react with untreated but not denatured beads whereas antibodies detectable by treated and untreated beads are considered to be directed against denatured antigens.

Another drawback of the Luminex® assay lies in the applied standard detection antibodies (i.e., anti-human IgG) which assess pan-IgG irrespective of the subclasses and complement-binding capability. We therefore developed two adaptations of the standard assay that facilitate the discrimination between complement-fixing and non-complement-fixing HLA-specific antibodies and IgG subclasses (IgG1-4) to further define the effector functions of the detected HLAab and correlate the results with allograft outcome (3.1.2).

It is known not only from our own published data that NDSA posttransplant are associated with impaired renal allograft survival (90, 97). It was then hypothesized that NDSA are possibly a surrogate marker for DSA adsorbed to the graft and thus are thought to share epitopes. To investigate this hypothesis we, together with Nadim ElAwar from the Terasaki Foundation, established methods to determine the potential functional epitope of HLAab (3.1.3 and 3.1.4).

3.1.1 Denaturation of HLA on LABScreen® Beads

Denaturation of HLA on LABScreen® beads, i.e., dissociation of β2m and MHC-bound peptide from HLA molecules, can be achieved by mild glycine acid or heat treatment. Thereby HLA molecules become unfolded and reveal linear neoepitopes. HLA and MICA antigens are thermally unstable and can be subjected to heat denaturation. HLA class I antigens only can additionally be denatured by mild acid treatment. The efficacy of the denaturation process was confirmed by monoclonal antibodies specific for the native and denatured isoform of the HLA molecule.

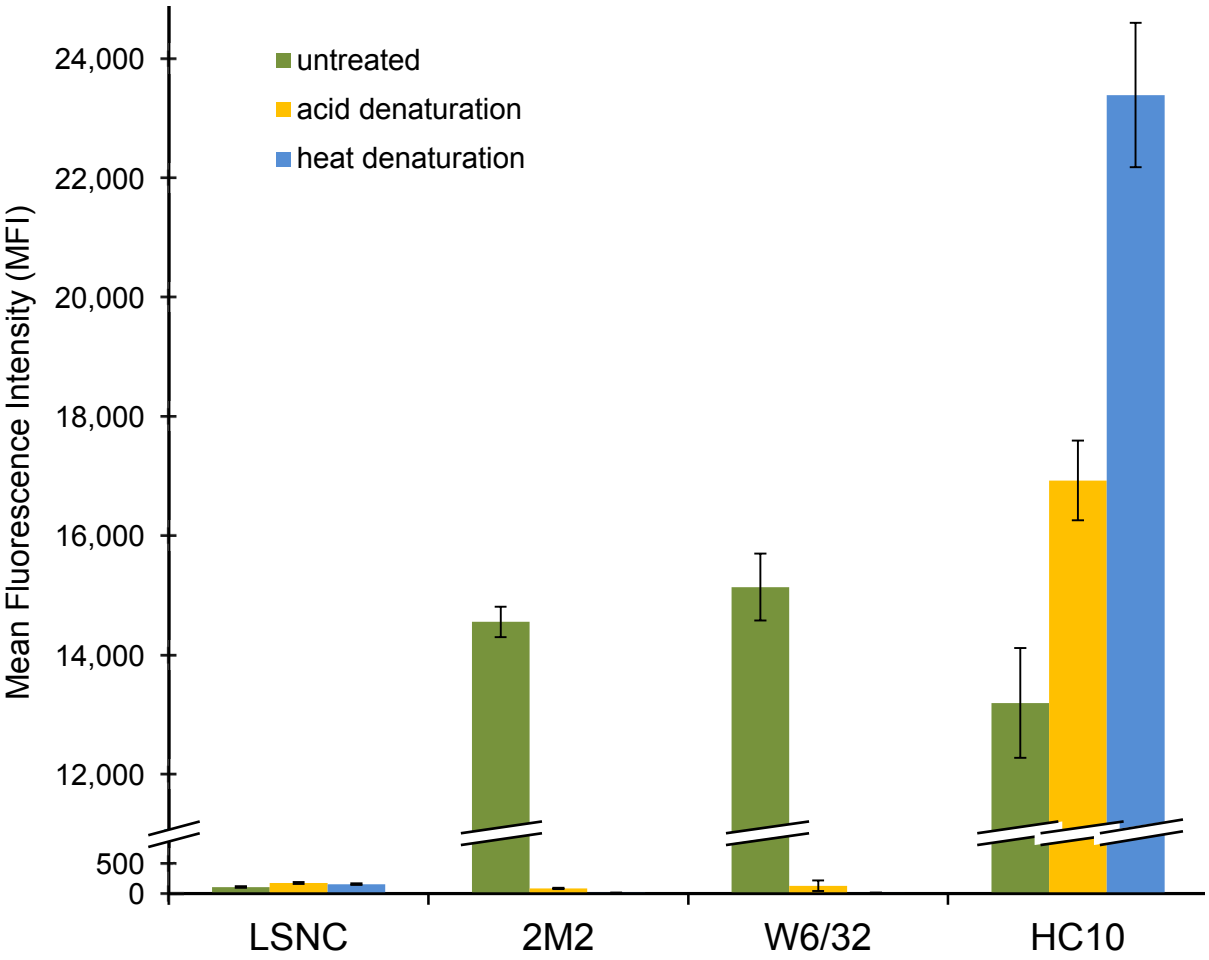


Figure 10. Efficacy of denaturation of LABScreen® Mixed HLA class I beads. Beads were denatured by mild acid or heat treatment. The efficacy of denaturation was verified by incubating untreated (green), acid-denatured (orange) and heat-denatured beads (blue) with negative control serum (LSNC), anti-β2-microglobuline (2M2), anti-HLA-A/B/Cw (W6/32), and anti-HLA-class-I-heavy-chain-specific monoclonal antibody (HC10).

Denaturation of HLA class I molecules

Binding of the monoclonal β2m-specific antibody 2M2 and native HLA class I-specific antibody W6/32 to LABScreen® class I beads was completely abolished by mild acid and heat treatment (**Figure 10**). Consistent with its conformational nature, the W6/32 epitope depends

on AA residues from all four domains of HLA class I molecules, i.e., the three immunoglobulin-like HC domains (α1-α3) and the non-covalently bound β_2m (98). The efficacy of denaturation using mild acid and heat treatment assessed by 2M2 and W6/32 was on average 98% and >99%, respectively.

The spectrum of commercially available antibodies specific for denatured HLA is very limited. In the present work the monoclonals HC10, MEM-136 and two not commercially available antibodies (F2 and FJ) from OneLambda have been proven by extensive testing to be appropriate as positive control.

The monoclonal antibody HC10 was raised against β2m-free HLA-B locus HC (87). HC10 reacted partially with untreated LABScreen® Mixed class I beads due to denaturation of a limited amount of molecules during the bead manufacturing process as indicated by the green bar in **Figure 10**. However, after acid and heat denaturation binding of HC10 to beads was increased dramatically demonstrating the efficacy of the denaturation process by either technique. Heat treatment led to a higher proportion of denatured antigen than acid treatment as indicated by the height of the blue bar in comparison to the orange bar in **Figure 10**. Successful antigen denaturation could be demonstrated not only by using LABScreen® pooled antigen but also SAB. In detail, HC10 revealed reactivity with 76% of denatured HLA class I antigens present in the single antigen test panel. That corresponds to 99% of PRA with the German donor population. HC10 reacted with 100% (15/15) of HLA-C antigens (i.e., Cw1, Cw2, Cw4, Cw5, Cw6, Cw7, Cw8, Cw9, Cw10, Cw12, Cw14, Cw15, Cw16, Cw17 and Cw18) as well as 41 of 43 (95%) HLA-B antigens in the panel (i.e., B7, B8, B13, B18, B27, B35, B37, B38, B39, B41, B42, B44, B45, B46, B47, B48, B49, B50, B51, B52, B53, B54, B55, B56, B59, B60, B61, B62, B63, B64, B65, B67, B71, B72, B73, B75, B76, B77, B78, B81, B82). The reactivity of HC10 with HLA-A heavy chain is limited to A25, A26, A33, A34, A66, A68 and A69. AA sequence alignment of the reactive HLA antigens revealed AA arginine (R) on position 62 of the HLA α chain, which is exclusively shared among all reactive β2m-free HLA class I antigens, as the determinant of reactivity. Indeed, the motif PxxWDR that could be aligned with AA 57P, 60W, 61D, and 62R of the first domain of the HLA heavy chain was found to be involved in the binding of HC10 (99).

Denaturation of HLA class II molecules

The commercially available monoclonal antibody MEM-136 reacts with the β-chain of HLA-DR/DP α/β heterodimers. When tested with heat denatured LABScreen® class II beads it revealed also some reactivity with dissociated β HC (**Figure 11**). The two antibody clones F2

and FJ (kind gift of OneLambda) were raised against EBV-transformed cells expressing HLA class II fusion proteins and react with the majority of HLA-DR/DQ antigens. Clone F2 more than FJ has been proven in our experiments to react not only with HLA-DR/DQ heterodimers but also dissociated HC as indicated by the blue bars in **Figure 11**. Nevertheless, all three monoclonals were able to verify the denaturation of HLA-DR/DQ and DP molecules on LABScreen® Mixed class II beads by heat treatment. The efficacy of heat denaturation could not properly be determined due to the reactivity of the control monoclonal antibodies with dissociated class II molecules but could be estimated by MEM-136 to be at least 80%.

Taken together, the complete denaturation of HLA antigens on LABScreen® beads by heat and acid treatment is technically feasible. With respect to efficacy heat is superior to acid denaturation.

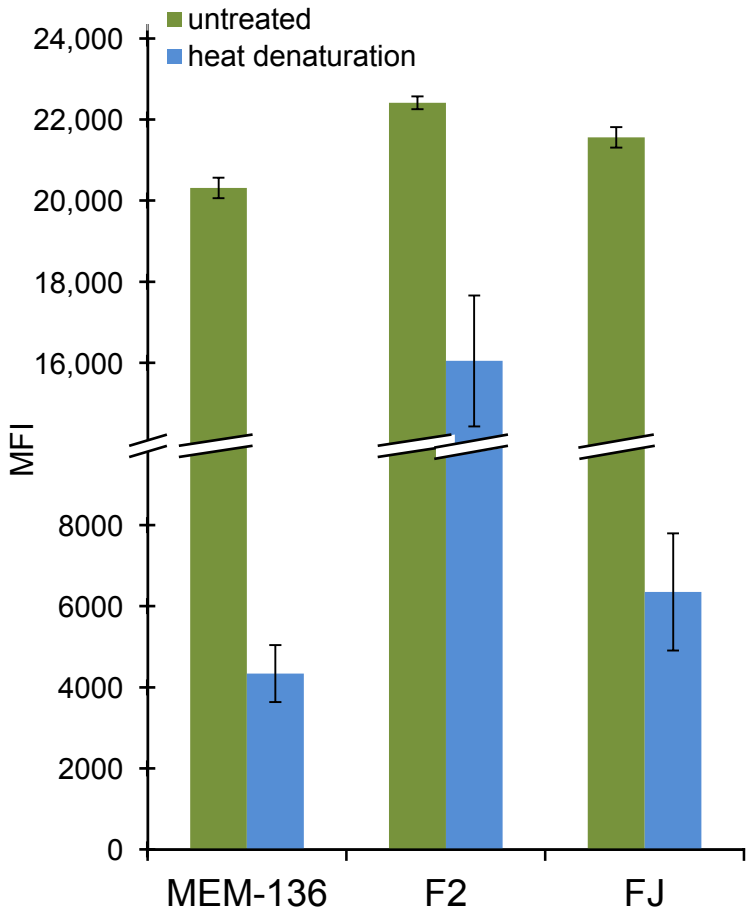


Figure 11. Denaturation of LABScreen® HLA class II beads by heat treatment. The ability of heat treatment to denature HLA class II molecules on LABScreen® pooled antigen beads was confirmed by using the HLA- class-II-specific monoclonal antibodies MEM-136, F2 and FJ, which showed reduced binding to denatured (blue) as compared to untreated beads (green).

3.1.2 Assessment of IgG Subclass and Complement Binding

Not only the specificity but also the effector function is hypothesized to determine the deleteriousness of HLAab. Therefore, two major IgG antibody effector functions (i) binding

to effector cells of the immune system, and (ii) complement binding were both assessed in this study by the determination of the IgG subclass as well as the binding capability of the initial complement component C1q to IgG antibodies.

IgG Subclasses (IgG1-4 Assay)

The standard LABScreen® assay was modified by replacing the generic reporter antibody pan-IgG by monoclonal secondary antibodies specific for IgG1-4 subclasses. The specificity of secondary IgG subclass-specific monoclonal antibodies was confirmed with chimeric IgG1-4 antibodies kindly provided by Michael Dechant from the University of Kiel (100). The murine Fab domain derived from hybridomas F3.3 was pan-HLA-class-II-specific, whereas the Fc domain was of human origin (i.e., human constant regions for κ–light chain, and γ1, γ 2, γ 3, γ 4).

Table 2. Specificity of anti-human IgG subclass specific secondary antibodies. The average MFI for all LABScreen® pooled antigen class II beads was determined under saturation conditions for both the chimeric (xi) pan-HLA class II IgG subclass and anti-human IgG subclass-specific secondary antibodies. MFI values >500 were considered to be specific.

MFI (signal/noise ratio)	Anti IgG1 (HP6069)	Anti IgG2 (HP6002)	Anti IgG3 (HP6050)	Anti IgG4 (HP6025)	Anti pan-IgG (OLI)
xi pan-HLA II IgG1	**7078 (59)**	113 (1)	103 (2)	102 (2)	20,321 (295)
xi pan-HLA II IgG2	137 (1)	**9995 (124)**	86 (2)	82 (2)	21,544 (317)
xi pan-HLA II IgG3	117 (1)	126 (2)	**16,256 (287)**	91 (2)	22,860 (431)
xi pan-HLA II IgG4	98 (1)	245 (3)	66 (1)	**15,196 (322)**	20,204 (331)

MFI = mean fluorescence intensity; xi pan-HLA II IgG1-4 = chimeric HLA-class-II-specific IgG1-4 antibodies

All chimeric antibodies were tested under saturated conditions on LABScreen® pooled antigen class II beads against a dilution series of the individual IgG subclass-specific secondary antibodies (**Table 2**). The maximum signal/noise ratio was determined as the ratio between the signal (average MFI of HLA class II beads) and background noise (average MFI of HLA class I beads). Unspecific binding of the PE-conjugated reporter mouse monoclonal antibody was determined with the appropriate isotype control antibody and was found to be negligible (i.e., 0.07% of the average signal MFI). There was only minimal cross reaction between the IgG subclass-specific and chimeric antibodies notable ranging from 0.4% to

2.5% for xiIgG4/αIgG3 and xiIgG4/αIgG2, respectively. On the contrary, the secondary IgG1 specific antibody clone 4E3 revealed substantial cross reactivity of 91% and 86% with the chimeric IgG2 and IgG3 antibodies bound to the beads, and was therefore excluded from further testing (data not shown).

To exclude the possibility that the chimeric character of the antibodies had an impact on our detection system we independently confirmed the results by applying four human monoclonal IgG1 and IgG3 antibodies produced and kindly provided by Frans Claas and Arend Mulder from Leiden University Medical Center (LUMC). These human monoclonal antibodies are characterized by certain HLA specificities and Fc domains of either γ1 or γ3 isotype. The following clones were used: (i) ZEL4F11 (IgG3,κ anti-HLA-B60/7/55/18), (ii) SN230G6 (IgG1,λ anti-HLA-A2/B57/B58), (iii) HDG8D9 (IgG1,λ anti-HLA-B51/35), and (iv) ROU9A6 (IgG3,λ anti-HLA-B12/13/40/2141). The reported specificity as determined by CDC could be confirmed using our IgG1-4 assay. However, 3 of 4 monoclonal antibodies (ZEL4F11, HDG8D9 and ROU9A6) revealed additional specificities, which in turn could be plausibilized by HLA epitope analysis. Antibody ROU9A6 e.g. reacted to HLA-B12/13/40/21/41 known from CDC and additionally with HLA-B47 when used in our IgG1-4 assay. This could be explained by reactivity to the threonine residue at position 41 (41T) of the HLA α chain well exposed on the molecule surface, and exclusively shared among all reactive HLA antigens including B47. In fact, the female serum donor with HLA type A1, A25, B8, B18 and Cw7 was immunized by pregnancy with B44 carrying the immunogenic non-self epitope 41T. Therefore, we contend that additional HLA specificities revealed by the IgG1-4 assay are plausible and represent true-positive reactions that remained undetected by classical CDC due to a lack of sensitivity and specificity.

Figure 12 shows a representative reaction pattern of antibody SN230G6 with pan-IgG as well as IgG1-4 subclass-specific secondary antibodies. The specificity of the human monoclonal antibody reported as HLA-A2/B57/B58 as well as the Fc-specificity of the antihuman IgG1 secondary antibody could be confirmed. There was no cross reactivity between the IgG1 antibody SN230G6 and the IgG2-4 subclass-specific reporter antibodies detectable.

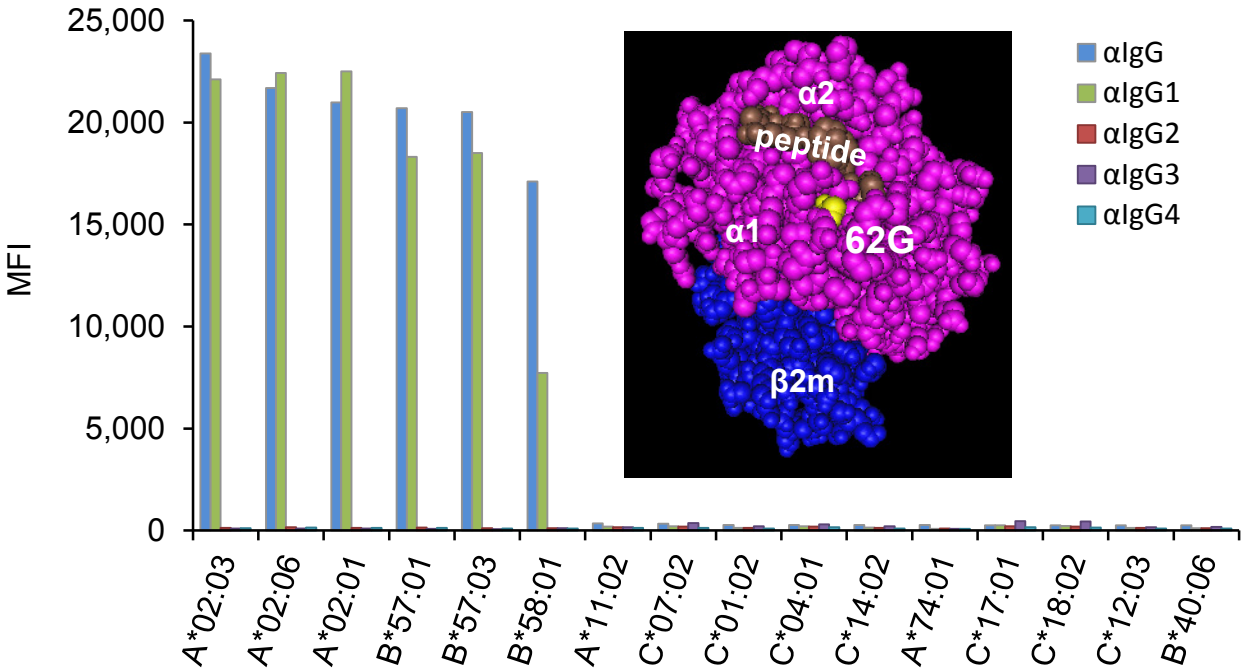


Figure 12. Human monoclonal antibody SN230G6 reaction pattern towards IgG1-4 subclass-specific secondary antibodies. The human monoclonal (IgG1, κ anti-HLA-A2/B57/B58) was directed against the HLA epitope containing the residue glycine on position 62 of the HLA α chain (62G) (depicted in the right small panel) without false reaction. Cross reaction with anti-human IgG2, IgG3 or IgG4 was not detected. MFI greater than 500 was considered as positive.

Complement-binding Capacity (sC1qA)

The detection of complement-binding HLAab by SPA was first described by Wahrmann et al. (101) who described an assay to assess the deposition of the complement degradation product C4d on HLA-coated beads. Recently, Chin et al. (85) published an approach to detect the complement component C1q using LABScreen® beads, which was demonstrated to be more sensitive than the C4d assay. Complement activation occurs by binding of C1q to the Fc domain of IgG or IgM in complex with an antigen. At least two of the globular heads of C1q must be bound simultaneously to the Fc domain of IgG or IgM in order to initiate the complement cascade (102).

We adapted the complement binding assay described by Chin et al. (C1qScreen, commercially available from OneLambda) to meet our requirements on sensitivity and specificity. In contrast to Chin et al. we applied an antibody sandwich technique instead of using directly labeled detection antibodies. The comparison between the in-house sC1qA and commercially available C1qScreen shown in **Figure 13** revealed a much higher sensitivity of the sC1qA in detecting especially complement-binding IgG1 antibodies.

As expected, in comparison with the gold standard CDC for detection of complement-activating HLAab sC1qA was proven to be superior with respect to sensitivity and specificity

(see **Table A1** in **Appendix I**). A more detailed systematic comparison between CDC and sC1qA was recently submitted by our group and is now accepted for publication (see **Appendix II**).

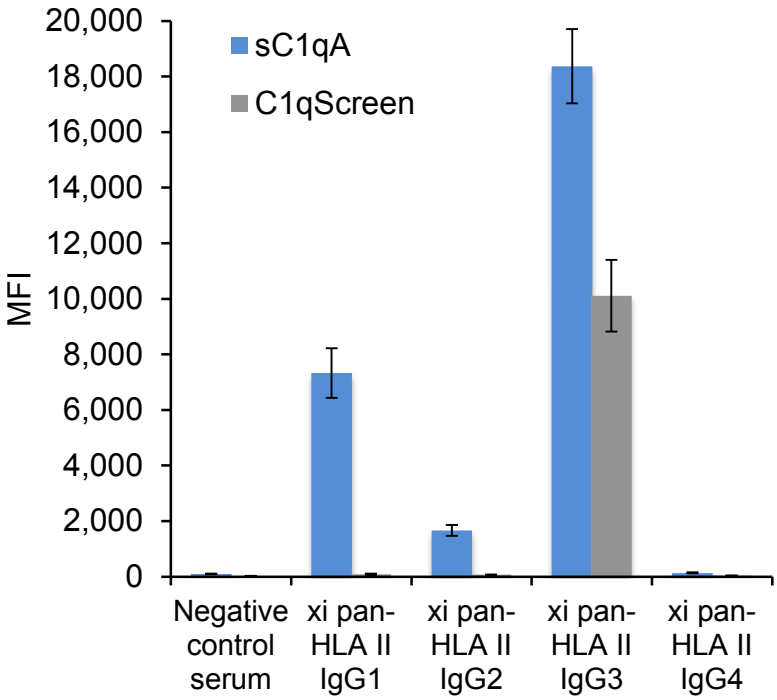


Figure 13. C1q binding of chimeric (xi) pan-HLA class II IgG1-4 antibodies. MFI derived from the in-house sC1qA are depicted by blue bars. The assay is shown to be more sensitive than the commercially available C1qScreen (85) whose results are depicted by grey bars. The C1q binding ability decreased from IgG3 >> IgG1 >> IgG2. IgG4 was not able to fix complement.

As shown in **Figure 13** the chimeric IgG1-4 antibodies showed the following hierarchy in the ability to bind complement: IgG3 > IgG1 > IgG2 which is in agreement with the literature (103).

Human monoclonal antibodies (clones: HDG8D9 and ZEL4F11) were used to confirm the results of chimeric antibodies (**Figure 14**). The sC1qA demonstrated 100% specificity in detecting C1q-binding IgG with similar sensitivity as compared to pan-IgG detection.

Consequently, detection of complement-activating HLAab can be achieved with high sensitivity and specificity by our developed in-house sC1qA.

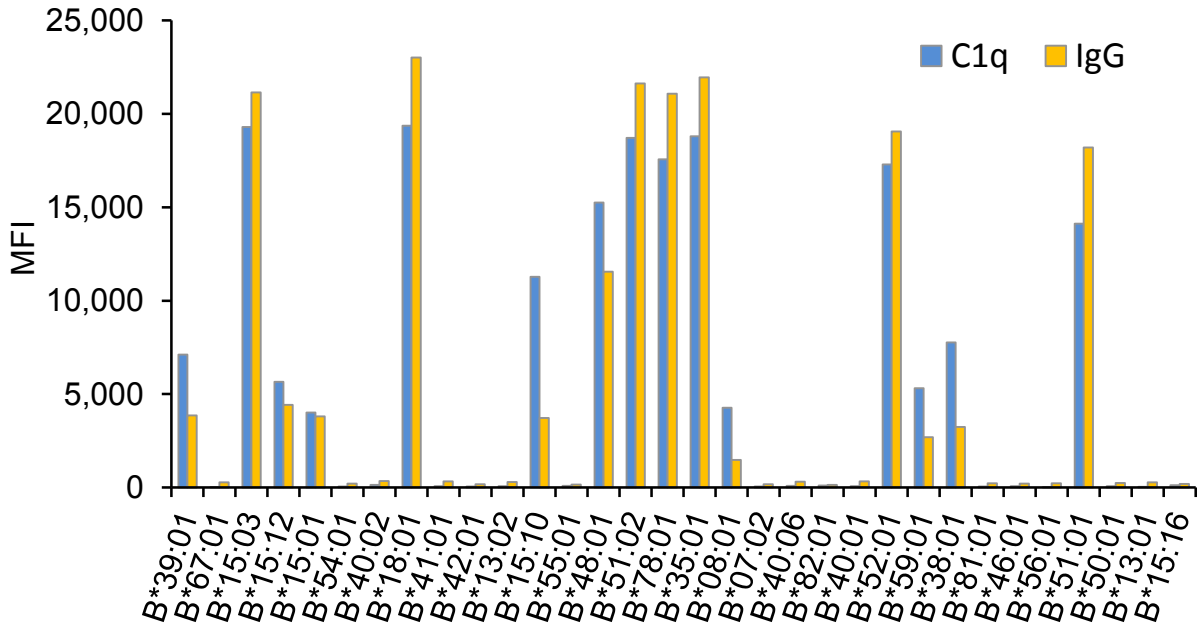


Figure 14. C1q binding of human monoclonal IgG1 antibody clone HDG8D9. The C1q assay showed 100% specificity in the detection of C1q bound to HLA-specific human IgG1 monoclonal antibody.

The C1q concentration in the test serum has been shown as being the critical determinant for the sensitivity of the sC1qA (**Table 3**). In order to achieve a standardized concentration of C1q in test serum, all intrinsic complement activity has to be extinguished by heat inactivation and subsequently spiked with purified human C1q to a final concentration of 200 µg/mL.

MFI (signal/noise ratio)	C1q (200 µg/mL)	C1q (100 µg/mL)	C1q (50 µg/mL)
xi pan-HLA II IgG1 1:5	9,830 (111)	2,634 (29)	686 (14)
xi pan-HLA II IgG1 1:10	2,713 (27)	580 (9)	131 (2)
xi pan-HLA II IgG1 1:50	129 (1)	106 (2)	122 (2)
xi pan-HLA II IgG1 1:100	112 (1)	108 (2)	109 (2)

Table 3. Specificity of complement binding in the C1q assay (sC1qA). The binding of C1q in the sC1qA was shown to be dependent on the concentration of the complement component C1q and the concentration of HLA-specific complement-binding IgG antibodies. MFI values >500 were considered as specific.

Applying a standardized C1q concentration in our assay results in a linear correlation of MFI values with the complement-binding immunoglobulin concentration.

In summary, we succeeded in establishing two novel IgG1-4 and C1q detection assays to assess known effector function of HLAab.

3.1.3 Functional Epitope Determination

In an attempt to further characterize the specificity of HLAab to the epitope level we developed a computer algorithm to determine immunogenic HLA epitopes (cooperation with Nadim ElAwar and Nori Sasaki from the Terasaki Foundation). The determination of functional HLA class I epitopes was based on observations that single AA mismatches may lead to HLA-specific alloantibodies (104).
In contrast to the approach of Rene Duquesnoy (18) who generated a limited number of 63 fixed patches of AA in the proximity of polymorphic residues (designated as eplets) we considered each single AA as well as combinations of up to 3 residues as potential epitopes. The computer algorithm developed by Nori Sasaki (105) considered all polymorphic AA residues from position 1 to 300 of the mature HLA molecule (n = 169) and built associations between common HLA specificities among all AA combinations.

The algorithm was verified by the analysis of well-defined so-called cross-reactive HLA groups (CREG) - very common reaction patterns of HLAab – which are ascribed to public epitopes shared among HLA molecules (106). An example of a very prominent public epitope called Bw4 is shown in **Figure 15**. Arginine (R) at position 83 (83R) represents the Bw4 epitope (107) which is expressed on the following HLA: A23, A24, A25, A32, B13, B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63 and B77. The computer algorithm generated correctly 83R as one possible epitope but also 82L and five additional but redundant epitopes.

