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Finland

The relevance of donor-specific HLA antibodies in renal transplantation

Juha Peräsaari

ACADEMIC DISSERTATION

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To my family

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1 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. Haimila K, Peräsaari J, Linjama T, Koskela S, Saarinen T, Lauronen J, Auvinen MK, Jaatinen T. HLA antigen, allele and haplotype frequencies and their use in virtual panel reactive antigen calculations in the Finnish population. Tissue Antigens. 2013 Jan;81(1):35-43

II. Peräsaari J, Jaatinen T, Merenmies J. Donor-specific HLA antibodies in predicting crossmatch outcome: Comparison of three different laboratory techniques. Transpl Immunol. 2018 Feb;46:23-28

III. Miettinen J*, Peräsaari J*, Lauronen J, Qvist E, Valta H, Pakarinen M, Merenmies J, Jalanko H. Donor-specific HLA antibodies and graft function in children after renal transplantation. Pediatr Nephrol. 2012 Jun;27(6):1011-1019

IV. Peräsaari JP, Kyllönen LE, Salmela KT, Merenmies JM. Pre-transplant donor-specific anti-human leukocyte antigen antibodies are associated with high risk of delayed graft function after renal transplantation. Nephrol Dial Transplant. 2016 Apr;31(4):672-678

The original publications I-IV are referred to in the text by their roman numerals, and are reproduced with the kind permission of their copyright holders. Also some previously unpublished data are presented.

*Authors with an equal contribution on the article.

2 ABBREVIATIONS

AMR	antibody-mediated rejection
ARR	acute reversible rejection
AUC	area under curve
BMI	body mass index
CDC	complement-mediated lymphocytotoxicity test
CDCXM	complement-mediated lymphocytotoxicity crossmatch
CI	confidence interval
CIT	cold ischemia time
CPRA	calculated PRA
DGF	delayed graft function
DSA	donor-specific HLA antibodies
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESRD	end-stage renal disease
FCXM	flow cytometric crossmatch
GFR	glomerular filtration rate
HLA	human leukocyte antigen
IF/TA	interstitial fibrosis and tubular atrophy
LXM	Luminex crossmatch
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
OR	odds ratio
PE	R-phycoerythrin
PRA	panel reactive antibody
ROC	receiver operating characteristic
RR	relative risk
RRT	renal replacement therapy
RTx	renal transplantation
SBT	Sanger sequencing
SRR	steroid-resistant rejection
SSO	sequence-specific oligonucleotide probes
VXM	virtual crossmatch

3 ABSTRACT

Renal transplantation is a preferred choice of treatment for patients who have lost their kidney function due to end-stage renal disease (ESRD). Transplantations in Finland have been centralized to Helsinki University Hospital for both paediatric and adult patients. The results of the kidney transplantation program in Finland have improved over the years and are now excellent, with a one-year graft survival of over 90 %. The major improvements concern short-term problems, and the main challenge today is chronic rejection and long-term survival. The key to further improvements is prevention of chronic rejection and the adequate level of immunosuppression.

This thesis was designed to study the prevalence of human leukocyte antigen (HLA) antibodies and to evaluate the relevance of identified donor-specific antibodies (DSA) in the graft outcome. The focus was on graft function, as assessed by the glomerular filtration rate (GFR) and occurrence of delayed graft function (DGF). Another goal for this study was to evaluate various techniques for measuring the immunisation status of a patient and the sensitivity and specificity of different crossmatch techniques with the aim of developing practices in histocompatibility testing.

Several cohorts were used in this study. HLA allele frequency, haplotype and panel reactive antibody (PRA) calculations included the data provided by the Finnish Bone Marrow Donor Registry (19807 individuals) and the Finnish Cord Blood Bank (2699 individuals). In addition, 30 immunised patients were included. A total of 235 patients waiting for kidney transplant and 40 deceased donors were used in the prediction of crossmatch outcome. Retrospective clinical studies included 123 pediatric and 771 adult kidney transplant patients.

In our study of the Finnish population, a limited amount of allelic diversity was found. For many HLA antigens, practically only one allele is identified. For example, it is extremely unlikely that A3, A11 and A24 are found to be alleles other than *A*03:01, A*11:01 or A*24:02*, respectively. Also, the most common Finnish HLA haplotypes have very high frequencies when compared to other populations and some haplotypes are unique to the Finnish population. In virtual PRA, HLA antibodies identified against all potential donors were assessed and reported as a PRA% value. This value describes the percentage of donors that present antigens

that the patient is immunised against. Due to the uniqueness of the Finnish HLA composition, the use of a calculated population-specific PRA provides a more accurate and reliable estimate of the level of immunisation against available donors

Three different crossmatch methods were compared against virtual crossmatch (VXM) results. The flow cytometric crossmatch (FCXM) and Luminex crossmatch (LXM) proved to be the most accurate methods according to the receiver operating characteristic (ROC) analysis, with area under curve (AUC) values of 0.861 and 0.805, respectively. The performance of the complement-mediated lymphocytotoxicity crossmatch (CDCXM) was not as good (AUC: 0.724). There was no clear correlation between the serum samples providing false positive and negative results in each crossmatch technique, which indicates that the main reason for the differences is that each method identifies a different type of antibodies.

In the pediatric cohort, HLA antibodies were detected in half of the samples. During the follow-up, one third of the patients presented antibodies against the transplanted kidney. We did not find any association between DSA and poor GFR at the time of sampling or later during the follow-up.

In the adult cohort one third (265/771) of the patients were immunised. DSA was detected in 13% (103/771) of the patients at the time of transplantation, even with a negative CDCXM. DGF was more common in patients with DSA than in non-immunised patients (48% and 26%, respectively). DSA against all loci contributed a risk for DGF, but DRB1 seemed to provide the highest relative risk (RR) individually (RR 2.4). Also, the number of DSA and the strength of DSA as measured by cumulative mean fluorescence intensity were significant factors.

4 INTRODUCTION

The human kidneys are a pair of organs situated at the back of the abdominal cavity. The role of the kidneys is to excrete soluble waste and regulate body fluids, electrolytes and acid-base balance and to produce hormones. When kidney function is lost due to ESRD, renal replacement therapy (RRT) is needed. For this, there are two options: dialysis or renal transplantation (RTx). As RTx improves both quality of life and life expectancy compared to dialysis, it is therefore the preferred form of treatment (Tennankore et al. 2014). The first kidney transplantation in Finland was performed in 1964 (Salmela et al. 2004). In recent years, the average annual rate has been 250 transplantations (Scandiatransplant 2017). The number of pediatric RTx is approximately ten to twenty per year (Salmela et al. 2004).

The assessment of histocompatibility has played a critical role in RTx allocation since the beginning. Ideally, the recipient and the kidney donor harbor similar tissue types, in other words are HLA identical. As this is rarely achievable due to the wide variety of possible HLA combinations and the limited number of transplants, mismatching must be tolerated. The key to successful RTx is to use transplants with accepted immunological mismatches and avoid the ones with unfavorable outcome (Claas et al. 2004).

In addition to the blood group barrier, immunisation against foreign HLA is one of the greatest obstacles to receive a transplant with a good prognosis. Laboratory techniques for assessing the preformed humoral immunity of the recipients in the terms of its capability to damage the potential transplant have been used since the beginning of transplantation (Patel and Terasaki 1969).

CDCXM has been the gold standard in the detection of preformed donor-directed HLA antibodies (Graff et al. 2010). Although CDCXM enables the identification of a compatible donor for a patient and ensures safe transplant, it has some drawbacks, and therefore other crossmatch methods utilizing different technologies, e.g. flow cytometry and bead array, have been developed (Ho et al. 2008). As it is not possible to perform crossmatch between a donor and all potential recipients, a pre-selection of most suitable recipients is necessary. This is done through HLA matching and clinical estimation of the patients

Patients waiting for a transplant are screened regularly for HLA antibodies. Today, bead array is the preferred method to screen pre-existing HLA antibodies from the patient sera (Minucci et al. 2017). Predetermined antibody specificities allow virtual crossmatching to be performed as soon as the donor candidate has been typed for HLA. Prediction of a crossmatch test result based on HLA antibodies identified with solid phase assays is not optimal as the sensitivity of these methods is very different (Eng et al. 2008; Wahrmann et al. 2013).

The current practice in Finland is to perform CDCXM-negative transplantations without VXM assessment, except when the patient is prioritized due to high immunisation level. The value of VXM, however, is under debate as many of the patients with pre-formed HLA antibodies against the graft, have acceptable transplant outcome after all (Gupta et al. 2008). The challenge is to identify preformed DSA with clinical relevance. Pretransplant DSA have been reported to associate with several clinical complications such as antibody-mediated rejection, graft loss and DGF (Caro-Oleas et al. 2012a; Gilbert et al. 2011; Willicombe et al. 2017). However, the clinical course for individual patient with positive VXM is poorly predicted.

5 REVIEW OF THE LITERATURE

5.1 Renal transplantation

RTx is the best treatment for patients with ESRD. Both the life expectancy and quality of life of transplanted patients is better than for patients remaining on dialysis (Lloveras et al. 2015; Tennankore et al. 2014). The incidence of Finnish patients in RRT was 84 per million population (total n=461) in 2014 (Pippias et al. 2017). This was the lowest incidence in the Nordic countries, while in Europe as a whole, the incidence was 133 per million population.

The kidney diseases leading to ESRD differ in adults and children. In Finland, the most common diseases in adults, are diabetic kidney disease, glomerulonephritis, polycystic kidney disease, and nephrosclerosis (Finnish registry for kidney diseases 2016). For children, the most common causes are congenital nephrotic syndrome of the Finnish type, other hereditary kidney diseases, and congenital urological anomalies (Holmberg and Jalanko 2016). The graft survival of transplanted kidneys has improved over the years (Collaborative transplant study 2017). The half-life of kidney transplants is approximately 22 years (Fig. 1).

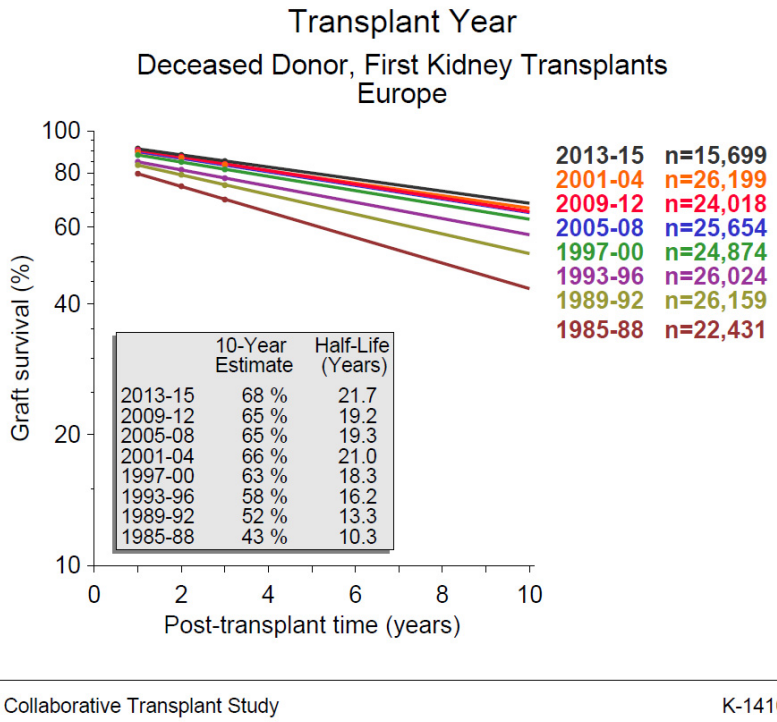

Figure 1. Graft survival according to transplantation year in Europe. Reproduced with permission from the publisher (Collaborative transplant study 2017).

5.2 Transplantation immunology

5.2.1 Human leukocyte antigens

HLA complex is located in the chromosome 6p21.3 and it encodes major histocompatibility complex (MHC) proteins. With more than 17,000 alleles identified, the HLA genes are highly polymorphic (Robinson et al. 2015). Classical HLA molecules are divided into class I and class II molecules. HLA class I molecules (HLA-A, -B, -C) are heterodimers formed by two subunits: the membrane bound α chain encoded by the HLA gene and the non-polymorphic ß2-micglobulin that stabilizes the structure. HLA class II molecules (DR, DQ, DP) are heterodimers formed by two transmembrane chains, α and ß (Fig. 2). These chains are encoded by separate HLA genes, with *DRA1, DQA1, DPA1* encoding α chains

and *DRB1, DRB3, DRB4, DRB5, DQB1, DPB1* encoding ß chains. There is a strong linkage disequilibrium in the HLA complex and the entire HLA complex is usually inherited as a haplotype. With a low recombination frequency, some allelic combinations are more frequent than expected by chance.

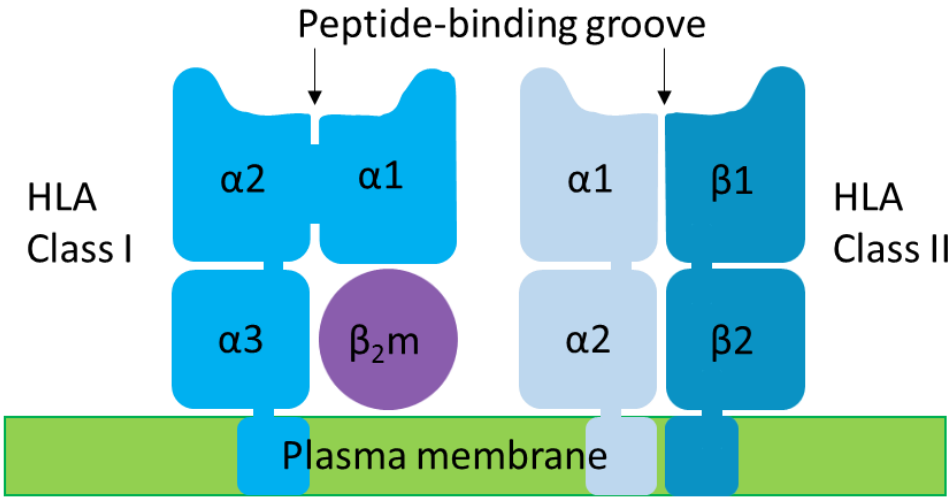

Figure 2. Structure of HLA molecules. The HLA class I molecule is a heterodimer formed by the α chain and the non-polymorphic ß2-microglobulin. The peptide binding groove of the HLA class I molecules is formed by α1 and α2 domains. The HLA class II molecule is a heterodimer of α and ß chains. The peptide binding groove of the HLA class II molecule is formed by α1 and ß1 domains encoded by different HLA genes.

HLA class I and II differ in their expression. Class I molecules are expressed on the cell surface of all nucleated cells whereas class II molecules are expressed only in antigen presenting cells such as dendritic cells, macrophages, activated T cells, and B cells. The role of HLA molecules is to present peptides to T lymphocytes. HLA class I molecules present peptides derived from degraded intracellular proteins of the cell. These include self-peptides and peptides derived from viral infection. HLA class II molecules present peptides derived from extracellular proteins that are endocytosed into the cell. These extracellular proteins can be derived from extracellular pathogens, e.g. from bacterial infection. Presentation of

peptides to T cells is required for the activation of adaptive immune response (Klein and Sato 2000). The repertoire of peptides presented by each HLA molecule depends on the structural properties of the peptide binding groove of each allelic variant. Each HLA molecule has the capability to present all peptides fulfilling the peptide binding pocket's criteria related to structural, electrostatic, hydrophobicity, and hydrogen bond donor/acceptor properties (Doytchinova and Flower 2002).

5.2.2 Allorecognition

HLA molecules contribute to individuals' ability to distinguish their own tissues from foreign tissue. There are two main forms of allorecognition, direct and indirect (Marino, Paster, Benichou 2016). In direct allorecognition, transplanted donor-derived HLA-peptide complexes presented by donor dendritic cells are directly recognized by the T cells of the recipient, leading to a response against the cells expressing the foreign molecule complex. This response may be formed against the allopeptide presented by the foreign HLA or the foreign HLA molecule itself (Boardman et al. 2016). In indirect allorecognition, allopeptides from the transplant are presented by the recipient's own antigen presenting cells (Benichou and Thomson 2009). The direct pathway has been considered more important in rejection. However, the direct alloresponse weakens gradually, possibly due to the tolerogenic properties of alloantigen presentation by the parenchymal cells of the transplant. In contrast, the indirect pathway is expected to be permanently active, due to the presence of recipient dendritic cells in the graft (Rogers and Lechler 2001).

5.2.3 HLA antibodies

The primary cause of antibody formation is exposure to foreign HLA antigens as a result of pregnancy, blood transfusion, or organ transplantation (Table 1). When antibodies were measured by bead array (Luminex), subjects without known immunisation events had a positive HLA-antibody rate of 12.3%, while patients with a history of blood transfusion, pregnancy or transplantation showed an immunisation rate of 22.8%, 53.1% and 88.5%, respectively; antibodies induced by previous transplantation or pregnancy produced higher mean fluorescence intensity (MFI) values than antibodies induced by transfusion (Lopes et al. 2015).

In addition, antibodies induced primarily by pregnancy exhibit significantly stronger response against restimulation by the new graft than antibodies induced by previous transplant or transfusion (Higgins et al. 2015).

Table 1. Predictors of allosensitisation

	OR	95% CI	P Value
Age	1.011	0.991–1.031	0.29
Female sex	3.633	1.904–6.932	<0.001
Sensitisation			
None	reference		
Pregnancy only	2.702	1.243–5.874	0.012
Transfusion only	1.973	1.121–3.472	0.018
Transplantation only	60.084	23.338–156.684	<0.001

OR, Odds ratio; CI, confidence interval (Lopes et al. 2015).

Antibody formation is a result of a cascade where foreign cells shed alloantigens, including soluble HLA molecules. B cells of the recipient have membrane bound antibodies on their surface that act as B cell receptors (BCR) for extracellular (donor) antigens. Alloantigens are gathered through B cell receptor-mediated uptake (Valenzuela, Hickey, Reed 2016). Then, the activated host B cells present allogenic peptides using the HLA class II molecules on their cell surface to associated T cells that recognize the MHC peptide complex through their T cell receptor. Interaction between CD40 ligand and CD28 on T cells and CD40 and CD80/86 on B cells, combined with the production of several cytokines, facilitates differentiation of both T cells and B cells into effector and memory subsets (Karahan, Claas, Heidt 2017). While T cells can become activated as effector cells or differentiate into memory T cells, B cells convert to antibody-producing plasma cells and memory B cells (El-Awar, Jucaud, Nguyen 2017; Karahan, Claas, Heidt 2017) (Fig. 3).

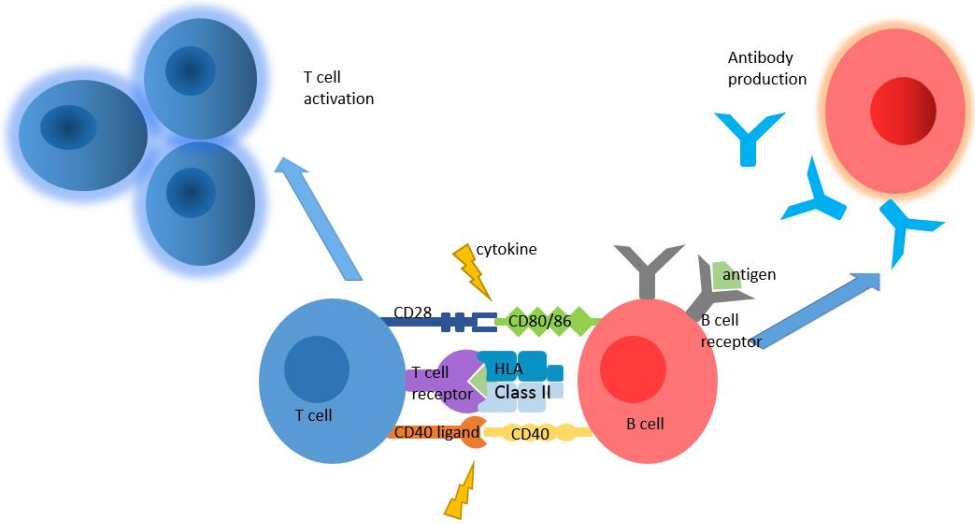

Figure 3. Antibody production. Alloantigens gathered through B cell receptor-mediated uptake are processed and then presented by HLA class II molecules. T cells recognize the MHC peptide complex through their T cell receptor. Costimulatory factors and cytokines enable the activation of T cells and differentiation of B cells into antibody-producing plasma cells. Modified from (Karahan, Claas, Heidt 2017).

The structure in the HLA molecule that is recognized by an antibody is called epitope (Fuller et al. 1990). This three-dimensional structure can be public, meaning that it is common for different HLA antigens or alleles. A good example of a public epitope is Bw4, which is present in half of the HLA B molecules and in some HLA A molecules. Other epitopes are private, meaning that they are found only in one antigen or one allele (El-Awar, Jucaud, Nguyen 2017). The broadness of the immunization largely depends on whether the target of an alloantibody is a public or private epitope.

The antibodies formed can be of different immunoglobulin classes, of which IgM and IgG are of importance primarily in organ transplantation. While IgM antibodies are considered irrelevant for the survival of the graft (Everly et al. 2014), they are not irrelevant in the activities of a histocompatibility laboratory as special measures must be taken to exclude them from various tests, such as

complement-mediated cytotoxicity and bead array tests. It is the IgG antibodies that mediate the destructive effect of immunisation and most antibody screening techniques measure the anti HLA IgG antibodies. Successive IgG subclass switching takes place during the progression of antibody production, typically from IgG3 to IgG1 to IgG2 to IgG4 (van Zelm 2014). Not all IgG antibodies are equal. It has been shown that complement-binding subtypes of IgG antibodies cause most of the problems in organ transplantation. IgG3 shows the strongest complement-binding capacity, followed by IgG1. While IgG2 activates complement only weakly, and IgG4 does not activate complement at all, both of these subclasses are able to recruit effector cells through the Fc receptor (Zhang 2017).

5.2.4 Histocompatibility in renal transplantation

The immunological assessment of a donor and potential recipients consists of three major components. The first of these is based on ABO blood group matching. In Finland, ABO compatibility is required for deceased donor transplants. Secondly, histocompatibility of the recipient and the donor is determined at the level of shared HLA antigens. In RTx, this is usually determined by the shared HLA-A, -B and -DRBI antigens. Patients fulfilling the criteria set for a sufficient HLA match (or prioritized by clinical parameters) will proceed to a prospective crossmatch, in which the presence of preformed antibodies against the donor cells is determined. This is crucial as compatibility between recipient and donor is rarely a full match due to the high level of polymorphism at the HLA. Therefore, preformed HLA antibodies are considered as a part of histocompatibility assessment with crossmatching.

In addition to the standard protocol, based on ABO-compatibility, HLA-A, -B, -DRB1 matching and crossmatching, there are other protocols, especially for highly immunised patients, such as the Scandiatransplant acceptable mismatch program (Koefoed-Nielsen et al. 2017). This program utilizesinformation on routinely screened and identified HLA antibodies and the HLA type of the donor in virtual crossmatch.

5.2.5 Immunosuppression

Even with all efforts made to achieve good histocompatibility between recipients and donors, the immune system recognizes the new kidney as foreign tissue and initiates a response to destroy it, unless prevented from doing so by immunosuppressive medication. Immunosuppressive drugs lower the ability of the immune system to reject a transplanted organ. Immunosuppressive drugs are classified into several categories: calcineurin inhibitors, antiproliferative agents, corticosteroids, and targeted antibodies. Most of these drugs are targeted against T cell function. Calcineurin inhibitors prevent the release of calcineurin and interleukin-2 transcription; mycophenolic acid prevents purine (nucleotide) synthesis; inhibitors of the mammalian target of rapamycin induce cell cycle arrest in T cells; azathioprine inhibits DNA synthesis; and steroids inhibit transcription of inflammatory cytokines, while monoclonal and polyclonal antibodies have a range of targets (Lim, Kohli, Bloom 2017). In addition to the desired properties, immunosuppressive drugs have many side-effects, such as nephro toxicity, dyslipidemia, glucose intolerance, and increased risk for infections and cancer (Halloran 2004). Usually combination therapy allowing reduced doses of individual drugs is used to avoid side effects.

5.2.6 Rejections

The process leading to rejection involves three stages: 1. Recognition of the transplant as non-self, 2. Generation of immune response, 3. Destruction of transplanted organ (Becker, Morath, Suesal 2016). The underlying mechanism in rejection can be either T cell-mediated or antibody-mediated. Rejections can be classified according to timing and histological findings into hyperacute (minutes to hours), acute (days to months), and chronic (months to years) (Mehra and Baranwal 2016).

Hyperacute kidney rejection takes place immediately after the kidney transplantation as a consequence of high levels of preformed complement-activating DSA. This is very rare today, but may occur if HLA immunisation has been missed in pre-transplant crossmatch and antibody screening processes. In hyperacute rejection, binding of DSA to the vascular endothelium of the transplant activates the complement system. This leads to a massive inflammatory response and vascular thrombosis (Becker, Morath, Suesal 2016)

Acute rejection may occur anytime within the first months of the transplantation procedure. A T cell-mediated response against foreign HLA is the key factor in acute cellular rejection, with infiltrates of CD4+ and CD8+ T cells found in the renal interstitium, tubuli and blood vessels. In acute antibody-mediated rejection (AMR), donor-specific HLA antibodies attach especially to peritubular capillaries and induce damage in the endothelium (Chalasani et al. 2004).

Chronic rejection develops after the acute rejection episodes have subsided. Chronic rejections are both antibody- and T cell-mediated (da Silva et al. 2017). As the most common type of rejection, chronic rejection is the reason for the majority of transplant failures seen today (Moreau et al. 2013).It develops in transplants that are exposed to recurring or continuous cellular and humoral responses resulting from indirect recognition of alloantigens (Joosten et al. 2005). Mononuclear infiltrates with a high number of plasma cells are present. Inflammatory events and insufficient immunosuppression can lead to the formation of de novo DSA, resulting in long-term damage to the transplant (Becker, Morath, Suesal 2016)

Another way to classify rejections is to use the response to steroid administration (methylprednisolone) as a criterion. Overall, 60-70% of acute rejections respond to steroid treatment and are therefore classified as acute reversible rejections (ARR), with the rest of the rejections regarded as steroid-resistant rejections (SRR) (Bock 2001). Rejections responsive to steroid treatment usually have a cellular origin, while rejections without response are more likely of humoral background (Rekers et al. 2016).

5.3 Histocompatibility and transplant outcome

5.3.1 Effect of mismatches

Good matching provides several benefits in kidney transplantation, such as longer graft survival, better graft function, and a lower risk for infection and malignancy due to reduced level of immunosuppression (Opelz and Dohler 2013). However, well-matched transplants are not available for all patients within a reasonable waiting time, due to the rarity of some tissue types. Therefore, transplants with

differing degrees of HLA mismatching are transplanted consistently. According to the early findings of Collaborative Transplant Study in 1985, transplantations with no mismatch in HLA-B and –DR show a 20% higher success rate than mismatched transplants (Opelz 1985). This difference is still seen in the modern era with advanced immunosuppression (Collaborative transplant study 2017) (Fig. 4).

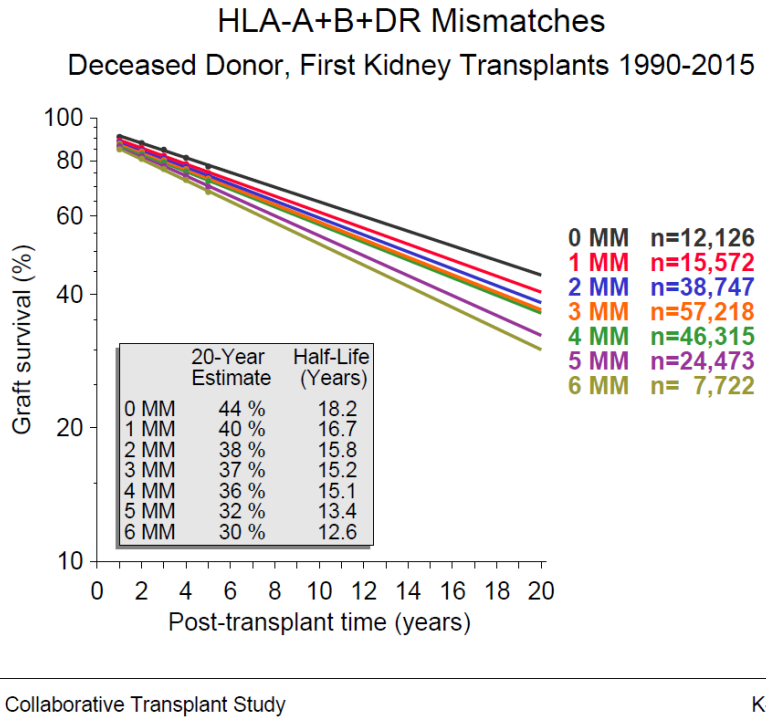

Figure 4. Graft survival according to mismatches in HLA-A, -B, -DR. Reproduced with permission from the publisher (Collaborative transplant study 2017).

HLA mismatches sustain the risk of sensitisation when the graft is lost. For patients waiting for a retransplant, prior mismatches can decrease the chance of finding a suitable organ and thus increase the waiting time.

As mismatches carry a risk for complications they also have an effect on the medical costs. In the U.S. patients receiving zero mismatch transplants, the

average three year medical costs are $60,436, while in patients with a full mismatch the transplant costs rise to $80,807 (Schnitzler et al. 1999).

5.3.2 Effect of preformed antibodies

Immunisation against HLA antigens lowers the likelihood of receiving a transplant as patients with preformed antibodies face a risk of a positive crossmatch result preventing transplantation. Since the sensitive solid phase assays, like bead array, were implemented in routine antibody screening, there has been debate over the relevancy of identified DSA in crossmatch-negative situations. There are conflicting reports on the importance of such antibodies (Amico et al. 2011; Aubert et al. 2009; Caro-Oleas et al. 2012a; Lefaucheur et al. 2010). It has become clear that preformed DSA have an effect on the population level but the prediction of the clinical course for an individual patient is not that simple.

In a study by Riethmuller et al., immunised patients with negative T cell CDCXM were transplanted. The 1 year cumulative incidence of AMR was lower in the negative VXM than in the positive VXM group (6% vs. 35%, respectively). No difference was seen in T cell-mediated rejections (41% vs. 40%, respectively) (Riethmuller et al. 2010). It has also been reported that both class I and class II DSA increase the risk for graft loss. In patients with class I DSA alone, the relative risk (RR) of graft loss was 5.174 (95% CI 2.416–11.079), whereas in patients with class II DSA alone RR was 2.576 (95% CI 1.236–5.368) (Caro-Oleas et al. 2012a). The MFI value of the detected DSA has a great impact as shown by Lefaucheur et al. The prevalence of AMR rises to 0.9%, 18.7%, 36.4%, 51.3% as the MFI of highest DSA increases <465, 466-3000, 3001-6000, >6000 MFI, respectively. Similarly, the eight year graft survival decreases to 82.5%, 78.4% 60.6% with the rising DSA MFI values of <465, 466-3000, >3000 MFI, respectively (Lefaucheur et al. 2010).

5.3.3 Effect of *de novo* DSA

HLA mismatches between the graft and recipient predisposes the patient to produce antibodies against the graft. HLA-DR mismatches in particular are of importance as due to the strong linkage equilibrium they are often combined with DQ mismatches. In addition, HLA class II antibodies have a stronger impact on

AMR and graft loss (Willicombe et al. 2011). In a study of 505 patients without preformed DSA, 18% developed *de novo* DSA. DSA against antigens: HLA-A, HLA-B, HLA-DR, and HLA-DQ associated with AMR and transplant glomerulopathy, while HLA-Cw associated only with transplant glomerulopathy. HLA-DQ DSA associated with an inferior 40-month allograft survival than non-DQ DSA (76% vs. 95%) (Willicombe et al. 2012). It must be noted that antibody formation itself is not a risk factor if the antibodies are not directed against antigens present on the graft. No significant differences in rejection episodes have been observed between non-immunised patients and patients with third-party antibodies (Caro-Oleas et al. 2012b).

In a study by Everly et al., patients tested post-transplant with single antigen beads for DSA IgG, IgG3 and IgM showed additive effect between immunoglobulin classes and subtypes. Almost all (95%) of the patients that developed alloimmune response presented IgM DSA and half (47%) had IgG DSA. IgM DSA alone did not increase the risk for graft loss. However, patients showing IgG isotype switch to IgG3 with the persistent IgM (19%) had the highest risk (47%, median 72 month follow-up) for graft loss (Everly et al. 2014).

5.3.4 Non-immunological factors

There are known donor-related factors involved in transplant outcome, such as donor age and cause of brain death. The use of donors with expanded criteria has highlighted the importance of donor factors to graft survival. These criteria include age either above 60 years or between 50 and 59 years combined with two of the following additional characteristic: donor history of cerebrovascular accident, hypertension, and a serum creatinine level higher than 1.5 mg/dL (132.6 μmol/L) (Johnston et al. 2004). A three year graft survival of 75% was reported for expanded criteria donors as compared to 84% for standard criteria donors (Ferrer et al. 2009). The recipient and transplant related factors predisposing to unfavorable outcome are hepatitis C virus-positivity, high weight, diabetes, hypertension, and long waiting time (Caro-Oleas et al. 2013; Khalkhali et al. 2010). A study by Gibling et al. shows that cold ischemia time (CIT) has a clear effect on graft survival when the first and second kidneys of the donor are compared (Giblin et al. 2005). With a mean CIT of 19.9 h for the first kidney and 25.7 h for the second kidney, a significant difference was observed in the graft survival rates. Graft survival at 1 year was better for the first kidney than for the second one (88.5% vs.

84.7%), and the difference persisted at 10 years (55.2% vs. 40%, respectively). Altough CIT is a non-immunological factor, a prolonged CIT may elevate humoral immunogenicity of the transplant, and promote higher production of HLA class I antibodies (Bryan et al. 2001).

5.4 Other complications and their relation to immunisation

5.4.1 Delayed graft function

In a study by Humar et. al., an elevated PRA of >75% resulted in an elevated risk for DGF, (RR 3.4, p= 0.0001) (Humar et al. 2002). Other reported recipient-related risk factors for DGF according to multivariate analysis are male gender, BMI>30 kg/m^2, primary cause of ESRD (diabetes), and longer maintenance dialysis. Transplant-related factors include HLA mismatches, donor-recipient size-mismatch, CIT, and low transplant center volume (Doshi et al. 2011).

5.4.2 Impaired glomerular filtration

The data on the relation between DSA and GFR is limited. It is known that chronic antibody-mediated rejection is caused by DSA and eventually leads to increased proteinuria and decreased GFR. Adult patients with DSA have been shown to have significantly higher proteinuria levels, and proteinuria seems to be an important factor in determining rapid GFR decline (DeVos et al. 2012; Immenschuh et al. 2015). The direct association between DSA and GFR is somewhat inconclusive. In a study by Rusai et al., no association between DSA status and poor GFR was found (Rusai et al. 2016). Conflicting results were reported by Chaudhuri et al., describing decline of graft function at the presence of DSA (Chaudhuri et al. 2013).

5.5 Laboratory techniques measuring immunisation

5.5.1 Complement-mediated lymphocytotoxicity test (CDCXM)

This landmark technique was introduced and published in 1964 by Terasaki and McClelland in the first histocompatibility workshop (Terasaki and McClelland 1964). Until then, leukoagglutination had been the most commonly used test to identify HLA antigens. This new method, which used only 0.1% reagents compared to agglutination test, made it possible to perform the groundwork for identifying HLA antigens in HLA workshops (Terasaki 2012). The clinical relevance of this test as a measure of immunisation was demonstrated by Patel and Terasaki in a study where only eight of 195 (4%) crossmatch-negative kidneys failed, whereas 24 out of 30 (80%) crossmatch-positive kidneys were lost immediately (Patel and Terasaki 1969). Ever since, this test has been used to detect preformed antibodies against the graft. In addition to HLA typing, this technique can be used to screen and identify pre-formed antibodies or for CDCXM (Fig. 5).