The developed algorithm revealed the potential to determine functional epitopes of HLAab. However, as shown in the example a critical review and manual selection of the most probable epitope is indispensable. It is obvious that the quality of epitope prediction using the algorithm depends on the string of specificities to be analyzed. In general, the more specificities are considered the more potential epitopes are revealed and the selection of reasonable epitopes is concomitantly hampered. Especially allosera are characterized by HLAab with broad reactivities. Noteworthy, an antibody epitope is known to be very complex in structure and the algorithm does not assert the claim to be able to identify the structural

epitope. The algorithm is rather considered to identify the critical elements (single AA residues) that substantially contribute to the far more complex antibody structural epitope.

input		22	A23	A24	A2403	A25	A32	B13	B27	B37	B38	B44	B4403	B47	B49	B51	B52	B53	B57	B5703	B58	B59	B63	B77
p082	L	22	A23	A24	A2403	A25	A32	B38	B13	B77	B63	B27	B37	B44	B4403	B47	B49	B51	B52	B53	B57	B5703	B58	B59
p083	R	22	A23	A24	A2403	A25	A32	B38	B13	B77	B63	B27	B37	B44	B4403	B47	B49	B51	B52	B53	B57	B5703	B58	B59
p004	F	1	B63																					
p065	G	3	A23	A24	A2403																			
p070	S	4	B63	B57	B5703	B58																		
p080	I	16	A23	A24	A2403	A25	A32	B38	B77	B63	B49	B51	B52	B53	B57	B5703	B58	B59						
p081	A	19	A23	A24	A2403	A25	A32	B38	B13	B77	B63	B44	B4403	B49	B51	B52	B53	B57	B5703	B58	B59			

Figure 15. Epitope search program output for the Bw4 epitope. The entered HLA specificities shown in the first row led to the assignment of 82L, 83R, 4F, 65G, 70S, 80I and 81A as potential epitopes for Bw4. Each of the two epitopes 82L and 83R (highlighted in the red frame) alone might explain all 22 Bw4-reactive HLA specificities.

3.1.4 Adsorption and Elution of HLA Antibodies

The humoral immune response to an alloantigen is characterized by polyclonal expansion of allospecific B cells followed by terminal differentiation to plasma cells which thereupon produce alloantibodies. However, deconvolution of polyclonal alloantibodies is necessary to correctly determine the underlying immunogenic epitope.

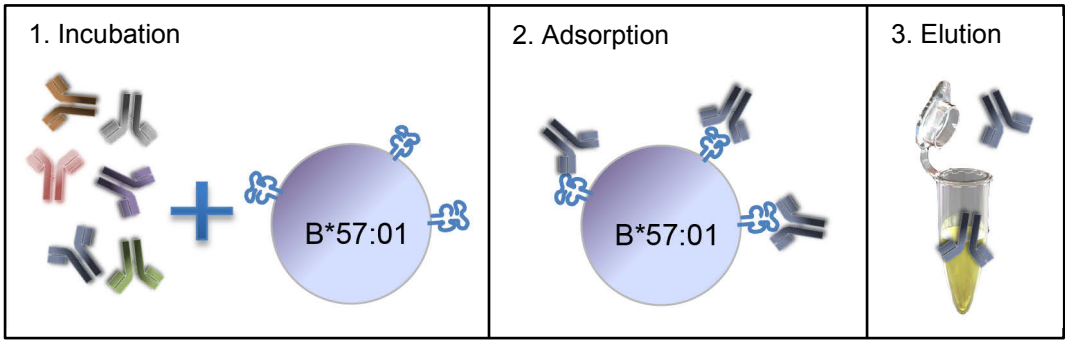


Figure 16. Process of adsorption and elution. Alloserum with different subsets of HLAab was incubated with a recombinant cell line expressing a single HLA allelic antigen. The HLAab was eluted and retested by Luminex® SAB.

Therefore, the A&E technique which was developed in cooperation with Nadim ElAwar (108) was used in this thesis. During the adsorption process alloantibodies are bound under physiological conditions to cells expressing recombinant single HLA allelic antigens and eluted after mild acid treatment as a consequence of conformational changes of the antigen (**Figure 16**). Thereby alloantibodies directed against numerous epitopes are deconvoluted into antibodies against a limited number of epitopes expressed by single HLA allelic antigens. As a proof of principle the A&E technique was applied to a limited number of cases. In the

example of a patient serum shown in **Figure 17** the polyclonal alloantibodies directed against 34 HLA alleles could be deconvoluted by a single A&E experiment using an HLA-B*15:14 expressing cell line into antibodies with a distinct reaction pattern of now only 10 HLA allelic antigens.

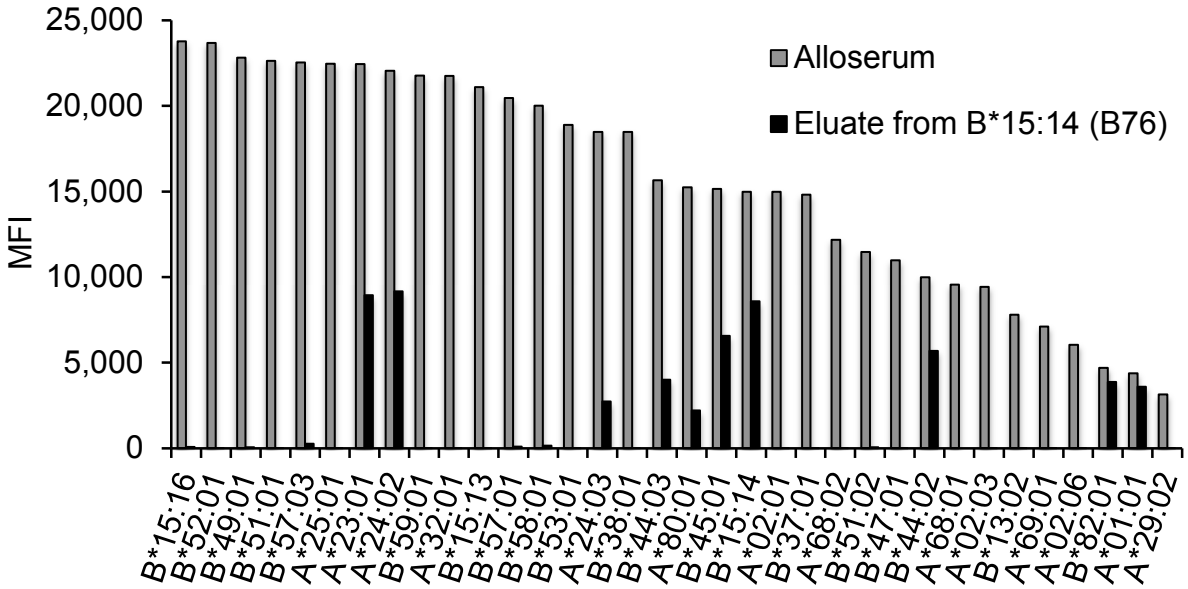


Figure 17. Deconvolution of alloserum broadly reactive with HLA class I SAB by the A&E technique. Human alloserum from highly immunized patient N5 reacted with a broad spectrum of class I SAB. Serum was first adsorbed onto and then eluted from a cell line expressing the single HLA allelic antigen B*15:14 (B76). The eluate revealed a distinct reaction pattern (A*01:01, A*23:01, A*24:02, A*24:03, A*80:01, B*44:02, B*44:03, B*45:01, B*82:01, B*15:14).

In some cases a single A&E assay was not sufficient for proper epitope analysis. Thus, a second adsorption was performed with a different HLA expressing cell line using the eluate from the first adsorption resulting in single epitope-specific antibodies. **Figure 18** demonstrates that each A&E assay yields in merely 10-50% antibody recovery due to insufficient adsorption.

Taken together the A&E technique is the only method described to date for the deconvolution of a pool of polyspecific antibodies with respect to HLA epitopes into monospecific antibodies. By the development of the A&E technique we were able to show that the epitopes of HLAab found in allosera after Nx are characterized by very broad reactivities (detailed analysis can be found in section 3.2.3).

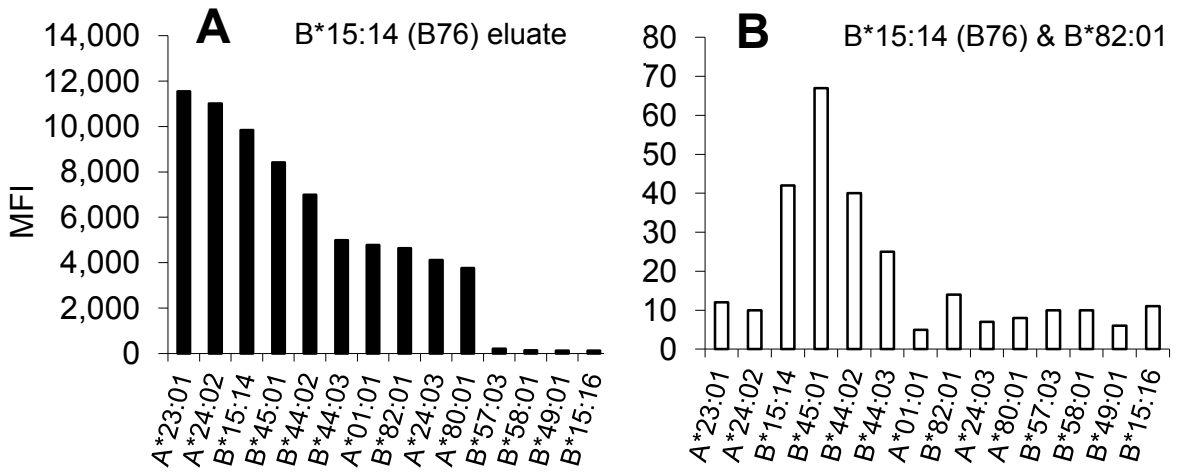


Figure 18. Sequential A&E technique. Alloserum from patient N5 was sequentially adsorbed onto and eluted from B*15:14 and B*82:01 expressing cell lines. The reaction pattern of the eluate from B*15:14 (**A**) was further deconvoluted into an antibody reactive with the epitope 167S (B*15:14, B*44:02, B*44:03, B*45:01) and 167G (A*01:01, A*23:01, A*24:02, A*24:03, A*80:01) (**B**).

These newly developed methods described in section 3.1 are essential means which enabled us to perform the detailed analysis on the significance of HLAab posttransplant further elaborated in this thesis. Denaturation of HLA on detection beads allowed the discrimination of truly HLA-specific from unspecific antibody reactivity. IgG subclass determination and assessment of complement binding of HLAab offered the possibility to elucidate the impact of antibody effector function on allograft survival. Finally, determination of the epitope specificity of HLAab from polyspecific allosera became available only by the development of the A&E technique in combination with the epitope search algorithm.

3.2 Incidence of Antibodies Late After Renal Transplantation

The cohort comprising 1075 renal transplant recipients was analyzed in a cross-sectional manner for the presence of HLA and MICA IgG antibodies using today's most sensitive Luminex® bead-based detection assay to assess the incidence of antibodies late posttransplant.

General characteristics of the cohort

We found that 33% (n = 357) in our cohort of patients were positive for IgG HLAab. The general and immunological characteristics of patients stratified according to the posttransplant HLAab status are summarized in **Table 4**. The patient characteristics were representative of the total kidney transplant population of the Charité transplant center.

Table 4. Patient characteristics. Patient characteristics stratified according to HLAab status at time of testing.

	HLAab pos (n = 357)	**HLAab neg (n = 718)**	
Recipient gender, % male (count)	45.1% (161)	65.5% (470)	p < 0.001
Recipient age at time of TX, yrs (IQR)*	42.1 (33.8 - 50.4)	45.2 (35.1 - 54.9)	p = 0.05
Median time from TX to enrollment, yrs (IQR)	5.1 (2.1 - 10.4)	3.9 (1.7 - 9.2)	p = 0.04
Median time of follow-up, yrs (IQR)	6.1 (3.0 - 8.3)	7.3 (4.9 - 8.6)	p < 0.001
Median SCr* at enrollment, mg/dl (IQR)	1.4 (1.1 - 1.9)	1.4 (1.1 - 1.8)	p = 0.4
eGFR** at enrollment, ml/min/1.73m^2 (IQR)	47.1 (35.9 - 61.6)	51.4 (39.0 - 66.2)	p = 0.01
Maintenance immunosuppression (count)***			
CSA-based therapy	50.7% (181)	49.9% (358)	p = 0.8
TAC-based therapy	41.5% (148)	44.0% (316)	p = 0.4
w/ AZA	24.6% (88)	19.8% (142)	p = 0.1
w/ MPA	37.8% (135)	40.7% (292)	p = 0.4
w/ steroids	70.3% (251)	70.5% (506)	p = 1.0
Type of kidney transplantation (count)			
retransplantation	33.0% (118)	4.7% (34)	p < 0.001
living donation	6.1% (22)	13.0% (93)	p = 0.001
single kidney	94.0% (336)	89.9% (639)	p = 0.006
Number of HLA mismatches, mean (SD)*			
class I: A, B locus	1.9 (1.1)	2.0 (1.2)	p = 0.1
class II: DR locus	0.9 (0.7)	1.0 (0.7)	p = 0.3

* TX = transplantation; SD = standard deviation; IQR= interquartile range; SCr = serum creatinine level
** estimated glomerular filtration rate (eGFR) according to the abbreviated MDRD equation (78)
*** ciclosporin (CSA), tacrolimus (TAC), azathiorpine (AZA), mycophenolic acid (MPA)

As expected, a significantly higher proportion of female patients showed reactivity to HLA alloantigens compared to male recipients. Similarly, the ratio of regrafted transplant recipients was significantly higher in the group of HLAab positive patients. Recipients of kidney transplants from a living donor showed less HLAab compared to recipients of a deceased donor transplant. The median SCr levels of HLAab positive and negative patients at time of testing did not differ significantly. However, the superior eGFR values indicated a slightly

better renal function for HLAab negative patients. The median time of follow-up for patients with HLAab was marginally lower compared to HLAab negative patients. On average, renal transplants were functioning for 4.2 years (IQR = 9.8 to 1.8 years) prior to the beginning of this study.

There was a slight but statistically significant difference in the time between TX and enrollment in this study notably between the HLAab positive and negative group (i.e., 5.1 vs. 3.9 years, P = 0.04). The maintenance immunosuppression (IS) at time of testing in both groups was predominantly a triple therapy based on calcineurin inhibitor (CNI) ciclosporin (CSA) with mycophenolic acid (MPA) and steroids. The mean number of HLA class I antigen mismatches (i.e., HLA-A, -B) was two, and the number of class II HLA-DR mismatched antigens was one, without significant differences between the groups.

HLAab against class I or II were exclusively detected in 15% and 7% of patients, respectively, whereas simultaneous class I and II HLAab were found in 12% (**Figure 19**).

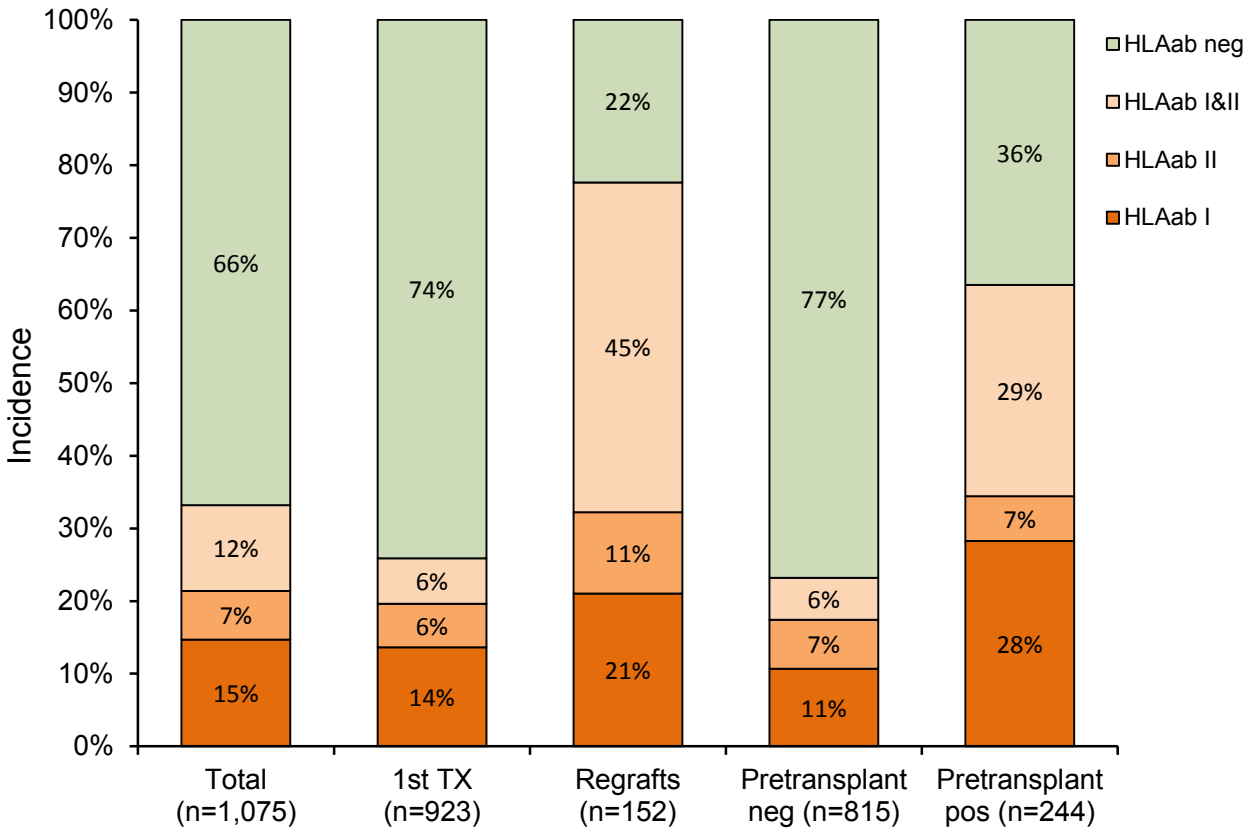


Figure 19. HLAab status posttransplant stratified according to previous transplants and pretransplant antibody status. Patients preimmunized by transfusions, gravidities and previous transplants showed as expected the highest incidence of HLAab posttransplant (64%) predominantly directed against HLA class I and II antigens (29%) whereas first transplants and pretransplant HLAab negative patients had a lower incidence of HLAab (i.e., 26% and 23%, respectively) that were primarily directed against HLA class I (14% and 11%, respectively).

neg = negative; pos = positive

Pretransplant immunization against HLA

Patients were enrolled regardless of their pretransplant antibody status, so some of them might have been preimmunized against HLA by transfusions, gravidities and/or previous transplants.

These HLAab pretransplant were assessed by CDC and ELISA. In 815 patients (75%) HLAab could not be detected pretransplant, whereas 150 (14%) were tested positive by CDC and additional 94 (9%) positive only by ELISA. *De novo* produced HLAab including those that went undetected pretransplant were found in 189 of 357 (53%) HLAab positive patients and 18% of the whole cohort. All pretransplant detected HLAab were considered as unacceptable HLA mismatches and therefore avoided for TX. For a total of 16 first transplant recipients (1.5%) there was no information available about pretransplant antibodies. *De novo* HLAab were predominantly directed exclusively against class I antigens. A proportion of 36% (89/244) of preimmunization could be attributed to prior TX. Conversely, 59% (89/152) of regrafted patients were preimmunized but only 17% (155/907) of first transplant recipients showed HLAab detected by CDC and ELISA pretransplant ($P < 0.0001$).

As expected preimmunization is gender-specific with a higher prevalence of females among pretransplant positive than negative patients (52% vs. 38%, $P = 0.001$). For 33% (145/444) female patients gravidities were recorded. Consequently, the incidence of posttransplant HLAab in regrafted patients was much higher than among first transplant recipients accompanied with a shift towards a higher proportion of combined HLA class I and II antibodies, i.e., 45% vs. 6% (**Figure 19**).

Noteworthy, patients who received a regraft were demonstrated to produce *de novo* HLAab posttransplant with a similar frequency as first transplant recipients (22% vs. 17%, $P = 0.1$). In 83 of 244 (36%) preimmunized patients HLAab were transient and could not be detected posttransplant. This group of patients consisted of mainly male, first transplant recipients immunized by transfusions only (59%, 49/83) as compared to only 18% (29/161) among patients preimmunized and HLAab positive posttransplant ($P < 0.0001$). There was no correlation between the presence HLAab detectable by CDC or ELISA pretransplant and HLAab posttransplant.

Using LABScreen® SAB we were able to clearly identify the HLAab reactivity to single HLA-A, -B, -DRB1, -DRB3/4/5, -DQB and -DPB antigens. The reactivity to the different HLA antigens among first kidney transplant recipients (n = 239) was: HLA-A 42% (n = 102), HLA-B 40% (n = 96), HLA-DRB1/3/4/5 26% (n = 63), HLA-DQB 33% (n = 80) and HLA-DPB 5% (n = 11). In contrast, the incidence of HLA-A, -B, -DRB1/3/4/5, -DQB and -DPB

antibodies among regrafted patients with HLAab (n = 118) was significantly increased to 62% (n = 73), 57% (n = 67), 53% (n = 62), 54% (n = 64) and 19% (n = 22), respectively. Especially, HLA-DP specific antibodies result predominantly from TX rather than from gravidities or transfusions.

Impact of the recipient's HLA on de novo antibodies

Late *de novo* production of HLAab is hypothesized to occur via the indirect allorecognition pathway which involves the presentation of the alloantigen by the recipient's HLA. Therefore, the impact of the recipient's HLA on the *de novo* production of HLAab in our cohort was analyzed by means of a logistic regression model. Interestingly, patients who received a mismatched renal transplant and were typed HLA-DR4 or -DR1 positive (including DR10 and DR103) showed an increased risk of producing *de novo* HLAab (**Figure 20**). On the contrary, HLA-DR3 (DR17 and DR18) revealed a protective effect (odds ratio (OR) = 0.57, 95% CI = 0.36 to 0.89, $P = 0.01$). None of the other HLA alleles significantly contributed to the regression model.

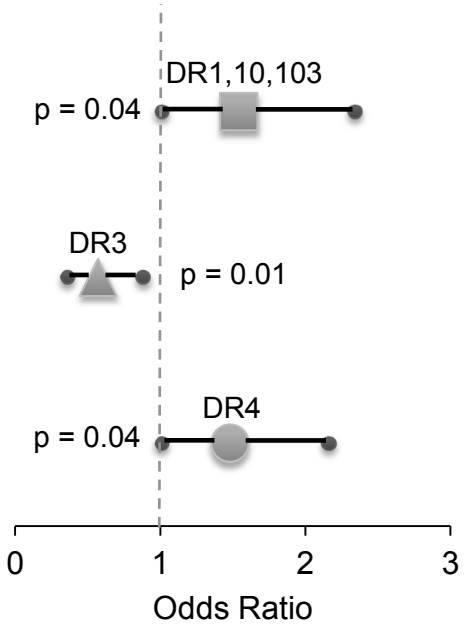


Figure 20. Relative risk of patients typed as HLA-DR1, -DR3 or -DR4 to produce *de novo* HLAab. Logistic regression analysis revealed patients having HLA-DR1, -DR10, -DR103 and -DR4 on an increased risk of producing *de novo* HLAab. HLA-DR3 had a protective effect on HLAab production. The squared, rectangular and circle markers of the forest plot each indicate the OR. The bars represent the 95% CI.

Immunization against MICA

The incidence of MICAab among 994 patients who could be tested with SAB covering the most frequent MICA antigens was 14% (n = 137). MICAab were found in 69 patients without HLAab (10%, 69/662) but 68 (20%, 68/332) in the presence of HLAab ($P < 0.0001$). In particular, patients with HLA class II and combined class I and II antibodies showed a higher prevalence of MICAab of 29% (20/70) and 25% (30/121), respectively, than those with HLA class I antibodies only (13%, 18/141) ($P = 0.01$). There is some correlation between the

presence of MICAab and immunization due to a previous transplant. A proportion of 25% (36/142) regrafted patients vs. 11% (101/852) first transplants showed MICAab. However, neither presensitization with HLAab nor genders were independent prognostic factors for the presence of MICAab (data not shown).

Taking together, the cohort analyzed in this thesis is representative for the total renal transplant population at Charité hospital with expected disequilibrium in gender, type of transplant and follow-up time between the HLAab positive and negative patient group (consecutive transplants in unmatched groups). Pretransplant immunization against HLA was mainly driven by previous transplants.

3.3 Specificity of Antibodies Following Renal Transplantation

The donor specificity of HLAab posttransplant is analyzed in the following section based on the traditional approach of HLA antigen mismatches supplemented by innovative epitope analysis. In addition, the incidence of antibodies directed against denatured HLA, which is partly present on the detection beads, will be assessed as these antibodies are anticipated to be clinically irrelevant.

HLA antigen specificity

The incidence of DSA among HLAab positive patients was 32% (113/357) of which 61% (69/113) was directed solely against donor class II antigens. DSA against both HLA class I and II antigens were rather rarely detected with an incidence of 8% (9/113). In addition, class II DSA revealed a higher MFI with a median of 6500 (IQR = 3000 to 14,000) compared to class I DSA with a median of 4500 (IQR = 2000 to 6500). Antibodies against donor HLA-DQB antigens accounted for the majority of 74% (58/78) of class II DSA. Notably, reactivity of antibodies against HLA-DQA, -DPA and -DPB has not been considered in this analysis due to the missing donor and recipient typing for these loci. The incidence of class I DSA against A-, B- and C-locus antigens was 61% (27/44), 36% (16/44) and 2% (1/44). On the contrary, NDSA were mainly directed against HLA class I only (53%). Logistic regression analysis revealed male gender (OR = 1.8; 95% CI = 1.1 to 3.0; P = 0.02), first TX (OR = 2.0; 95% CI = 1.2 to 3.1; P = 0.005) and HLA-A, -B, -DR mismatches (OR = 18.2; 95% CI = 2.1 to 159.5; P = 0.009) as independent predictors for the development of DSA posttransplant. Preimmunization did not contribute significantly to the regression model.

HLA epitope specificity

Antibodies are directed against distinct areas of the HLA molecule called epitope rather than an HLA antigen as a whole. Epitopes are formed from the 3-dimensional structure of AA exposed on the surface and somewhat mismatched between organ donor and recipient. The number of single AA mismatches can be predicted from the number of HLA-A, -B, -DR split antigen mismatches as determined by a linear regression analysis. The slope of the regression line indicated that for each antigen mismatch the number of AA mismatches increased by 8 (coefficient = 8.0; 95% CI = 7.8 to 8.3; $P < 0.0001$; $y = 8.0x$; $r^2 = 0.85$). By its conventional definition DSA are present when a detected antibody is directed against the antigen of the donor. In fact, DSA are specific only for a limited number of mismatched donor epitopes (109).

Conventionally defined NDSA among first transplant recipients without presensitization (n = 52) were analyzed in detail with respect to the epitope specificity. In 28 patients (54%) all detected HLAab conventionally designated as NDSA (range = 1 to 15 specificities) have been demonstrated to be potentially directed against donor-specific epitopes (DSE) (**Table A2** in **Appendix I**). In another 14 patients DSE could explain only a proportion of on average 66% of NDSA (i.e., 242/363 cumulative specificities). These results indicate that the majority of NDSA are potentially derived from donor mismatches and thus might have some power to predict alloreactivity which will be further illustrated in section 3.6.1.

HLA heavy-chain-specific antibodies

As demonstrated earlier in section 3.1.1 and from personal communications with the manufacturer of LABScreen® beads it is known that the immobilized HLA molecules are partially present in a denatured state on the beads without β2m and/or peptide. In addition, it was shown in recent studies that antibodies against β2m- and peptide-free HC molecules can be found in non-alloimmunized individuals and transplant patients (45, 110). However, the clinical relevance of these antibodies is still a matter of contention (111).

We hypothesize from our own previous data on a similar cohort of transplant recipients that HC-specific HLAab are clinically irrelevant (110). In an attempt to further decipher the specificity of detected antibodies in our cohort into those with HC- and without HC-specificity, HLA and MICA molecules on LABScreen® pooled antigen beads underwent a complete denaturation process and were tested in parallel with untreated beads. Based on the results three distinct antibody groups could be defined as illustrated in **Table 5**.

Table 5. Definition of HLAab and MICAab with and without HC-specificity. Classification is based on parallel testing with untreated and denatured beads.

Untreated Beads	Denautred Beads	Interpretation
positive	positive	HLA/MICAabs with (partial) HC-specificity
positive	negative	HLA/MICAabs without HC-specificity
negative	positive	HC-specific (non-HLA/MICA) antibodies

HC = heavy chain

We tested 788 sera of 1075 (73%) total renal transplant recipients on untreated, acid- and heat-denatured beads. This approach allows discriminating intact HLA/MICA- from HC-specific antibodies. The major characteristics did not differ significantly between patients from both groups (**Table A3** in **Appendix I**). As shown in **Figure 21** for heat denaturation, 525 (67%) of them had no antibodies, while 263 (33%) patients harbored HLA class I and/or class II antibodies. The majority (68%) of detected total HLAab (180/263) also reacted with heat-denatured beads. In contrast, 33% (263/788) of antibodies reacted exclusively with HC molecules on denatured beads. These antibodies have been regarded as non-HLA/MICA-specific. However, 180 patients were tested positive against both untreated and denatured beads and were defined as “HLAab with HC-specificity”, while in 83 cases (11%) patients were tested positive against untreated but not denatured beads and were defined as “HLAab without HC-specificity”. Interestingly, HLAab specificities detected in non-alloimmunized male blood donors by Morales-Buenrostro et al. (45) were found with a higher frequency in patients with HC-specific antibodies than without (i.e., A80 with 14% vs. 8%; B76 30% vs. 16% and B37 17% vs. 9%). Except for B76 with a *P* value of 0.04 the differences did not reach statistical significance. Thus, detection of these specificities by untreated beads might in fact indicate reactivity against denatured antigen.

Heat and mild acid treatment were applicable only to class I HLA. The concordance between both methods was 55%. Heat denaturation seemed to be more effective as 306 of 788 (39%) sera tested negative with mild acid-treated beads revealed positive reaction with heat-denatured beads and 50 (6%) vice versa. These discrepancies might reflect the methodical disparity between dissociation of β2m and peptide by a chemical versus a thermic process.

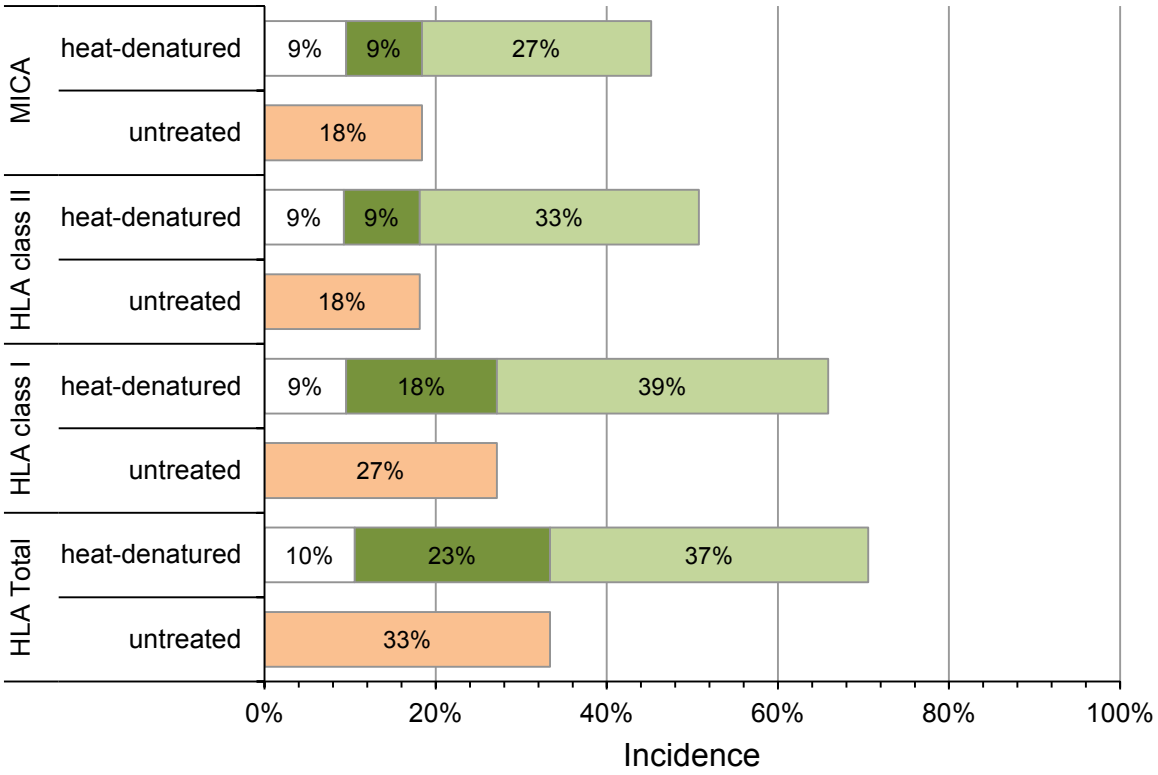


Figure 21. Distribution of antibodies reacting with untreated and heat-denatured LABScreen® pooled antigen class I, II and MICA beads. Of the 788 patients tested the majority of antibodies reacted solely with heat-denatured LABScreen® pooled antigen beads (light green bars). A relatively constant proportion of 9-10% revealed antibodies directed against intact HLA class I, II and MICA antigens only (white bar). Antibodies against untreated and heat-denatured beads are depicted in dark green.

This analysis provided a detailed insight into the different specificities of HLAab late after renal TX. In summary, one third of HLAab are directed against donor mismatched antigens designated as classical DSA. Among the NDSA, antibodies specific for donor-derived epitopes (i.e., DSE) could be suspected. Antibodies specific for denatured HLA are not uncommon and could be detected in another third of patients. The individual impact of each of these subgroups of antibodies on renal allograft survival will be analyzed in section 3.6.

3.4 Antibodies after Graft Failure and Nephrectomy

The amount and specificity of HLAab detectable in peripheral blood is thought to be affected by the allograft itself in a way that a substantial amount of DSA might be adsorbed by antigens present in the donor organ and not detectable as free antibodies in the serum. This phenomenon also referred to as the "sponge-effect" (112) is thought to become evident when IS is withdrawn or the graft is removed by Nx. In fact, it is a common observation that after Nx the PRA, as the indicator of amount and specificity of HLAab, increases dramatically within a short time period revealing DSA but also a multiple NDSA. In the following section

we analyzed in detail the dynamic of HLAab specificities before and after graft failure and subsequent Nx as a model for the concept of NDSA predicting the presence of DSA. Evidence of *de novo* DSA appearance after Nx in the presence of NDSA sharing common epitopes would suggest a "sponge effect".

We selected 9 patients who underwent Nx following late allograft rejection on average 7 (5.9) years posttransplant. All 9 patients were first kidney transplant recipients without HLAab pretransplant but known transfusions and/or gravidities summarized in **Table 6**. Sera taken at three different time points per patient were tested for the presence and specificity of HLAab: (a) immediately pretransplant, (b) before graft dysfunction, and (c) on average 6 months after Nx. HLAab after Nx underwent A&E experiments (see 3.1.4) followed by an epitope analysis to uncover potential DSE-specific antibodies among NDSA and thereby prove the hypothesis of the "sponge-effect".

Table 6. Immunologic characteristics of patients who underwent Nx following allograft rejection. Nine patients with known immunizing events but without any preformed HLAab (0% PRA as tested by Luminex® SAB) underwent Nx following late allograft dysfunction. The cause of allograft failure was determined by biopsy.

ID	TX Type	HLA Mismatches	Nephrectomy Biopsy	Immunization
N1	liv kidney	A24,B7,DR15,DQ6	AMR	2 TF, 0 grav
N2	kidney	B51,B39,Cw1,DR11	sAMR	2 TF, 0 grav
N3	kidney	A1,A23,B44,B27,Cw5,DR11,DR13,DQ6,DQ7	sAMR	2 TF, 0 grav
N4	kidney/liver	A2,Cw9,DR11,DQ6	sAMR	10 TF, 0 grav
N5	kidney	A24,Cw1,DQ2	sAMR	12 TF, 5 grav
N6	kidney	A3,B62,B27,Cw9,DR1,DQ5	sAMR	0 TF, 0 grav
N7	kidney	A2,A32	AMR	22 TF, 1 grav
N8	kidney	A1,B8,B39,DR1,DR17,DQ5	sAMR	2 TF, 0 grav
N9	kidney	A30,B13,B55,Cw1,Cw6,DR11,DQ2,DQ7	sAMR	6 TF, 4 grav

TX = transplant; TF = transfusion; grav = gravidity; liv = living; %PRA = percent panel-reactive antibodies; n.t. = not tested; AMR = antibody-mediated rejection; sAMR = suggestive for AMR according to Banff (113)

The mean PRA of detected HLAab increased significantly from 30% before to 91% after Nx ($P < 0.001$). All 9 patients revealed DSA after Nx but in 3 of them DSA were already detected in sera before failure and Nx. A representative example of alloreactivity of HLA class I antibodies in patient N1 before and after Nx is illustrated in **Figure 22**. Patient N1 received a kidney transplant from a living donor. The donor organ was mismatched for HLA-A24, -B7, -DR15, -DR51 and -DQ6. No HLAab could be detected up to 1.5 years posttransplant. At first DSA against DR51 became detectable followed one year later by the presence of DSA and NDSA against multiple HLA class I and II antigens accompanied with an increase in SCr.

Finally, 2.7 years posttransplant the graft failed with detectable cytotoxic HLAab. After Nx HLA class I PRA increased from 40% to 95%.

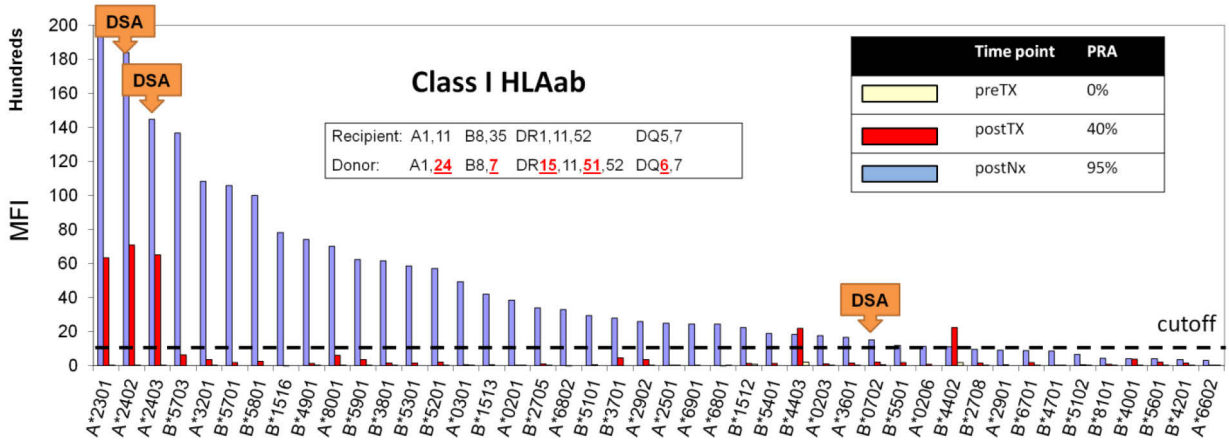


Figure 22. Alloreactivity of patient N1 before and after Nx. MFI of HLA class I antibodies as revealed by Luminex® SAB is indicated on the y-axis in hundreds over antibody specificity. Three serum samples of patient N1 have been tested: (i) pretransplant (preTX, yellow), (ii) 853 days posttransplant (postTX, red) and (iii) after Nx (postNX, blue). The PRA increased dramatically from 0% via 40% to 95% after Nx. Presence of DSA is indicated by arrowed boxes.

For all Nx patients A&E experiments were performed to deconvolute the high PRA polyclonal antibodies after Nx (e.g. blue bars in **Figure 22** for patient N1) into monospecific antibodies which made epitope determination of antibodies from allosera possible in the first place. A total of four different single HLA antigen-expressing cell lines were used for patient N1 to deconvolute six distinct class I HLAab subsets from alloserum (**Figure 23**). Considering the specificities of all six epitopes together, they explain the reactivity of alloantibodies against 82% (32/39) of positive HLA antigens. The antibody reactivity against A*29:01, A*29:02, A*36:01, B*07:02, B*27:08, B*55:01 and B*67:01 could not be attributed to any of the determined epitopes. It is apparent that all these specificities with exception of A*29:02 revealed relatively low fluorescence intensities (MFI < 1500). Due to the process of A&E a substantial proportion (80-95%) of the initial fluorescence intensity is lost, which makes it difficult to detect low level HLAab after A&E. Interestingly, DSA always revealed the highest MFI values following A&E experiments.

In addition to our findings outlined above, 5 of 6 epitopes were present on the mismatched donor antigen HLA-A24 (A*24:02 or A*24:03) but not on the recipient and thus referred to as DSE. In conclusion, application of the A&E-assay to this patient sample could attribute 82% of NDSA after Nx as DSEab recognizing epitopes shared with incompatible donor antigens.

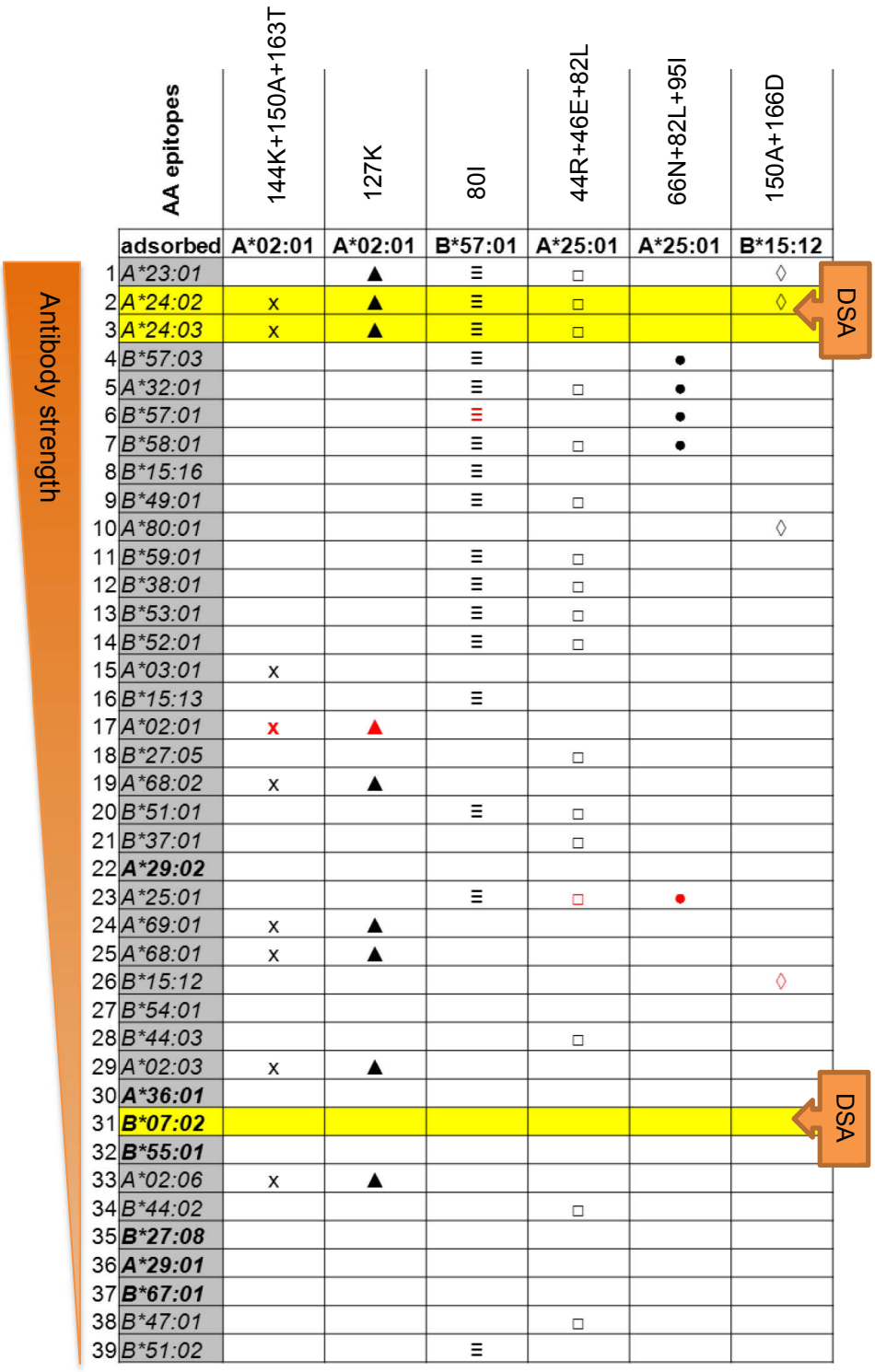


Figure 23. Reaction patterns of HLA class I alloantibodies of patient N1 after A&E experiments. The reaction patterns of eluted antibodies after Nx were analyzed with respect to potential epitopes listed on top. Each symbol represents a positive reaction with SAB as listed on the left hand site. Specificities are ordered with descending MFI. DSA are highlighted in yellow whereas antigens used for A&E are highlighted by a red symbol.

A summary of the epitope analysis following A&E experiments for all 9 Nx patients is shown in **Table 7**. Thus, 67% of all 243 HLA class I specificities could be attributed to DSE.

The majority of HLAab specificities detected by standard Luminex® SAB after Nx are de facto derived from a limited number of DSE. This finding supports the hypothesis of a "sponge-effect" and suggests that DSA as well as NDSA might be adsorbed by the graft in situ. In conclusion, NDSA recognizing DSE can therefore be considered as potential biomarkers for DSA.

Table 7. Experimentally identified HLA class I epitopes in patients after Nx. Epitopes determined by A&E experiments accounted for 67% of all 243 HLA class I specificities found among 9 patients after Nx of the failed allograft.

ID	Epitopes count	DSE count	Specificities count	Epitope accountable specificities, count(%)	Epitopes*
N1	6	5	39	32 (82%)	(144K+150A+163T); (127K); (80I); (44R+46E+82L); (66N+82L+95I); (150A+166D)
N2	2	2	40	20 (50%)	(80I); (45T+65Q/66I/69T/70N+99Y)
N3	5	5	34	26 (76%)	(163E+166E); (69A+82L/83R+116D); (167S); (152A); (167G)
N4	2	2	8	6 (75%)	(127K); (66K+76V)
N5	3	3	51	35 (69%)	(77N); (70H); (166D)
N6	2	2	7	6 (86%)	(56G+62Q+70Q); (70K)
N7	2	2	19	11 (58%)	(41A+62R/65Q/66I+97S); (52I+65Q+69A)
N8	1	1	13	5 (38%)	(166D)
N9	4	4	32	22 (69%)	(46A+76E); (99Y+131S+163E); (70Q+77D); (62Q+76V)
Total	27	26	243	163 (67%)	

DSE = donor-specific epitope(s)
* (80I) epitope consisting of AA isoleucine (I) at position 80 of HLA class I molecule
(144K+150A+163T) composite epitope consisting of three single epitopes
(69A+82L/83R+116D) two equivalent composite epitopes separated by "/"

3.5 Assessment of Antibody Effector Function

Along with the specificity the antibody effector function conducted by the Fc domain of the antibody molecule plays an eminent role in the pathogenicity of antibodies. The analysis of the IgG effector functions will be presented in the following chapter.

IgG1-4 subclass

The four IgG subclasses are distinguished by relatively few AA exchanges in the hinge region of the molecule but differ substantially among one other with respect to the elicited effector functions (i.e., opsonization and complement activation). During opsonization antibodies serve as flexible adapters to bridge alloantigens via Fc receptors (FcR) with designated accessory effector cells of the immune system (e.g. macrophages, dendritic and NK cells) leading to phagocytosis or cytotoxicity. The different FcR are signaling receptors with a specific affinity for certain IgG subclasses. FcγRIII (CD16) for example expressed on NK cells can be activated exclusively by IgG1 or IgG3 leading to destruction of the alloantigen bearing target cells by a process referred to as antibody-dependent cell-mediated cytotoxicity. In general, the relative concentration of total IgG1-4 in the serum is IgG1>IgG2>IgG3=IgG4. The relative composition of HLA-specific IgG1-4 antibodies late after TX and consequences with respect to the pathogenicity of HLAab is not yet well determined. The questions remain how IgG subclass composition contributes to the complement activation capability in complex allosera.

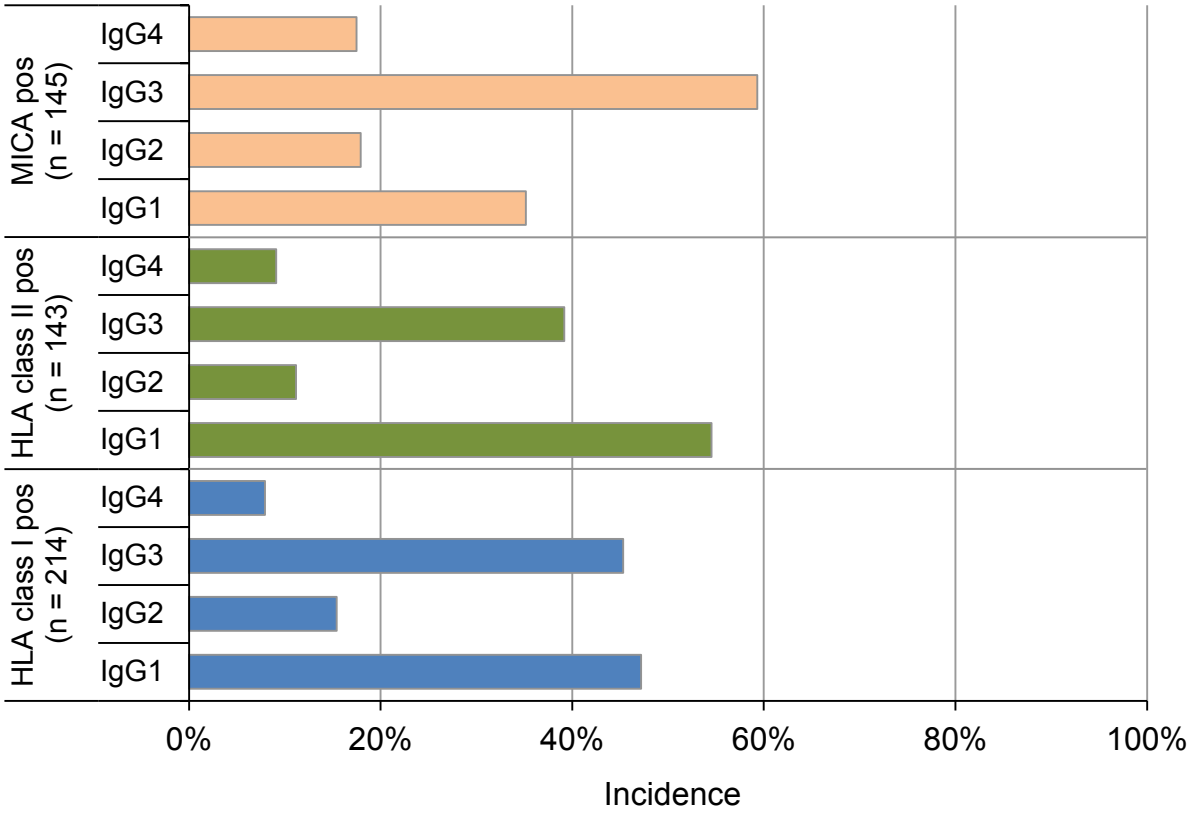


Figure 24. Composition of HLA and MICAab with respect to IgG subclasses. HLA class I and II antibodies were dominated by IgG1 and IgG3. IgG2 and IgG4 were rarely detected. Among MICA antibodies IgG3 was preferably detected.

pos = positive

Opposed to the above described distribution pattern of total IgG1-4 subclass antibodies in serum of normal humans, HLA class I and II antibodies of transplant recipients are dominated by IgG1 and IgG3 followed with quite a gap by IgG2 and IgG4. Thus, the hierarchy of IgG subclass HLAab detectable in the late phase after renal TX can be described as IgG1=IgG3>>IgG2=IgG4 (**Figure 24**), which represents a clear shift to complement-activating IgG. This finding is in contrast to the IgG subclass composition found in other immune responses, e.g. directed against bacterial proteins dominated by IgG1 (114), bacterial polysaccharides IgG2 (115), or viral infections IgG1 and IgG4 (116). MICAab showed a similar pattern but are clearly dominated by IgG3. However, the impact of antibodies is rather determined by synergism between IgG subclasses. As summarized in **Figure 25** HLA and MICAab are primarily formed by combinations of strong cytotoxic IgG1 and/or IgG3. Exclusively non-cytotoxic IgG2 and/or IgG4 antibodies were rarely detected. Approximately half of the sera consisted of mixed cytotoxic and non-cytotoxic HLA and MICAab (IgG1/2/3/4).

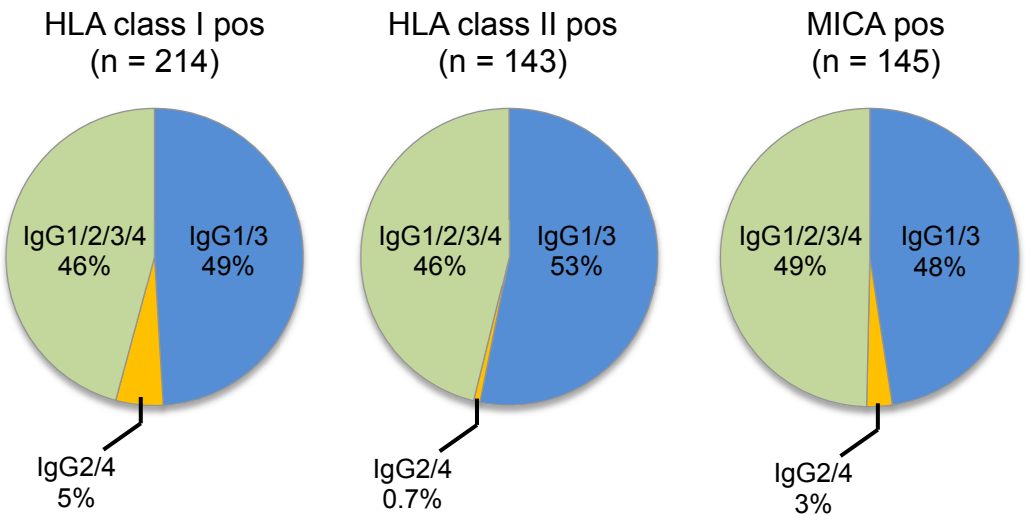


Figure 25. Distribution of cytotoxic IgG1/3 and non-cytotoxic IgG2/4 antibodies among HLA and MICAab positive patients. Approximately half of the detected HLA class I, class II and MICAab were exclusively of the IgG1 and/or IgG3 subclass (blue) without the presence of any non-cytotoxic IgG2 or IgG4 antibodies (orange). Mixtures of cytotoxic and non-cytotoxic antibodies (green) were present in proportions ranging from 46-49%.

Complement activation

HLAab can activate complement by the classical pathway. The initial complement component C1q binds to the antibody-antigen complex and activates a series of proteolytic cleavage reactions. As indicated by the pace of HAR in the presence of cytotoxic HLAab leading to allograft loss within minutes (117), complement activation is presumably the strongest antibody effector function.

The complement binding capability of HLA and MICAab in this thesis was assessed by the newly developed in-house sC1qA described in section 3.1.2. As illustrated in **Figure 26** proportions of 28%, 20% and 25% of HLA class I, class II and MICAab, respectively, were found to bind C1q. This corresponds to as little as 11% (90/788) of analyzed total patients but 30% of HLAab positive patients.

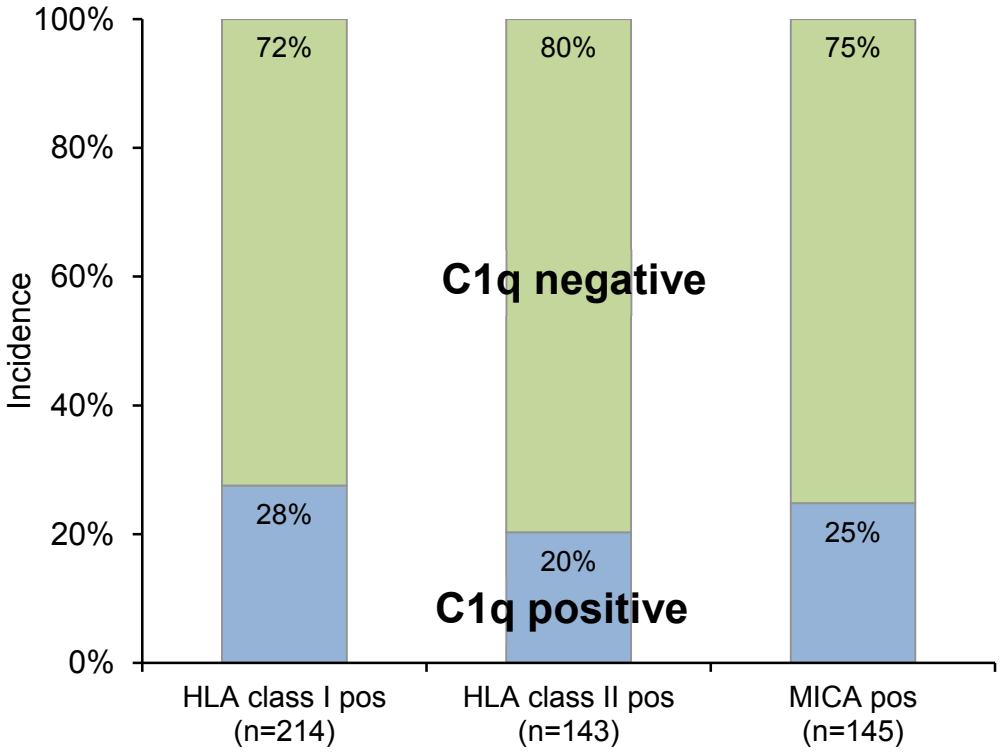


Figure 26. Proportion of complement C1q-fixing HLAab and MICAab. The incidence of C1q-fixing HLA class I, class II and MICAab (blue) was 28%, 20% and 25%, respectively.