When this test is used for antibody screening, a panel of cells with known HLA types is incubated with the serum of interest in the presence of rabbit complement. The presence of complement-binding antibodies launches a cascade where a membrane attack complex of complement is formed on the target cell surface. As a result, channels to the cell surface are formed, allowing staining with otherwise non-permeable stains. The readout of the test is the percentage of permeable (dead) cells of the total cell population on the scale of 1-8, with the higher scores representing a stronger reaction. The proportion of different cells in the panel that are subject to killing by the serum antibodies are represented as the percentage of PRA. This PRA percentage is used as a measure of the immunisation level. Patients with a high PRA are therefore at a higher risk of having antibodies against the potential donor.

In the crossmatch, the recipient serum is tested only against the potential organ donor. This test detects all antibodies capable of cytotoxicity in the presence of complement, being therefore either IgG or IgM. A variant of the test excludes IgM antibodies with the use of reducing agent dithiothreitol (DTT). The DTT treatment clears disulfide bonds of the IgM molecule tertiary structure, while leaving other immunoglobulin classes unaffected (Taylor et al. 1989). The biggest impediment of the CDCXM method is the need for viable cells. CDCXM still holds its place as, in

this test, the donor antigens are presented in a native form on target cells close to their natural expressional proportions.

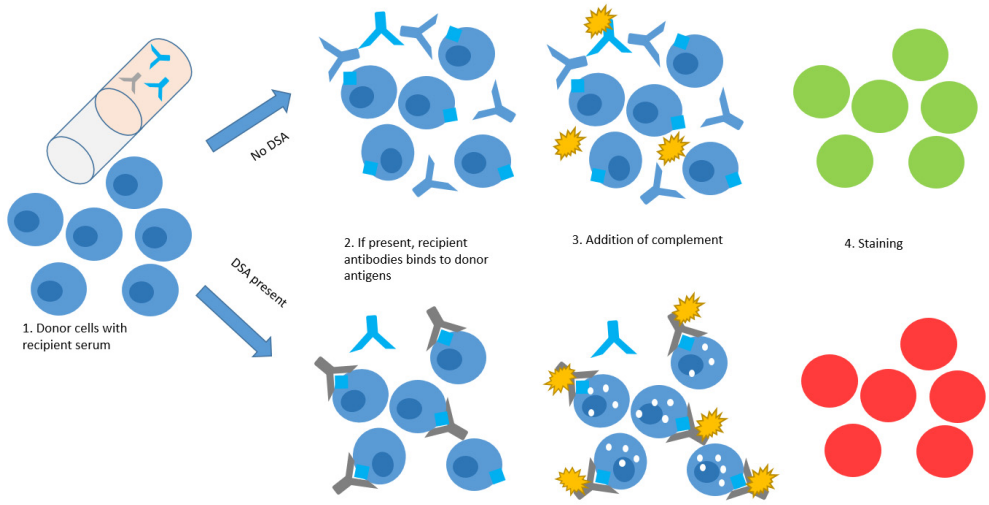

Figure 5. CDCXM. 1. Donor cells are incubated with the recipient serum. 2. Antibodies identifying donor antigens bind to the lymphocytes. 3. with addition of complement cells with complement fixing recipient antibodies lyse. 4. Lysed cells are identified with viability staining.

5.5.2 Flow Cytometry (FCXM)

Flow cytometric crossmatch was introduced to kidney transplantation protocols in 1983 (Scornik et al. 1994). This method is considered to be more sensitive, quantitative, and objective than CDCXM. In this method, donor lymphocytes are incubated with the recipient serum. Recipient antibodies bound to the donor cells are identified with a secondary fluorochrome-conjugated antibody against human IgG. The use of different fluorochrome-labelled secondary antibodies being easy makes it possible to detect different immunoglobulins, such as IgM and different IgG subtypes. The cell type of interest can be detected with another set of fluorochrome-labelled antibodies, for example T cells with anti-CD3 antibodies and B cells with anti-CD19 antibodies. The labelled cells are analyzed with a flow cytometer and the results are compared to a negative control (a serum pool from non-immunised individuals). Whenever fluorescence against an immuniglogulin is

detected at at a level that exceeds the threshold for a positive result, the test is considered positive (Fig. 6). Similarly to CDCXM, FCXM is not specific to HLA antibodies and false positive results occur due to non-HLA antibodies (Kerman et al. 1999). As a secondary antibody directed against human IgG is generally used, this method detects also non-complement activating antibodies. When B cells are used as a target, false positivity is caused by the formation of irrelevant antigen-antibody complexes through binding of Fc-fragments to the Fc-receptors (Altermann et al. 2006; Delgado and Eckels 2008). Eng et al and Le Bas-Bernardet et al have reported that at least as many as two thirds of positive B cell crossmatches were false positives as no donor-specific HLA antibodies were present in the tested sera (Eng et al. 2009; Le Bas-Bernardet et al. 2003). This suggests that B cell crossmatch should always be interpreted in combination with the antibody identification results.

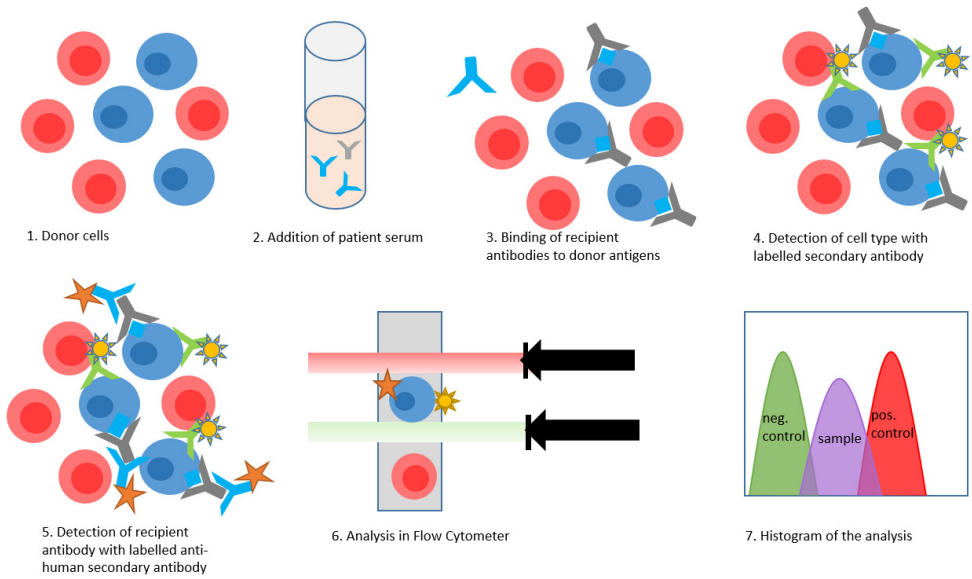

Figure 6. FCXM. 1. Donor cells are isolated. 2. Recipient serum is added. 3. Antibodies identifying donor antigens bind to the lymphocytes. 4. Target cells are identified with labelled antibody. 5. Binding of recipient antibodies to the donor cells is determined with labelled secondary antibodies. 6. Cells and the binding of recipient antibodies are analysed with flow cytometer. 7. Crossmatch result presented as a histogram.

5.5.3 Bead array

In solid phase assays, solubilized HLA molecules are bound to a surface e.g. microtiter plate (enzyme-linked immunosorbent assay) or polystyrene beads (bead array) (Hwang et al. 2012; Lachmann et al. 2013). Bead array refers to a single commercial platform known as "Luminex" and it is currently the most widely used solid phase assay. The Luminex platform utilizes a technology where polystyrene beads are filled with two fluorescent dyes in different proportions creating 100 colour combinations. These staining variants can be detected through the emitted fluorescent pattern excited by the red laser of the Luminex analyzer. The binding of antibodies to the HLA molecules on the beads is determined with a secondary antibody conjugated with R-phycoerythrin (PE). The emission following excitation with green laser is directly measured as the fluorescent intensity (Lachmann et al. 2013) (Fig. 7).

5.5.3.1 Antibody detection

The antibody detection protocol with Luminex consists of two stages: screening and identification. The screening kit contains a mixture of beads coated with purified HLA antigens pooled from multiple individuals. The identification is performed with single-antigen beads, where the antigens are purified from recombinant cell lines, one bead carrying only a single antigen.

The threshold for a positive result in a single-antigen analysis has been under debate as long as bead array techonology has been available (Liu et al. 2012; Piazza et al. 2014; Riethmuller et al. 2010). The assay was originally developed for qualitative use only, but as it provides fluorescent intensity against each antigen, the fluorescent value is used as a surrogate to address antibody strength in many studies (Chung et al. 2014; Ellis et al. 2012; Wu et al. 2013). Different values for clinically relevant thresholds for different conditions have been described as varying from 500 to 5000 MFI, while the generally used threshold for reporting a positive result is 1000 MFI. Separate clinical endpoints might be differentially affected by various quantities of antibodies (AMR, DGF, Graft loss) and the clinically relevant antibody level may vary depending on the endpoint measured (Bradley et al. 2011; Lefaucheur et al. 2010).

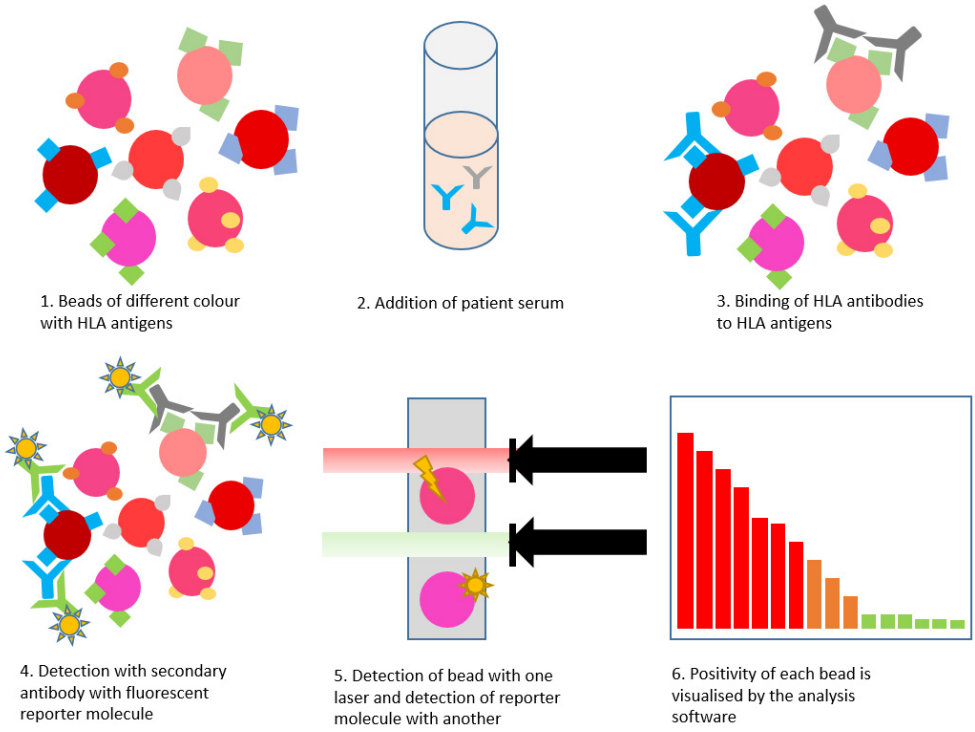

Figure 7. Antibody detection by bead array. 1. Polystyrene beads covered with HLA antigens are used. 2. Patient serum is incubated with the beads. 3. HLA antibodies present at the serum will bind to the beads. 4. HLA antibodies are identified with labelled secondary antibody against human IgG. 5. The bead carrying the HLA antigen is identified by the bead analyzer by one laser and the amount of IgG antibodies bound to the bead is identified by another laser. 6. The result is visualised by the analysis software.

There are some technical limitations to the assay. First, the amount of antigen on each bead is not standardized, resulting in differences in the amounts of various antigens on the bead surface. An antigen that is saturated on a bead will produce higher fluorescence values than antigens present at a lower density. Second, the densities of antigens on the beads are not in proportion to the densities found on the cell surface (Lachmann et al. 2013). HLA-Cw has markedly lower expression than other class I antigens and yet its density on the beads is similar to that of other class I antigens (Apps et al. 2015). Thus, antibodies against HLA-Cw are over-detected. Third, it has been reported that the conformation of HLA molecules

on the beads is sometimes affected by the manufacturing process leading to abnormal positivity (El-Awar et al. 2009). In addition, as the number of beads in the analysis is limited, the extensive variation seen in the HLA region cannot be represented comprehensively and therefore some antibodies may not be identified.

The sample itself may cause limitations. It has been reported that a high level of HLA-IgM antibodies may block the binding of IgG antibodies, resulting in false negative results (Kosmoliaptsis et al. 2009). Also, complement components can act as blocking agents for the secondary antibody used in the assay (Schnaidt et al. 2011). This phenomenon is now widely recognized and measures have been taken to eliminate the problem (Kosmoliaptsis et al. 2010; Zachary et al. 2009a). Treatment of sera with ethylenediaminetetraacetic acid (EDTA) has proven to be the most effective solution for false negative results and is now widely used in HLA laboratories worldwide (Schnaidt et al. 2011).

The main advantage brought by the bead array is the possibility to modify the standard assay with alternative secondary antibodies to enable the detection of different immunoglobulin classes or IgG subtypes. With additional steps it is also possible to detect capacity forcomplement binding (Loupy et al. 2013).

5.5.3.2 Luminex crossmatch (LXM)

The most innovative variant of the bead array is the Luminex crossmatch. In this assay, donor cells are lysed and HLA antigens are captured on the beads by HLA antibodies to produce beads with all HLA antigens of the individual donor. Therefore LXM enables an HLA-specific crossmatch. One of the advantages of LXM is that the preformed antibodies are routinely identified with the same technique and therefore it should carry a high predictive value. Indeed, it has been shown that there is a good correlation between the screening and crossmatching for HLA- A, -B, -C, DRB1 (Caro-Oleas et al. 2010). However, some findings suggest that the beads may not capture HLA-DQ and HLA-DP antigens as efficiently as other antigens (Billen et al. 2008; Caro-Oleas et al. 2010).

5.5.4 Calculated PRA (CPRA)

Calculated PRA, based on identified antibodies, has replaced the traditional cell panels in many laboratories. CPRA is calculated as the percentage of donors

identified in the population by the antibodies present in the potential recipient´s serum. This gives a much better estimate than the traditional PRA assessed with cell panels. These cell panels were not representative of the donor population as rare antigens were over-represented at the expense of common antigens. On the other hand, the Luminex technology used in CPRA shows a much higher sensitivity than the traditional complement-mediated lymphocytotoxicity test (CDC) method. As antibodies used in the PRA calculations are detected with a higher sensitivity than before, the CPRA of the patients on the waitlist has increased (Baxter-Lowe et al. 2016).

5.5.5 Virtual crossmatch (VXM)

A virtual crossmatch is performed by comparing the antibodies identified in the recipient serum with the HLA antigens of a particular donor. The presence of DSA indicates a positive crossmatch. The standard procedure for VXM is to identify antibodies with a sensitive method (Luminex) and HLA type the donor for every loci against which antibodies are present in the recipient serum (EFI Standards 2017). However, the term VXM is used in two very different scenarios. Usually VXM is performed together with a prospective CDCXM or FCXM, giving additional risk stratification for a negative "physical" crossmatch. In Finland, this strategy is used for living donor transplants. VXM is also used on its own, omitting a prospective "physical" crossmatch before entering the operating theatre. A common practice is to combine VXM and prospective crossmatch strategies. Patients with a negative VXM will be transplanted without a prospective crossmatch, while patients with a positive VXM will be transplanted only if the prospective crossmatch is negative. With this approach, a lower 1 year cumulative incidence of clinical/subclinical AMR rate was observed in the negative VXM group compared with the positive VXM group (8% vs. 42%, respectively), along with superior death censored allograft survival at 2 years (98% vs. 91%, respectively) (Amico et al. 2011).

6 AIMS OF THE STUDY

The goal of this thesis was to study the prevalence of HLA antibodies and evaluate the relevance of DSA for the graft outcome. Also, the performance of different methods used for evaluating the immunisation status of kidney transplant patients were compared.

The specific aims of the study were:

1. To assess the usefulness of population-based virtual PRA in comparison with the traditional panel in addressing immunisation status.

2. To study incidence of HLA antibodies in pediatric kidney transplant patients and their significance on graft function.

3. To analyze prevalence of preformed HLA antibodies and their impact on early kidney graft function.

4. To compare the predictive value of antibody screening results with different crossmatch methods and develop practices in histocompatibility testing.

7 MATERIALS AND METHODS

7.1 Study subjects

Study I. The HLA data from the Finnish Bone Marrow Donor Registry and Finnish Cord Blood Bank (19,807 and 2,699) were included for HLA allele frequency calculations. Haplotypes were determined for 504 individuals genotyped at a high-resolution level. For virtual PRA calculations, 30 serum samples from HLA antibody positive patients were used.

Study II. The work included 288 latest serum samples from 235 patients waiting for a kidney transplant in Finland. The patient sera were crossmatched against splenocytes from 40 consecutive deceased donor.

Study III. Altogether 123 pediatric kidney transplant patients transplanted at Children´s Hospital, Helsinki University Hospital, between 1989 and 2004, were included. Patients were selected if serum samples were available for HLA antibody screening and identification, and sufficient follow-up data were available.

Study IV. The study group included a total of 771 adult (≥16 years) patients who had received a deceased donor transplant at Helsinki University Hospital between 2000 and 2004. Only patients without surgical complications or thrombosis related to transplantectomy were included.

7.2 HLA typing (I, II, III, IV)

HLA typing was performed in the Histocompatibility Testing Laboratory of the Finnish Red Cross Blood Service. Results were reported according to the current World Health Organization nomenclature. Deceased donors were initially typed with the CDC (Biotest Rockaway, NJ, USA) and polymerase chain reaction with sequence-specific primers (One Lambda Inc., Canoga Park, CA, USA). Further supplementary typing was performed with polymerase chain reaction with sequence-specific oligonucleotide probes (SSO) (One Lambda) or with Sanger sequencing (SBT) (Atria Genetics, South San Francisco, CA, USA) in situations where donor-specificity of an antibody was unclear. The typing methods for the HLA data of the Finnish Bone Marrow Donor Registry and Finnish Cord Blood

Bank vary depending on the time of initial typing. At least CDC, SSO and SBT were used for typing registry donor candidates.

7.3 HLA antibody screening and identification (I, II, III, IV)

HLA antibodies were screened with commercial bead array kits (LABScreen® Mixed, One Lambda). Antibodies detected in the screening were identified with LABScreen® single antigen kits (One Lambda), with a cut-off value of the baseline normalized value of 500/1000 MFI. Antibodies were determined against all classical HLA class I (HLA-A, -B and -Cw) and class II (HLA-DR, -DQ and -DP antigens. In study II, also LABScreen® PRA kits were used. Antibody specificities were assigned with the HLA Visual™ or HLA Fusion™ software.

7.4 Complement-mediated lymphocytotoxicity crossmatch (II, III, IV)

CDCXM was performed on-call with donor splenocytes (Amos et al. 1969). Parallel crossmatches with different volumes of recipient serum (0.1 µl, 1 µl, 5 µl) were performed in duplicate. International workshop scoring was used to score the percentage of cell death with the modification that any cell death above background considered as positive: score 1: <1%; 2: 1-20%; 4: 21-50%; 6: 51-80%; 8: 81-100%. No DTT was used to neutralize IgM antibodies.

7.5 Flow cytometric crossmatch (II)

Retrospective T cell IgG, T cell IgM, and B cell IgG FCXM were performed with frozen deceased donor splenocytes (Matinlauri et al. 2004). In each test, 500 000 splenocytes with 50 µl of serum were used. T and B cell populations were identified with PE-anti-CD3 and PE-anti-CD19 (BD Biosciences, San Jose, CA). The binding of IgG antibodies was detected with FITC-conjugated F(ab)2 anti-human IgG (Jackson ImmunoResearch, West Grove, PA). Crossmatches were analyzed using FACScan instrument (BD Biosciences). A cut-off of 40 channel shifts for T cells and 60 channel shifts for B cells was set for a positive test result.

7.6 Luminex crossmatch (II)

LXM was performed according to the manufacturer's protocol, with the exception of using frozen splenocytes for the lysate preparation. For individual tests, a lysate of 2.2 x10^6 splenocytes was used. Donor antigens of the lysate were captured on the bead array beads with anti HLA class I or class II antibodies. The serum of interest was incubated with the beads coated with donor class I or class II HLA antigens. Binding of HLA antibodies to captured donor antigens was detected with PE conjugated anti-human IgG. Samples were run on Luminex (LabScan 200) and analysed with LifeMatch software (Tepnel, Lifecodes). A user-specific threshold for a positive result with MFI values above 1000 against all three negative control values was used.

7.7 Virtual crossmatch (II, IV)

For VXM, DSA were assigned by comparing detected antibodies to the donor's HLA. When DSA were identified, VXM was considered positive. The strength of VXM was described as cumulative DSA. The sum of all individual DSA above 1000 MFI was reported as the cumulative DSA.

7.8 Calculated (virtual) PRA (I)

For calculated PRA, the HLA fusion™ software with imported population data was used for the calculations of the Finnish population and the EuroTransplant calculator representing the European population (Eurotransplant Reference Laboratory 2018).

7.9 Clinical data (III, IV)

Clinical data were collected retrospectively from hospital records. This data included: kidney disease leading to RTx, recipient's age, and gender, BMI (Study IV), age of donor (Study III), HLA of recipient and donor, date of transplantation, transplant number, rejection episodes, CIT, DGF, kidney graft function with GFR, graft outcome, cytomegalovirus status (Study III), and allograft biopsy results at 1.5 and 3 years post-RTx (Study III).

7.10 Clinical endpoints

7.10.1 Delayed graft function (IV)

DGF was defined as fulfilment of one of the following criteria: serum creatinine >500 μmol/L throughout the first perioperative week, more than one dialysis session during the first week after transplantation, or oliguria (<1 L/day) two days after transplantation (Halloran et al. 1988). Conventional DGF was defined as a need of dialysis on the first perioperative week and slow graft function as an impaired creatinine clearance without need for dialysis (Humar et al. 1997).

7.10.2 Glomerular filtration rate (III)

GFR was measured as the total plasma clearance of Chromium-51 labelled ethylenediaminetetraacetic acid (Jodal and Brochner-Mortensen 2009). GFR values were corrected according to modified Brochner-Mortensen equation. GFR was measured at 3 and 6 months after RTx, and then annually with an average of 9 measurements per patient.

7.11 Bioinformatics and statistical analysis

Categorical variables were analysed with the Fisher's exact test. Binary logistic regression analysis or Mann–Whitney U-test was used to compare continuous variables. Sensitivity and specificity were assessed with the ROC analysis. The accuracy of the test was classified as the AUC with 0.9-1: excellent; 0.8-0.9: good; 0.7-0.8: fair; 0.6-.07: poor; 0.5-.06: fail. Results with a P value of ≤0.05 were considered statistically significant. All presented P values are uncorrected for possible multiple testing. Analyses were performed with the SPSS statistics version 21.0 software (IBM, Armonk, NY, USA).

7.12 Ethics

The Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry of the Helsinki and Uusimaa Hospital District approved the study protocol (86/2007,

126/2007, 269/2009, 22/E7/2007). The Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District approved the study protocol 42/13/03/00/2011. The Ethics Committee for Surgery of the Helsinki and Uusimaa Hospital District approved the study protocol 297/13/03/02/2008.

8 RESULTS

8.1 HLA haplotype frequencies and calculated PRA (I)

HLA allele and antigen frequencies of the Finnish population were studied. Also, the most common Finnish high-resolution haplotypes for five HLA loci were determined. Calculated population specific PRA was determined by correlating identified antibodies to the Finnish HLA population data.

8.1.1 HLA haplotypes

The genotypes of the Finnish patients waiting for stem cell transplantation were used for HLA haplotype analysis. A total of 372 different haplotypes were identified from 504 individuals. The most common HLA haplotypes and their frequencies are presented in Table 2. The most common Finnish haplotype: *A*03:01-B*35:01-C*04:01-DRB1*01:01-DQB1*05:01* dominated, being much more common than any other haplotype. The frequency of the second most common haplotype *A*01:01-B*08:01-C*07:01-DRB1*03:01-DQB1*02:01* was 37% lower.

8.1.2 Calculated PRA

PRA values were measured with a panel (LabScreen PRA) and by virtual calculation with either the Finnish or Eurotransplant population data (Eurotransplant Reference Laboratory 2018) as the point of comparison. PRA values obtained via the population-based calculation were clearly higher than values obtained with the panel, resulting in twice (15/30 vs. 7/30) the proportion of highly immunised patients (PRA I or II ≥80 %). The calculated PRA values for Finnish patients were determined in accordance with both the Finnish and Eurotranplant haplotype frequencies. The differencies seen between these values are due to the different allele and haplotype frequencies in the populations (Table 3).

Table 2. The 40 most common Finnish HLA haplotypes and their frequencies (2n=1008).

A*	B*	C*	DRB1*	DQB1*	n	Frequency
03:01	35:01	04:01	01:01	05:01	72	0.0714
01:01	08:01	07:01	03:01	02:01	46	0.0456
03:01	07:02	07:02	15:01	06:02	23	0.0228
02:01	13:02	06:02	07:01	02:02	22	0.0218
02:01	07:02	07:02	15:01	06:02	21	0.0208
02:01	15:01	03:03	13:01	06:03	21	0.0208
02:01	15:01	03:04	04:01	03:02	20	0.0198
02:01	27:05	02:02	08:01	04:02	20	0.0198
03:01	07:02	07:02	13:01	06:03	13	0.0129
02:01	15:01	04:01	08:01	04:02	12	0.0119
68:01	08:01	07:01	03:01	02:01	11	0.0109
24:02	40:01	03:04	13:02	06:04	11	0.0109
03:01	15:01	03:03	08:01	04:02	11	0.0109
02:01	40:02	02:02	04:05	03:01	10	0.0099
24:02	07:02	07:02	15:01	06:02	10	0.0099
24:02	39:01	07:02	04:04	03:02	9	0.0089
03:01	15:01	03:04	04:01	03:02	9	0.0089
02:01	27:05	01:02	04:08	03:01	9	0.0089
03:01	18:01	07:01	04:04	03:02	8	0.0079
02:01	56:01	01:02	04:01	03:02	8	0.0079
02:01	44:02	05:01	12:01	03:01	7	0.0069
02:01	27:05	02:02	01:01	05:01	7	0.0069
02:01	40:01	03:04	08:01	04:02	7	0.0069
02:01	27:05	02:02	04:04	03:02	6	0.0060
02:01	44:02	05:01	04:01	03:01	6	0.0060
31:01	18:01	07:01	15:01	06:02	6	0.0060
25:01	18:01	12:03	15:01	06:02	6	0.0060
02:01	56:01	01:02	04:01	05:03	6	0.0060
02:01	08:01	07:01	03:01	02:01	6	0.0060
02:01	40:01	03:04	13:02	06:04	6	0.0060
24:02	07:02	07:02	13:01	06:03	6	0.0060
25:01	18:01	12:03	01:01	05:01	6	0.0060
02:01	35:01	04:01	01:01	05:01	6	0.0060
24:02	39:01	07:02	08:01	04:02	6	0.0060
02:01	44:27	07:04	16:01	05:02	5	0.0050
03:01	35:01	04:01	04:01	03:02	5	0.0050
32:01	44:02	05:01	04:01	03:02	5	0.0050
03:01	35:01	04:01	15:01	06:02	5	0.0050
02:01	51:01	15:02	09:01	03:03	5	0.0050
23:01	44:03	04:01	07:01	02:02	5	0.0050
						Total 0.4790

Table 3. Calculated PRA percentages for immunised patients in Finnish and Eurotranplant populations.

Patient	Antibodies against	Panel PRA%	cPRA% Fi	cPRA% Eu	Fi% vs. Eu%
1	B76,B45,B44,B82,A1	16	28	48	-20
1	DQ7,DQ2,DR17,DR8,DQ4,DQ8,DQ9,DR13,DR14,DR16,DR11,DR18,DR7,DR12 ,DR15,DR52,DQ6,DR103,DR4,DR51	97	95	98	-3
2	DR51,DR103,DR1,DR16,DR10,DR15,DQ4,DR9,DQ2,DQ5,DQ6,DR8,DR11,DR1 7,DR13	77	97	96	1
3	Cw6,Cw18,Cw15,B45,B44,Cw2,B76,B82,Cw5,Cw8,Cw17,B37,B47,Cw4,B13,B27, B51,B49,B53,B41,B61	62	67	73	-6
3	DR53,DQ8,DR4,DQ9,DQ7,DQ4,DR51,DR15,DQ2,DR16,DR9,DR7,DR10,DR8	86	89	91	-2
4	A3,A34	11	42	29	13
5	DR9,DR51,DR103,DR10,DR1,DQ5,DQ6,DR7,DR15	49	83	77	6
6	B45,B76,B44,B59,B51,Cw4,B27,B63	15	52	55	-3
7	A32,A25,A66,B51,A26,B52,B59,B49,B53,B63,A23,A24	18	40	48	-8
7	DR8,DR12,DQ2,DQ8,DQ4,DQ7,DR7,DQ9,DR52,DR17,DR9,DR14,DR11,DR13, DR18,DR4	86	90	93	-3
8	A25,A26,A66,B62,B76,A34,A43,B77,A11,B63,B75,B57,B13,A31,B72,A29,A30,A33, A74,B50,A32,B58	49	47	59	-12
8	DQ5,DQ2,DR10,DR1,DQ6	40	84	81	3
9	A25,A26,Cw18,Cw17,Cw6,Cw5,Cw2,A69,Cw8,A34,Cw15,A33,A66,A24,A68,Cw4, A2,B63,B54,B39	62	90	90	0
9	DR1,DR103,DR9,DR51,DR16,DR10,DR15,DR53,DR8,DR11	49	94	88	6
10	DR4	14	26	26	0
11	A2,B27,B37,B47,A69,A68,B44,B51,A23,A24,B53,B59	55	83	79	4
11	DR1,DR4,DP9,DR103,DR8,DP17,DP18,DR13,DP2,DR10,DP3,DP4,DP14,DR11, DP28,DR51	49	99	88	11
12	A33,A31	4	8	8	0
13	A26,A25,A66,A43,A34,A33,A11,A68,A29,A69	47	31	31	0
13	DR15,DR51,DR13	34	52	48	4
14	A3,A34,A66,A26,A11,A33,A29,A30,A25,A32,A31,A1,A36	16	79	79	0
14	DR8,DR16,DR11,DR103,DQ8,DR15,DR12,DR51,DQ4,DR13,DQ7,DQ6,DR7, DQ5,DQ9,DR1,DR4,DR10,DR9,DR53	91	99	98	1
15	DR103,DR16,DR14,DR53,DR4,DR7,DR15,DR1,DR9,DR10,DR51,DR8,DR11	89	95	92	3
16	A2,A69,B82,A68,Cw5,B76,A32	11	66	63	3
17	A69,A24,B57,A68,B58,A2,B45,B44,A23,B82,B76,B13,B41	62	81	78	3
18	DR51,DR9,DQ6,DR1,DR103,DR10,DR15,DR53	26	91	85	6
18	A24,A23,B53,B38,B63,B57,A25,B77,A32,B59,B76,B51,B58,A80,B62,B52,B49,B75, B13,A1,B46	62	64	75	-11
19	Cw7,B73,Cw18,Cw6,B8,Cw4,A25	24	87	77	10
20	DR8,DR12	14	26	10	16
21	B41,B45,B44,B61,B50,B47,B13,B49,B60,B27,B81,B48,B7,B76,B82,B73,Cw12, A66,B37	44	72	70	2
22	DR8,DR12,DR13,DR16,DR11,DR7	89	62	68	-6
23	B62,B52,B72,B48,B50,B49,B37,B13,B77,B63,B76,B60,B44	25	49	54	-5
24	B27,B81,B61,A66,B7,B13,B60,B48	13	59	45	14

Patient	Antibodies against	Panel PRA%	cPRA% Fi	cPRA% Eu	Fi% vs. Eu%
25	B63,A2,A69,A68	5	61	55	6
26	DR9,DP19,DP20,DR52,DP2,DP28,DR14,DP15,DQ9,DQ6,DR4,DR11,DP5	46	88	92	-4
27	A33,A31,B78,Cw17,B72,B55	4	10	11	-1
27	DR12,DR13,DR11,DR15	20	59	65	-6
28	A32,B51,B59,B53,B44,B27,B13,B38,B49,B77,B37,B47,B52,B63,B57,A26,A66,B58,A24,A23	55	63	72	-9
29	A23,A24	22	23	22	1
30	B47,B56,B58,B44,Cw9,A69,Cw10,B59,A30,A26,Cw17,A29,B57,A1,A66,B81,B55,B64,A31,A33,B60,B77,Cw18,A43,A80,B61,B65,A11,A34,A36,B46,B75,Cw6,B48,A74,B38,B52,B63,B71,A68,B18,B41,B51,B78,B54,B82,B8,B67,A3,Cw15,B62,B72,A32,A25,B73,B53,Cw5,B37,B35,B76,B49,B13,B39,B45,A24,B42,B50	80	99	100	-1

PRA, panel reactive antibody; cPRA, calculated panel reactive antibody; Fi, Finnish population; Eu, Eurotransplant population. Modified from publication I Table 5

8.2 DSA predicting crossmatch outcome (II)

The results of the actual crossmatches, performed with different methods, were evaluated against the VXM results. The aim was to define appropriate cut-off values for DSA that would predict the crossmatch results generated by different crossmatch techniques. This study included routine on-call crossmatches, and the immunisation status of the samples represented the situation in actual clinical practice.

8.2.1 HLA antibody status

Of the performed crossmatches, 32% (118/364) were performed with sera from sensitised patients while 23% (83/364) of the crossmatches were performed with serum containing antibodies against donor HLA. Antibodies were present against all HLA loci, but most commonly against HLA-B antigens (28% (101/364)). Correspondingly DSA were most frequently directed against donor HLA-B (13% (48/364)) (Fig. 8).