HLA class I, class II and MICAab with a mixed composition of IgG subclasses were preferably detected in C1q positive sera with incidences of 54%, 55% and 72% (**Table 8**). As expected, high frequencies of strong cytotoxic IgG (IgG1/3) and very low frequencies of low- to non-cytotoxic IgG2 and IgG4 antibodies were found. Surprisingly, there was no statistically significant difference in the IgG subclass composition patterns between sera with and without C1q-fixing antibodies. Based on this observation, it becomes clear that the differential C1q-fixing capability associated with certain IgG subclasses on its own cannot fully explain the complement fixation observed in this thesis. Presumably, the titer of each antibody determines the cytotoxic potential in addition to its isotype and subclass. However, there was a tendency to a more mixed IgG subclass phenotype of HLA and MICAab in C1q-positive sera. This finding suggests that complement and non-complement fixing antibodies can act synergistically to fix complement more effectively.

Taken together, HLAab of IgG1 and IgG3 subclasses were most frequently found in this thesis. However, their cytotoxic potential was rather determined by the synergism between the IgG subclass composition patterns and antibody level. Assessment of the IgG subclass of HLAab only was not sufficient to predict complement binding.

Table 8. Distribution patterns of IgG1-4 among complement C1q-fixing HLA and MICAab. Mixed IgG subclass HLA and MICAab (IgG1/2/3/4) were found in the majority of C1q positive sera. There was no statistically significant difference in the IgG subclass composition between C1q negative and positive sera.

	HLA class I pos (n=214)			
	IgG1/3	IgG1/2/3/4	IgG2/4	Total
C1q pos	43% (25)	54% (32)	3% (2)	100% (59)
C1q neg	52% (80)	42% (66)	6% (9)	100% (155)
	HLA class II pos (n=143)			
	IgG1/3	IgG1/2/3/4	IgG2/4	Total
C1q pos	45% (13)	55% (16)	0% (0)	100% (29)
C1q neg	55% (63)	44% (50)	1% (1)	100% (114)
	MICA pos (n=145)			
	IgG1/3	IgG1/2/3/4	IgG2/4	Total
C1q pos	25% (9)	72% (26)	3% (1)	100% (36)
C1q neg	55% (60)	42% (46)	3% (3)	100% (109)

3.6 Patient and Graft Survival

The overall patient survival probability among the 1075 renal allograft recipients after 8 years of follow-up was 85% (95% CI = 82.0 to 87.1%). Patients with and without HLAab revealed a survival probability of 87% (95% CI = 81.4 to 90.1%) and 84% (95% CI = 80.8 to 86.9%) ($P = 0.7$). Among 136 patients with reported death during follow-up the prevailing cause was of cardiovascular origin (35%, n = 48), followed by malignancies (23%, n = 31), infections (17%, n = 23), and suicide (1%, n = 2). For 32 patients (24%) no information could be retrieved from the records. In total, 108/136 patients (79%) were censored for death with a functioning allograft.

3.6.1 HLA Antibodies Predict Impaired Allograft Survival

The prevalence of HLAab among patients with a still functioning allograft at 8 years of follow-up was 25%. In contrast, half of all patients with failed allografts and censored for death were tested HLAab positive ($P < 0.001$). In other words, antibodies were more frequently present in sera of failed transplants. The average time to organ failure among patients with HLAab was 1597 days (IQR = 779 to 2481 days), in contrast to 1843 days (IQR = 948 to 2423 days) for patients without HLAab ($P = 0.5$). As depicted in **Figure 27** the proportion of DSA among patients whose allograft failed was significantly increased to 40% as compared to 24% in those with a still functioning graft after follow-up ($P = 0.002$). We

found no difference in the antibody profile with respect to HLAab class or DSA. Exclusive class I HLAab were most frequently present in both groups, each with 44%. As stated earlier it is again apparent that although class II HLAab account for only 20% of total HLAab they add up to 60% among DSA.

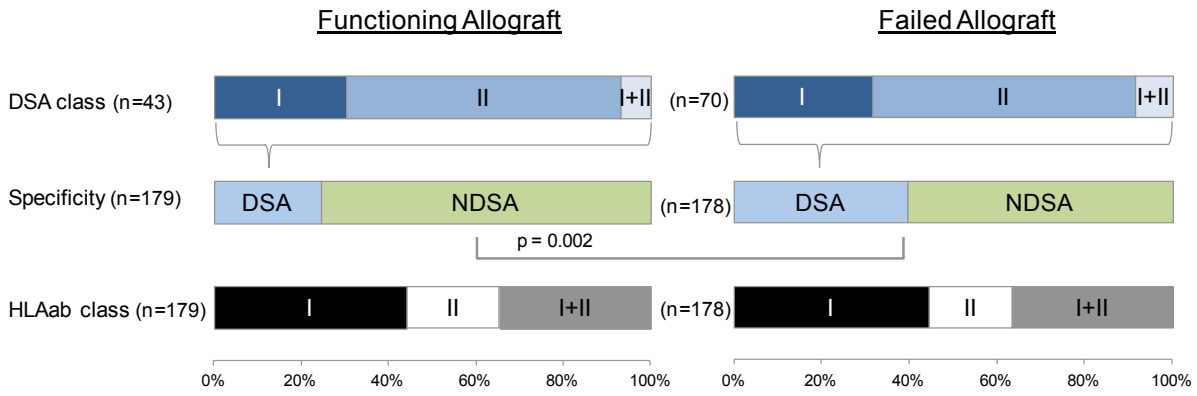
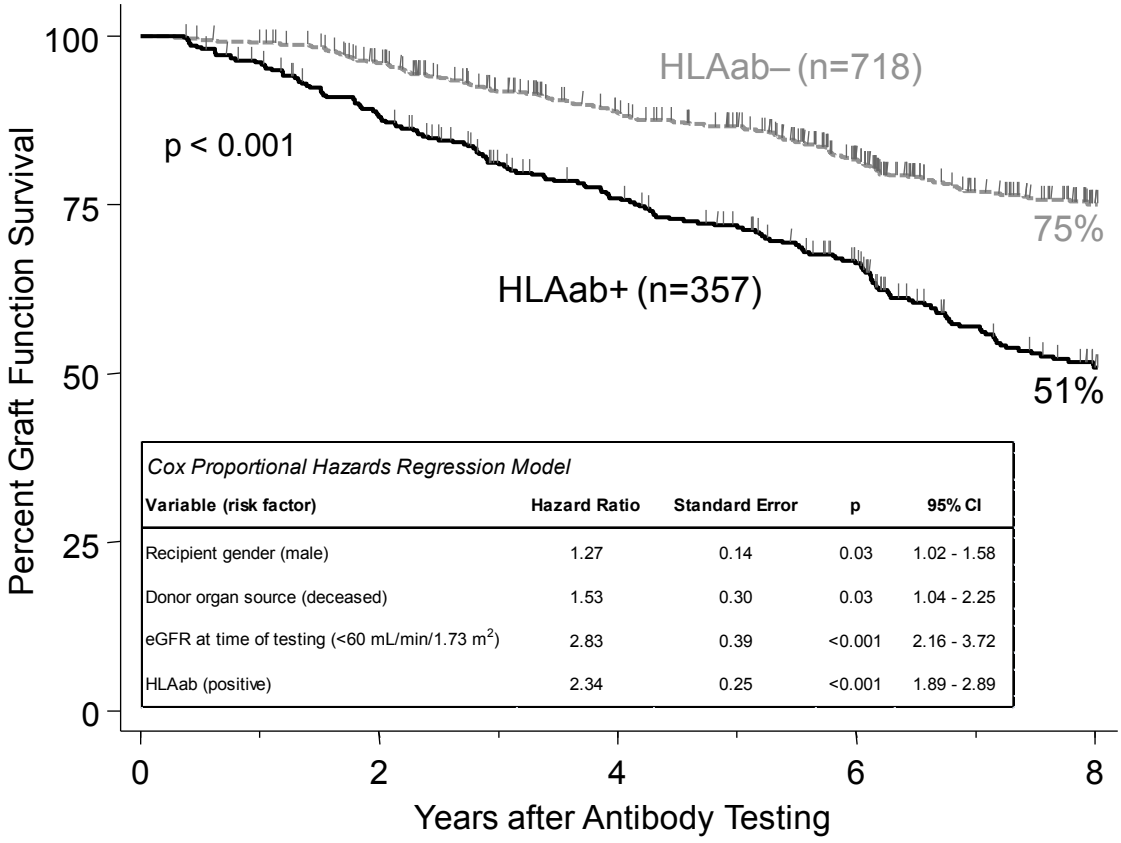


Figure 27. Antibody profile in patients stratified according to allograft outcome at 8 years of follow-up. The antibody profile with respect to the HLAab class and DSA did not differ significantly between patients with functioning versus failed allografts. Solely an increased prevalence of DSA (40% vs. 24%) was characteristic for patients with allograft failure ($P = 0.002$).

The overall graft function survival was 67% (95% CI = 63.7 to 69.9%). This observed annual failure rate of approximately 4% is equivalent to the overall failure rate in large registry data (10, 118).



Cox Proportional Hazards Regression Model

Variable (risk factor)	Hazard Ratio	Standard Error	p	95% CI
Recipient gender (male)	1.27	0.14	0.03	1.02 - 1.58
Donor organ source (deceased)	1.53	0.30	0.03	1.04 - 2.25
eGFR at time of testing (<60 mL/min/1.73 m^2)	2.83	0.39	<0.001	2.16 - 3.72
HLAab (positive)	2.34	0.25	<0.001	1.89 - 2.89

Figure 28. Allograft function survival probability of patients stratified according to the presence of HLAab at time of testing including a Cox proportional hazards regression model. The presence of HLAab was highly prognostic for late allograft dysfunction within 8 years of follow-up. The presence versus the absence of HLAab was associated with a 2.3 fold increased risk for allograft dysfunction as revealed by a Cox proportional hazards regression model corrected by known confounders. Only explanatory variables with statistical significance were included.

HLAab- = HLAab negative; HLAab+ = HLAab positive

For patients with detectable HLAab the Kaplan-Meier estimates of the 8-year allograft function survival rate (**Figure 28**) was 51% (95% CI = 45.0 to 56.5%) in contrast to 75% (95% CI = 71.4 to 78.4%) for patients without HLAab ($P < 0.001$). Cumulative hazard ratios were calculated using a Cox proportional hazard regression model under consideration of potential confounders. According to this model, patients with HLAab faced a 2.3 fold increased risk for allograft failure as compared to patients without antibodies ($P < 0.001$) (**Figure 28**). Other explanatory variables that contributed significantly to the model comprised recipient gender, donor organ source, and allograft function at time of antibody testing. As expected and revealed by the Cox model patients with a decreased allograft function indicated by an eGFR value <60 mL/min/1.73m^2 were 2.8 times more likely to lose their allograft.

Table 9. Graft function survival probability of patients with mutually exclusive HLA class I and/or class II or MICAab. There was no survival difference between patients with HLA class I or class II or class I+II antibodies. The relative risk for graft failure assessed by univariate Cox proportional hazard analysis was almost equally distributed. The presence of MICAab was not associated with an increased risk of graft failure.

	% Graft Function Survival in Year of Follow-Up					Cox Proportional Hazard Model (univariate)		
	0	2	4	6	8	Hazard Ratio	p	95% CI
HLAab neg (n=718)	100	96	89	82	75	1.0	n/a	n/a
class I HLAab pos (n=158)	100	88	74	59	48*	2.5	p<0.001	1.91 - 3.23
class II HLAab pos (n=72)	100	94	83	76	57*	2.3	p<0.001	1.58 - 3.26
class I+II HLAab pos (n=127)	100	84	74	69	51*	2.5	p<0.001	1.92 - 3.35
MICAab pos (n=91)	100	98	92	85	72	0.8	p=0.2	0.51 - 1.14

*$P < 0.001$ as compared to HLAab neg (log-rank test)
HLAab = HLA antibody; MICAab = MICA antibody; neg = negative; pos = positive; n/a = not applicable

Concerning the discussion on the differentially detrimental effect of class I or class II HLAab we were not able to demonstrate a statistically significant difference in the relative risk for allograft failure (**Table 9**). The presence of exclusively MICAab was not shown to increase the risk for allograft failure. On the contrary, when HLAab were accompanied by MICAab graft survival decreased from 51% to 38% ($P = 0.2$) (data not shown).

Detrimental impact of donor-specific antibodies

Allograft outcome 8 years after HLAab determination was significantly impacted by the incidence of DSA. As shown in **Figure 29** graft survival of patients with DSA, with NDSA, and without HLAab was 40%, 55%, and 75%, respectively ($P < 0.001$). The mean time span from antibody testing to graft failure between patients with DSA and NDSA did not differ significantly with means (SD) of 4.4 (2.7) years and 4.7 (2.6) years, respectively ($P = 0.2$).

The presence of NDSA was primarily impacted by the history of presensitizing events. As shown earlier (see 3.2.1) among 152 patients who received a regraft a proportion of 78% (n = 118) were alloimmunized whereas only 26% (n = 239) of first transplant recipients revealed HLAab ($P < 0.001$). The majority of antibodies in regrafts were directed against antigens of the previous transplants. Although gravidities predispose to produce more likely HLAab (53% vs. 40%, $P = 0.008$) they cannot be made responsible for a higher proportion of NDSA (80% vs. 73%, $P = 0.2$). Unfortunately, transfusions - as the third main pillar of

alloimmunization - could not be analyzed systematically in this cohort due to incomplete information and was therefore omitted from further consideration.

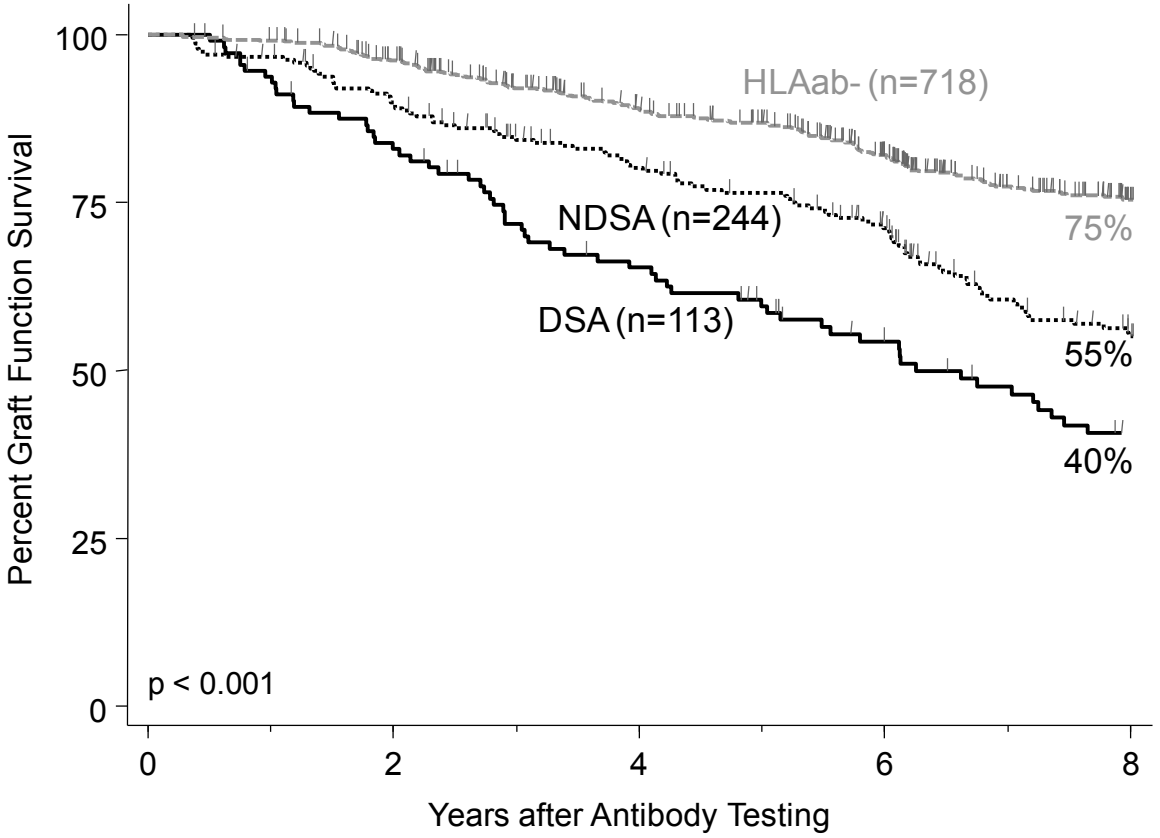


Figure 29. Allograft function survival was negatively impacted by presence of DSA and NDSA. The presence of DSA was associated with a statistically significant decrease in allograft function survival probability as compared to HLAab negative patients (40% vs. 75%, $P < 0.001$). NDSA also revealed a negative impact on 8-year graft survival.

HLAab- = HLA antibody negative

Despite the mentioned predisposing factors for NDSA production none of the variables analyzed including pretransplant HLAab status, number of previous transplants and maximum MFI value of detected NDSA was able to discriminate between harmful and harmless NDSA with respect to allograft survival. In addition, the proposed determination of DSE specificities among 52 patients harboring exclusively class I NDSA (see 3.2.2) did not provide any potential for graft outcome prediction (**Table A4** in **Appendix I**).

3.6.2 Predicting the Pathogenicity of HLA Antibodies

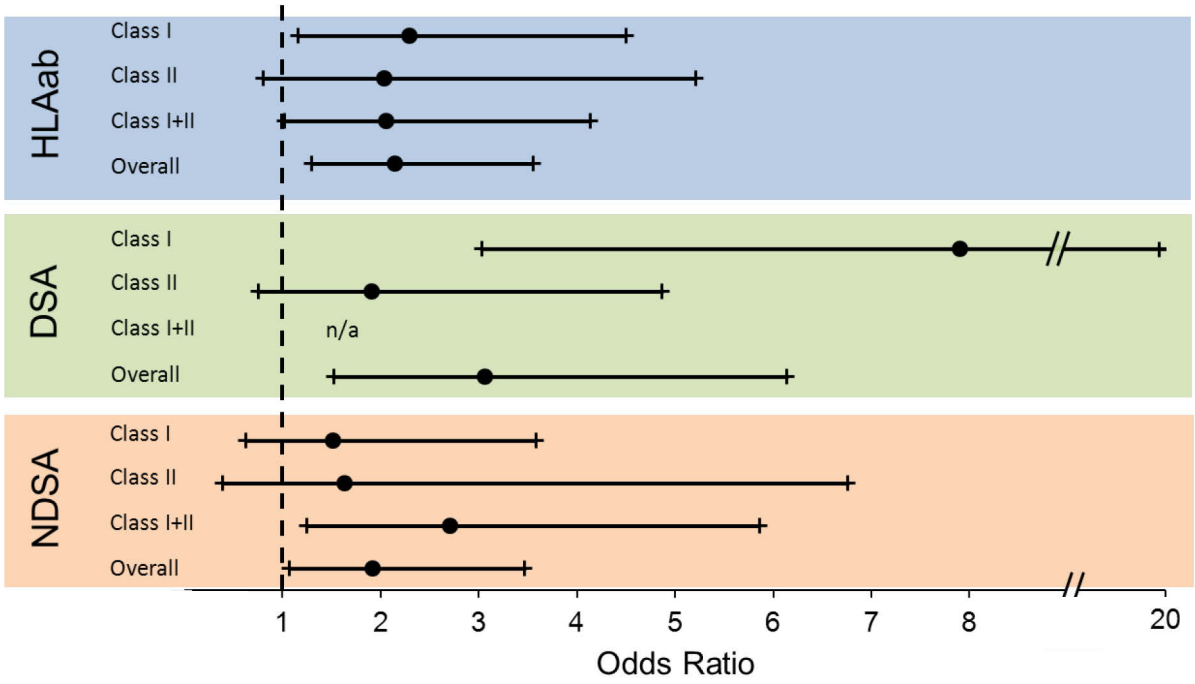


Figure 30. Relative risk (odds ratio) of patients with eGFR ≥ 60 mL/min/1.73m² for allograft failure stratified according to the specificity of HLAab. Patients with normal allograft function at time of antibody testing but detectable HLA class I, HLA class I+II, donor-specific class I, and non-donor-specific class I+II antibodies revealed a statistically significant increase of risk for allograft failure within 8 years of follow-up. The odds ratio of patients with DSA class I+II could not be determined due to the low numbers of observations in this stratum. The filled circles (•) of the forest plot indicate odds ratios and the bars the 95% confidence interval (95% CI).

n/a = not applicable

Since all patients were tested at one single time point after TX and followed for a median of 8 years the cohort comprised individuals at different levels of graft function. Of special interest for the nephrologists in the outpatient clinic are those patients with unimpaired graft function but detectable HLAab. Consequently, according to a multivariate Cox proportional hazard regression model including HLAab status, donor specificity, source of organ donor, number of previous transplants, HLAab status pretransplant, recipient gender, year of transplant and allograft function at time of enrollment, only an eGFR value <60 mL/min/1.73m² revealed an eminent confounder for allograft outcome. We therefore analyzed the stratum of patients with unimpaired graft function at time of antibody testing with respect to the impact of HLAab on allograft survival. **Figure 30** illustrates that especially HLA class I, class I+II, DSA class I, and NDSA class I+II were associated with a statistically significant risk for allograft failure despite an excellent graft function at time of testing. Class I DSA were proven to have the most eminent negative impact on late allograft survival with an OR of 7.9 (95% CI = 3.0 to 20.6; $P < 0.001$). Exclusive class II HLAab irrespective of donor specificity did

not reveal any prognostic value for the prediction of allograft outcome. Due to a low number of observations of patients with class I+II DSA the OR could not be determined. However, class I accompanied with class II HLAab or NDSA were associated with poorer graft outcome.

Mean fluorescence intensity (MFI)

The MFI is an indicator for the amount of bound fluorophore-conjugated anti-human IgG detection antibody on the beads. Consequently, MFI is anticipated to be an indirect measure of the amount of HLAab. Although knowing that the MFI does not directly correlate with the antibody titer it was used here as the only available surrogate marker for the level of antibodies. A logistic regression analysis failed to demonstrate a direct correlation between the MFI value of DSA or NDSA and graft failure (data not shown).

A multivariate Cox proportional hazard model was used to elucidate the prognostic power of MFI using arbitrary cutoff values of >1000, >5000 and >10,000 MFI. According to these data summarized in **Table 10** using a MFI cutoff >1000 is the most appropriate way of analyzing the SAB assay. DSA and NDSA above this cutoff already revealed a statistically significant increase in risk for graft failure. The NDSA associated hazard ratio slightly increased using a cutoff >5000 MFI but decreased >10,000 MFI. For class II and class I+II DSA there is a steady increase in the hazard ratio from low to high cutoff values. Applying a cutoff >1000 MFI revealed an appropriate balance between sensitivity, specificity, and positive predictive value. The relatively poor sensitivity of the assay with respect to prediction of graft failure resulted from the fact that only 50% of patients with a failed allograft revealed HLAab at the time of testing. In addition, the cause of allograft loss is multifactorial including immunological but also non-immunological factors. As expected with increasing cutoff values sensitivity decreases in favor of increasing specificity reaching close to 100%.

Taken together the data presented here imply that the MFI value is not the appropriate indicator to distinguish between harmless and harmful NDSA. On the other hand, with increasing MFI of DSA (applies only partly to class I DSA) the relative risk for graft failure increases.

Table 10. Predictive value of DSA and NDSA with respect to MFI for overall graft failure. DSA with a cutoff >5000 MFI and irrespective of HLA class revealed the highest positive predictive value for subsequent graft failure but with low sensitivity. NDSA in general revealed a lower predictive value. Both NDSA and DSA detectable at a cutoff >1000 MFI were associated with an increased risk.

		MFI Cutoff	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Cox Proportional Hazard Model (multivariate*)		
						Hazard Ratio	p	95% CI
NDSA	Class I	>1000	32	84	45	1.9	p<0.001	1.5 - 2.5
		>5000	23	88	43	1.7	p<0.001	1.3 - 2.3
		>10,000	14	92	42	1.6	p=0.01	1.1 - 2.2
	Class II	>1000	20	90	42	1.7	p=0.001	1.2 - 2.4
		>5000	14	94	45	1.9	p=0.001	1.3 - 2.8
		>10,000	8	95	38	1.6	p=0.05	1.0 - 2.5
	Class I+II	>1000	15	93	45	1.9	p=0.001	1.3 - 2.8
		>5000	9	96	49	2.2	p=0.001	1.4 - 3.5
		>10,000	4	97	35	1.7	p=0.2	0.8 - 3.4
DSA	Class I	>1000	13	98	58	3.3	p<0.001	2.2 - 4.9
		>5000	5	99	71	3.3	p<0.001	1.7 - 6.3
		>10,000	1	99	40	2.6	p=0.2	0.6 - 10.7
	Class II	>1000	22	96	67	3.0	p<0.001	2.2 - 4.1
		>5000	12	98	70	3.1	p<0.001	2.1 - 4.7
		>10,000	9	98	69	3.6	p<0.001	2.3 - 5.7
	Class I+II	>1000	4	100	100	7.2	p<0.001	3.5 - 15.0
		>5000	2	100	100	12.0	p<0.001	3.7 - 38.6
		>10,000	1	100	100	48.7	p<0.001	6.0 - 392.8

*Covariates: glomerular filtration rate at time of enrollment and year of transplant
CI = confidence interval; MFI = mean fluorescence intensity; DSA = donor-specific antibodies; NDSA = non-donor-specific antibodies

Complement activation

Antibody binding can initiate the classical complement activation pathway leading to target cell lysis. Complement binding strongly depends on the isotype and subclass of antibody but also on titer.

In order to define whether our established sC1qA (section 3.1.2) can further predict the pathogenicity of IgG HLAab, we took advantage of sera from 788 patients selected from the total cohort solely based on sufficient serum availability. Sera were tested on C1q-fixing HLAab. We anticipated that the C1q assay will facilitate further stratification of IgG antibodies based on their complement binding capability. We first confirmed that the subgroup was representative for the total cohort according to the following parameters:

recipient gender and age, length of follow-up, graft function at time antibody testing, number of retransplants and antigen mismatches as well as the prevalence of HLAab and DSA (see **Table A3** in **Appendix I**). Moreover, graft survival did not differ between both groups with 74% vs. 75% and 49% vs. 51% for HLAab negative and positive patients. It turned out that 10% (79/788) of all patients in the subgroup or 30% (79/263) of HLAab positive patients revealed C1q-fixing antibodies. Surprisingly, in 11 sera C1q-fixing non-IgG antibodies could be detected. This finding suggests the presence of IgM antibodies in the sera. This observation was really unexpected as sera underwent several freezing and thawing cycles as well as heat inactivation for 30 min at 56°C that which is thought to degrade IgM. Nevertheless, these C1q positive but IgG negative patients revealed a 6-year graft survival probability of 90% as compared to 81% for HLAab negative patients and indicate that these antibodies do not contribute to graft dysfunction. Notably, there was no follow-up beyond 6 years available due to the low number of observations.