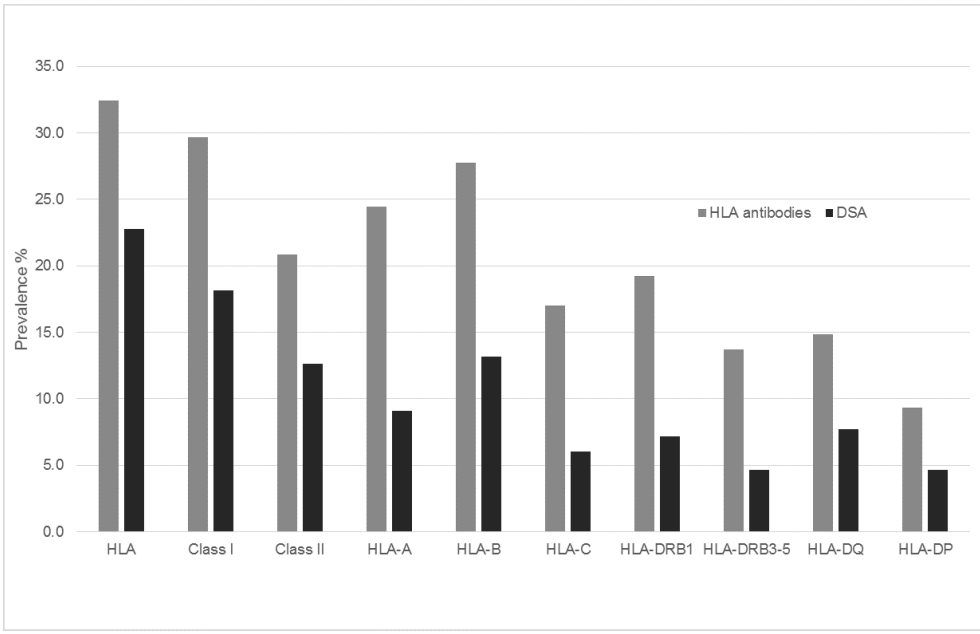

Figure 8. Prevalence of HLA antibodies and DSA in crossmatches. Modified from publication II Figure 1

8.2.2 DSA detected by different crossmatch methods

Eighty-three of the crossmatches were performed with DSA-positive sera. The detection rates for DSA in FCXM, LXM and CDCXM were 83%, 71% and 66%, respectively. Reverse results were seen in DSA-negative crossmatches where the highest rate of false positive tests was seen in CDCXM (20%), followed by LXM (11%), and FCXM (10%). The true positive crossmatches correlated well between the techniques (Fig. 9A). However, the false positive crossmatches seemed to be different in each method, as only one sample was false positive with all methods (Fig. 9B).

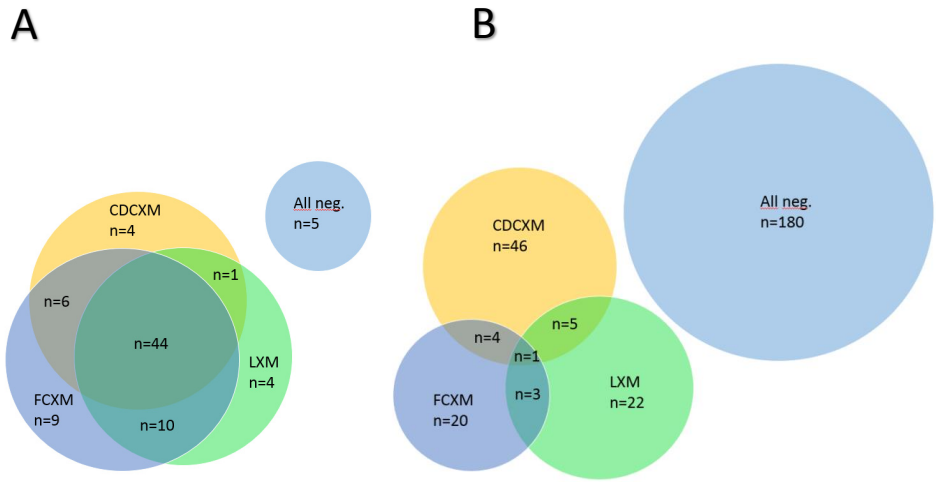

Figure 9. Crossmatch positivity of individual samples in each technique. (A) Crossmatches of DSA positive samples. (B) Crossmatches of DSA negative samples. The sizes of the areas are directional only. Modified from publication II Figure 3

As CDCXM was performed with a mixed T and B cell population, separation for class I and II antibodies was not possible. Similarly, FCXM performed with T and B cells was not able to separate class I and II antibodies. For samples with only class I antibodies, T cell FCXM and B cell FCXM detected the DSA with very similar rates of 78% and 68%, respectively. For class II antibodies, a better

detection rate was achieved with B cell FCXM than with T cell FCXM (76% and 41%, respectively). LXM achieved a clear separation between class I and II antibodies, with class I LXM detecting 62% of class I and 12% of class II DSA. Correspondingly, class II LXM detected 65% of class II DSA and only 5% of class I DSA (Table 4).

8.2.3 Sensitivity, specificity and accuracy

The diagnostic ability of each crossmatch method was studied by ROC analysis. The predictive accuracy for CDCXM was fair (AUC 0.724). LXM and FCXM showed good performance, with AUC of 0.805 and 0.861, respectively (Fig. 10).

With a routine cut-off level of 1000 MFI, the best sensitivity for DSA detection was reached with FCXM (0.722). While LXM produced almost similar sensitivity (0.667), considerably lower sensitivity was obtained with CDCXM (0.495). In terms of specificity, less variation was seen between the methods. Again, FCXM performed better, offering higher specificity than LXM and CDCXM (0.940, 0.905 and 0.803, respectively). Similar results were seen with regard to accuracy as FCXM outperformed LXM and CDCXM (0.885, 0.849 and 0.769, respectively).

Table 4. DSA status in different crossmatch techniques

	DSA- (n=281)		DSA+ (n=83)		Class I DSA (n=66)		Class II DSA (n=46)		Only Class I DSA (n=37)		Only Class II DSA (n=17)		Class I and II DSA (n=29)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
CDCXM	56	20	55	66	45	68	34	74	21	57	10	59	24	83
T cell FCXM	20	7	61	73	54	82	32	70	29	**78**	7	41	25	86
B cell FCXM	25	9	64	77	51	77	39	85	25	68	13	**76**	26	90
Total FCXM	28	10	69	**83**	56	**85**	40	**87**	29	**78**	13	**76**	27	**93**
Class I LXM	13	**5**	41	49	39	59	18	39	23	62	2	12	16	55
Class II LXM	20	7	30	36	19	29	28	61	2	5	11	65	17	59
Total LXM	31	11	59	71	48	73	36	78	23	62	11	65	25	86

The best detection rate in each DSA category is highlighted. CDCXM, Complement-dependent cytotoxicity crossmatch; DSA, donor-specific antibody; FCXM, Flow cytometric crossmatch; LXM, Luminex crossmatch. Modified from publication II Table 1

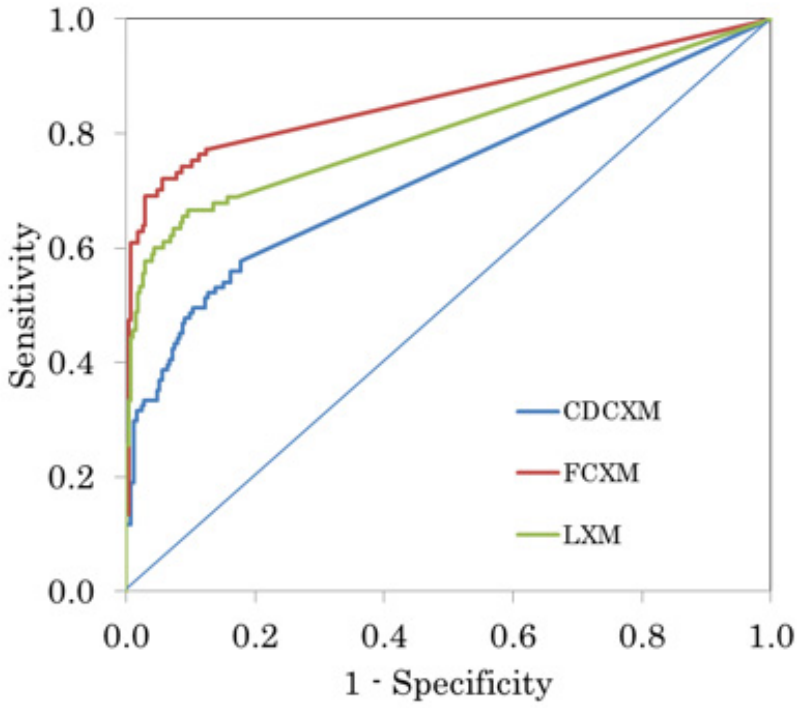

Figure 10. ROC curve analysis of DSA in comparison with different crossmatch methods. CDCXM, AUC 0.724; FCXM, AUC 0.861; LXM, AUC 0.805. The linear part of the curves represents repeated MFI values of 0 (no DSA identified). Modified from publication II Figure 2

8.3 DSA and graft function (III)

8.3.1 Immunisation status

HLA antibody levels were retrospectively analysed in 123 pediatric kidney transplant patients. Of the 294 routinely collected post-transplant serum samples, 48% (140/294) had pre-existing HLA antibodies, and 21% (62/294) of the samples

presented antibodies directed against the allograft (Fig. 11). A third of the patients (42/123, 34%) had DSA at some time point after transplantation. Class I DSA were detected in 10% (12/123) and class II DSA in 19% (23/123) of the patients, while both class I and II DSA were detected in 6% (7/123) of the patients. The fraction of DSA-positive samples increased over time from 15% at ≤2 years to 20% at 3-5 years and to 36% at 6-10 years after RTx.

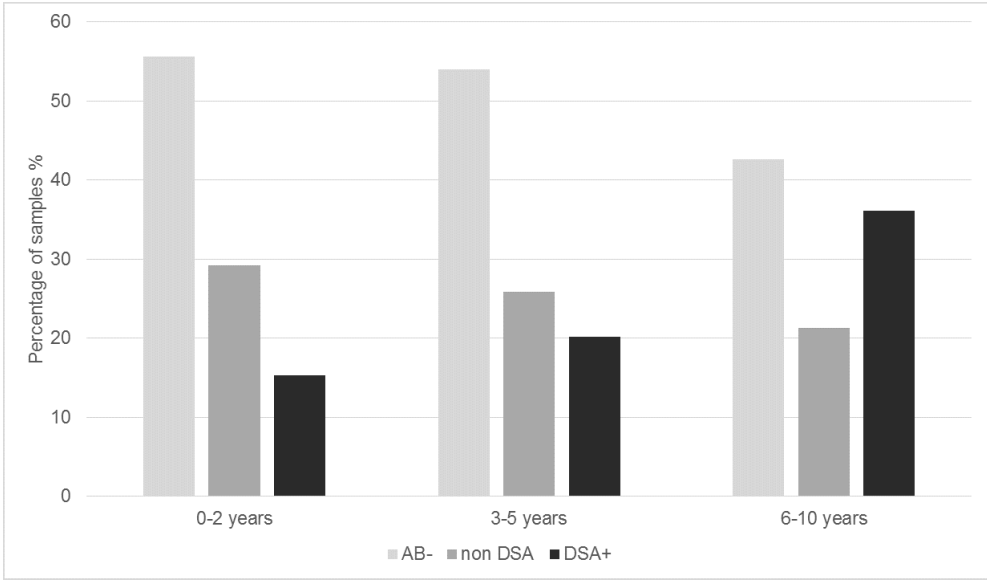

Figure 11. Percentage of HLA antibodies in pediatric kidney transplant patients at three time points after transplantation. A total of 294 serum samples were collected and analyzed for HLA antibodies. AB, no HLA antibodies; non DSA, HLA antibodies not specific against donor antigens; DSA+, donor specific HLA antibodies. Modified from publication III Figure 1

8.3.2 DSA and GFR

A total of 1152 GFR measurements, with an average of 9 measurements per patient, were included in this study. There were no differences in measured GFR at three years post-RTx or at any time point during the follow-up in four subgroups formed on the basis of immunisation status. (Fig. 12).

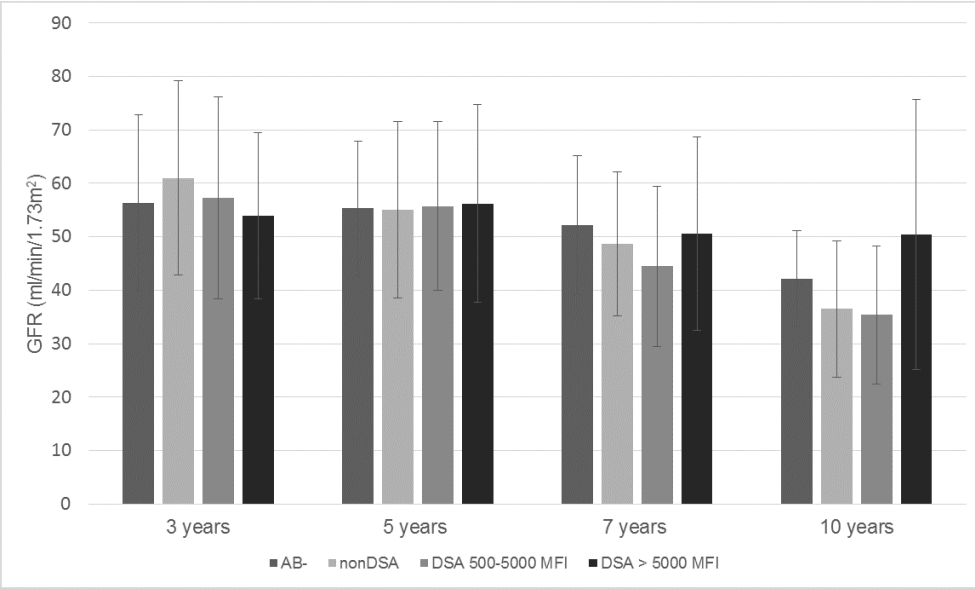

Figure 12. GFR values 3 to 10 years after kidney transplantation according to HLA-antibody status of the patients and strength of cumulative DSA determined 0-2 years after transplantation (DSA 500-5000 MFI and DSA > 5000 MFI). No statistical difference was observed between patients with no HLA antibodies (AB-) and patients with either HLA antibodies not specific against donor antigens (nonDSA) or patients with donor-specific HLA antibodies (DSA) at any time point (p>0.05). Modified from publication III Table 4

Surveillance allograft biopsies from kidney transplants were taken simultaneously with HLA antibody screening at 1.5 and 3 years after transplantation from 64 and 75 patients, respectively. Chronic lesions were detected after 1.5 and 3 years in 31% (20/64) and 47% (35/75) of the patients, respectively. At 3 years after transplantation, chronic lesions were seen in 51% (36/71) of the patients without DSA, while 54% (13/24) of the patients with DSA had chronic lesions (P=0.8166).

At 7 years, there were no differences in the presence of chronic lesions between patients without and with DSA (51% (23/45) and 44% (7/16), respectively (P=0.7723)). Patients showing both DSA and a chronic histological lesion during the first three years after transplantation had lower GFR values (49 mL/min/1.73m^2) than patients without DSA and with normal histological findings (64 mL/min/1.73m^2). A decrease in GFR during the follow-up was seen with both DSA and chronic histological lesions (Fig. 13). However, statistical difference was only seen between DSA-positive patients with and without chronic lesions at 3 years (P=0.040), suggesting that biopsy findings play a larger role in graft function than DSA.

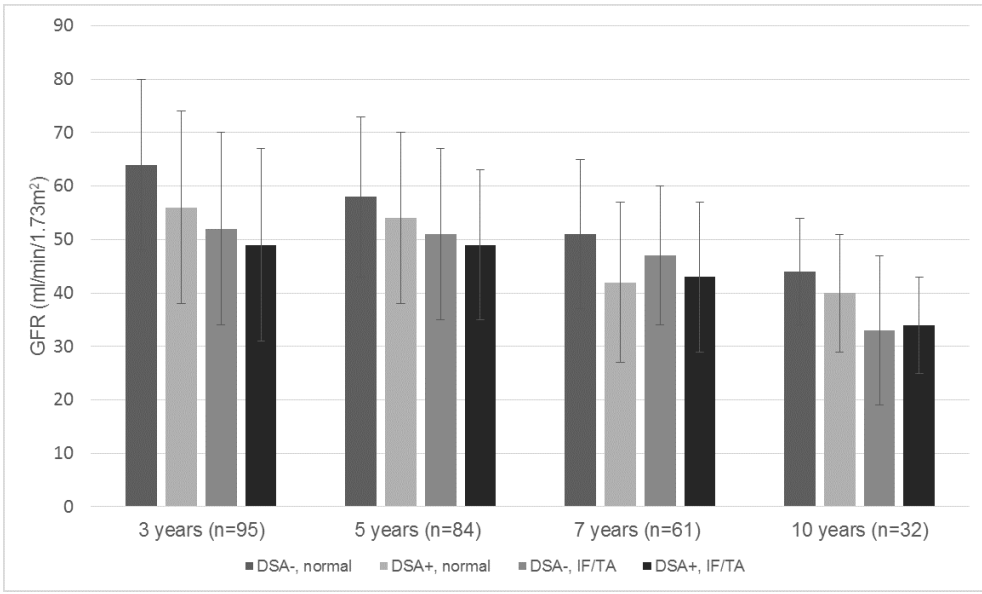

Figure 13. Comparison of GFR values at 3 to 10 years post-transplant with HLA antibody and allograft biopsy findings taken at time point of 1.5 and 3 years after transplantation. GFR at 3 years after transplantation: DSA+, normal vs. DSA+, IF/TA, P=0,040. DSA, donor specific HLA antibodies; IF/TA, interstitial fibrosis and tubular atrophy.

8.4 Delayed graft function (IV)

Many patients have DSA prior to transplantation when measured with bead array, even if the prospective CDC crossmatch gives a negative result. In this study, we examined the impact of pretransplant DSA of RTx patients on DGF.

8.4.1 Characteristics of recipients according to DGF status

As seen in Table 5, both the recipients and donors were older in the DGF group. Patients with early graft function were less immunised than patients with DGF and had a significantly lower amount of DSA. Among patients with DGF, the cold ischemia time was longer and the rate of previous transplants was higher (Table 5).

Table 5. Characteristics of recipients with early and delayed graft function

	EF (n=544)	DGF (n=227)	P-value
Mean recipient age, yearsr	48	50	0.0348
Mean donor age, years	44	51	<0.0001
Male recipients, n (%)	344 (63)	152 (67)	0.3646
Average HLA mismatch	2.18	2.17	0.8296
HLA immunised, n (%)	172 (32)	93 (41)	0.0157
DSA positive, n (%)	54 (10)	49 (22)	<0.0001
Retransplants, n (%)	42 (8)	38 (17)	0.0004
Cold ischemia time, hoursr	21.53	23.06	<0.0001

EF, early function; DGF, delayed graft function; DSA, Donor-specific antibody; HLA, human leukocyte antigen. Modified from publication IV Table 1

8.4.2 Independent predictors for DGF

Several independent predictors were found for DGF in multivariate analysis (Table 6). The highest relative risks for DGF were the presence of DSA and previous RTx. The strongest statistical association was found for donor age and CIT.

Table 6. Factors associating with DGF in multivariate logistic regression analysis

Characteristic	RR	95% CI	P
Recipient age, years	1.001	0.987-1.016	0.8611
Recipient BMI, kg/m^2	1.047	0.995-1.102	0.0758
Re-Tx	1.879	1.064-3.319	0.0297
Rejection ≤ 1 week	0.808	0.177-3.687	0.7830
Donor age, years	1.037	1.022-1.053	<0.0001
Donor BMI, kg/m^2	1.049	1.004-1.095	0.0331
CIT, hours	1.068	1.025-1.112	0.0015
DSA	2.039	1.246-3.335	0.0046

BMI, body mass index; CIT, cold ischemia time; DGF, delayed graft function; DSA, Donor-specific antibody; Re-Tx, previous kidney transplant; RR, relative risk. Modified from publication IV Table 2

There were some differences in the frequency of DGF and presence of DSA (yes/no) depending on the primary cause of ESRD. The highest rate of DGF (45%) was detected in recipients with unknown etiology of kidney failure, while recipients with hypertension/large vessel disease had the lowest DGF rate (19%). DSA were most often seen in patients with pyelonephritis or interstitial nephritis (Fig. 14).

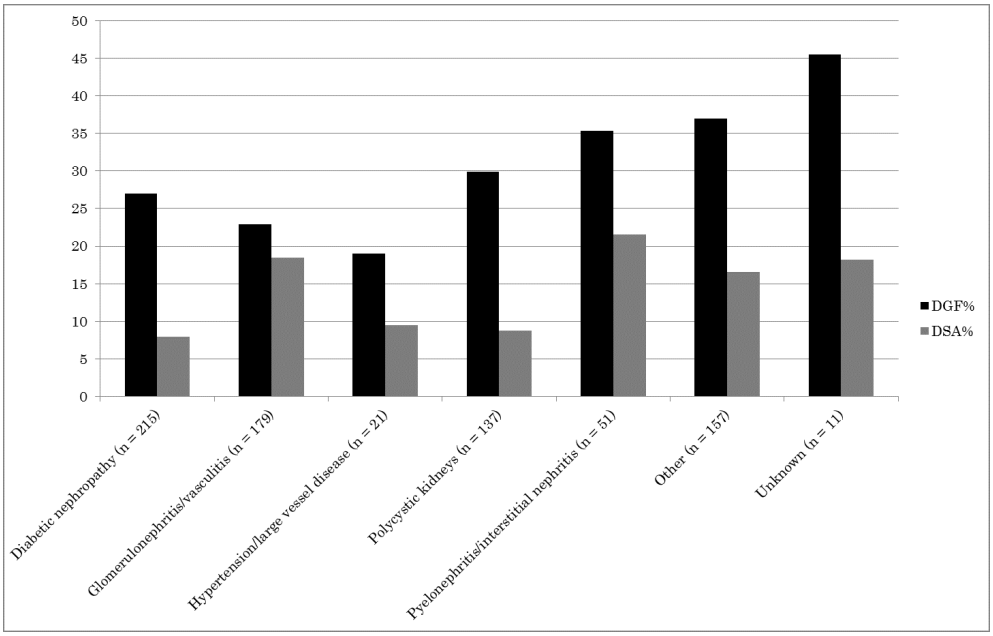

Figure 14. Primary cause of ESRD and incidence of DGF and DSA. DGF, delayed graft function; DSA, Donor-specific antibody, ESRD, end-stage renal disease.

8.4.3 Immunisation status and DGF

One third of the patients (265/771; 34%) had pre-existing HLA antibodies before kidney transplantation. Class I antibodies were detected in 225 (29%) patients and class II antibodies in 131 (17%). The majority (162/265; 61%) of the immunised patients presented non-DSA antibodies. In total, DSA were detected in 103/771 (13%) of the patients.

The risk of pre-immunisation is largely due to donor-specificity of the antibodies as HLA antibodies against third-party antigens had no effect on the DGF rate when compared to non-immunized patients (28% vs. 26%, $p = 0.6098$) (Fig. 15). Conversely, patients with DSA had a significantly higher incidence of DGF when compared to patients without DSA (48% vs. 27%, $p < 0.0001$).

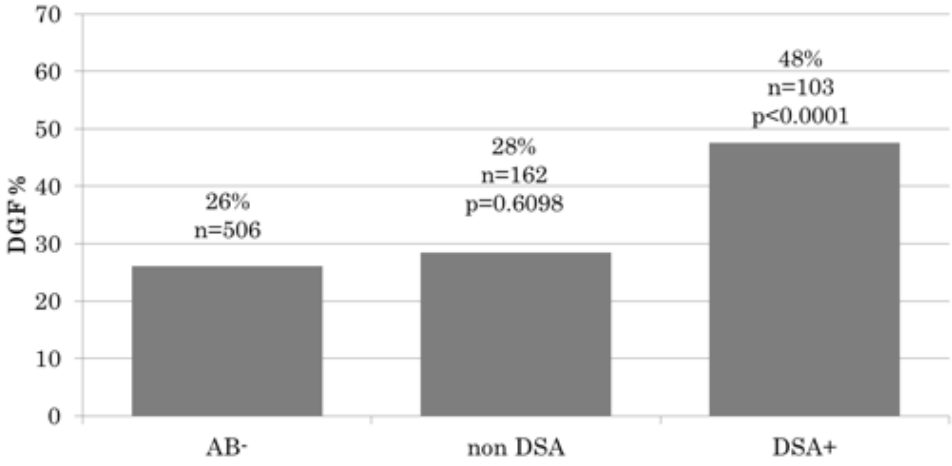

Figure 15. Comparison of immunisation status with DGF. AB-, no detected HLA antibodies, DGF, delayed graft function; DSA, Donor-specific antibody; non-DSA, antibodies against a third party. Modified from publication IV Figure 1 © 2016 Oxford University Press on behalf of ERA-EDTA. All rights reserved

The number of antigens detected as DSA had an effect on that DGF rate as recipients with a single DSA had a DGF incidence of 43%, whereas patients with two or more DSA had DGF rates of 57% and 53%, respectively ($P < 0.0001$). The strength of DSA as measured by cumulative DSA MFI showed a good predictive value as well. The incidence of DGF was more than twice as high in patients with DSA of 3000–5000 MFI when compared to patients with DSA of 1000-3000 MFI or patients without DSA (65%, 31% and 27% respectively; $P = 0.0351$) (Table 7).

8.4.4 DSA and rejections

Of the patients, 17% (129/771) were diagnosed with at least one episode of acute rejection during the follow-up. Of the rejections, 15% (19/129) were steroid resistant. No association with DSA positivity or the total DSA MFI value was seen in ARR. However, steroid-resistant rejections were more common in patients with a cumulative DSA MFI of > 5000 than in patients without DSA (14% vs. 2%, $P = 0.0005$) (Fig. 16).

Table 7. The incidence of DGF according to the number of DSA and the cumulative MFI value

		Delayed Graft Function		
DSA status	**N**	**n**	**(%)**	**P-value**
DSA number				<0.0001
no DSA	668	178	27	
1 DSA	65	28	43	
2 DSA	21	12	57	
≥3 DSA	26	9	53	
Cumulative DSA				<0.0001
no DSA	668	178	27	
1000-3000 MFI	22	10	31	
3000-5000 MFI	18	11	65	
>5000 MFI	43	23	53	

DSA, donor-specific antibody; MFI, mean fluorescent intensity. Modified from publication IV Figure 1 © 2016 Oxford University Press on behalf of ERA-EDTA. All rights reserved

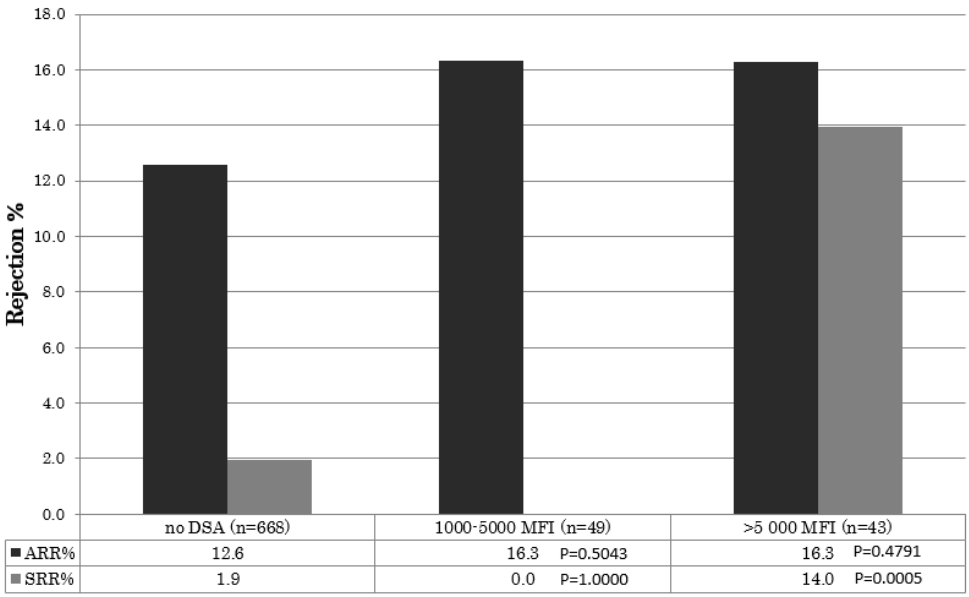

Figure 16. Association of cumulative DSA MFI with rejections. Univariate analysis performed with two-tailed Fischer's exact test. ARR, acute reversible rejection; SRR, steroid resistant rejection.

9 DISCUSSION

This thesis evaluated the role of HLA antibodies in kidney transplantation from two different angles. The first two publications analysed various techniques used to measure the immunisation status of patients and the sensitivity and specificity of different crossmatch techniques. The last two publications evaluated the importance of HLA antibodiesin the transplant outcome. The study endpoints were long-term graft function as measured by GFR and early graft function as assessed by DGF. These retrospective clinical studies were performed in pediatric and adult kidney transplant patients.

9.1 The importance of knowing your population

This study provided new information on the distinctive features of HLA antigens in the Finnish population. Although the allelic diversity in the Finnish population is limited when compared to other European populations, the enrichment of certain haplotypes makes the population unique. The most common Finnish haplotype: *A*03:01-B*35:01-C*04:01-DRB1*01:01-DQB1*05:01* is much more frequent in Finland than in the population of European background in the US (frequency of 0.0714 and 0.0126, respectively) (Maiers, Gragert, Klitz 2007). The most common European haplotype *A*01:01-B*08:01-C*07:01-DRB1*03:01-DQB1*02:01* is particularly common in North West England, where its frequency is twice as high as that seen in Finland (0.0950 and 0.0456, respectively) (Gonzalez-Galarza et al. 2011). In addition, there are population-specific haplotypes, such as the Finnish haplotype *A*31:01-B*51:01-C*01:02-DRB1*13:01-DQB1*03:03,* that do not exist at all in the population of European background in the USA (Maiers, Gragert, Klitz 2007). There are also some common European haplotypes that were not detected in the Finnish population at all, such as *A*33:01-B*14:02-C*08:02-DRB1*01:02-DQB1*05:01*, which is the second most common haplotype in the Italian population (Rendine et al. 2012).

The dominance of the most common Finnish haplotype: *A*03:01-B*35:01-C*04:01-DRB1*01:01-DQB1*05:01* is evident, as this haplotype is much more frequent in Finland than anywhere else. After the publication of study I, other studies describing the unique features of the Finnish population have also been carried out (Linjama et al. 2017). Because of low allelic variation, the risk of a patient

being HLA homozygous is elevated in Finland. This phenomenon is particularly evident for HLA-A for which a homozygosity rate of 11% is seen in the American population, while in the Finnish population, this rate is 24% (Linjama T. personal communication; Shah et al. 2011). Homozygosity has several implications. Firstly, homozygous patients become more broadly immunised because the number of structures that are foreign to the immune system is higher in blood transfusion, pregnancy and transplantation, as compared to heterozygous patients (Lucas, Leffell, Zachary 2015). Secondly, these patients regularly receive a full haplotype mismatched graft. Often, these patients are immunised against all non-self HLA. The most common Finnish haplotype is carried by 14% of the population, of which 0.5% are homozygous carriers. Therefore, a donor homozygous for this haplotype is compatible with 14% of the entire waiting list of patients as there are no HLA mismatches with those patients. In some organ transplant programs, homozygous patients are given priority with regard to homozygous donors as these patients are prone to becoming immunised against foreign HLA (De Meester et al. 1998). However, there is high demand for these donors as they are compatible with many already immunised patients too.

The broadness of HLA immunisation is measured with the PRA test. Traditionally this has been done with a cell panel representing as many HLA antigens as possible. The problem with these panels is that rare antigens are overrepresented while common antigens are underrepresented, therefore the results produced by the panel are not representative of the donor population. Today, many laboratories use calculated PRA, where the identified antibodies are correlated with the donor population, producing a more accurate result regarding the broadness of immunisation. Differences in the haplotype frequencies in various populations lead todifferences also in the probability of finding a donor for an individual patient from different countries through the international organ exchange (Glorie et al. 2014). For most patients, this is not a problem, but for a patient with a PRA of nearly 100%, the chances of finding a donor from a certain population may be higher. For example, patient 30 in our study I had 1% probability of a HLA-compatible Finnish donor and 0% of a Eurotransplant donor. The drawback of the calculated PRA is that the method used for antibody detection is much more sensitive than the PRA determined by a traditional cell panel, thus a larger proportion of immunised and highly immunised patients is seen when the calculated PRA is used (Cecka 2010). Therefore, the patient often has a much better chance of a crossmatch-negative transplant than is presumed based on the PRA percentage.

9.2 The value of DSA in histocompatibility testing

The routine protocol for selecting patients to undergo a final crossmatch is based on ABO and HLA compatibility and clinical factors, such as the time on the waiting list, and immunisation level, with weighting of the variables varying depending on the allocation program (Chopra and Sureshkumar 2015; Mayer and Persijn 2006). According to the local selection criteria, one third of the patients were immunised at the time of the crossmatch. As negative virtual crossmatches are not required for a final crossmatch, about 25% of the patients were HLA-antibody incompatible because of DSA. Many patients selected for a crossmatch were broadly immunised and had antibodies against HLA antigens encoded by different HLA loci. Therefore, the risk of a positive virtual crossmatch for an immunised patient was 70%.

The ability of different crossmatch methods to detect DSA determined by a single antigen varies greatly, since these methods do not measure exactly the same thing. Only 53% of the serum samples containing DSA were positive with all three crossmatch methods, 74% were positive with two methods, and 94% with one method. The samples giving false positive crossmatches were predominantly different in each crossmatch method. Only one sample gave a false positive result with all the methods tested. However, 36% of the crossmatches where DSA were not present were false positives with at least one method. This lack of concordance in samples producing false negative and positive results with each of the crossmatch methods indicates that the difference in the results is based on the type of antibodies detected rather than the cut-off used for each technique. The highest false positive rate was seen for CDCXM (20%), while FCXM and LXM produced better results (10% and 11%, respectively). The high rate of false positives for CDCXM is in agreement with an earlier study where false positive rates of 18% and 23% were detected for T and B cells, respectively (Alheim et al. 2015). The DSA detection rate of 83% achieved with FCXM in our study was particularly good as the rates in previous studies have ranged from 52 to 65% (Reinsmoen et al. 2016; Zachary et al. 2009b). The advantage of LXM is that it can distinguish between class I and class II DSA. The advantage offered by FCXM and LXM is that an objective measurement of fluorescence values by flow cytometer or bead analyzer is performed, instead of a subjective cytotoxicity reading by microscope.

In our study, CDCXM had the lowest and FCXM the highest sensitivity, specificity and accuracy of the tested crossmatch methods when compared to identified

antibodies. Does this mean that FCXM is the best method for crossmatching and the routinely used CDCXM should be replaced? This is a key question in the antibody compatibility and there are several issues that need to be discussed. First, what are the properties that make an antibody clinically relevant? Secondly, how do we identify it? Thirdly, how do we define what is clinically relevant. And finally, what are the goals of our crossmatch protocol?

What properties do clinically relevant antibodies have? Most immunologists agree that their capacity for complement-mediated cytotoxicity is a crucial characteristic. On the other hand, pre-transplant IgM antibodies that are capable of cytotoxic killing are widely considered as clinically irrelevant (Taylor et al. 1989). The high rate of false positives found in our study for CDCXM is mostly explained by IgM antibodies, as DTT was not used in this study protocol to neutralize IgM antibodies. In our clinical transplant protocol, DTT treatment has been performed for samples of selected patients with known IgM antibodies for years. The current practice is to perform all CDCXM with DTT treated serum to gain the maximum benefit. Some IgG subgroups have higher potency for complement binding and they are therefore considered more relevant (Lefaucheur et al. 2016). IgG3 has been reported to be the most detrimental IgG subpopulation (Everly et al. 2014). The titer and affinity of an antibody are also of importance, but these are not commonly tested in clinical protocols due to the complexity of the assays. However, it has been shown that the affinity of an HLA antibody can have an effect on the level of complement-dependent cytotoxicity (Daga et al. 2017).

Different antibody testing methods identify different types of antibodies. Complement-binding activity can be measured by CDC and with modified bead array. IgG antibodies can be measured with FCXM and LXM while specific HLA antibodies can be measured with bead array and LXM. Antibodies identified with CDCXM are regarded as clinically relevant. Somewhat less faith is placed in FCXM as this method has been suggested to be oversensitive, thus preventing transplants with good outcome (Vlad et al. 2009). There is also consensus that single antigen bead array identifies antibodies with very high sensitivity and thus some antibodies identified might not be clinically relevant (Aubert et al. 2009).

In terms of clinical relevancy, we have several possible categories that may differ in their classification of antibodies. In an organ transplantation setting, there is an agreement that antibodies able to cause hyperacute rejection are clinically relevant. Also, antibodies able to promote chronic rejection are considered

clinically relevant. How about antibodies predisposing to DGF, are they clinically relevant?