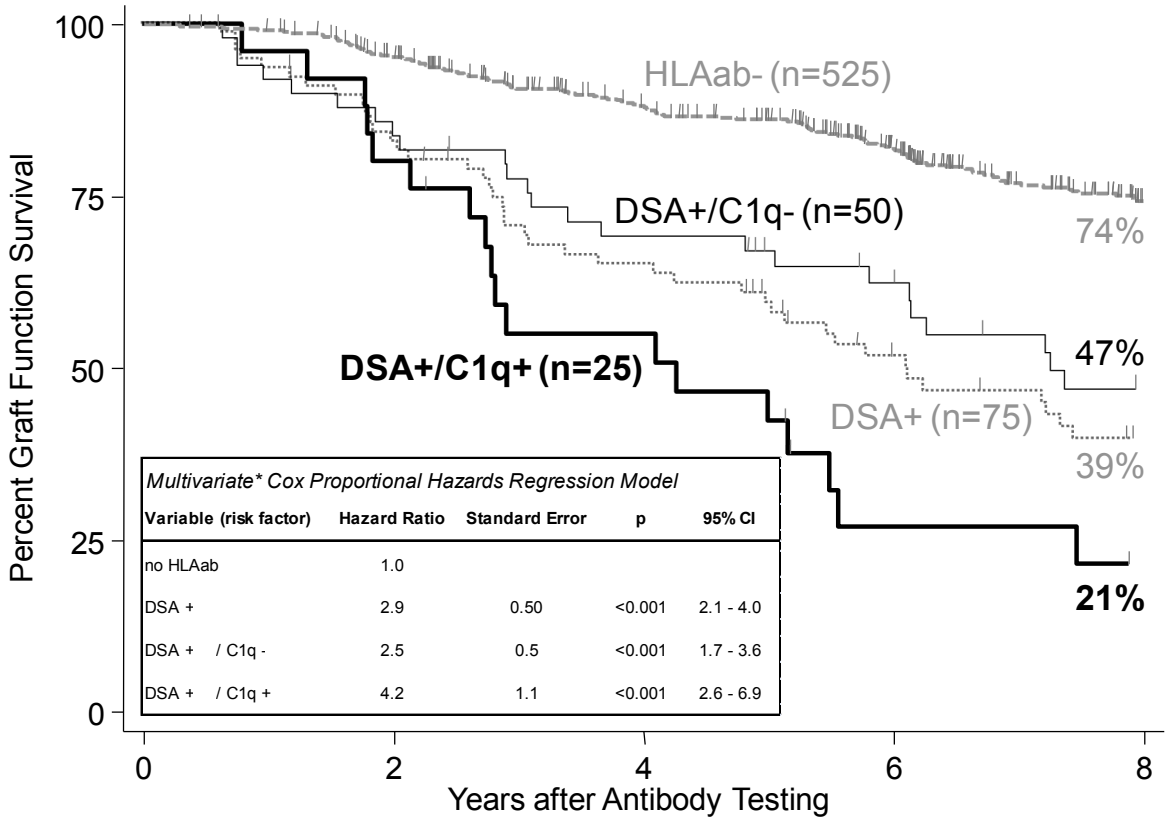


Figure 31 . Allograft function survival of DSA stratified according to their complement C1q-fixing ability. C1q-fixing DSA revealed a stronger adverse effect on allograft function survival as compared to DSA not capable of fixing C1q. The independent effect was confirmed by a multivariate Cox proportional hazard model (box).

*Covariates: year of transplant, graft function at time of antibody testing
"+" = positive; "-" = negative; DSA = donor-specific antibody(ies); HLAab = HLA antibody; CI = confidence interval

However, C1q-fixing IgG HLAab revealed a very strong negative impact on graft survival with a survival probability of 45% as compared to 74% among HLAab negative patients ($P < 0.001$). Taking only HLAab positive patients into consideration there was no statistically significant difference in graft survival between those with or without C1q-fixing antibodies (45% vs. 51%, $P = 0.7$). **Figure 31** illustrates a survival difference of 26 percent points (47% vs. 21%) between DSA without and with C1q-fixing capability. Although not yet statistically significant ($P = 0.06$, Wilcoxon-Breslow-Gehan Test) the difference is clinically important as to define the pathogenicity of DSA.

IgG subclass

As an important feature of antibody effector function the IgG subclass distribution patterns of HLAab were analyzed with respect to graft function survival. Based on the results summarized in **Table 11** for HLA class I and II antibodies there was no statistically significant difference in functional graft survival detectable. In addition, the same analysis considering class I and II DSA revealed analog results (data not shown).

The IgG subclass distribution pattern was not capable of further determine antibody pathogenicity.

Table 11. IgG subclass-specific graft function survival probability. There was no statistically significant difference in graft function survival detectable between cytotoxic (IgG1 and/or IgG3), non-cytotoxic (IgG2 and/or IgG4) or mixed (IgG1/2/3/4) HLA class I and II antibodies. Graft function survival for 525 HLAab negative patients was 74%.

		% Graft Function Survival at 8-year follow-up (count)	
		HLAab class I pos	**HLAab class II pos**
IgG1/3	cytotoxic	52% (105)	54% (76)
IgG2/4	non-cytotoxic	51% (11)	n/a* (1)
IgG1/2/3/4	mixed	45% (98)	48% (66)
Total IgG		49% (214)	51% (143)

*low number of observations; pos = positive; neg = negative

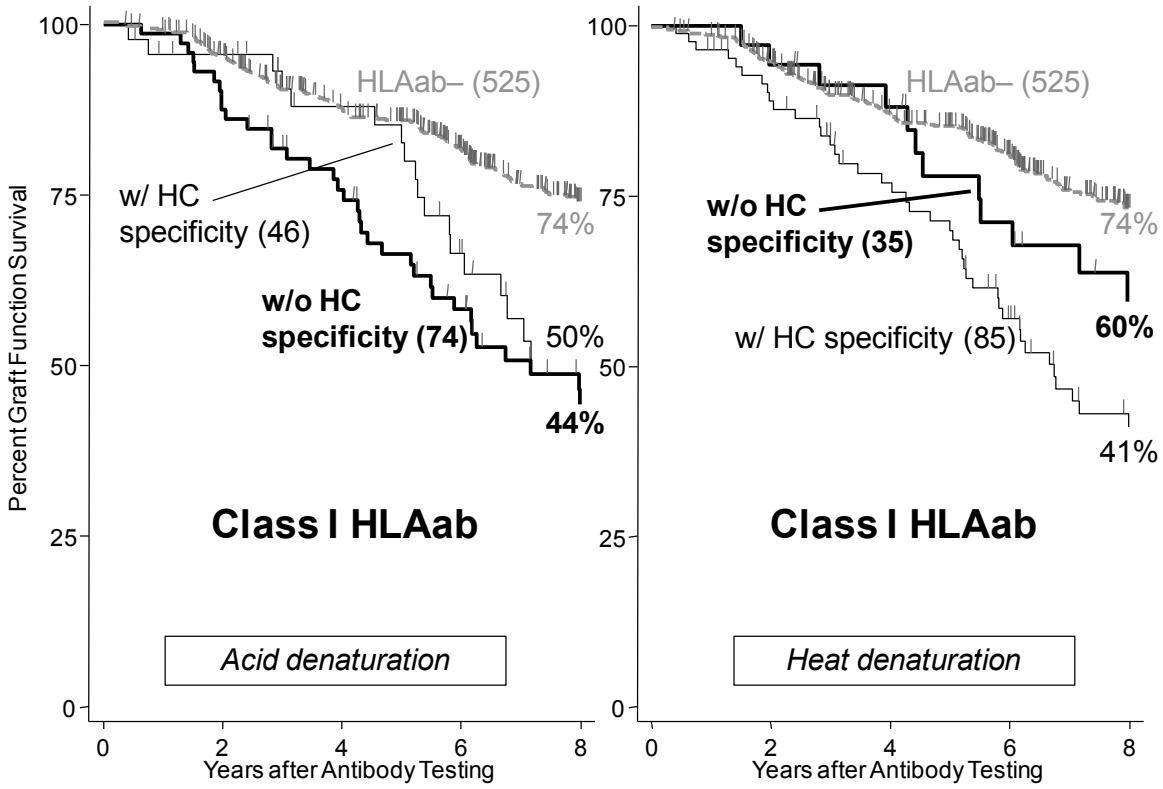


Figure 32. Functional allograft survival of class I HLAab detected by β2m-free HC-coated beads. No statistically significant difference in allograft survival probability between HC-specific and non-HC-specific class I HLAab could be revealed by either testing sera using beads denatured by acid or heat treatment. Class II HLAab positive patients have been excluded from the analysis.

w/ = with; w/o = without; HC = heavy chain; HLAab– = HLAab negative

HLA heavy-chain specificity

It is nowadays well accepted that a certain indefinable proportion of antigen on Luminex® beads is in fact partially denatured missing β2m and/or the peptide. This results in a conformational change of the molecule exposing neoepitopes. The clinical significance of antibodies directed against these denatured antigens is not yet well defined. In a previous study of our group using acid-denatured beads we could demonstrate that class I HLAab with HC-specificity at 4 years of follow-up are clinically irrelevant (110).

On the contrary, in this thesis with a follow-up of 8 years neither acid nor heat denaturation could significantly contribute to the definition of antibody pathogenicity (**Figure 32**). As pointed out already in section 3.3 the concordance in class I HLAab detection between acid- and heat-treated beads was only 54%. Indeed, in 39% of discordant tests additional HC-specific antibodies were detected using heat-denatured beads which opens the discussion on false-positive reaction due to manipulation of detection beads. In fact, reanalysis of data under consideration of only concordant results between both tests basically verified the findings

shown in **Figure 32** (left panel) for acid denaturation with graft survival of 43% and 54% for HLAab without and with HC specificity (data not shown).
The discriminative power of testing class I HLAab in parallel with β2m-free denatured and regular beads is limited due to missing significance with respect to graft outcome and conflicting data depending on the method used to denature the beads. Acid treatment is ineffective to denature class II antigens. Consequently, class II HLAab could be tested only by using heat-denatured beads and revealed no significant difference between antibodies with and without HC specificity (46% vs. 56% graft function survival at 8 years of follow-up).

In summary, graft function survival was especially negatively impacted by complement-fixing DSA followed by non-complement-fixing DSA but also NDSA. Detection of class I DSA revealed the highest prognostic value for subsequent graft failure. On the contrary, the presence of mutually exclusive class II DSA among patients with a good renal allograft function (>60 mL/min/1.73m^2) was not associated with an increased relative risk for graft failure. Neither the determination of the IgG subclass distribution patterns nor the exclusion of HC-specific antibodies contributed significantly to the risk assessment of HLAab with respect to their impact on graft function survival.

3.6.3 Longitudinal Follow-up

So far, the experimental design of this study allowed us to elucidate the impact of HLAab on renal allograft survival based on one single antibody testing. However, to address the clinical significance of *de novo*, persistent and transient HLAab it is important to have information over time. We therefore decided to partially extend the study design by adding more antibody tests. We aimed to define whether regularly performed antibody testing posttransplant can provide additional information to predict allograft outcome.

In extending the cross-sectional study to a longitudinal design we were able to recruit a median of three serum samples (IQR = 3 to 4) for almost half of the individuals (531/1075) from the total cohort. This fraction was representative for the total cohort with respect to the following variables: recipient gender, graft function at the time of first antibody testing, number of retransplants and antigen mismatches as well as the prevalence of HLAab and DSA (**Table A5** in **Appendix I**). However, there was a slight difference in the demographics with respect to recipient age at time of transplant. Patients enrolled in the longitudinal follow-up group were younger with a median age of 41.9 years (IQR = 34 to 50 years) compared to

all patients (median = 44.1 years; IQR = 35 to 54 years). Serum samples were collected on average 970 days (IQR = 890 to 2221 days) after the initial and with an average interval of 258 days (IQR = 90 to 462 days) afterwards for subsequent samples. 60/531 patients (11%) developed HLAab after initial antibody testing which corresponds to 16% of the 371 patients without HLAab at time of initial antibody testing with a graft function survival of 81%.

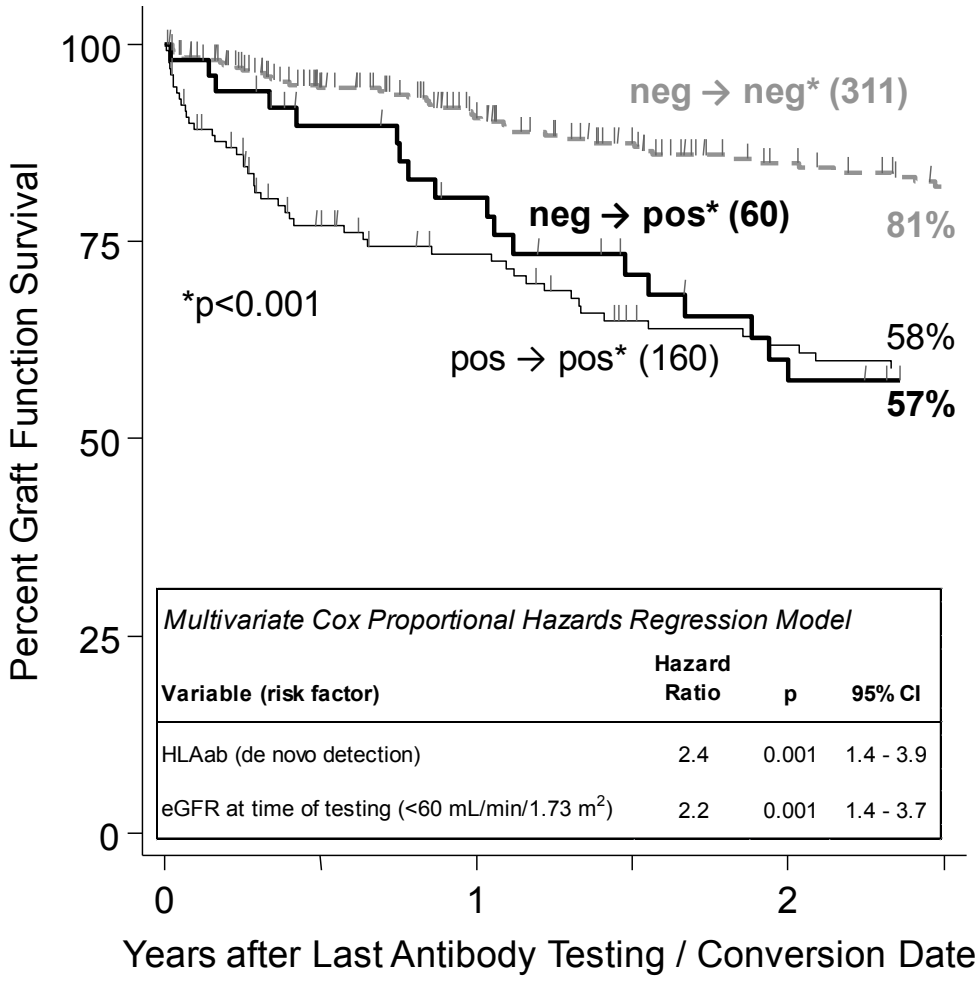


Figure 33. Adverse impact of *de novo* HLAab detection on allograft function survival of 531 patients with longitudinal follow-up. Graft survival probability plotted from the time of seroconversion (neg→pos) and last HLAab testing (neg→neg, pos→pos), respectively. *De novo* HLAab were associated with a 2.4-fold increased risk of allograft failure within 2.5years after detection.

HLAab = HLA antibody; neg = HLAab negative; pos = HLAab positive; CI = confidence Interval; eGFR = estimated glomerular filtration rate

Patients were classified into 4 groups according to the presence or absence of antibodies at different time points of antibody testing: (A) neg → neg, (B) pos → pos etc. Of special interest were patients with seroconversion (C): neg → pos or (D) pos → neg. When analyzing those patients who become HLAab positive during follow-up (neg → pos) compared to those

who never produced HLAab (neg → neg) a statistically significant difference in graft survival was observed with 65% vs. 85% ($P < 0.001$). Worse graft survival was only revealed by patients with persistent HLAab (pos → pos, 55%, $P < 0.001$) (data not shown). Consequently, as shown in **Figure 33** from the day of conversion (neg → pos) graft survival is reduced to 57% after 2.5 years whereas patients without HLAab have a graft survival probability of 81% ($P < 0.001$). HLAab conversion was associated with a 2.4-fold increased relative risk of graft. It was noted that in 24 of 60 (40%) patients seroconversion was accompanied by a simultaneous rise in SCr level (i.e., +25% above the individual baseline). Of those, 79% (19/24) experienced subsequent graft loss vs. 19% (7/36) among patients without an increase ($P < 0.001$).

Since serum samples were collected and tested with an initial interval of 970 days and 258 days interval thereafter the time point of *de novo* HLAab production could be narrowed down. Antibodies were detected *de novo* on average 1947 days (IQR = 1034 to 2625 days) after first antibody testing. In detail, **Figure 34** illustrates the observed dynamics of HLAab production with annual increases ranging from 0 to 28 percentage points. The incidence of *de novo* HLA class I, class II and class I+II antibodies in a total of 60 samples was 40% (n = 24), 43% (n = 26), and 16% (n = 10). Graft function survival did not differ significantly according to a Kaplan-Meier survival analysis. Similarly, patients with *de novo* HLAab lost their graft on average 386 days (IQR = 272 to 688 days) after conversion, but patients with persistent and without HLAab failed within 313 days (IQR = 72 to 922 days) and 462 days (IQR = 180 to 1008 days) after last antibody testing. None of these differences reached statistical significance.

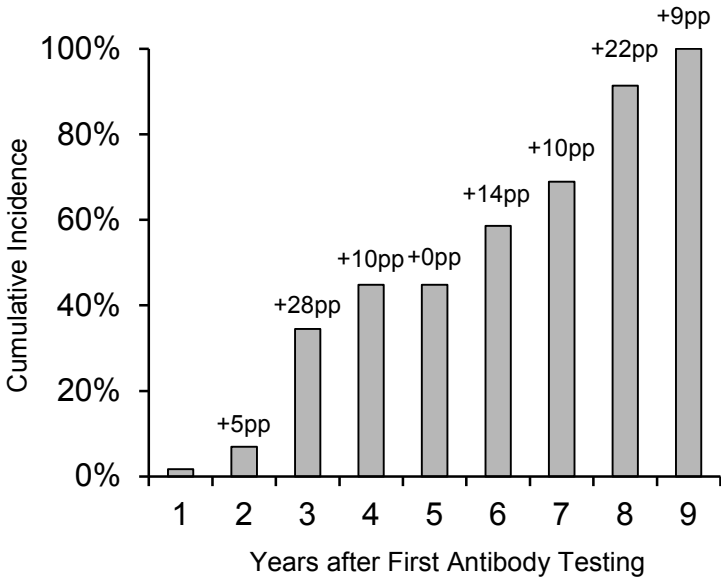


Figure 34. Cumulative incidence of patients with *de novo* HLAab during follow-up. In the subgroup of 531 patients enrolled for longitudinal follow-up a total of 60 (11%) developed HLAab at various time points after first antibody testing. The annual increment ranged from 0 to 28 percentage points (pp).

The longitudinal follow-up provided more insight into the dynamics of HLAab production. Thus, HLAab can appear *de novo* at any time point posttransplant and are associated with an increased risk for subsequent graft loss. Especially, when the newly developed antibodies are accompanied with an increase in SCr level the prognosis is poor. Posttransplant HLAab monitoring at least once per year using highly sensitive and specific Luminex®-based SPA is essential to identify patients at risk for allograft failure.

3.7 The Etiology of Allograft Failure

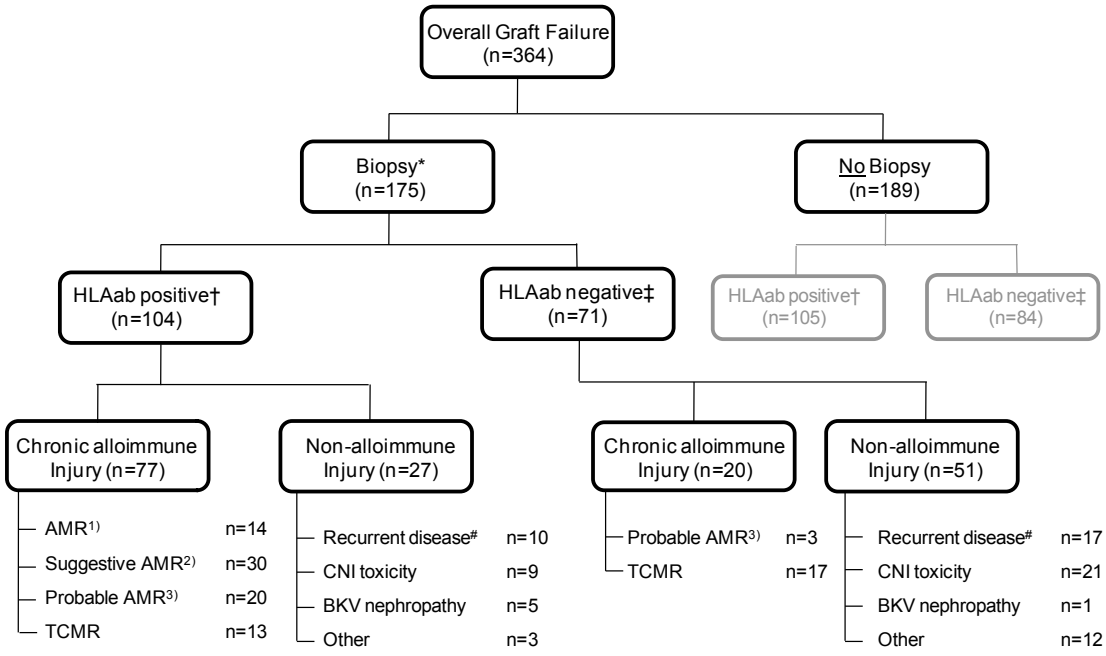


Figure 35. Classified causes of allograft failure stratified according to the HLAab status. Indication biopsies of 175 patients with and without HLAab were analyzed with respect to the etiology of graft loss and categorized into alloimmune-mediated and non-alloimmune causation.

*median -77 days (IQR = -259 to 6 days)
†HLAab positive at any time point during follow-up
‡HLAab negative at all time points tested during follow-up
[1]fulfilled 2/4 histological features of chronic AMR: (a) glomerular double contours, (b) PTC basement membrane multilayering, (c) IF/TA and/or (d) fibrous intimal thickening in arteries plus diffuse C4d staining of peritubular capillaries plus DSA
[2]transplant glomerulopathy (TG) (cg>0) and C4d deposits and NDSA or TG and DSA without C4d
[3]TG and NDSA without C4d deposits or TG with C4d deposits but without HLAab
#recurrent or *de novo* renal diseases (malignancies, IgA nephropathy, FSGS, glomerulonephritis)
HLAab = HLA antibody; AMR = antibody-mediated rejection; TCMR = T-cell-mediated rejection; CNI = calcineurin inhibitor; BKV = human polyomavirus 1

The etiology of late renal allograft dysfunction is always described to be multifactorial including immunologic (antigen-dependent) and non-immunologic (antigen-independent) factors, e.g. cellular and humoral immunity vs. drug toxicity, diabetes and hypertension. In

the following the emphasis is placed on biopsy and morphological assessment of the failed graft to provide more evidence for a causal relationship between HLAab and late allograft failure. We sought to identify whether our test results correlate with those from biopsies. A positive correlation could in the future allow a non-invasive diagnostic approach and thus show the power of our assay system.

Of the 364 patients with allograft failure during follow-up indication biopsies were performed for 175 (48%) on average on day 77 before loss of graft function. Only biopsies taken in close timely proximity to graft failure were considered, and classified according to the current Banff criteria (113) including C4d-negative (suggestive) and DSA-negative (probable) AMR (pAMR) according to Sellarés et al. (75). The category of suggestive AMR (sAMR) also included HLAab-negative but C4d-positive biopsies with histological features of chronic AMR. The primary bioptic diagnoses of failed allografts were any form of AMR (n = 67, 38%), T-cell-mediated rejection (TCMR) (n = 30, 17%), CNI toxicity (n = 30, 17%), and recurrent/*de novo* renal diseases (n = 27, 15%). Other histological abnormalities without known etiology (summarized as other) (n = 15, 9%) and BKV nephropathy due to polyoma virus infections (n = 6, 3%) were rarely diagnosed.

As illustrated in **Figure 35** the group of patients with biopsy data did not differ significantly from the group of patients without indication biopsies stratified according to the HLAab status ($P = 0.5$). Based on the histological findings the cause of graft loss among HLAab positive patients (persistent or *de novo*) was dominated by chronic alloimmune injuries with 74% (77/104) vs. 28% (20/71) among HLAab negative patients ($P < 0.001$). In detail, AMR-associated histological lesions were the leading cause of graft failure when HLAab were detected (62%, 64/104) where sAMR, pAMR and true AMR individually account for 29% (30/104), 19% (20/104) and 13% (14/104), respectively. It is worth mentioning that in most cases AMR, sAMR and pAMR were accompanied by histological signs of TCMR (i.e., interstitial infiltration, arteritis and/or tubulitis). When DSA and NDSA were detected, 92% and 67% of biopsies revealed chronic alloimmune injuries, respectively. Only in 10 out of 36 (28%) patients with DSA the Banff criteria for true AMR were fulfilled. More common were histological findings without either C4d deposits or DSA.

CNI toxicity, recurrent/*de novo* renal diseases and TCMR were most common for HLAab negative patients with incidences of 30% (21/71), 24% (17/71) and 24% (17/71), respectively. In three patients (4%) without HLAab biopsy data revealed pAMR. All three patients were tested only once for the presence of HLAab during follow-up and antibody production after initial testing cannot be ruled out as possible explanation. Likewise, among 61 patients with

graft failure and repeatedly tested negative for HLAab 33 (54%) of them had an indication biopsy without any AMR-specific histological lesions. Main cause of allograft dysfunction here were CNI toxicity (36%, n = 12), recurrent/*de novo* renal diseases (24%, n = 8) and others with unknown etiology (21%, n = 7). On the other hand, the cumulative incidence of AMR (including sAMR and pAMR) among patients with conversion from HLAab negative to positive during follow-up and indication biopsy was 44% (7/16) ($P < 0.001$).

Taken together, the biopsy data suggest to some extent a causal relationship between the presence of HLAab especially DSA and chronic alloimmune-associated injuries leading to allograft dysfunction. HLAab and DSA detection posttransplant could be shown to correlate well with allograft loss due to alloimmune injuries and therefore could be used as non-invasive means to predict late allograft failure.

4 Discussion

Without a doubt, solid organ TX is a history of resounding success (119). However, the perpetual enigma of late renal allograft dysfunction is still unsolved. In this thesis we addressed this issue from a diagnostical point of view and could clearly demonstrate that (i) HLAab as detected by Luminex® SAB posttransplant are in fact valuable biomarkers to predict long-term outcome (**Figure 29**). Consequently, we established an effective posttransplant HLAab monitoring scheme currently in place at our institution which will be presented in one of the following sections. (ii) We successfully modified the standard Luminex® assay to facilitate the discrimination between complement-fixing and non-complement-fixing HLAab. Thereby, we could further improve the prediction of the antibodies' pathogenicity much better than using the standard approach of MFI (**Figure 31**). (iii) Epitope analyses and longitudinal follow-up of patients helped gaining more insight into the natural history of the humoral alloimmune response after TX. HLAab can appear *de novo* at any time point posttransplant (**Figure 34**) and are directed against immunogenic HLA epitopes resulting in a broad reactivity against multiple antigens with shared epitopes.
These results will be further discussed in the context of the humoral theory of TX which suggests a causal relationship between HLAab and graft dysfunction. Finally, we take the chance on perspectives of HLAab monitoring by SPA in the future.

4.1 Compliance with the Humoral Theory of Transplantation

In the 1950s, with his exceptionally outstanding work on immune tolerance the Nobel-prize winner Sir Peter Medawar established the dogma of cellular immunity and allograft rejection. Ever since then the humoral theory has fallen into oblivion. Terasaki and colleagues then demonstrated that preformed cytotoxic DSA can immediately deteriorate the transplant within minutes after reperfusion (i.e., HAR) and introduced the pretransplant CDC crossmatch which is still the decisive test for kidney TX (33). Despite this early recognition of the power of HLAab the focus was still on the cellular immune response indicated by the IS treatment posttransplant which successfully suppresses cellular immunity leaving humoral reactivity unrecognized (illustrated in **Figure 36**). Without a doubt, the introduction of powerful IS reagents such as CNI and the anti-metabolite azathioprine significantly improved transplant outcome (120). It is also undisputable that humoral immunity is dependent on costimulation by T helper cells; however, the missing second defensive line targeting humoral immunity

could make the overall IS regime ineffective. In fact, in an elegant study by Sellarés et al. it was shown that the risk for the development of AMR significantly increases over time (75).

Late with the advent of the modern SPA in the 1990s especially driven by the Luminex® technology which enabled the detection of HLAab with an unparalleled sensitivity shifted the focus towards the humoral arm of the immune system. HLAab could be associated with rejections which led Paul Terasaki to revive the humoral theory of TX. As outlined in his publication (121) the evidences are quite strong that HLAab: (i) cause HAR, (ii) lead to C4d deposits associated with early kidney graft failures, (iii) are an appropriate indicator of presensitization leading to early acute rejections, (iv) were present in 96% of patients who rejected a kidney graft, and (v) are associated with chronic rejection in 33 studies of kidney, heart, lung and liver grafts.

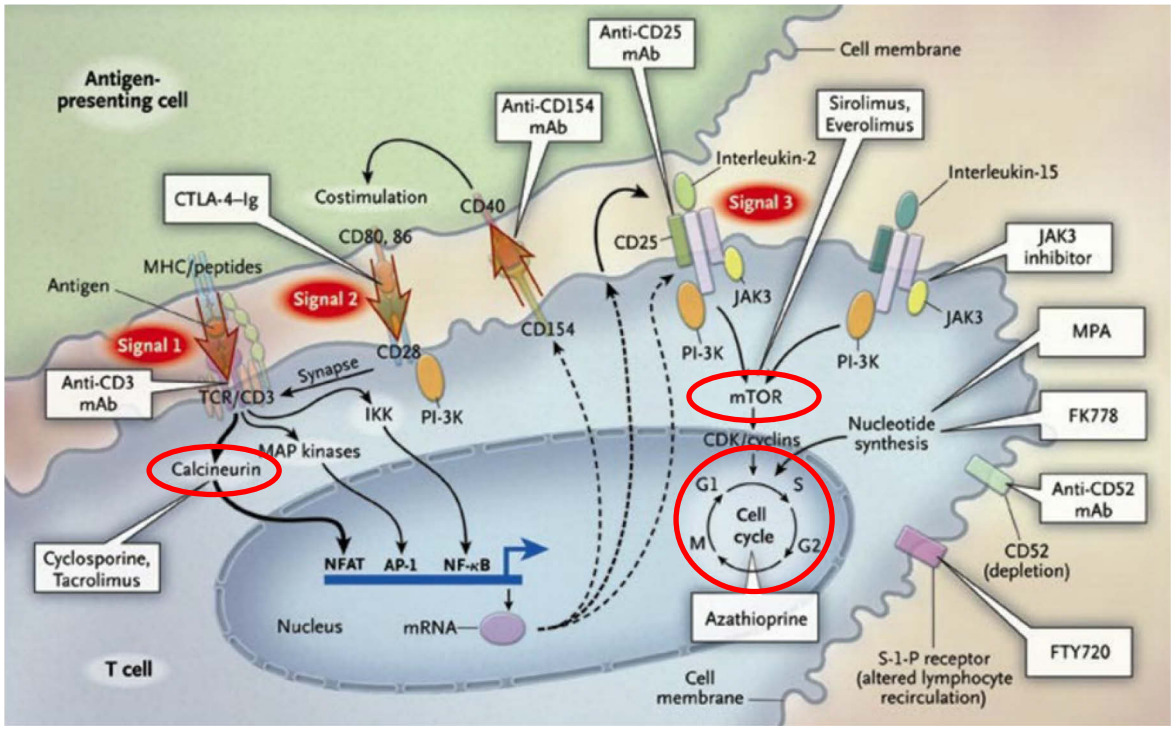


Figure 36. Current IS drugs and their site of action on T cell signaling. Current IS medication involves CSA, tacrolimus, mammalian target-of-rapamycin (mTOR) inhibitor, azathioprine, MPA as well as some depleting and non-depleting therapeutic antibodies. Targets of the most frequently applied IS reagents are calcineurin, mTOR and the cell cycle (highlighted by red frames). Picture was taken from (122) and adapted.

This thesis is intended to provide further evidence for a causal relationship between HLAab and late allograft dysfunction in support to the humoral theory of TX. We designed a cross-sectional study in which 1075 renal transplant recipients were enrolled and screened for HLA and MICAab once posttransplant. Graft function was followed for a median of 8 years from

the time of antibody testing. Determination of donor specificity was achieved by using Luminex® SAB. In addition, several novel methods were established and applied to further improve the predictive value of HLAab by determining (a) the specificity with respect to epitopes and denatured antigens as well as (b) the IgG subclass and C1q-binding capability of the antibodies as publications demonstrated in contradiction to our hypothesis that DSA do not inevitably cause graft injury (123, 124).

Defining the bad and the ugly - DSA

The first key finding of this thesis is that 718 patients without antibodies had an 8-year graft survival probability of 75% in comparison to 40% survival for the 113 patients who revealed DSA, and additionally reduced to 21% when DSA were capable of fixing C1q.

These results share a number of similarities with the publications of Piazza et al. (125) and Cardarelli et al. (126), in which HLAab and especially DSA posttransplant have been demonstrated to exhibit an eminent risk for long-term renal allograft survival. Compared to this thesis both published studies comprised a much smaller cohort with 120 and 251 patients, and DSA were determined by different techniques: FC crossmatch (FCXM) and ELISA. FCXM requires vital donor T and B lymphocytes and suffers from the same drawbacks as other cell-based methods including false-positive reactions due to non-HLA antibodies as reviewed by Bray et al. (32). ELISA on the other hand detects exclusively HLAab but is known to be less sensitive than Luminex® (127).

Other related studies published since 2001 are summarized in **Table 12.** They differ in their design, cohort size, endpoint and antibody detection assay. However, except for the publication by Bartel et al. (discussed later in this section) all authors *in unison* reported significantly impaired long-term allograft survival or increased incidences of late AHR, C4d positive PTC and TG in the presence of HLAab. The largest study with more than 4000 kidney transplants by Ozawa et al. (128) is the only multi-center study included in this literature review. Despite the diversity of detection assays used by each of the participating 45 transplant centers worldwide, the association between HLAab and detrimental graft outcome could be elucidated. However, the study suffers from the limitation that donor specificity of HLAab was not determined.

However, our comprehensive analysis using SAB as the most sensitive HLAab detection assay qualitatively confirmed results of previous studies that DSA are detrimental to long-term allograft outcome.

Table 12. Studies published between 2001 and 2012 on the clinical relevance of HLAab posttransplant. All but one publication by Bartel et al. *in unison* reported significant differences in long-term graft survival (GS), eGFR, incidence of acute (humoral) rejections (AR, AHR) and histological signs of graft injury (C4d, TG) between HLAab positive and negative patients. The publication by Ozawa et al. is the only multi-center study included.

First author	Year of publication	Study design	Cohort size, count	HLAab positive, count	Outcome			HLAab detection method
					Endpoint	HLAab positive	HLAab negative	
Piazza[1)]	2001	PL	120	29 dnDSA	2-year GS	66%	99%	FCXM & FlowPRA
Lee[2)]	2002	RL	139	59	8-year GS	51%	80%	ELISA
Cardarelli[3)]	2005	PC	251	28	AHR	31%	2%	ELISA
Zhang[4)]	2005	PL	49	30	AHR	27%	0%	ELISA
Campos[5)]	2006	PC	512	91	1-year GS	88%	95%	ELISA
Mao[6)]	2007	RL	54	32 dnHLA	5-year GS	47%	86%	Luminex SAB
Ozawa[7)]	2007	PC	4144	948	3-year GS	90%	95%	ELISA / FlowPRA / Luminex
Panigrahi[8)]	2007	RC	185	29	AR	55%	17%	ELISA
					2-year GS	24%	87%	
Bartel[9)]	2008	RC	34	9 dnHLA	5-year eGFR	no significant difference		FlowPRA & Luminex SAB
Viana[10)]	2009	PC	132	33	C4d+ve PTC	39%	8%	FlowPRA
Lachmann[11)]	2009	PC	1014	302 HLA	5-year GS	63%	83%	Luminex SAB
				93 DSA	5-year GS	49%	83%	
Sánchez-Fructuoso[12)]	2010	RC	267	7 dnDSA	AR	83%	23%	Luminex SAB
Eng[13)]	2011	RC	71	29 dnDSA	TG	72%	41%	Luminex SAB
Lee[14)]	2012	R	216	44	5-year GS	59%	99%	ELISA
					10-year GS	44%	91%	
					15-year GS	30%	65%	
Wiebe[15)]	2012	RL	315	47 dnDSA	10-year GS	57%	96%	FlowPRA & Luminex SAB

RL = retrospective longitudinal; PL = prospective longitudinal; PC = prospective cross-sectional; RC = retrospective cross-sectional; R = retrospective; dnHLA = *de novo* HLA antibodies; dnDSA = *de novo* donor-specific HLA antibodies; GS = graft survival; C4d+ve PTC = C4d positive peritubular capillaries; eGFR = estimated glomerular filtration rate; AR = acute rejection; AHR = acute humoral rejection; TG = transplant glomerulopathy; SAB = single antigen bead assay

[1)] Transplantation. 71(8):1106. [2)] Transplantation. 74(8):1192. [3)] Transpl Int. 18(5):532.
[4)] Transplantation. 79(5):591. [5)] Am J Transplant. 6(10):2316. [6)] Am J Transplant. 7(4):864.
[7)] Clin Transpl. 2007:255. [8)] Hum Immunol. 68(5):362. [9)] Am J Transplant. 8(12):2652.
[10)] Transplant Proc. 41(3):859. [11)] Transplantation. 87(10):1505. [12)] Transplant Proc. 42(8):2874.
[13)] Hum Immunol. 72(5):386. [14)] Transplant Proc. 44(1):264. [15)] Am J Transplant. 12(5):1157.

As mentioned earlier, Bartel et al. published controversial data (124). In their study the authors could associate *de novo* DSA with an excellent 1-year clinical renal allograft outcome and devoid of a decline in graft function during 6 years of follow-up. It is worth mentioning that among the described 9 patients with HLAab detected by FlowPRA pooled antigen beads only 5 could be confirmed by SAB to have DSA whereas only 3 showed a positive donor crossmatch by FCXM and can be defined as true DSA (2 for class I and 1 for class I + II). The relatively low number of observations and the intentional bias by selection of stable kidney transplant recipients make the generalization to the overall transplant cohort inappropriate. It is known also from our data that there is a proportion of 21% of patients with persistent DSA at 8 years after detection without impaired allograft function (**Figure 31**).

A possible mechanism often discussed in this context is transplant accommodation which describes a state of acquired resistance to antibody-mediated injury (129). Interestingly,

especially HLA class I antibodies are discussed to promote accommodation by ligation of MHC class I molecules and initiation of the survival pathway (130). Some highly sensitized recipients subjected to pretransplant desensitization were found to maintain stable graft function despite postoperative recurrence of DSA (131).

4.2 The Prediction of Antibody Pathogenicity

Accurate assessment of antibody pathogenicity still remains a major challenge in clinical TX. Pathogenicity is determined on one hand by the specificity and on the other hand by the effector function of immunoglobulins.

Complement-fixing ability

We could demonstrate in this thesis that in fact the ability to activate complement is the critical antibody effector function. By modifications to the standard Luminex® SAB, we were able to reveal a subset of DSA, which fix complement C1q and exhibit the worst long-term allograft survival probability (**Figure 31**). To date two divergent SPA to discriminate between complement-binding and non-complement-binding HLAab have already been described in the literature.

i) C4d assay

In 2005 Wahrmann et al. (101) published the first cell-independent, in vitro assay to detect complement-binding HLAab. The authors made use of HLA-coated FlowPRA microbeads on which they detected alloantibody-triggered C4 complement split product C4d depositions using a flow cytometer. In their most recent study Wahrmann et al. could demonstrate a trend towards adverse 5-year graft survival of pretransplant C4d-positive DSA among 338 renal allograft recipients (132).

A similar method published by Smith et al. (133) detects C4d-fixing HLAab on Luminex® SAB. Among 565 cardiac transplant recipients this method was able to demonstrate a strong association between the detection of pretransplant C4d-fixing DSA and poor allograft outcome (134). However, from our own experiences and personal communications with other experts it turned out that the detection of C4d on microbeads (FlowPRA or Luminex®) suffers from a relatively moderate sensitivity as MFI values barely exceed 2000 units in cases whereas total IgG antibodies in the same serum are detected with 10,000 – 20,000 MFI. Human C4d, a split product of the activated complement factor C4, is typically initiated by binding of antibodies to an antigen. In vivo, C4d binds covalently to endothelial cell surfaces and extracellular matrix components of vascular basement membranes near the sites of C4

activation (135). Thus, the appropriate binding sites for C4d are sparsely present on latex microbeads. In addition, in our hands the performance of the C4d Luminex® assay was critically dependent on the source of human complement reflecting individual differences in the concentration of essential complement components but also soluble complement inhibitors (dissertation by Kremena Todorova, Charité).

ii) C1q assay

The detection of C1q on Luminex® SAB, first demonstrated by Chin et al. (85), revealed to be the superior alternate assay to detect complement-fixing antibodies in vitro. The principle of the test was adapted to an in-house C1q assay (manuscript accepted for publication) which was then used in this thesis. The assay makes use of purified C1q instead of a complex human complement preparation eliminating potential inhibitory effects by serum proteins. In addition, the detection of antibody bound C1q was accomplished by a sandwich design whereby the signal of the anti-C1q detection antibody is amplified using a fluorophore-conjugated reporter antibody. The C1q assay combines the sensitivity and specificity of the Luminex® technique with the ability to detect the complement-binding potential of antibodies all in one single assay.

C1q is the initial component of the classical complement activation pathway. Formation of the antibody-antigen complex leads to conformational changes of the antibody constant domain exposing the C1q binding site. The four IgG subclasses exhibit differing abilities to fix complement (136). The C1q binding site for human IgG1 e.g. comprises the AA Asp-Lys-Pro-Pro at position 270, 322, 329 and 331 of the constant heavy chain 2 (CH2) domain (102). In addition, the multivalent C1q molecule must cross link at least two IgG molecules before it is firmly fixed. This illustrates that the determinants of the complement activation ability of IgG alloantibodies are complex. In line with previous findings, this study could confirm that the IgG subclass of alloantibodies other than monoclonal antibodies only moderately correlated with the observed C1q-fixing ability (137). Sera with a mixed composition of IgG subclass antibodies including IgG1 and/or IgG3 (regarded as strong complement-binding) together with IgG2 and/or IgG4 (non-complement-binding) revealed more likely a positive C1q assay result than IgG1 and IgG3 alone. Synergistic effects between non-complement-fixing and complement-fixing antibodies are one possible explanation for this observation. Indeed as expected, exclusively non-complement binding IgG2 and IgG4 antibodies revealed virtually no C1q-fixing ability. In their elegant study Bartel et al. found that the concentration of antibodies was a crucial variable which determines the complement-fixing ability of antibodies. Especially IgG1 rather than IgG3 alloantibodies were demonstrated to be more

dependent on a high antibody concentration in the serum to elicit complement fixation. To further complicate the discussion, it is less known that also the glycosylation pattern of the constant domain of IgG molecules, which varies with age, gender and disease status, directly influences C1q binding. Briefly, structural studies indicate that the presence or absence of specific terminal sugars may affect hydrophilic and hydrophobic interactions between sugar residues and AA residues in the constant domain of immunoglobulins, which in turn impact antibody effector functions (138).

To date, the only available data on the differential impact of C1q-binding and non-C1q-binding HLAab on renal transplants was recently published by Otten et al. (139). The outcome of 837 renal allograft recipients transplanted between 1990 and 2008 was analyzed in correlation with pretransplant DSA. By using the commercially available C1q-Luminex assay the prevalence of C1q-binding DSA was fairly low with only 10% among the IgG-DSA which hampered the analysis of the clinical significance. However, 10-year allograft survival was negatively impacted by the C1q-binding capability of pretransplant DSA (35% vs. 60%) which did not reach statistical significance due to the low number of cases but strongly indicates the detrimental effect of complement activation on allograft outcome. As shown in section 3.1.2 the sensitivity of the commercially available C1q assay is lower as compared to our in-house assay. It is thereby a matter of debate whether Otten et al. could have missed some weak but clinical relevant C1q-binding DSA in their study. In addition, posttransplant HLAab were not analyzed as this was beyond the scope of the study but is essential to understand the contribution of complement-activating HLAab on renal allograft survival.
Otten's and our results strongly indicate an enhanced clinical relevance of C1q-binding DSA over IgG-DSA pre- and posttransplant. However, further studies are mandatory to confirm these findings and allow more comprehensive risk stratification for renal transplants in the future.

Impact of non-donor-specific HLA antibodies

Complement fixation can only have an effect on the transplant if the antibody binds to donor-derived alloantigens. However, in agreement with Hourmant et al. (140) this thesis could confirm that NDSA also were somewhat predictive for long-term survival (**Figure 29**). A reasonable explanation for the observed significance of NDSA is that DSA were adsorbed by the allograft, while excess NDSA circulated in the blood stream. Results of recent studies support this theory. DSA could be detected in eluates from rejected transplants (141), needle core biopsy specimens (142), and in sera after Nx (143) in frequencies ranging from 70% to

97%. Adeyi et al. (143) reported that only in 11% of cases these DSA could be detected before Nx compared to 97% after removal of the failed graft, again suggesting that adsorbed DSA damaged the allograft. Another possible explanation is the local production of DSA by plasma cell infiltrates (144).

Three important findings within this thesis and outlined in our publications (90, 145) support this notion: First, only 3/10 nephrectomized patients had DSA before but all of them after Nx. Second, with progressive deterioration of the graft the proportion of patients with DSA to those with NDSA increased from 1:3 to 1:1. This means that DSA appear more frequently in the periphery compared to NDSA when a substantial nephron and antigen loss already occurred. Third, accompanied with decreasing graft function the adverse impact of NDSA and DSA on graft survival was leveled. In addition Hourmant et al. could show in their study that NDSA tend to be produced earlier (1-5 years) than DSA (5-10 years) after TX.

Taken together, all these findings suggest the following stages of late AMR with respect to alloantibody detection:

(i) ***De novo*** detectable **NDSA** in serum and adsorption of DSA by the graft,
(ii) **Progressive graft deterioration** and antigen loss accompanied by an increased level of alloantibodies in the serum,
(iii) **NDSA and DSA** detectable in serum, and finally
(iv) **Allograft dysfunction**.

Early identification of AMR holds promise for improving the prognosis of allograft survival. Thus, it is of great clinical importance to identify as early as possible NDSA which might be indicative for DSA currently undetectable in the serum (see (i) in stages of late AMR). We analyzed the epitope specificity of NDSA with respect to mismatched donor epitopes. Thus, NDSA with DSE specificity (DSEab) were regarded as a surrogate marker of adsorbed DSA and considered potentially harmful. The results (**Table A4** in **Appendix I**) were somewhat disappointing since DSEab could not be demonstrated to exhibit an increased risk of graft failure compared to non-DSEab. The most likely reason for this prima facie rather contradictory result might be the fact that DSE analysis was performed on 52 first transplant recipients with exclusively *de novo* class I NDSA. The *de novo* production of NDSA already suggests the initiation on an alloimmune response among these patients. In total 87% of NDSA were defined as DSEab. Thus, it is not surprising that graft survival of this selective group per se was decreased compared to the total cohort of NDSA positive patients (42% vs. 55%). The reevaluation of the concept of DSEab as a surrogate marker for DSA is in

preparation using an independent cohort of renal transplant patients with NDSA who received a regraft.

4.3 Clinical Relevance of non-HLA Antibodies

Recent studies proposed that MICAab have an adverse effect on renal graft survival (25, 146). Therein, MICAab have been proven to be associated with AR episodes and independently correlated with decreased graft function. On the contrary, this present study does not support these findings as exclusively detectable MICAab did not adversely affect graft survival in our cohort (**Table 11**). Our findings are rather in line with the publication by Lemy et al. (147) on 10-year graft survival of 425 patients with and without MICAab pretransplant. Contradictory to Lemy's and our study, both publications in favor of clinical significance of MICAab used an in-house MICAab detection assay developed by the co-author Peter Stastny (University of Texas, Dallas, TX). This method makes use of recombinant MICA antigens expressed by insect cells and coated to Luminex® beads (148). The panel of antigens did not differ between the in-house and commercially available Luminex® MICAab detection assays. All three methods identified antibodies against the most common MICA alleles. However, as stated by Cox et al. there was substantial but not further detailed disconcordance between the results of the in-house and commercial assays. Furthermore, the restricted use of the commercial MICAab screening assay in this thesis as well as missing donor and recipient MICA typing to distinguish between allo- and autoantibodies could account for the discrepancies with the findings by Cox and Zou. Nevertheless, we were able to show that MICAab could potentiate the detrimental effect of HLAab among patients with concomitant detection of both types of antibodies. As MICA is expressed on endothelial cells but not lymphocytes this may indicate the vascular endothelium as the primary target of the antibody-mediated alloimmune response. Thus, *de novo* MICAab posttransplant accompanied by HLAab could potentially be used as another surrogate marker for vascular rejection. However, this hypothesis needs further confirmation by additional studies which will have to incorporate a consensus method and MICA donor and recipient typing to identify allo- and autoreactive MICAab.

In addition, other tissue specific non-HLA antigens have been identified as major targets of the humoral immune response in TX (149) including non-polymorphic autologous antigens such as vimentin, cardiac myosin, collagen V, AT_1R, heat shock proteins and glutathione-S-transferase T1. The diagnostic possibilities of these non-HLA antibodies are still limited as there are only a few commercial ELISA kits available for the detection of autoantibodies.

However, only vimentin and AT_1R have been suggested to be implicated in renal allograft rejection so far.

Briefly, vimentin is a non-polymorphic intermediate filament expressed by endothelial and vascular smooth muscle cells as well as coronary arteries. The *de novo* tubular expression of vimentin was demonstrated to be associated with renal allograft rejection (150). Moreover, vimentin-specific antibodies were correlated with the development of CAN in a rat transplant model (151).

AT_1R is the main receptor for angiotensin II in the kidney and regulates arterial blood pressure and salt balance. In the initial report by Dragun et al. a functional bioassay using cardiomyocytes that express AT_1R was shown to detect antibodies (21). To date, a commercial ELISA (CellTrend GmbH, Luckenwalde, Germany) has been developed which employs extracts from Chinese hamster ovary cells overexpressing the human AT_1R. Special care is taken to maintain the conformational epitopes of the receptor. The ELISA has been reported to reveal results equivalent to the initial cardiomyocyte assay (152). Further studies suggesting the clinical relevance of AT_1R-specific antibodies in renal TX are about to be published by our group (manuscript in preparation).

From our point of view and under consideration of the current published data, monitoring for non-HLA antibodies is indicated when AMR is suspected from histological findings but HLAab are not detectable. Data describing the close relationship between allo- and auto-reactivity directed against renal transplants was recently reviewed by Nath et al. (153) which provides further directions for the future as non-HLA antibody monitoring could supplement the search for HLAab.

4.4 Antibodies Predict Pathology

Clinically-indicated biopsies of patients with failed allografts were evaluated to demonstrate the cause of graft loss and thereby provide supportive evidence for a causal correlation between the presence of HLAab and allograft deterioration.

Thus, patients with HLAab demonstrated predominantly alloimmune-mediated injuries of the graft (74%) including any form of AMR but also TCMR. Although only 28% of them showed deposits of C4d in PTC and/or glomerular capillaries indicating the sites of presumed antibody-antigen interaction, its absence does not rule out the participation of antibodies in the pathogenesis of allograft deterioration. On one hand, there are important technical issues regarding the detection of C4d, including which histological preparation should be used (frozen vs. paraffin-embedded sections), how the extent of immunostaining should be

measured (72), and how much C4d has to be found to allow a biopsy to be regarded as positive (154). On the other hand, alloantibodies have been shown to be involved in complement-independent mechanisms of rejection by transducing intracellular signals leading to cell proliferation and/or apoptosis (155, 156). Alternatively, chronic exposure to low levels of HLAab may produce injuries due to levels of complement insufficient to result in C4d deposition (157). Sis et al. (73) recently reviewed further evidence of a new entity of C4d-negative AMR which has not been recognized by the Banff classification yet. Thus, C4d-negative AMR is characterized by high intragraft endothelial gene expression, alloantibodies, histology of chronic AMR (less frequently acute AMR), and poor outcomes. To account for this new entity we used an extended definition of AMR. Histological features of AMR in the presence of alloantibodies but absence of C4d was recognized as "probable AMR".
Care has to be taken by any generalization based on our results because protocol biopsies have not been performed and 189 patients with allograft loss missed biopsy data. Thus, the observed spectrum of histological findings in the presence of HLAab is probably incomplete as the prevalence of subclinical rejections with features of AMR could not be estimated. However, as indicated by the Edmonton group (75) following 315 renal transplants for a median of 31 months by protocol biopsies all rejections (n = 36) had evidence of AMR which was the leading cause of late allograft loss (64%). Therefore, our data seem to underestimate the true prevalence of AMR in the overall transplant cohort.

Important evidence for a causal relationship between alloantibodies and allograft loss comes from studies elucidating the effect of antibody removal on graft outcome. There are several therapeutic options for AMR treatment available, however a standard of care has not yet been established (reviewed in (158)). One of the most promising candidates, according to recent studies, is bortezomib, a proteasome inhibitor widely used for the treatment of multiple myeloma. In contrast to other compounds, bortezomib selectively induces apoptosis of plasma cells and is thereby able to abrogate the secretion of alloantibodies (159). The first series of six patients diagnosed with refractory AMR and undergoing bortezomib treatment was published by Everly et al. (160). Herein, the authors showed that bortezomib therapy provided (i) prompt rejection reversal as indicated by protocol biopsies, (ii) significant and sustained reductions in DSA levels, (iii) suppression of recurrent rejections for at least 5 months, and ultimately (iv) improved renal allograft function. In our own study on a larger series of 10 patients treated with bortezomib the sustained reduction of the strongest DSA as indicated by the highest MFI (DSA_{max}) was predictive for improved 18-month allograft survival and reversal of AMR (161). Interestingly, the relative decrease of DSA levels after treatment but

not the absolute value of DSA at diagnosis seemed to predict the success of treatment (**Figure 37**). Worth mentioning, bortezomib treatment was not able to completely eradicate the existing DSA.

These findings emphasize the versatile applications of Luminex® SAB for the prediction of rejection but also monitoring of therapeutic interventions.

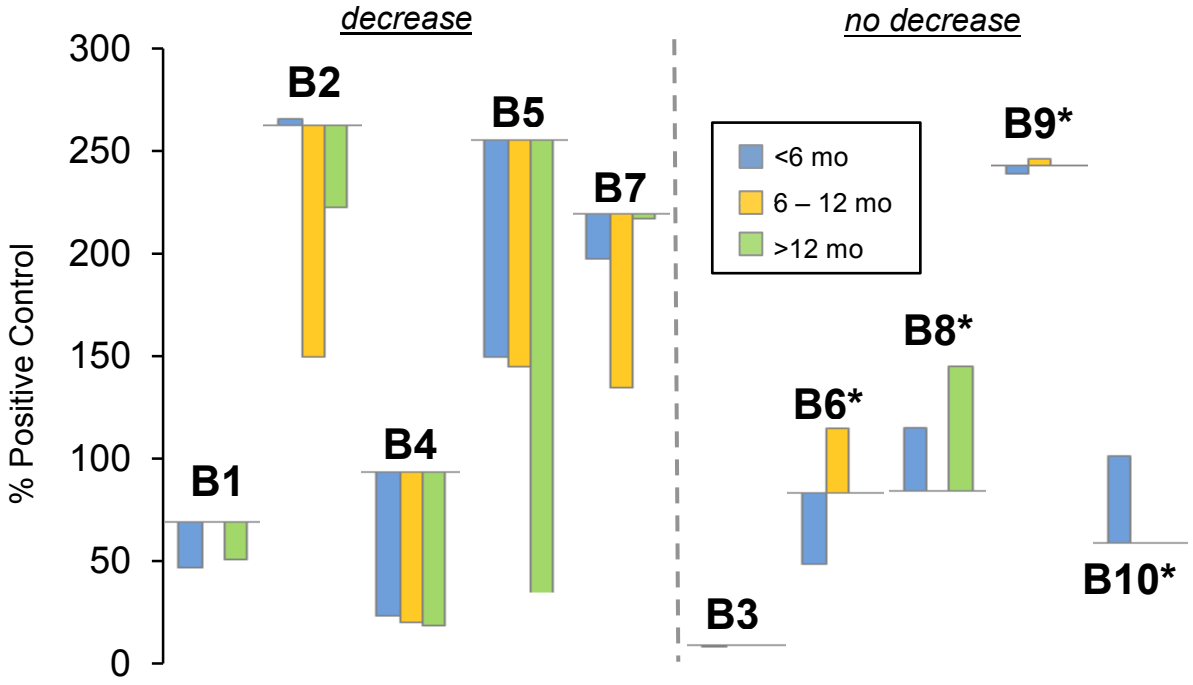


Figure 37. Monitoring of DSA following anti-humoral rejection therapy is predictive of final allograft outcome. The success of the therapeutic intervention of 10 patients diagnosed with AMR using bortezomib was monitored by recording the MFI of DSA. After 18 months of follow-up in intervals of 6 months after treatment a sustained decrease in the MFI values of DSA was predictive for graft survival. The horizontal line represents the starting MFI value of DSA at the time of AMR diagnosis for each patient B1-B10. The bars indicate the MFI values of DSA measured within 6 months (blue), between 6 and 12 months (orange), and between 12 and 18 months (green) after AMR treatment with bortezomib. Details are published by our group (161).

4.5 Prediction of *De Novo* HLA Antibody

The identification of risk factors associated with the *de novo* production of HLAab is important as acute or chronic AMR is still hardly controllable by any treatment with rather moderate prognosis for long-term allograft survival (162). As pointed out earlier in the introduction and illustrated by **Figure 3** in section 1.2 the degree of HLA antigen match has a significant impact on long-term renal allograft survival: The better the match the better the probability for good clinical outcome. This observation holds true for both pretransplant sensitized but also for unsensitized transplant recipients (163) which indicates the significance

of the *de novo* immune response to alloantigens as trigger for allograft rejection. Here we could show that the prevalence of HLAab in regrafted patients was significantly increased as compared to first transplant recipients (**Figure 19** in section 3.2). Moreover, a study by Meier-Kriesche et al. (164) pointed out that in fact the panel reactivity significantly increased by increasing HLA mismatch of the first transplant. However, the number of HLA-A, -B, -DR mismatches in our cohort turned out to have only limited power to predict HLAab (class II DSA only) which in one hand could potentially be attributed to the missing information on alloimmunizing events and pretransplant HLAab as the major contributing confounders. Details can be found in **Appendix I, Table A6** as supplemental data.