We may see different clinical scenarios, depending on the type of exposure and antibody. A low titer antibody with a low MFI value may be able to promote chronic rejection. A high MFI antibody (if able to bind complement) might be able to promote hyperacute rejection. It has been shown that the incidence of acute AMR in patients with pretransplant DSA is higher than in patients without DSA (41.7% vs. 1.6%). Further, CDSA above 4300-5300 MFI is predictive of acute AMR and shorter 5-year graft survival (Salvade et al. 2016).

An antibody that remains undetected in CDCXM but is detected by more sensitive methods, while unable to cause hyperacute rejection, could still cause DGF, as shown in study IV. In our adult study cohort, patients transplanted with a negative CDCXM crossmatch, and showing presence of DSA by the bead array, had a higher incidence of DGF. Later, another study has described similar findings with a higher DSA rate in patients with DGF (13 %) than in those without (6%) (P =0.03) (Willicombe et al. 2017). However, DSA was not associated with poor GFR, consistent with our study III with pediatric patients producing similar results. This finding has been confirmed later with adult patients, in whom no difference in the GFR was seen in DSA positive and negative patients (Zecher et al. 2017). This study, however, reported a significant difference in proteinuria levels between patients with and without DSA.

Thus, HLA antibodies identified by the sensitive bead array are clinically relevant in some situations but not in all. The immunological state of a patient waiting for the first transplant is completely different from that of a patient already transplanted and under immunosuppression. It has been shown that the impact of DSA is greater in patients with a pre-activated immune system, as determined by elevated soluble CD30 levels (Susal et al. 2016). For patients with non-activated immune system, pre-transplant DSA status did not affect the 3-year graft survival, as DSA-positive and DSA-negative patients had graft survival rates of 83% and 84%, respectively. However, patients with an activated immune system had significantly lower graft survival of 62% when DSA were present (Susal et al. 2016). This finding shows that graft outcome is influenced by multiple immunological factors and DSA is just one of those. Interestingly, only 38% of the DSA-positive patients were found to have a pre-activated immune system, explaining why in some patients, the presence of DSA is not harmful to the graft

(Susal et al. 2016). The IgM DSA, regarded as irrelevant in the prospective crossmatch, may not be irrelevant if detected after transplantation. Persistent IgM DSA may be the first sign that the patient is inefficiently immunosuppressed (Everly et al. 2014). Also, class II antibody IgM to IgG isotype switching strongly correlates with cellular rejection and poor long-term outcome (Lietz et al. 2005).

If we aim at hyper acute rejection-free transplantations, we can reach this goal with pre-transplant CDCXM. If we also want to perform an immunological risk assessment for each patient, a virtual crossmatch is needed. For a stable non-immunised patient, a good match is preferable. However, for a patient with a homozygous or rare HLA, the goal might have to be set differently. For a patient with moderate immunisation, avoidance of antibody-incompatible transplants may give better outcome. For highly-immunised patients, a crossmatch-negative transplant, even with DSA, might be the target to aim for.

Via pretransplant HLA antibody screening and identification, we can predict the crossmatch result to some extent. Most of our assays use donor cells as the target, and there is intra-individual variation in HLA expression and significant variation in HLA expression levels between different individuals, whichimpact the sensitivity of the crossmatch depending on donor factors (Badders et al. 2015; Honger et al. 2015). Therefore, there will always be some uncertainties involved in the prediction.

9.3 Limitations

The estimation of the Finnish HLA haplotypes was performed with the phase 2.1 software. The strong linkage disequilibrium and low recombination frequency in the HLA complex were taken into consideration in the settings of the analysis. As no haplotype phasing method performs with absolute certainty, some rare haplotypes may have been missed with the analysis.

HLA antibody identification with the Luminex technique has several limitations. The amount of antigens on the beads varies and they are not present in natural proportions found on the cell surface. Therefore, different antibodies are identified with different sensitivities. Also, the number of beads is limited, thus the entire repertoire of possible antigens is not represented, possibly resultingin some antibodies remaining undetected. In recent years, it has become evident that IgM

antibodies and the natural complement present in the serum may block the secondary antibody used in the bead array, leading to false negative results. This can be prevented by adding EDTA, but this procedure was not yet in use when these studies were performed (Schnaidt et al. 2011).

The donor material also brings some limitations as HLA expression varies between individuals. In CDCXM, one limitation is that in our study protocol, the total lymphocyte population from the spleen was used. There is also variation in T and B cell levels between the donors. Some donors have a higher number of B cells with higher HLA density, leading to variation in assay sensitivity. The lack of DTT treatment in the CDCXM raises the number of false positive results in our study. As CDCXM positive transplantations were not performed it was not possible to analyse the clinical relevance of DSA-negative CDCXM-positive results.

Due to the study's retrospective nature and the lack of pre-transplant serum samples in our study III, we were unable to perform survey of *de novo* DSA. The detailed evaluation of the relevance of DSA with our routine samples was challenging as many samples showed multiple DSA.

9.4 Future perspectives

As immunosuppression may not resolve all immunological incompatibilities between recipients and their donors, further studies are needed to understand the correlation between different factors and the transplant outcome. Today, we try to predict the outcome of the transplant with a very limited number of assays. In the future, understanding the synergistic effect of different factors may help us to evaluate better the immunological risk of an individual patient and to improve the overall graft survival. Most importantly, we may be able to perform individual pre-transplant risk assessments for each patient based on urgency, rarity of the HLA type, and activity of the immune system, and with improved understanding of HLA antibody compatibility. Also, understanding the synergistic effect of HLA antibodies and other factors may help us to follow transplants with higher efficacy and identify those for which damage is still treatable. There is room for further development in assays measuring HLA immunisation. Currently, we are only able to measure one antibody type at a time, which hampers the clinical use of these assays. Identification of the antibody subtype, complement binding, affinity and

titer in an individual sample is possible, but, so far, not cost nor time-effective; therefore a full analysis is not feasible.

10 CONCLUSIONS

On the basis of the present study the following conclusions can be drawn:

1. The Finnish population has unique allele and haplotype frequencies. The population-specific calculated PRA provides an accurate estimate of immunisation against the local donor population. As calculated PRA is based on a very sensitive bead array, the number of highly immunised patients will rise with this approach and some adjustments to the current definition of highly immunised patient is needed.

2. The predictive value of antibody screening results from different crossmatch methods varies. The best accuracy was reached with FCXM. The differences seen for samples producing false negative and positive results in each crossmatch method indicates that the difference in the results stems from the type of antibodies detected rather than the cut-off used for each technique.

3. HLA antibodies are common in pediatric kidney transplant patients, but they do not correlate with graft function measured by GFR.

4. Pre-transplant DSA in CDCXM-negative adult kidney transplant recipients is associated with DGF. Patients with DSA have twice as high risk for DGF than patients without DSA. Third-party antibodies had no effect on the risk for DGF. The strength of DSA as measured by cumulative DSA MFI provided a good predictive value. The incidence of DGF was more than twice as high in patients with DSA of 3000–5000 MFI when compared to patients with DSA of 1000-3000 MFI or patients without DSA.

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12 REFERENCES

Alheim M, Paul PK, Hauzenberger DM, Wikstrom AC. 2015. Improved flow cytometry based cytotoxicity and binding assay for clinical antibody HLA crossmatching. Hum Immunol 76(11):849-57.

Altermann WW, Seliger B, Sel S, Wendt D, Schlaf G. 2006. Comparison of the established standard complement-dependent cytotoxicity and flow cytometric crossmatch assays with a novel ELISA-based HLA crossmatch procedure. Histol Histopathol 21(10):1115-24.

Amico P, Hirt-Minkowski P, Honger G, Gurke L, Mihatsch MJ, Steiger J, Hopfer H, Schaub S. 2011. Risk stratification by the virtual crossmatch: A prospective study in 233 renal transplantations. Transpl Int 24(6):560-9.

Amos DB, Bashir H, Boyle W, MacQueen M, Tiilikainen A. 1969. A simple micro cytotoxicity test. Transplantation 7(3):220-3.

Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M. 2015. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. J Immunol 194(8):3594-600.

Aubert V, Venetz J, Pantaleo G, Pascual M. 2009. Low levels of human leukocyte antigen donor-specific antibodies detected by solid phase assay before transplantation are frequently clinically irrelevant. Hum Immunol 70(8):580-3.

Badders JL, Jones JA, Jeresano ME, Schillinger KP, Jackson AM. 2015. Variable HLA expression on deceased donor lymphocytes: Not all crossmatches are created equal. Hum Immunol 76(11):795-800.

Baxter-Lowe LA, Kucheryavaya A, Tyan D, Reinsmoen N. 2016. CPRA for allocation of kidneys in the US: More candidates >/=98% CPRA, lower positive crossmatch rates and improved transplant rates for sensitized patients. Hum Immunol 77(5):395-402.

Becker LE, Morath C, Suesal C. 2016. Immune mechanisms of acute and chronic rejection. Clin Biochem 49(4-5):320-3.

Benichou G and Thomson AW. 2009. Direct versus indirect allorecognition pathways: On the right track. Am J Transplant 9(4):655-6.

Billen EV, Voorter CE, Christiaans MH, van den Berg-Loonen EM. 2008. Luminex donor-specific crossmatches. Tissue Antigens 71(6):507-13.

Boardman DA, Jacob J, Smyth LA, Lombardi G, Lechler RI. 2016. What is direct allorecognition? Curr Transplant Rep 3(4):275-83.

Bock HA. 2001. Steroid-resistant kidney transplant rejection: Diagnosis and treatment. J Am Soc Nephrol 12 Suppl 17:S48-52.

Bradley JA, Baldwin WM, Bingaman A, Ellenrieder C, Gebel HM, Glotz D, Kirk AD. 2011. Antibody-mediated rejection?an ounce of prevention is worth a pound of cure. American Journal of Transplantation 11(6):1131-9.

Bryan CF, Luger AM, Martinez J, Muruve N, Nelson PW, Pierce GE, Ross G, Shield CF,3rd, Warady BA, Aeder MI, et al. 2001. Cold ischemia time: An independent predictor of increased HLA class I antibody production after rejection of a primary cadaveric renal allograft. Transplantation 71(7):875-9.

Caro-Oleas JL, Gonzalez-Escribano MF, Gentil-Govantes MA, Acevedo MJ, Gonzalez-Roncero FM, Bernal-Blanco G, Nunez-Roldan A. 2013. Influence of donor specific HLA antibodies detected by luminex in kidney graft survival: A multivariate analysis. Hum Immunol 74(5):545-9.

Caro-Oleas JL, Gonzalez-Escribano MF, Gonzalez-Roncero FM, Acevedo-Calado MJ, Cabello-Chaves V, Gentil-Govantes MA, Nunez-Roldan A. 2012a. Clinical relevance of HLA donor-specific antibodies detected by single antigen assay in kidney transplantation. Nephrol Dial Transplant 27(3):1231-8.

Caro-Oleas JL, Gonzalez-Escribano MF, Gentil-Govantes MA, Acevedo MJ, Gonzalez-Roncero FM, Blanco GB, Nunez-Roldan A. 2012b. Clinical relevance of anti-HLA donor-specific antibodies detected by luminex assay in the development of rejection after renal transplantation. Transplantation 94(4):338-44.

Caro-Oleas JL, Gonzalez-Escribano MF, Toro-Llamas S, Acevedo MJ, Martinez-Bravo MJ, Aguilera I, Nunez-Roldan A. 2010. Donor-specific antibody detection: Comparison of single antigen assay and luminex crossmatches. Tissue Antigens 76(5):398-403.

Cecka JM. 2010. Calculated PRA (CPRA): The new measure of sensitization for transplant candidates. Am J Transplant 10(1):26-9.

Chalasani G, Li Q, Konieczny BT, Smith-Diggs L, Wrobel B, Dai Z, Perkins DL, Baddoura FK, Lakkis FG. 2004. The allograft defines the type of rejection (acute versus chronic) in the face of an established effector immune response. J Immunol 172(12):7813-20.

Chaudhuri A, Ozawa M, Everly MJ, Ettenger R, Dharnidharka V, Benfield M, Mathias R, Portale A, McDonald R, Harmon W, et al. 2013. The clinical impact of humoral immunity in pediatric renal transplantation. J Am Soc Nephrol 24(4):655-64.

Chopra B and Sureshkumar KK. 2015. Changing organ allocation policy for kidney transplantation in the united states. World J Transplant 5(2):38-43.

Chung BH, Choi BS, Oh EJ, Park CW, Kim JI, Moon IS, Kim YS, Yang CW. 2014. Clinical impact of the baseline donor-specific anti-human leukocyte antigen antibody measured by luminex single antigen assay in living donor kidney transplant recipients after desensitization therapy. Transpl Int 27(1):49-59.

Claas FH, Witvliet MD, Duquesnoy RJ, Persijn GG, Doxiadis II. 2004. The acceptable mismatch program as a fast tool for highly sensitized patients awaiting a cadaveric kidney transplantation: Short waiting time and excellent graft outcome. Transplantation 78(2):190-3.

Collaborative transplant study K-14103E-Aug2017. 2017. www.ctstransplant.org (Nov 19, 2017).

Collaborative transplant study K-21103-Aug2017. 2017. www.ctstransplant.org (Nov 19, 2017).

da Silva MB, da Cunha FF, Terra FF, Camara NO. 2017. Old game, new players: Linking classical theories to new trends in transplant immunology. World J Transplant 7(1):1-25.

Daga S, Moyse H, Briggs D, Lowe D, Evans N, Jones J, Buchli R, McMurtrey C, Mulder A, Hildebrand W, et al. 2017. Direct quantitative measurement of the kinetics of HLA-specific antibody interactions with isolated HLA proteins. Hum Immunol.

De Meester J, Persijn GG, Wujciak T, Opelz G, Vanrenterghem Y. 1998. The new eurotransplant kidney allocation system: Report one year after implementation. eurotransplant international foundation. Transplantation 66(9):1154-9.

Delgado JC and Eckels DD. 2008. Positive B-cell only flow cytometric crossmatch: Implications for renal transplantation. Exp Mol Pathol 85(1):59-63.

DeVos JM, Gaber AO, Knight RJ, Land GA, Suki WN, Gaber LW, Patel SJ. 2012. Donor-specific HLA-DQ antibodies may contribute to poor graft outcome after renal transplantation. Kidney Int 82(5):598-604.

Doshi MD, Garg N, Reese PP, Parikh CR. 2011. Recipient risk factors associated with delayed graft function: A paired kidney analysis. Transplantation 91(6):666-71.

Doytchinova IA and Flower DR. 2002. Physicochemical explanation of peptide binding to HLA-A*0201 major histocompatibility complex: A three-dimensional quantitative structure-activity relationship study. Proteins 48(3):505-18.

EFI standards for histocompatibility & immunogenetics testing v7.0. 2017. http://www.efi-web.org/efi-committees/standards-committee.html (Nov 19, 2017).

El-Awar N, Jucaud V, Nguyen A. 2017. HLA epitopes: The targets of monoclonal and alloantibodies defined. J Immunol Res 2017:3406230.

El-Awar N, Terasaki PI, Nguyen A, Sasaki N, Morales-Buenrostro LE, Saji H, Maruya E, Poli F. 2009. Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood. Hum Immunol 70(10):844-53.

Ellis TM, Schiller JJ, Roza AM, Cronin DC, Shames BD, Johnson CP. 2012. Diagnostic accuracy of solid phase HLA antibody assays for prediction of crossmatch strength. Hum Immunol 73(7):706-10.

Eng HS, Bennett G, Tsiopelas E, Lake M, Humphreys I, Chang SH, Coates PT, Russ GR. 2008. Anti-HLA donor-specific antibodies detected in positive B-cell crossmatches by luminex predict late graft loss. Am J Transplant 8(11):2335-42.

Eng HS, Bennett G, Bardy P, Coghlan P, Russ GR, Coates PTH. 2009. Clinical significance of anti-HLA antibodies detected by luminex®: Enhancing the interpretation of CDC-BXM and important post-transplantation monitoring tools. Hum Immunol 70(8):595-9.

Eurotransplant Reference Laboratory Virtual PRA Calculator https://www.etrl.org/Virtual%20PRA/Default.aspx (Feb 28 2018)

Everly MJ, Rebellato LM, Haisch CE, Briley KP, Bolin P, Kendrick WT, Kendrick SA, Morgan C, Maldonado AQ, Harland RC, et al. 2014. Impact of IgM and IgG3 anti-HLA alloantibodies in primary renal allograft recipients. Transplantation 97(5):494-501.

Ferrer F, Mota A, Alves R, Bastos C, Macario F, Figueiredo A, Santos L, Roseiro A, Parada B, Pratas J, et al. 2009. Renal transplantation with expanded criteria donors: The experience of one portuguese center. Transplant Proc 41(3):791-3.

Finnish registry for kidney diseases. Report 2016. 2018. www.muma.fi/files/3360/Finnish_Registry_for_Kidney_Diseases_Report_2016.pdf (Feb 28, 2018).

Fuller AA, Rodey GE, Parham P, Fuller TC. 1990. Epitope map of the HLA-B7 CREG using affinity-purified human alloantibody probes. Hum Immunol 28(3):306-25.

Giblin L, O'Kelly P, Little D, Hickey D, Donohue J, Walshe JJ, Spencer S, Conlon PJ. 2005. A comparison of long-term graft survival rates between the first and second donor kidney transplanted--the effect of a longer cold ischaemic time for the second kidney. Am J Transplant 5(5):1071-5.

Gilbert M, Paul S, Perrat G, Giannoli C, Pouteil Noble C, Morelon E, Rigal D, Dubois V. 2011. Impact of pretransplant human leukocyte antigen-C and -DP antibodies on kidney graft outcome. Transplant Proc 43(9):3412-4.

Glorie K, Haase-Kromwijk B, van de Klundert J, Wagelmans A, Weimar W. 2014. Allocation and matching in kidney exchange programs. Transpl Int 27(4):333-43.

Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. 2011. Allele frequency net: A database and online repository for immune gene frequencies in worldwide populations. Nucleic Acids Res 39(Database issue):D913-9.

Graff RJ, Buchanan PM, Dzebisashvili N, Schnitzler MA, Tuttle-Newhall J, Xiao H, Schadde E, Gheorghian A, Lentine KL. 2010. The clinical importance of flow cytometry crossmatch in the context of CDC crossmatch results. Transplant Proc 42(9):3471-4

Gupta A, Iveson V, Varagunam M, Bodger S, Sinnott P, Thuraisingham RC. 2008. Pretransplant donor-specific antibodies in cytotoxic negative crossmatch kidney transplants: Are they relevant? Transplantation 85(8):1200-4.

Halloran PF. 2004. Immunosuppressive drugs for kidney transplantation. N Engl J Med 351(26):2715-29.

Halloran PF, Aprile MA, Farewell V, Ludwin D, Smith EK, Tsai SY, Bear RA, Cole EH, Fenton SS, Cattran DC. 1988. Early function as the principal correlate of graft survival. A multivariate analysis of 200 cadaveric renal transplants treated with a protocol incorporating antilymphocyte globulin and cyclosporine. Transplantation 46(2):223-8.

Higgins R, Lowe D, Daga S, Hathaway M, Williams C, Lam FT, Kashi H, Tan LC, Imray C, Fletcher S, et al. 2015. Pregnancy-induced HLA antibodies respond more vigorously after renal transplantation than antibodies induced by prior transplantation. Hum Immunol 76(8):546-52.

Ho EK, Vasilescu ER, Colovai AI, Stokes MB, Hallar M, Markowitz GS, D'Agati VD, Cohen DJ, Ratner LE, Suciu-Foca N. 2008. Sensitivity, specificity and clinical relevance of different cross-matching assays in deceased-donor renal transplantation. Transpl Immunol 20(1-2):61-7.

Holmberg C and Jalanko H. 2016. Long-term effects of paediatric kidney transplantation. Nat Rev Nephrol 12(5):301-11.

Honger G, Krahenbuhl N, Dimeloe S, Stern M, Schaub S, Hess C. 2015. Inter-individual differences in HLA expression can impact the CDC crossmatch. Tissue Antigens 85(4):260-6.

Humar A, Ramcharan T, Kandaswamy R, Gillingham K, Payne WD, Matas AJ. 2002. Risk factors for slow graft function after kidney transplants: A multivariate analysis. Clin Transplant 16(6):425-9.

Humar A, Johnson EM, Payne WD, Wrenshall L, Sutherland DE, Najarian JS, Gillingham KJ, Matas AJ. 1997. Effect of initial slow graft function on renal allograft rejection and survival. Clin Transplant 11(6):623-7.

Hwang HS, Sun IO, Yoon HE, Choi BS, Oh EJ, Kim JI, Moon IS, Kim YS, Yang CW. 2012. Antibody monitoring system to support the single-antigen luminex assay in donor-specific antibody detection. Hum Immunol 73(4):370-5.

Immenschuh S, Zilian E, Dammrich ME, Schwarz A, Gwinner W, Becker JU, Blume CA. 2015. Indicators of treatment responsiveness to rituximab and plasmapheresis in antibody-mediated rejection after kidney transplantation. Transplantation 99(1):56-62.

Jodal L and Brochner-Mortensen J. 2009. Reassessment of a classical single injection 51Cr-EDTA clearance method for determination of renal function in children and adults. part I: Analytically correct relationship between total and one-pool clearance. Scand J Clin Lab Invest 69(3):305-13.

Johnston TD, Thacker LR, Jeon H, Lucas BA, Ranjan D. 2004. Sensitivity of expanded-criteria donor kidneys to cold ischaemia time. Clin Transplant 18 Suppl 12:28-32.

Joosten SA, Sijpkens YW, van Kooten C, Paul LC. 2005. Chronic renal allograft rejection: Pathophysiologic considerations. Kidney Int 68(1):1-13.

Karahan GE, Claas FH, Heidt S. 2017. B cell immunity in solid organ transplantation. Front Immunol 7:686.

Kerman RH, Susskind B, Buyse I, Pryzbylowski P, Ruth J, Warnell S, Gruber SA, Katz S, Van Buren CT, Kahan BD. 1999. Flow cytometry-detected IgG is not a contraindication to renal transplantation: IgM may be beneficial to outcome. Transplantation 68(12):1855-8.

Khalkhali HR, Ghafari A, Hajizadeh E, Kazemnejad A. 2010. Risk factors of long-term graft loss in renal transplant recipients with chronic allograft dysfunction. Exp Clin Transplant 8(4):277-82.

Klein J and Sato A. 2000. The HLA system. first of two parts. N Engl J Med 343(10):702-9.

Koefoed-Nielsen P, Weinreich I, Bengtsson M, Lauronen J, Naper C, Gabel M, Sorensen SS, Wennberg L, Reisaeter AV, Moller BK, et al. 2017. Scandiatransplant acceptable mismatch program (STAMP) a bridge to transplanting highly immunized patients. HLA 90(1):17-24.

Kosmoliaptsis V, O'Rourke C, Bradley JA, Taylor CJ. 2010. Improved luminex-based human leukocyte antigen-specific antibody screening using dithiothreitol-treated sera. Hum Immunol 71(1):45-9.

Kosmoliaptsis V, Bradley JA, Peacock S, Chaudhry AN, Taylor CJ. 2009. Detection of immunoglobulin G human leukocyte antigen-specific alloantibodies in renal transplant patients using single-antigen-beads is compromised by the presence of immunoglobulin M human leukocyte antigen-specific alloantibodies. Transplantation 87(6):813-20.

Lachmann N, Todorova K, Schulze H, Schonemann C. 2013. Luminex((R)) and its applications for solid organ transplantation, hematopoietic stem cell transplantation, and transfusion. Transfus Med Hemother 40(3):182-9.

Le Bas-Bernardet S, Hourmant M, Valentin N, Paitier C, Giral-Classe M, Curry S, Follea G, Soulillou JP, Bignon JD. 2003. Identification of the antibodies involved in B-cell crossmatch positivity in renal transplantation. Transplantation 75(4):477-82.

Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, Gautreau C, Charron D, Glotz D, Suberbielle-Boissel C. 2010. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. J Am Soc Nephrol 21(8):1398-406.

Lefaucheur C, Viglietti D, Bentlejewski C, Duong van Huyen JP, Vernerey D, Aubert O, Verine J, Jouven X, Legendre C, Glotz D, et al. 2016. IgG donor-specific anti-human HLA antibody subclasses and kidney allograft antibody-mediated injury. J Am Soc Nephrol 27(1):293-304.

Lietz K, John R, Burke E, Schuster M, Rogers TB, Suciu-Foca N, Mancini D, Itescu S. 2005. Immunoglobulin M-to-immunoglobulin G anti-human leukocyte antigen class II antibody switching in cardiac transplant recipients is associated with an increased risk of cellular rejection and coronary artery disease. Circulation 112(16):2468-76.

Lim MA, Kohli J, Bloom RD. 2017. Immunosuppression for kidney transplantation: Where are we now and where are we going? Transplant Rev (Orlando) 31(1):10-7.

Linjama T, Eberhard HP, Peräsaari J, Muller C, Korhonen M. 2018. A European HLA isolate and its implications for hematopoietic stem cell transplant donor procurement. Biol Blood Marrow Transplant.24(3):587-593

Liu C, Wetter L, Pang S, Phelan DL, Mohanakumar T, Morris GP. 2012. Cutoff values and data handling for solid-phase testing for antibodies to HLA: Effects on listing unacceptable antigens for thoracic organ transplantation. Hum Immunol 73(6):597-604.

Lloveras J, Arcos E, Comas J, Crespo M, Pascual J. 2015. A paired survival analysis comparing hemodialysis and kidney transplantation from deceased elderly donors older than 65 years. Transplantation 99(5):991-6.

Lopes D, Barra T, Malheiro J, Tafulo S, Martins L, Almeida M, Pedroso S, Dias L, Castro Henriques A, Cabrita A. 2015. Effect of different sensitization events on HLA alloimmunization in kidney transplantation candidates. Transplant Proc 47(4):894-7.

Loupy A, Lefaucheur C, Vernerey D, Prugger C, Duong van Huyen JP, Mooney N, Suberbielle C, Fremeaux-Bacchi V, Mejean A, Desgrandchamps F, et al. 2013. Complement-binding anti-HLA antibodies and kidney-allograft survival. N Engl J Med 369(13):1215-26.

Lucas DP, Leffell MS, Zachary AA. 2015. Differences in immunogenicity of HLA antigens and the impact of cross-reactivity on the humoral response. Transplantation 99(1):77-85.

Maiers M, Gragert L, Klitz W. 2007. High-resolution HLA alleles and haplotypes in the united states population. Hum Immunol 68(9):779-88.

Marino J, Paster J, Benichou G. 2016. Allorecognition by T lymphocytes and allograft rejection. Front Immunol 7:582.

Matinlauri IH, Kyllonen LE, Eklund BH, Koskimies SA, Salmela KT. 2004. Weak humoral posttransplant alloresponse after a well-HLA-matched cadaveric kidney transplantation. Transplantation 78(2):198-204.

Mayer G and Persijn GG. 2006. Eurotransplant kidney allocation system (ETKAS): Rationale and implementation. Nephrol Dial Transplant 21(1):2-3.

Mehra NK and Baranwal AK. 2016. Clinical and immunological relevance of antibodies in solid organ transplantation. Int J Immunogenet 43(6):351-68.

Minucci PB, Resse M, Sabia C, Esposito A, De Iorio G, Napoli C. 2017. Anti-HLA antibodies testing on solid phase: Comparative evaluation of different kit vendors through luminex technology. Exp Clin Transplant 15(6):636-40.

Moreau A, Varey E, Anegon I, Cuturi MC. 2013. Effector mechanisms of rejection. Cold Spring Harb Perspect Med 3(11):10.1101/cshperspect.a015461.

Opelz G. 1985. Correlation of HLA matching with kidney graft survival in patients with or without cyclosporine treatment. Transplantation 40(3):240-3.

Opelz G and Dohler B. 2013. Ceppellini lecture 2012: Collateral damage from HLA mismatching in kidney transplantation. Tissue Antigens 82(4):235-42.

Patel R and Terasaki PI. 1969. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med 280(14):735-9.

Piazza A, Ozzella G, Poggi E, Caputo D, Manfreda A, Adorno D. 2014. Virtual crossmatch in kidney transplantation. Transplant Proc 46(7):2195-8.

Pippias M, Kramer A, Noordzij M, Afentakis N, Alonso de la Torre R, Ambuhl PM, Aparicio Madre MI, Arribas Monzon F, Asberg A, Bonthuis M, et al. 2017. The european renal association - european dialysis and transplant association registry annual report 2014: A summary. Clin Kidney J 10(2):154-69.

Reinsmoen NL, Patel J, Mirocha J, Lai CH, Naim M, Ong G, Wang Q, Zhang X, Liou F, Yu Z, et al. 2016. Optimizing transplantation of sensitized heart candidates using 4 antibody detection assays to prioritize the assignment of unacceptable antigens. J Heart Lung Transplant 35(2):165-72.

Rekers NV, de Fijter JW, Claas FH, Eikmans M. 2016. Mechanisms and risk assessment of steroid resistance in acute kidney transplant rejection. Transpl Immunol 38:3-14.

Rendine S, Ferrero NM, Sacchi N, Costa C, Pollichieni S, Amoroso A. 2012. Estimation of human leukocyte antigen class I and class II high-resolution allele and haplotype frequencies in the italian population and comparison with other european populations. Hum Immunol 73(4):399-404.

Riethmuller S, Ferrari-Lacraz S, Muller MK, Raptis DA, Hadaya K, Rusi B, Laube G, Schneiter G, Fehr T, Villard J. 2010. Donor-specific antibody levels and three generations of crossmatches to predict antibody-mediated rejection in kidney transplantation. Transplantation 90(2):160-7.

Robinson J, Halliwell JA, Hayhurst JD, Flicek P, Parham P, Marsh SG. 2015. The IPD and IMGT/HLA database: Allele variant databases. Nucleic Acids Res 43(Database issue):D423-31.

Rogers NJ and Lechler RI. 2001. Allorecognition. Am J Transplant 1(2):97-102.

Rusai K, Dworak J, Potemkina A, Fischer G, Csaicsich D, Arbeiter K, Aufricht C, Muller-Sacherer T. 2016. Donor-specific HLA antibodies and graft function in kidney-transplanted children - the vienna cohort. Pediatr Transplant 20(4):507-14.

Salmela K, Höckerstedt K, Salminen US, Hämmäinen P. 2004. Organ transplants, relevant operations already for 40 years. Duodecim 120(11):1359-69.

Salvade I, Aubert V, Venetz JP, Golshayan D, Saouli AC, Matter M, Rotman S, Pantaleo G, Pascual M. 2016. Clinically-relevant threshold of preformed donor-specific anti-HLA antibodies in kidney transplantation. Hum Immunol 77(6):483-9.

Scandiatransplant figures. 2017. http://www.scandiatransplant.org/data/scandiatransplant-figures (Nov 06 2017).

Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. 2011. HLA antibody specification using single-antigen beads--a technical solution for the prozone effect. Transplantation 92(5):510-5.

Schnitzler MA, Hollenbeak CS, Cohen DS, Woodward RS, Lowell JA, Singer GG, Tesi RJ, Howard TK, Mohanakumar T, Brennan DC. 1999. The economic implications of HLA matching in cadaveric renal transplantation. N Engl J Med 341(19):1440-6.

Scornik JC, Brunson ME, Schaub B, Howard RJ, Pfaff WW. 1994. The crossmatch in renal transplantation. evaluation of flow cytometry as a replacement for standard cytotoxicity. Transplantation 57(4):621-5.

Shah N, Decker WK, Lapushin R, Xing D, Robinson SN, Yang H, Parmar S, Tung SS, O'Brien S, Fernandez-Vina M, et al. 2011. HLA homozygosity and haplotype bias among patients with chronic lymphocytic leukemia: Implications for disease control by physiological immune surveillance. Leukemia 25(6):1036-9.

Susal C, Dohler B, Ruhenstroth A, Morath C, Slavcev A, Fehr T, Wagner E, Kruger B, Rees M, Balen S, et al. 2016. Donor-specific antibodies require preactivated immune system to harm renal transplant. EBioMedicine 9:366-71.

Taylor CJ, Chapman JR, Ting A, Morris PJ. 1989. Characterization of lymphocytotoxic antibodies causing a positive crossmatch in renal transplantation. relationship to primary and regraft outcome. Transplantation 48(6):953-8.

Tennankore KK, Kim SJ, Baer HJ, Chan CT. 2014. Survival and hospitalization for intensive home hemodialysis compared with kidney transplantation. J Am Soc Nephrol 25(9):2113-20.

Terasaki PI. 2012. A personal perspective: 100-year history of the humoral theory of transplantation. Transplantation 93(8):751-6.

Terasaki PI and McClelland JD. 1964. Microdroplet assay of human serum cytotoxins. Nature 204:998-1000.

Valenzuela NM, Hickey MJ, Reed EF. 2016. Antibody subclass repertoire and graft outcome following solid organ transplantation. Front Immunol 7:433.

van Zelm MC. 2014. B cells take their time: Sequential IgG class switching over the course of an immune response? Immunol Cell Biol 92(8):645-6.

Vlad G, Ho EK, Vasilescu ER, Colovai AI, Stokes MB, Markowitz GS, D'Agati VD, Cohen DJ, Ratner LE, Suciu-Foca N. 2009. Relevance of different antibody detection methods for the prediction of antibody-mediated rejection and deceased-donor kidney allograft survival. Hum Immunol 70(8):589-94.

Wahrmann M, Hlavin G, Fischer G, Marinova L, Schwaiger E, Horl WH, Zlabinger GJ, Kormoczi GF, Konig F, Bohmig GA. 2013. Modified solid-phase alloantibody detection for improved crossmatch prediction. Hum Immunol 74(1):32-40.

Willicombe M, Rizzello A, Goodall D, Papalois V, McLean AG, Taube D. 2017. Risk factors and outcomes of delayed graft function in renal transplant recipients receiving a steroid sparing immunosuppression protocol. World J Transplant 7(1):34-42.

Willicombe M, Roufosse C, Brookes P, Galliford JW, McLean AG, Dorling A, Warrens AN, Cook TH, Cairns TD, Taube D. 2011. Antibody-mediated rejection after alemtuzumab induction: Incidence, risk factors, and predictors of poor outcome. Transplantation 92(2):176-82.

Willicombe M, Brookes P, Sergeant R, Santos-Nunez E, Steggar C, Galliford J, McLean A, Cook TH, Cairns T, Roufosse C, et al. 2012. De novo DQ donor-specific antibodies are associated with a significant risk of antibody-mediated rejection and transplant glomerulopathy. Transplantation 94(2):172-7.

Wu P, Jin J, Everly MJ, Lin C, Terasaki PI, Chen J. 2013. Impact of alloantibody strength in crossmatch negative DSA positive kidney transplantation. Clin Biochem 46(15):1389-93.

Zachary AA, Lucas DP, Detrick B, Leffell MS. 2009a. Naturally occurring interference in luminex assays for HLA-specific antibodies: Characteristics and resolution. Hum Immunol 70(7):496-501.

Zachary AA, Sholander JT, Houp JA, Leffell MS. 2009b. Using real data for a virtual crossmatch. Hum Immunol 70(8):574-9.

Zecher D, Bach C, Staudner C, Boger CA, Bergler T, Banas B, Spriewald BM. 2017. Characteristics of donor-specific anti-HLA antibodies and outcome in renal transplant patients treated with a standardized induction regimen. Nephrol Dial Transplant 32(4):730-7.

Zhang R. 2018. Donor-specific antibodies in kidney transplant recipients. Clin J Am Soc Nephrol 13(1):182-192.

13 ORIGINAL PUBLICATIONS