HLA epitope matching

It is well known from AA sequence alignments that each HLA molecule shares common (public) epitopes with other HLA and thus cannot elicit an alloimmune response (104). Imagine a recipient with the HLA typing A1, A3, B7, B8 and Cw7 who is intended to be transplanted with an organ allowing only a single antigen mismatch. This single antigen mismatch in turn could represent as low as 0 (A36, B42) or as many as 14 (Cw5) epitope mismatches. Consequently, each antigen mismatch comprises a different epitope load. In fact, it has been shown by a Dutch group that the number of epitope mismatches is directly correlated with the likelihood of antibody production in women against the paternal antigens of their child (19). In our analysis each epitope mismatch statistically converts into an increase in relative risk to produce DSA by 4-8% (**Table A6**). It is worth mentioning that here only the absolute number of epitope mismatches has been considered. However, it is well accepted nowadays that the immunogenicity of a mismatched epitope is additionally affected by other determinants of which the recipient's own HLA repertoire has a profound impact (165).

It is hypothesized that the indirect pathway plays the major role in allorecognition late after TX as processed antigens from apoptotic/necrotic organ donor cells are presented by recipient APC via HLA. In line with this hypothesis the recipient's HLA class II repertoire determined *de novo* production of HLAab in our cohort. Thus, recipients with a HLA-DR4 antigen revealed a significantly increased prevalence of *de novo* HLAab. It is known from the literature that DR4 is capable of presenting several class I (A2, B35) and II (DR4) HLA antigens to CD4+ T lymphocytes, and thus can initiate the humoral immune response (166). HLA-DR4 presents peptides based on anchor positions in favor of the above antigens. Recognized motifs include AA positions 55-71 which are well exposed on native HLA molecules and accessible for antibodies. On the contrary, HLA-DR3 which was demonstrated

to have a protective effect has been shown to present for instance peptides from serum albumin and complement components but not HLA. Only HLA-A2-derived peptides from AA position 103-117 are known to be presented by DR3. However, these positions are mainly buried on native molecules and thus not accessible for antibodies. Little is known on peptides presented in context of HLA-DR1, DR10 and DR103 including transferrin receptor and HLA-A2. The differential prevalence of HLAab based on the recipient's HLA class II repertoire supports the hypothesized significance of the indirect allorecognition pathway which leads to *de novo* DSA production affecting late allograft outcome.

Compliance with medications

Most importantly, adherence to the lifelong IS therapy is indicated for renal transplant recipients to suppress the permanent immunogenic trigger exhibited by mismatched HLA epitopes. There are only a few cases of operational tolerance to the donor organ described in the literature where recipients do not depend on immunomodulating reagents (167). Most of them resulted from accidental or intended withdrawal of medications by the recipient. Reasonable arguments against lifelong IS therapy include that posttransplant quality of life is not only determined by graft function but is significantly impacted by medical and physical complications due to medication (168). Consequently, the overall non-compliance rate is estimated to vary from 20% to 50% (169) and is mostly prevalent among young adults (170). On the downside non-adherence leading to under-immunosuppression and *de novo* alloimmune response appears to be a significant cause of late AR episodes and transplant loss (171). Although well recognized as the major driving force for *de novo* alloimmune response means to record and potentially prevent non-adherence including patient questionnaires (172) and electronic devices (173) do not show great promise. Here, a routinely performed posttransplant HLAab monitoring could help to identify patients suspected for non-adherence. Similarly, posttransplant HLAab monitoring enabled us to identify an increased prevalence of *de novo* DSA among kidney transplant recipients who intentionally were converted from a CNI- to a less nephrotoxic everolimus-based IS therapy (174). In this publication we could demonstrate that the late conversion to a CNI-free regimen was associated with increased risk of *de novo* DSA formation and consequently increased risk for AMR. To our surprise everolimus targeting B cell proliferation was not potent to minimize DSA formation. On the contrary, CNI targeting T cells was more effective. These observations show the complex interplay between immunosuppressants and components of the immune system quite plainly. The impact of rearranged maintenance IS therapy was also shown by other authors where the withdrawal or weaning of corticosteroids was associated with appearance of DSA and

rejection (175, 176). In another interesting study we detected *de novo* DSA in 5% of transplant recipients who received the adjuvanted H1N1 vaccine (177). This shows that despite an unaltered IS regimen the *de novo* formation of DSA can be initiated by adjuvants. Other confounding determinants of antibody production and maintenance have been nicely reviewed by Clatworthy et al. (178).

4.6 Recommendations for Posttransplant HLA Antibody Monitoring

After we ascertained that posttransplant HLAab monitoring enables the identification of patients at increased risk for renal allograft failure it needs to be determined who should be tested, how often, and how to interpret antibody tests appropriately.

Based on the findings of this study we propose a monitoring scheme as illustrated in **Figure 38**. Accordingly, early posttransplant HLAab monitoring should be performed weekly until discharge of the patient from hospital. Long term follow-up starts with stratification of patients into those facing increased and normal immunological risk (NR). Increased risk (IR) is defined by immunization (DSA or high levels of NDSA), degree of mismatch in host-versus-graft direction and suspected non-adherence or rejection by an elevated SCr or proteinuria (as defined by the clinicians). IR patients are screened by pooled antigen beads on intervals of 6 months which allows interpretation of the level and breadth of antibodies in serial samples by side-by-side analyses. Specification of antibodies is performed by SAB when there is a change observed in comparison to previous samples, but at least once annually. NR patients are screened once per year, and if HLAab positive, specificities are determined by SAB. Independently from the initial risk stratification persistent or *de novo* DSA will lead to the decision-making of performing allograft biopsy in case renal function is impaired. The appropriate treatment will be initiated when rejection is proven. All posttransplant sera are stored at a minimum of -20°C for further retrospective analyses. The proposed schedule is now in place at all three Charité kidney transplant programs and under regular revision.

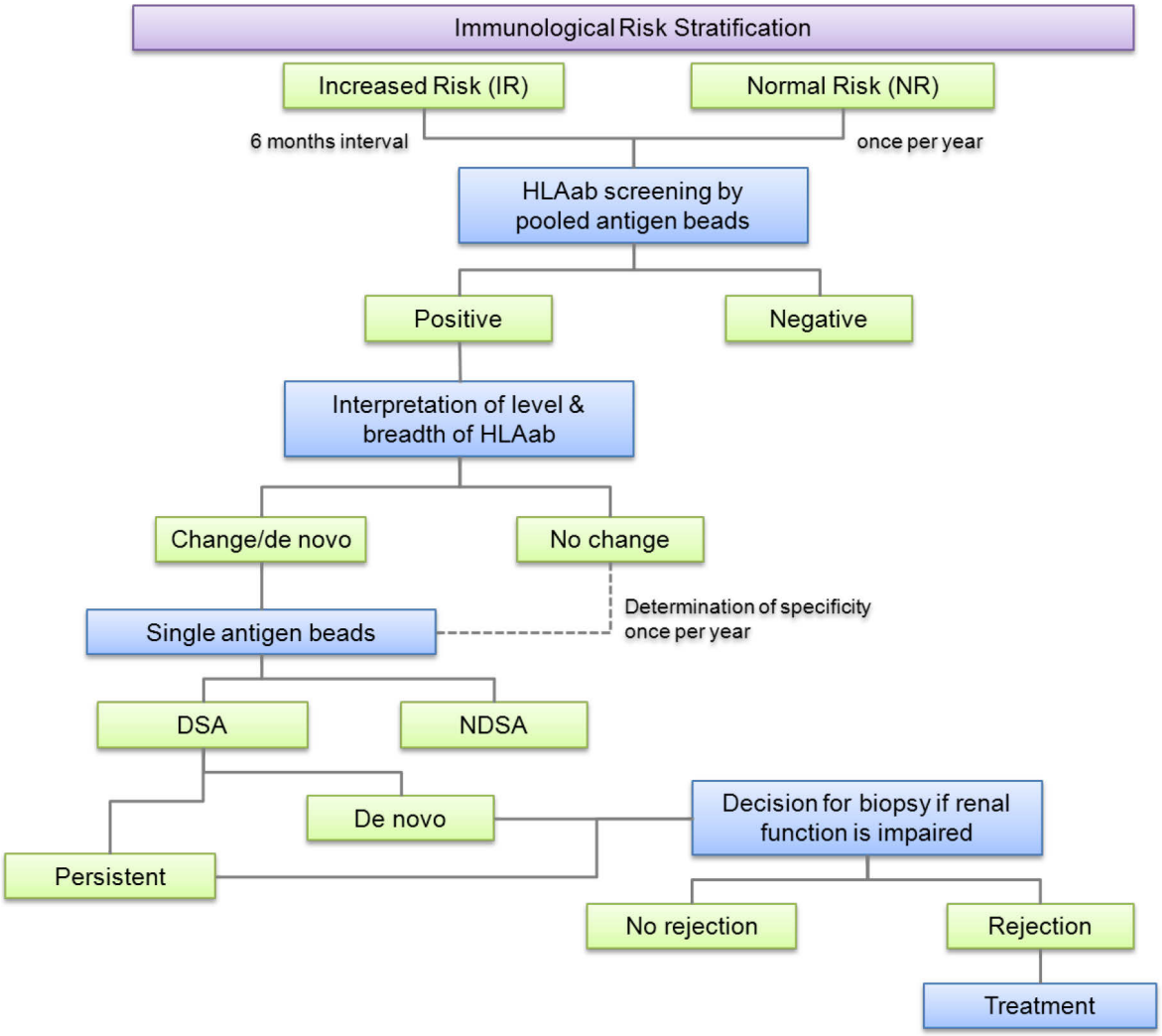


Figure 38. Proposed posttransplant HLAab monitoring scheme. Based on the immunological risk stratification HLAab monitoring for all transplant recipients visiting the outpatient clinic is initially performed by Luminex® pooled antigen beads. In case that there is no change in the level and breadth of HLAab, determination of the specificity is performed only once per year by SAB. *De novo* or persistent detection of DSA leads to a histological examination by biopsy when graft function is impaired. Treatment is initiated when rejection is diagnosed.

HLAab = HLA antibody(ies); DSA = donor-specific antibodies; NDSA = non-donor-specific antibodies

Economic considerations

Here we want to argue that also from an economic point of view posttransplant HLAab monitoring is justified. Usually cost calculations in the context of TX are published in US$ but are approximately transferable to the European market. Early detection of subclinical AMR by *de novo* DSA and thus adjustment/conversion of the maintenance IS therapy could prevent some patients from premature graft loss. A well-functioning renal allograft saves at least $25,000 each year on health care costs over hemodialysis (179). Likewise, anti-rejection treatment for instance using immunoglobulins (IVIG) or B-cell-targeting immunosuppressants like bortezomib adds costs to an uncomplicated transplant. A discussion of the potential treatment options for AMR can be found elsewhere (158, 161). However, a typical IVIG and

bortezomib treatment scheme is worth $12,000 (180) and $40,000 (personal communication with Johannes Waiser). In comparison HLAab screening and specification costs are estimated to range from $50 to $1000 per patient and year depending on the respective work-up (see **Figure 38**) which represents a maximum of 7% of the annual costs for a standard maintenance IS regimen (179). Specimens can be collected while routine outpatient visits without the need for hospitalization. A detailed calculation of the cost-effectiveness is beyond the scope of this thesis but warranted for further studies. Nevertheless, based on these numbers the cost-effectiveness of HLAab monitoring over rejection treatment and graft loss is obviously not a matter of debate.

4.7 Diagnostical and Technological Impact

The negative predictive value of a negative antibody test

One very important finding of this study is that if patients are tested once after TX for the presence of HLAab it is possible to identify those facing a low risk for late allograft failure, i.e. those patients without HLAab who harbor an 8-year graft survival probability of 75%. Graft loss in this cohort of patients was mainly attributed to the recurrence of the original kidney disease, TCMR or BK virus-mediated nephropathy. However, the most ubiquitous pathohistological finding could be associated with CNI toxicity.

Since the 1980s and the advent of CNI (CSA and tacrolimus) the incidence of acute renal allograft rejections could markedly be reduced (historical review provided by Casey et al. (181)). However, the other side of the coin is the nephrotoxic side effect of CNI leading to reversible, acute hemodynamically mediated renal graft dysfunction leading to chronic, irreversible graft deterioration (182). Consequently, review of the CNI dose to reduce nephrotoxic side effects was postulated to be beneficial for late renal allograft survival. Arteriolar hyalinosis, the histopathologic correlate of chronic CNI toxicity as defined by Nankivell et al. (183) was described to be present in literally all renal allografts at 10 years posttransplant (184). Strikingly, graft survival in this group of patients was excellent despite the diagnosis of CNI toxicity. In addition, the incidence of arteriolar hyalinosis was recently shown to be independent from the use of CNI (185). Consequently, it is currently a matter of contention to what extent and if at all CNI-reduced or -free IS regimens are able to prolong renal allograft survival.

A recently described issue inherent with the Luminex® technology is the potential underestimation of HLAab and DSA due to false-negative reactions. This phenomenon referred to a prozone-like effect is described to be due to interference of HLA-specific IgM antibodies (41) or blocking of the Fc-portion of human IgG by the complement component C1

(43). Adaptations of Luminex® assays to diminish the effect include the pretreatment of serum by heat or DTT, addition of a C1 inhibitor, EDTA or the dilution of test serum. All these measures have their drawbacks as DTT for example unspecifically breaks disulfide bonds and thus can inactivate IgG, especially IgG_3 which is rich in disulfide bonds. Serum dilution bears the risk of losing low level antibodies whereas heat treatment inactivates inter alia complement but also other serum components. The pros and cons of each of these modifications are still a matter of contention (44, 186). However, neither the prevalence of the effect is exactly known nor the mechanism fully understood. According to our unpublished observations a prozone-like effect might preferentially be detectable in a subgroup of highly-immunized patients and thus may affect less than 5% (i.e., proportion with >85% PRA on the waiting list). Such an effect cannot be excluded to have been present in the sera of this study since it was not tested for. However, to our best knowledge a prozone-like effect has not been described in posttransplant sera yet. Our preferred method in the daily routine is heat treatment which is used for example in sera of patients with suspected AMR but lacking HLAab as tested using the standard Luminex® assays. Here, adsorption of DSA by the allograft is more likely an explanation especially when cross-reactive *de novo* NDSA sharing epitopes with mismatched donor antigens are detected (see section 3.4). In addition, DSA of the IgM isotype cannot be excluded by our approach since heat treatment is not sufficient to eliminate IgM (data not shown). Thus, the frequency and the mechanism of the prozone-like effect need to be elucidated in future studies to elaborate effective approaches to deal with it in HLAab detection assays.

Taking into account that the humoral immune response is a very dynamic process with fluctuations in the level of HLAab it is somewhat unexpected that the snapshot taken by a single HLAab test provides the described predictive value for long-term allograft outcome. Lessons learnt from protocol biopsy studies support the notion of a slowly progressive but continuous process of late allograft deterioration (reviewed by Loupy et al. (187)). Chronic renal allograft injuries with unknown etiology were used to be described by the non-specific term of CAN which nowadays tends to be abandoned from the literature (89). CAN was described to be the major obstacle to successful allograft outcome present in 89% of patients at 10 years posttransplant (183) and included several histopathological features (IF/TA, subclinical rejection, TG and vasculopathy as well as arteriosclerosis). The underlying causes of each of these features have not been identified yet; however, it is thought that these lesions are the result of chronic inflammatory healing processes partially caused by the destructive power of HLAab (188). Recent revisions of the Banff classification significantly driven by the advent of the Luminex® technology for HLAab monitoring and refinements in biopsy

diagnosis led to the recognition of chronic AMR as one major entity of late allograft dysfunction previously attributed to CAN or CNI toxicity. Therefore, most sensitive and routinely performed HLAab monitoring is an essential, non-invasive means for posttransplant risk stratification. In the subgroup of patients with a longitudinal antibody monitoring we could demonstrate that those who were tested positive *de novo* during follow-up revealed a decreased allograft survival as compared to those repeatedly tested negative. These antibodies could have truly been developed *de novo* following the initial HLAab test. Also likely, fluctuation of the serum antibody level led to a delayed detectability of HLAab in peripheral blood. Nonetheless, we can summarize that sequential negative HLAab tests are highly predictive for an uneventful long-term renal allograft survival.

The importance of defining DSA correctly

The clinical relevance of HLAab as detected exclusively by Luminex® SAB has been questioned by a recent multi-center study in which DSA did not predict allograft outcome (77). Although in contrast to this thesis the authors of the study analyzed pretransplant sera we will discuss it here because it clearly demonstrates pitfalls in the appropriate assignment of HLAab specificities.

In the Süsal study the incidence of preformed HLAab among 118 patients with graft loss was as high as 45% (class I antibodies). Among the matched control group of another 118 patients without graft loss the incidence was even higher with 66%, which dramatically exceeds the values we and others have observed using SAB. According to the literature the prevalence of HLAab in unselected cohorts ranges from 24% (189, 190) to 27% (191). Similarly, we have a quite constant proportion of 29% HLAab-positive individuals on our waiting list of 1570 patients. Süsal et al. selected patients without antibodies as detected by both CDC and ELISA. In contrast to unselected cohorts we would expect a much lower incidence of HLAab based on the before mentioned selection criterion.

The most probable explanation for this discrepancy is that the authors inappropriately defined HLAab using SAB which might have led to the overestimation of the prevalence of DSA. Symptomatically, DSA targeting HLA-C or -DP were detected unexpectedly more frequently than against HLA-A, -B, -DR, -DQ and the majority of patients with HLAab had only a few specificities as indicated by a PRA of 1-5%. These observations very strongly resemble those initially shown by Morales-Buenrostro et al. (45), in which HLAab specificities were detected among healthy males without alloimmunizing events. These antibodies, referred to as “natural” HLAab, could be shown by epitope analyses to react with polymorphic AA residues usually not exposed to the molecular surface (192). Cryptic epitopes are accessible only on

dissociated antigens and comprise for instance of AA residues buried by the peptide within the MHC binding groove. Other epitopes can be found within the alpha-3 domain of HLA class I antigens in close proximity to the transmembrane domain and are thus in the native orientation of the molecule inaccessible by antibodies due to steric hindrance. Luminex® SAB are especially prone to detect these antibodies due to the unparalleled sensitivity of the assay and the nature of the used antigens. The proper conformation of HLA as regulator in the immune system highly depends on the heterodimeric nature (α- and β-chain), the bound peptide, and the orientation but also the glycosylation pattern of the molecule (193). All these parameters are directly or indirectly influenced by recombinant expression of single antigens. The purification and coating process leads partially to improper conformation and orientation which can give rise to the detection of unexpected specificities (194). One can assume that some of the HLAab detected in the study by Süsal et al. are in fact directed against cryptic epitopes which have been shown to be clinically irrelevant (111, 195).

Consequently, we consider antibodies with low to intermediate MFI (1000 to 8000) directed against a limited number of less frequent HLA (e.g. A80, B37 and B76) detected by SAB in patients without alloimmunizing events as suspicious. By using Luminex® pooled antigen beads first to screen for HLAab before considering testing by SAB we are able to eliminate the majority of "natural" HLAab. However, more evidence can only be gained by testing sera against acid-denatured antigens, as reactivity will be maintained or increased as demonstrated in section 3.1.1. The two vendors of HLA SAB are aware of this problem and at least one of them very recently released a product with significantly diminished proportions of denatured HLA. Future validation of this type of beads is certainly warranted.

Due to a lack of standardization the assignment of positive and negative reactions still remains somewhat problematic using SAB. Consequently, to date there is no consensus on the relevance of DSA as detected by Luminex®-based assays. The MFI has been suggested to be the determinative parameter. However, the MFI readout simply represents the level of antibody on the beads which greatly depends not only on antibody concentration in the serum but also density, conformation and orientation of antigen as well as on the antibody avidity toward the respective antigen. We and others are therefore convinced that HLAab determination solely based on MFI is inappropriate. Consequently, we consider specificities exceeding 1000 MFI above background as potentially positive. Most importantly, the plausibility of an antibody specificity is assumed if there is a classical immunizing event (i.e., transfusion, pregnancy or TX), and the antibody is repeatedly detected in at least two individual serum samples. Plausibility is further supported by HLAMatchmaker analysis or a simplified epitope determination using tabulated CREG. However, Süsal et al. used fixed

cutoffs of 1000, 2000 and 3000 MFI which potentially led to the overestimation of HLAab and biased this analysis. To aid antibody determination the manufacturers provide users with analysis software featuring different tools. Tail analysis is known to preferentially assign the most frequent leaving out less common and masked specificities. In particular HLA-DR is usually preferred over -DQ potentially leading to data misinterpretation. A more reliable tool therefore is an epitope analysis as featured by both vendors. As mentioned above, the interpretation of antibody specificities by considering CREG is in most cases absolutely sufficient to explain reaction patterns. However, CREG based interpretation has its shortcomings when analyzing HLA class II antibody reaction patterns as there are no published CREG defined for class II. In addition, CREG maps marginally describe interlocus reactions and completely lack allele-specific antibodies. Verification of these types of antibodies, progressively detectable since the advent of SAB, can be achieved by using epitope analysis as described in section 3.1.3.

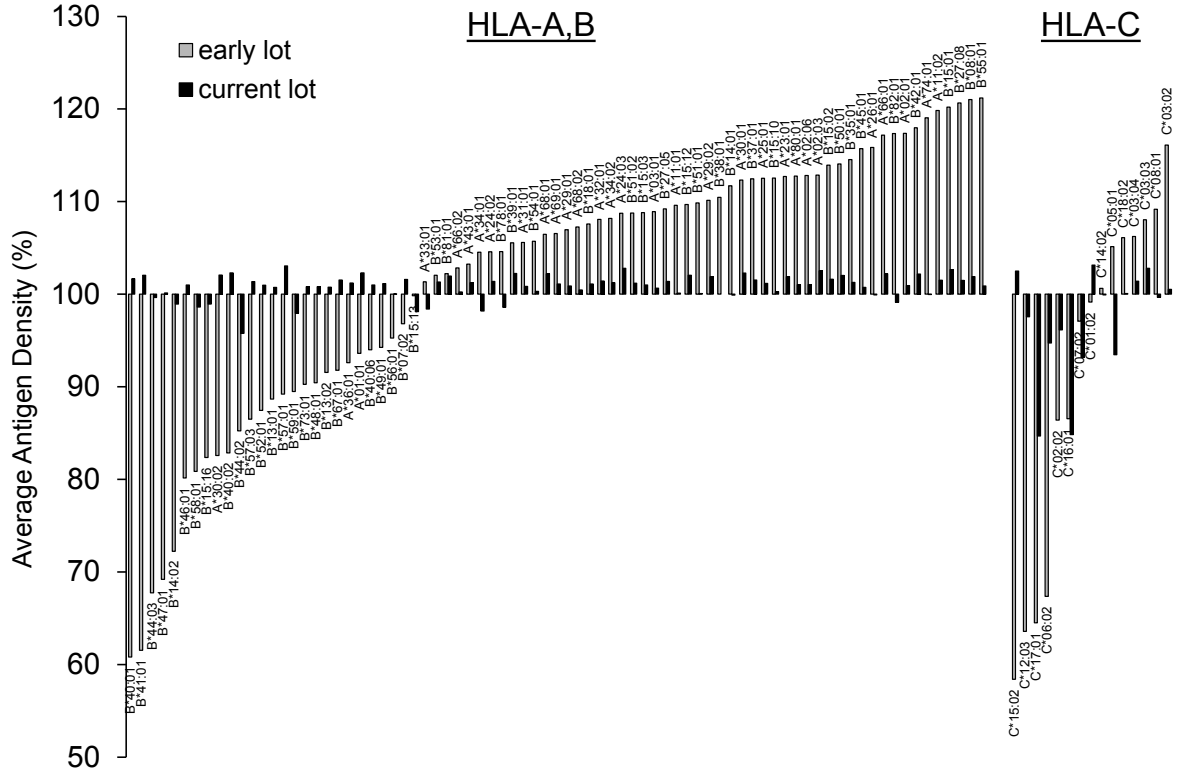


Figure 39. Decreasing variability of the relative antigen density on HLA class I SAB of one vendor comparing an early and the most current lot. Relative antigen density was assessed by the monoclonal antibody clone W6/32 which binds a conformational epitope carried by all HLA-A, -B and –C native antigens. Variability of antigen density could be significantly reduced from an early (LABScreen® lot#003) to a current lot (lot#007). HLA-C antigen density reveals the highest variability.

Assay variability

The following mentioned viewpoints have been compiled by me as a fundamental part of the antibody consensus paper by The Transplantation Society (accepted for publication).

There are several factors that need to be considered when interpreting Luminex® assays. The relative density of a particular antigen differs substantially between pooled antigen, phenotype and SAB. Especially, HLA-C but also -DQ and -DP on SAB are characterized by a higher relative antigen density as compared to phenotype beads and human cells. The relatively low expression of HLA-C as compared to HLA-A and -B on human cells was shown to be regulated by a designated non-coding region leading to faster degradation of mRNA. In contrast, cDNA-transfected cells, the source of recombinant single antigens for SAB, revealed equal surface expression (196). As a consequence, HLAab directed against these naturally low-expressed antigens run the risk of being detectable exclusively by SAB. This fact may contribute to the controversy on the clinical relevance of HLAab as there is an eminent discrepancy in antigen density between the antibody detection assay and the surface of endothelial cells as the antibody target of a renal allograft. Consequently, HLA-DQ antibodies detected exclusively by SAB revealed only a low immunological risk for renal transplant (197). Disparities in relative antigen quantity exist not only across different bead formats but also among SAB. Since the advent of SAB continuous improvements in the manufacturing process and quality assurance measures have contributed to a more uniform antigen density across all beads from lot to lot (**Figure 39**). In addition, the antibody analysis software includes features to normalize MFI raw data according to an average antigen density as determined by the manufacturer.

As for any immunoassay, Luminex® assays reveal variability of results on a day-to-day and tech-to-tech basis due to variability in ambient test conditions and technical handling. In particular, a standardized protocol contributes significantly to improved qualitative and quantitative reproducibility of test results. Nevertheless, these assays are robust. The coefficient of variation (CV) between experienced technicians taking into account quality control sera with defined antibody specificities is usually less than 10% (unpublished observations). Increased variability comes into play when considering deviations from lot to lot and between manufacturers. Interassay CV between lots of the same manufacturer may be as high as 100%. Similarly, nonspecific background for each bead as assessed by the manufacturer under optimal conditions varies substantially between lots with a CV ranging from 25% to 100% (comparison of negative control serum with 6 different lots of SAB from one vendor). Lot-to-lot variations are not restricted to the HLA-coated beads but are also inherent to the fluorophore-conjugated secondary antibody. All the above contribute to the

reproducibility of the Luminex® assays which is often criticized as being relatively poor. However, the unparalleled sensitivity of these assays is in part responsible for these deviations. Further, the degree of variability is similar if not comparable to that of cell-based assays. Therefore, the overall quality of the assays with respect to antigen condition and nonspecific background is still in need of improvement to assure minimal lot-to-lot variations. SAB are available from only two vendors to date. The panel composition of SAB is quite balanced with >80% concordance between manufacturers and covering the most common specificities. However the two kits may show discrepant MFI values for the same allelic antigen. HLAMatchmaker analyses show that one result is consistent with the epitope specificity of an antibody while the other is not (165). Differences between alleles might be related to the possibility that certain antibody-defined epitopes may include parts of the bound peptides (198). As revealed by extensive parallel testing some alleles in the kit of one particular vendor give unexpected positive reactions possibly because they have undergone significant denaturation giving rise to the exposure of cryptic epitopes (personal communication with R. Duquesnoy). The other kit tends to give lower MFI values and this might be related to the amount of serum tested, the amount of antigen on the beads, or other factors. Both commercial kits have been developed under different technical conditions and allelic antigens are derived from different sources. Detailed information is proprietary.

Future perspectives

The search for biomarkers to assess allograft function and predict rejection is of major interest for tailoring optimal IS to the individual patient profile. In an attempt to balance between negative side effects (overdose) and risk for rejection (underdose) the older principle of “one fits all” has become more and more obsolete (199). Potentially useful candidates for renal biomarkers at the protein, peptide or transcriptomic level of peripheral blood and urine have been reviewed by Bestard et al. (200). However, the high complexity of the approaches used to assess these biomarkers (e.g. mass-spectrometry-based peptide profiling) makes it unlikely that those become a routine testing strategy in the foreseeable future. In contrast, here we present convincing evidence that HLAab as detected by Luminex® SAB may function as a useful biomarker to predict long-term renal allograft outcome. In particular, pathogenicity of IgG alloantibodies was best described in this study by the assessment of the complement-binding capacity. On the one hand, activation of the complement cascade leads to rapid allograft injuries and graft loss. On the other hand, complement-independent pathways of antibody-mediated processes involve stimulation of cell proliferation and cell-mediated cytotoxicity which lead to a rather delayed loss of graft function (26). Thus, one single test to

facilitate the discrimination between complement-fixing and non-complement-fixing alloantibodies would be desirable. The simultaneous assessment of the complement activation ability and isotype of DSA in a flow-cytometric crossmatch approach was first shown by Schönemann et al. (201) using a human IgG-specific secondary detection antibody in combination with the vital dye 7AAD to detect complement-mediated cell lysis. We then further developed the assay by using simultaneously anti-human IgG and anti-C1q detection antibodies conjugated with orange and green fluorophores. As illustrated in **Figure 40** this approach facilitates the discrimination between complement-fixing (right panel) and non-complement-binding IgG DSA (middle panel) in a standard flow cytometer. Care has to be taken by selecting the appropriate antibody clones. Blocking of the anti-IgG binding site by the C1q molecule has to be avoided. Similarly, steric hindrance due to the Fc portion of the detection antibodies can be diminished when $F(ab')_2$ antibodies are used. However, the major obstacle to the implementation of a similar approach is the limitation of today's Luminex® instruments to only one single reporter channel. Recently, efforts have been undertaken to increase the multiplexing capability by adding a third detector to the innovative FlexMap 3D instrument which allows the discrimination of a maximum of 500 fluorescence-barcoded beads each impregnated with three different fluorophores (www.luminexcorp.com). Alternatively, beads of different sizes have been incorporated into the current fluorescence schema and thereby increased the number of analytes. However, the addition of a second reporter channel would be desirable for the future to further increase the flexibility of the instruments.

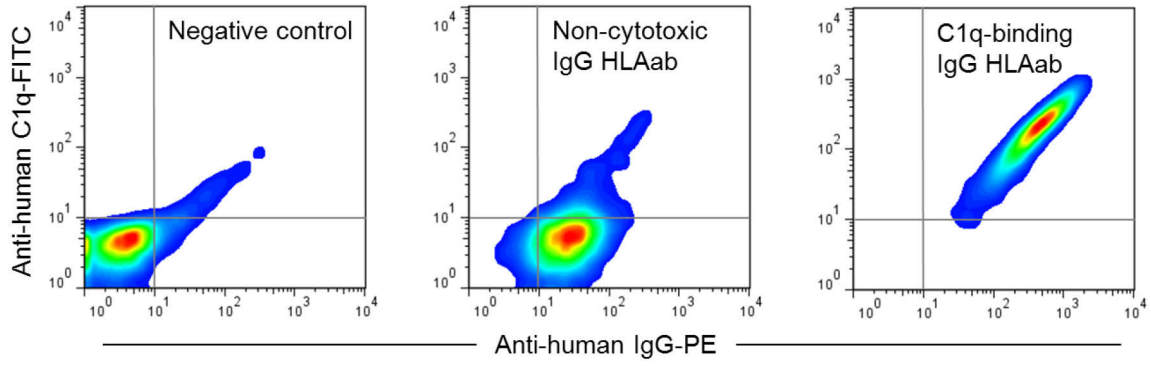


Figure 40. Simultaneous detection of the immunoglobulin isotype and C1q-binding capability of HLAab by flow cytometric crossmatch. The usage of anti-human IgG and C1q-specific secondary antibodies facilitates the discrimination between non-cytotoxic and C1q-binding antibodies in a single flow crossmatch assay. IgG not capable of fixing C1q are detected in the lower right quadrant (middle) whereas C1q-fixing IgG visualized in the right panel are detected in the upper right quadrant.

Although the Luminex® technology is already known to reveal a high degree of multiplexing capability and sensitivity (≥ 100 PE molecules per microsphere) there is still potential for improvements by using quantum dots (QD) replacing currently used standard organic fluorophores. QD known from FC are derived from semiconductor materials forming nanometer-scale crystals, which can emit a wide variety of fluorescent light upon excitement (202). Advantages of QD over standard fluorophores include (i) a broad excitation spectrum which would enable to save instrument costs and maintenance by using only one laser for excitation of a variety of QD. (ii) QD are relatively bright with a quantum yield of 90% vs. 30% for fluorophores. (iii) Emission spectra of QD are symmetrical and show only little overlap with other spectra; thus minimizing the need for extensive compensation to reduce fluorescence spillover. On the other side currently very few companies offer antibodies conjugated to QD. Fortunately, the conjugation of antibodies to QD is straightforward using simple equipment and low-cost reagents. Even production of QD-barcoded beads have been described allowing for dramatic multiplexing that is not possible with organic fluorophores (203).

Acknowledgment

I do not know anyone who has gotten to the top without hard work. That is the recipe. It will not always get you to the top, but it will get you pretty near.

Margaret Thatcher

I want to start off the acknowledgment with the above quote, which always provided me with a certain spark of confidence throughout the whole process of writing this thesis.

My first thank-you is due to Prof. Abdulgabar Salama and Prof. Axel Pruß in person for giving me the possibility to conduct my research at the facility of the tissue typing laboratory as part of the Charité, Institute of Transfusion Medicine.
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Caroline Mühle, Fay Jahr, Gabriele Adam, Hugo Kaneku, Karen Ng, Rene Castro, Viola Jacob and the whole technical staff from the Terasaki Foundation Laboratory in Los Angeles as well as the whole team from the Charité HLA Laboratory in Berlin.

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Most importantly, after completion of the thesis under the cost of great privations of my sons Janis & Joris as well as my beloved wife Nancy I owe my deepest gratitude to my family. I can barely imagine the sacrifices they had to make. Special thank-you is due to my parents and my brother for their never-ending patience with me. Thank you for your faith in me.

Lastly, I offer my regards and blessings to all of those anonymously who are not mentioned by name but supported me in any respect during the completion of the thesis. Thanks to all of you.

Appendix I: Supplemental Data

Table A1. C1q assay (sC1qA) revealed increased sensitivity and specificity in comparison with CDC in detecting complement-activating HLAab. Comparison of 5 patient sera with complement-activating HLAab tested in parallel with CDC and C1q assay. The C1q assay was proven to be superior to CDC with additional detected specificities (underlined) and thus increased %PRA.

Serum ID	CDC		sC1qA	
	%PRA	Specificities	%PRA	Specificities
IRV 1_2010_21	26	B44 B45	26	B44 B45 B76 B82
IRV 1_2010_22	7	B13	52	B13 B44 B45 B62 B75 B76 Cw6 Cw15 Cw17 Cw18
IRV 1_2010_23	45	A1 A36 A23 A24	87	A1 A36 A23 A24 A11 A25 A26 A29 A32 A43 A66 A80 B27 B13 B37 B38 B44 B45 B47 B49 B51 B52 B53 B57 B58 B59 B63 B76 B77 B82
IRV 1_2010_24	8	B57 B58	23	B57 B58 A25 A32 B63 B54 B55
IRV 1_2010_25	15	B62 B63 B75 B76	32	B62 B63 B75 B76 B13 B46 B49 B50 B57 B71 B72 B77

Table A2. Detailed information on DSEab defined among 52 patients with *de novo* HLAab. All 52 patients revealed exclusively *de novo* HLA class I antibodies without direct donor specificity. HLA-A and –B antigen mismatches (MM) and detected NDSA are listed for each patient. Single AA MM between donor and recipient were calculated and corresponding AA shared with NDSA defined as DSE. Probability of DSE antibodies was calculated based on the proportion of NDSA that could be explained by at least one DSE.

(Table follows on the next three page.)

ID	A locus MM	B locus MM	# AB MM	# aa MM	A locus NDSA	B locus NDSA	Propability	Unexplained NDSA	aa MM with Donor-Specific Epitopes (DSE)
689	-	B39 B49	2	13	-	B35 B51 B53 B75 B78	100	-	009Y 024S **063N 080I 081A 095W 103L** 116F 116L **152E** 158T **163L** 305T
2138	A32	B35	2	26	-	B44	100	-	062Q 066N 080I **081A 095I** 097M 114Q 246S 297M 298F 299A 307R **011A 012M** 045T 067F **069T 070N 071T 074Y 094I 095I** 103L 116S **163L** 305T
2562	A1 A23	B44 B27	4	30	A80	-	100	-	009S 044K **062E** 065G 066K 067M 095L 097M 099F 114H 116Y 127K 150V 152A 156R 158V **166D 167G** 282V 283H 311N **045E** 067C 070K 071A 156D **163E** 167S 199V 325C
3394	A3	B49	2	7	A2 A68 A69	-	100	-	009F **070H 076A 105S 156L** 161D **163T**
3737	A23 A31	B49 B51	4	33	A25 A26 A66	-	100	-	009T 062E 065G 066K 073I **080I** 097M 099F 114H **114Q** 127K **193A 194V** 207S 246S 253Q 283H 294F 298F 299A 307R 311N 009H 032L 045K 045T 080I 095W 097T **152E** 163L 171H 305T
3840	A1	B46 B57	3	35	A68	B67 B76 B8	100	-	044K 067M 076A 077N 090D 097I **114R** 150V 152A 156R 158V 163R **166D** 167G 276L 321T 009Y **011A 012M** 041T 045K 046A 067M 070S 077N 080I 081A 094I 097V **113H 114D 116S 163L 167S** 199V
7346	A32	B7 B35	3	19	A11	B75	100	-	**062Q** 080I 081A 082L 083R 297M 045T 067Y 067F **070Q** 071A **094I** 097S 103L **114D** 116S 156R **163L** 305T
7351	A2	B7	2	31	A24 A23	B8 B37	100	-	062G **066K** 074H 095V **105S** 107W **114H 116Y 127K** 142T 145H **163T** 184A 193A 194V 207S 253Q 294F **024S 045E 063N 067Y 077S 080N** 095L **097S** 116Y **163E 177D 178K 180E**
7646	A2	B51	2	17	A36 A1 A80 A23 A24	B76 B45	100	-	062G **066K** 074H 095V 107W **114H 127K** 142T 145H **011A 012M** 067F **077N** 095W 097T **114N 163L**
7667	A31	B44 B39	3	29	A26 A33 A66 A68 A69	B18 B35 B37 B51 B52 B53 B58 B78 B8 B81 B82	100	-	009T 056R 062Q **066N** 073I **095I 114Q 116D** 246S 298F 299A 307R **024S** 024T 032L 041T **045E** 045K **063N** 067C **094I** 095I **114N** 116F **156D** 158T **167S** 199V 325C
7702	A31	B49	2	12	A34	B8 B59	100	-	009H 024T 032L 041T 045K 062L 063Q **070Q** 076A **103L** 114R **116L**
7727	-	B37	1	10	-	B76 B62 B75 B46 B57 B63 B77	100	-	009H 045T **067S 074Y** 077D **097R** 099S 116F **131S** 305T
7877	-	B18 B41	2	15	-	B76 B45 B44	100	-	062Q 090D 097I 152A 156Q 163R 276L 321T 024S **069T 070N 071T** 097W 116F 171H
7919	A3 A11	B18 B52	4	13	-	B27 B41 B42 B55 B56 B67 B7 B81	100	-	**070Q 076V 077D** 161D **024S** 030G **045T 063N 077S 080N 097T 171H 194V**
7921	A1	-	1	13	A24	-	100	-	044K 067M **070H** 076A **077N** 090D 150V 152A 156R 158V 163R **166D 167G**
7955	A23	B44 B62	3	21	A1 A36	-	100	-	009S 062E 065G 080I 097M 099F **114R** 156W **166D 167G** 245V 283H 311N 004F 046A **067M** 070S 095W 103L 116S 305T
8008	A28 A32	B40 B21	4	16	A24	B82	100	-	009H 024T 032L 041T **070Q 079R** 097R **103L 116L 127K** 143S 145H 147L 156W 178K 184A
8042	A3 A11	B27 B60	4	23	A2 A68 A69	B57 B58	100	-	009F **090D 144K 151H 152E** 152A **156Q** 161D **163R** 009H 070K **071A 077S** 080N 097N **114H 116Y** 143S 147L 163E 177D 178K 180E
8174	A3	-	1	5	-	B18 B61 B72 B37 B60 B48 B76 B44 B41 B52 B13	100	-	**097I 114R 161D** 276L 321T
8250	A24	-	1	2	A68 A2	-	100	-	**166E 167G**
8275	-	B44	1	9	-	B13 B8	100	-	**011A 012M 094I 097R 131S 156D 163L 167S** 199V
8515	A2 A3	B8	3	19	-	B35 B44 B45 B49 B50 B51 B52 B53 B56 B62 B63 B71 B72 B75 B76 B82	100	-	009F 009D **011A 012M** 062Q 066K **067F** 074H 095V 099R 099I 099S 107W **113H 116Y 156D** 161D 178D 180E
8519	A28	B47	2	10	-	B46 B73	100	-	127K 142T 145H **156W** 245V **095L** 099F 114H **163E** 307V
8814	A3	B37	2	18	A23 A24	B35 B75	100	-	076V 077D **105S** 161D **163T** 009H **045T 067S 069T 070N 071T** 077D 099S 114N 116F 156D 163T 305T
8969	A1	B45	2	15	A25 A26 A34 A43 A66	-	100	-	044K 062Q **066N** 067M **076A 090D** 097I 114R 150V 152A 156R 158V **163R** 276L 321T

(continued)

ID	A locus MM	B locus MM	# AB MM	# aa MM	A locus NDSA	B locus NDSA	Propability	Unexplained NDSA	aa MM with Donor-Specific Epitopes (DSE)
9039	A24	B40	2	21	A80	-	100	-	009S **062E** 065G 066K 095L 097M 099F **105S** 114H 116Y 127K 163T 311N 009H 024T 032L **045E** 067C 070K 097N 163E
9084	A29	B43 B40	3	18	A34 A68	-	100	-	009T 024T 032L 041T 045K **062R** 063Q 076A **095I 097M 114R 116D** 142S 147L 163E 177D 178K 180E
9199	A30	B44	2	21	A2 A69 A68 A24	B7	100	-	**070H 076A 114Q 149T 152E 156W 184A 193A** 207S 246S 253Q 294F 299A 009H 032L 045K **095W 116Y** 156D **177D 180E**
3473	A24	B7	2	14	-	B13 B35 B49 B50 B51 B52 B53 B56 B62 B63 B71 B72 B75 B77	93	B35	009S 062E 065G **080I** 097M 099F 156Q 311N **067Y 070Q 071A 152E 163E** 178K
5682	-	B48	1	6	-	B7 B67 B4005 B39 B2708 B75 B54 B45 B8 B35 B42 B55 B73 B76 B18 B62 B41 B48 B72 B56 B39 B81 B61 B82 B50 B78 B46 B71 B65 B64 B41 B60	84	B4005 B39 B45 B41 B48	067M 070S **071A 080I** 097V **116S**
8722	A10	B7	2	16	-	B35 B53 B51 B44 B45	80	B45	009Y 024S **063N** 066N 067Y 070Q 071A 090D **095I** 097S 114Q **116D** 149T 156W 156R 163R
9075	A26	B42	2	6	-	B58 B63 B44 B57 B55	80	B55	**080I 081A** 024S 030G **067S** 171H
5171	-	B44	1	7	-	B7 B8 B18 B35 B39 B45 B48 B50 B54 B55 B56 B60 B61 B62 B64 B65 B67 B71 B72 B73 B75 B76 B78 B81 Bw6	79	B39 B55 B64 B65 B67	**032L 045K 114D 156D 163L 167S** 199V
1574	A3 A24	B63	3	29	-	B38 B39 B48 B58 B59 B64 B65 B8	75	B38 B48	009S 062Q 062E 065G **066N 070Q 074D 095I** 097I 097M **099F** 114R 116D 156Q 161D 166D 167G 276L 311N 321T 004F 046A 066N **067M 070S 071A 103L 114D 116S**
7396	-	B38	1	6	-	B13 B27 B37 B60 B61 B7 B73 B81	75	B73 B81	**074Y 077N 080I 081A 082L 083R**
8271	A32	B44	2	30	A1 A66 A43 A36 A25 A11	B7 B60 B2708 B27 B67 B48 B73 B81 B42 B54 B47 B61 B55 B56 B82 B13 B76	70	B47 B48 B81 B54 B55 B56 B76	009F **062Q 065R 066N 095I 114Q 116D 184A 193A 194V** 207S 246S 253Q 294F 297M 298F 299A 307R **024T 032L 041T 045K 080T 094I** 095I 116D 156D 167S 199V 325C
16985	A11	B51 B60	3	20	-	B37 B47 B76	66	B37	090D 152A 163R 009H **024T 032L 041T 045K** 067F 094I 103L **114D 116S** 143S 147L **163E** 177D 178K 180E 325C
7432	A33	B14 B7	3	19	A32 A23 A24 A25	B51 B52 B53 B44 B49 B57 B58 B63 B77 B37 B13 B47 B27 B38 B59	63	B51 B52 B53 B49 B37 B38 B59	009T 073I **097M 114Q 116D** 186R 246S 298F 299A 307R 067Y **071A** 097S 156R **163E** 177D 178K 180E 325C
7488	A29	B12	2	12	A1 A23 A24 A25 A80	B42 B55 B56 B67 B7 B76 B81 B82	62	A23 A24 B55 B67 B81	009T 045K 062L 063Q **076A 114R 114D 156D 163L 167S 193A** 199V
7878	A11 A26	B7 B38	4	13	-	B4005 B49 B62 B72 B56 B50 B52	43	B49 B62 B72 B50	076V 077D 156Q **067Y 070Q 071A 097S 116Y** 163E 177D 178K 180E 325C
9072	A24	B41 B18	3	26	-	B56 B82 B67 B55 B54 B42 B7 B81	38	B56 B55 B54 B42 B81	062E 065G 066K 097M **097R 099F** 114H 114Q 127K 149T 156Q **156W** 184A 193A 207S 246S 253Q 294F 299A 311N 009H 030G 067S 097R 114D 116S
8148	-	B18	1	5	A66 A26 A33 A68 A34 A69 A25 A33	-	25	A66 A26 A68 A34 A69 A25	009H 024S 030G 067S **171H**

(continued)

ID	A locus MM	B locus MM	# AB MM	# aa MM	A locus NDSA	B locus NDSA	Propability	Unexplained NDSA	aa MM with Donor-Specific Epitopes (DSE)
3188	A31	-	1	12	A2 A80	-	0	A80	009T 073I **097M 114Q 193A*** 207S 246S 253Q 294F 298F 299A 307R
3299	-	B53	1	1	A66	B7 B2708 B45 B48 B27 B47 B76 B44 B61 B81 B13 B60 B44 B4005 B73 B49 B41 B82 B50 B8 B41	0	B27 B45 B48 B27 B47 B76 B81 B60 B4005 B73 B41 B82 B50 B8 B41	**077N***
8653	A25	B27 B51	3	14	A23 A24	-	0	-	077S 009H **045E*** 045T 067C 067F 070K 095W 097N 097T 114N **116Y*** 171H 305T
857	A30	B18	2	22	-	B76 B45	0	B45 B76	009S 017S 056R 062Q 066N 070Q 097I 114E 116H 152W 276L 321T 024S 030G 045T 063N 077S 080N 114D 116S 171H 305T
5328	-	B60 B62	2	13	A23 A24 A32	-	0	A23 A24 A32	009Y 024T 032L 041T 045K 046A 143S 147L 152E 156W 163L 163E 178K
7628	A11	-	1	4	-	B41 B61 B37 B72 B41	0	B41 B61 B37 B72 B41	090D 152A 156Q 163R
7653	A10	B38	2	1	-	B48 B60	0	B48 B60	080I
8467	A3	-	1	1	A25 26 66	B76 44 45	0	A25 A26 A66 B76 B44 B45	161D
938	-	-	0	0	A2 A68 A69	B45 B76 B44 B82 B57 B58	0	A2 A68 A69 B45 B76 B44 B82 B57 B58	-
8517	-	-	0	0	A23 A24 A25 A32	B13 B27 B37 B38 B44 B47 B49 B51 B52 B53 B57 B58 B59 B63 B77 Bw4	0	A23 A24 A25 A32 B13 B27 B37 B38 B44 B47 B49 B51 B52 B53 B57 B58 B59 B63 B77 Bw4	-

Table A3. Consistency of the major characteristics of patients in the total cohort and the subgroup of patients with HC, C1q and IgG subclass testing. The subgroup of 788 patients who could be tested for HC-specific, C1q-fixing and IgG subclass antibodies did not differ significantly from the total cohort with respect to the characteristics listed below.

	total cohort (n = 1075)	**subgroup: HC-testing, C1q, IgG subclass (n = 788)**
Recipient gender, % male (count)	58.7% (631)	59.5% (469)
Recipient age at time of TX, yrs (IQR)	44.1 (35 - 54)	44.5 (35 - 55)
Median time of follow-up, yrs (IQR)	6.6 (4.0 - 8.5)	6.3 (3.7 - 8.5)
Median SCr at enrollment, mg/dl (IQR)	1.4 (1.1 - 1.8)	1.4 (1.1 - 1.9)
eGFR at enrollment, ml/min/1.73m^2 (IQR)	49.7 (38.1 - 64.9)	48.2 (37.0 - 64.0)
Type of kidney transplantation (count)		
retransplantation	14%	14%
single kidney	91%	100%
HLAab pos	33%	33%
DSA pos	11%	10%
Number of HLA mismatches (SD)*		
class I: A, B locus	2.0 (1.1)	1.9 (1.2)
class II: DR locus	0.9 (0.7)	0.9 (0.7)

IQR = interquartile range; eGFR = estimated glomerular filtration rate; TX = transplantation; neg = negative; pos = positive

Table A4. No discrimination of the harmfulness of NDSA based on presensitization, number of previous transplants and maximum fluorescence intensity (MFImax). Known risk factors for NDSA production (presensitization and retransplantation) as well as the maximum fluorescence intensity and potential donor specificity (DSE) were tested by Kaplan-Meier survival analysis for their potential to predict the harmfulness of exclusively class I and II NDSA. None of the variables analyzed was able to discriminate between harmful and harmless NDSA.

	8-year graft survival	95% CI	
Exclusively class I NDSA (n=131)	50%	42-61%	reference
pretransplant HLAab -ve (n=69)	51%	37-63%	
pretransplant HLAab +ve (n=60)	53%	39-66%	
n.t. (n=2)	n/a	n/a	
regrafts (n=28)	56%	32-74%	
first transplants (n=103)	51%	40-61%	
MFImax 1000-5000 (n=48)	50%	33-64%	
5001-10,000 (n=39)	55%	36-71%	
>10,000 (n=44)	52%	34-67%	
NDSA with DSE analysis (n=52)	42%	27-58%	reference
DSE +ve (n=26)	49%	26-69%	
DSE -ve (n=26)	38%	17-58%	
Exclusively class II NDSA (n=38)	65%	44-80%	reference
pretransplant HLAab -ve (n=27)	67%	42-83%	
pretransplant HLAab +ve (n=11)	61%	17-87%	
regrafts (n=13)	64%	22-87%	
first transplants (n=25)	66%	41-83%	
MFImax 1000-5000 (n=14)	46%	11-76%	
5001-10,000 (n=8)	83%	27-97%	
>10,000 (n=16)	70%	33-89%	

neg = negative; pos = positive; MFImax = maximum MFI; 95% CI = 95% confidence interval

Table A5. Equality of the major characteristics of patients in the total cohort and the subgroup with a longitudinal follow-up. The subgroup of 531 patients with a longitudinal follow-up did not differ significantly from the total cohort with respect to the characteristics listed below.

	total cohort (n = 1075)	**subgroup: longitudinal follow-up (n = 531)**
Recipient gender, % male (count)	58.7% (631)	58.9% (313)
Recipient age at time of TX, yrs (IQR)	44.1 (35 - 54)	41.9 (34 - 50)
Median SCr at enrollment, mg/dl (IQR)	1.4 (1.1 - 1.8)	1.4 (1.1 - 1.8)
eGFR at enrollment, ml/min/1.73m^2 (IQR)	49.7 (38.1 - 64.9)	50.7 (40.0 - 66.0)
Type of kidney transplantation (count)		
retransplantation	14%	14%
single kidney	91%	88%
HLAab pos	33%	31%
DSA pos	11%	11%
Number of HLA mismatches (SD)*		
class I: A, B locus	2.0 (1.1)	2.0 (1.2)
class II: DR locus	0.9 (0.7)	1.0 (0.7)

IQR = interquartile range; eGFR = estimated glomerular filtration rate; TX = transplantation; neg = negative; pos = positive

Appendix II: Prior Printed Publications

Parts of this thesis have been published in peer-reviewed journals and books as listed here. A complete list of publications is compiled in the curiculum vitae.

Peer-reviewed journals:

Lachmann N, Todorova K, Schulze H, Schönemann C. *Systematic comparison of four cell- and Luminex-based methods for assessment of complement-activating HLA antibodies.* Transplantation 2012 (accepted for publication).

Waiser J, Budde K, Schütz M, Liefeldt L, Rudolph B, Schönemann C, Neumayer HH, **Lachmann N**. *Comparison between bortezomib and rituximab in the treatment of antibody-mediated renal allograft rejection.* Nephrol Dial Transplant. 2012; 27 (3): 1246.

Cai J, Terasaki PI, Anderson N, **Lachmann N**, Schönemann C. *Intact HLA not beta2m-free HC-specific HLA class I antibodies are predictive of graft failure.* Transplantation. 2009; 88 (2): 226.

Lachmann N, Terasaki PI, Budde K, Liefeldt L, Kahl A, Reinke P, Pratschke J, Rudolph B, Schmidt D, Salama A, Schönemann C. *Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts.* Transplantation. 2009; 87 (10): 1505.

Books:

Lachmann N, Terasaki PI, Schönemann C. *Donor-Specific HLA Antibodies in Chronic Renal Allograft Rejection: A Prospective Trial with a Four-Year Follow-Up.* Clin Transpl 2006; 171.

Terasaki PI, **Lachmann N**, Cai J. *Summary of the Effect of de novo HLA Antibodies on Chronic Kidney Graft Failure.* Clin Transpl 2006; 455.

Affidavit/Eidesstattliche Erklärung

Herewith I declare on oath that I wrote this thesis independently and on my own. I clearly marked any language or ideas borrowed from other sources as not my own and documented their sources. Parts of this thesis, which have been published prior to completion, are documented in Appendix II: Prior Printed Publications as described in the Prüfungsordnung §5 sect. 1.6. Furthermore, I declare that I did not submit this dissertation elsewhere.

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Vorveröffentlichungen von Teilen der Arbeit sind gemäß §5 Abs. 1.6 der Promotionsordnung im Anhang II aufgeführt. Weiterhin versichere ich, weder anderweitig die Promotionsabsicht erklärt noch ein Promotionseröffnungsverfahren angestrebt zu haben.

Falkensee, den 21.12.2012

Nils Lachmann

Abbreviations

95% CI	95% confidence interval
A&E	Adsorption and elution
AA	Amino acid(s)
AMR	Antibody-mediated rejection
APC	Antigen-presenting cell(s)
AR	Acute rejection
AT_1R	Angiotensin II type-1 receptor
BSA	Bovine serum albumin
CAN	Chronic allograft nephropathy
CD	Cluster of differentiation
CDC	Complement-dependent lymphocytotoxicity test
CNI	Calcineurin inhibitor
CR	Chronic rejection
CREG	Cross-reactive group
CSA	Ciclosporin
CV	Coefficient of variance
DSA	Donor-specific antibody(ies)
DSE	Donor-specific epitope(s)
DSEab	Donor-specific epitope antibody(ies)
DTT	Dithiothreitol
EB	Elution buffer
eGFR	Estimated GFR
ELISA	Enzyme-linked immunosorbent assay
FC	Flow cytometry
FcR	Fc receptor
FCXM	FC crossmatch
GFR	Glomerular filtration rate
HAR	Hyperacute rejection
HC	Heavy chain
HLA	Human leukocyte antigen
HLAab	HLA antibody(ies)
IF/TA	Interstitial fibrosis and tubular atrophy
IFNγ	Interferon gamma
IQR	Interquartile range
IS	Immunosuppression/immunosuppressive

LSWB	LABScreen washing buffer
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA	Major histocompatibility class I-related chain A-antigen
MICAab	MICA antibody(ies)
MLR	Mixed lymphocyte reaction
MPA	Mycophenolic acid
NDSA	Non-donor specific HLAab
neg	Negative
NK	Natural killer
Nx	Nephrectomy
OR	Odds ratio
PBMC	Peripheral mononuclear cell(s)
PCR	Polymerase chain reaction
PE	R-phycoerythrin
pos	Positive
PRA	Panel reactive antibody
PTC	Peritubular capillary(ies)
QD	Quantum dots
RT	Room temperature
SAB	Single antigen bead(s)
sC1qA	Sandwich C1q assay
SCr	Serum creatinine
SD	Standard deviation
SPA	Solid-phase immunoassay
SSO	Sequence specific oligonucleotide
SSP	Sequence specific primer
TBE	Tris borat EDTA
TCMR	T-cell-mediated rejection
TG	Transplant glomerulopathy
TX	Transplantation
xi	Chimeric
XM	Crossmatch
β2m	Beta-2-microglobulin

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