Analysis and monitoring of anti-HLA antibodies in solid-organ and stem cell transplantation : What to do? When should they be tested?

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"Analysis and monitoring of anti-HLA antibodies in solid-organ and stem cell transplantation: What to do? When should they be tested?"

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I. Abbreviations

Abs:	antibodies
ABMR:	antibody mediated rejection
APC:	antigen presenting cell
CAN:	chronic allograft nephropathy
CBU:	Cord-Blood Unit
CDC:	complement-dependent cytotoxic crossmatch
CML:	chronic myeloid leukemia
DSA:	donor-specific antibodies
dnDSA:	de novo donor-specific antibodies
GVHD:	graft-versus-host disease
GWAS:	genome wide association study
HLA:	human leukocyte antigen
HSCT:	hematopoietic stem-cell transplantation
MFI:	mean fluorescence intensity
MHC:	major histocompatibility complex
MM:	mismatch
LSA:	Luminex® single antigen
LSM:	Luminex® screening
NMDP:	National Marrow Donor Program (USA)
PBMC:	peripheral blood mononuclear cells
PCR-SSOP:	polymerase chain reaction using sequence-specific oligonucleotide probes
PCR-SSP:	polymerase chain reaction using sequence-specific primers
qPCR:	quantitative real-time polymerase chain reaction
RT-PCR:	reverse transcriptase-polymerase chain reaction
SNP:	single nucleotide polymorphism
SOAS:	Swiss organ allocation system
SOT:	solid-organ transplantation
SPA:	solid phase assay
TCMR:	T-cell mediated rejection
TRM:	transplant-related mortality
VAD:	ventricular assist devices

II. **Summary**

Solid organ transplantation (SOT) offers hope and a new life to many patients. Due to the shortage of deceased donor the search for suitable kidney from living donors has intensified. As the outcome of kidney grafts from living donors is more favorable than that from deceased donors, preference is given to organs from living donors. Unfortunately, recipient-donor couples are often HLA-mismatched due to the high polymorphism of HLA loci. Moreover, the number of sensitized patients is on the increase, as nowadays patients often need more than one transplant during their lifetime. Therefore, previous transplants are a major risk factor for developing anti-HLA antibodies, and we have to deal with this problem by increasing the number of tests as well as their sensitivity, and by carrying out accurate HLA-typing to ensure more appropriate organ allocation and the efficient follow-up and treatment of recipients. In the light of these additional difficulties our aim was to facilitate SOT transplantation, particularly kidney transplantation; we accordingly increased our catalogue of assays and developed guidelines for physicians regarding the acceptance of organs and an adequate follow-up of their patients. With living-donor kidney grafts, ABO-incompatible kidney transplantation and compatible non-directed donor exchange, immunological compatibilities are mandatory to avoid both acute and antibody-mediated rejection. With these strategies, we have so far obtained 100% graft survival for all types of living-donor kidney transplantation, as well as an excellent survival rate for pancreas and islet grafts as compared to other centers.

III. Introduction

Considering that the presence of donor-specific antibodies (DSA) has a direct impact on organ allocation to the patient [1], it is essential to determine the repertoire of specific anti-HLA antibodies. Should DSA develop after transplantation, the immunosuppressive protocol has to be adapted accordingly [2, 3].

To ensure the efficient follow-up of transplant patients, various techniques have been developed since transplantation started to exist. Some are still used at present, some are the fruit of a better understanding of HLA molecules, the anti-HLA antibodies involved and their role in graft survival. New methods for detecting anti-HLA antibodies have been made available, some of which have been validated for accreditation while others are awaiting approval from clinicians and laboratory supervisors.
Since these highly specific technologies are also very sensitive, the clinical relevance of anti-HLA antibodies with low, but also with intermediate or high MFI, is a matter of intense debate in the transplantation community [4, 5]. The presence of such anti-HLA antibodies could result in a patient being denied a transplant on the basis of irrelevant DSA or, after transplantation, in over-treating a recipient having developed DSA [1].

Moreover, the high polymorphism of the MHC system is characterized by the sharing of epitopes between alleles not only of the same locus but also of different loci (Fig.1) [6]. The sensitivity and the cross-reactivity of shared epitopes on SPA could therefore ensure the detection of a wide range of anti-HLA antibodies, even in case of exposure to a limited number of HLA antigens [7-9]. To address the crucial question as to which among this wide range of anti-HLA antibodies are detrimental to the transplanted organ, and which are not, several techniques have been devised to assist clinicians to attribute the organ that is appropriate for a given recipient and to help them in the patient's follow-up.

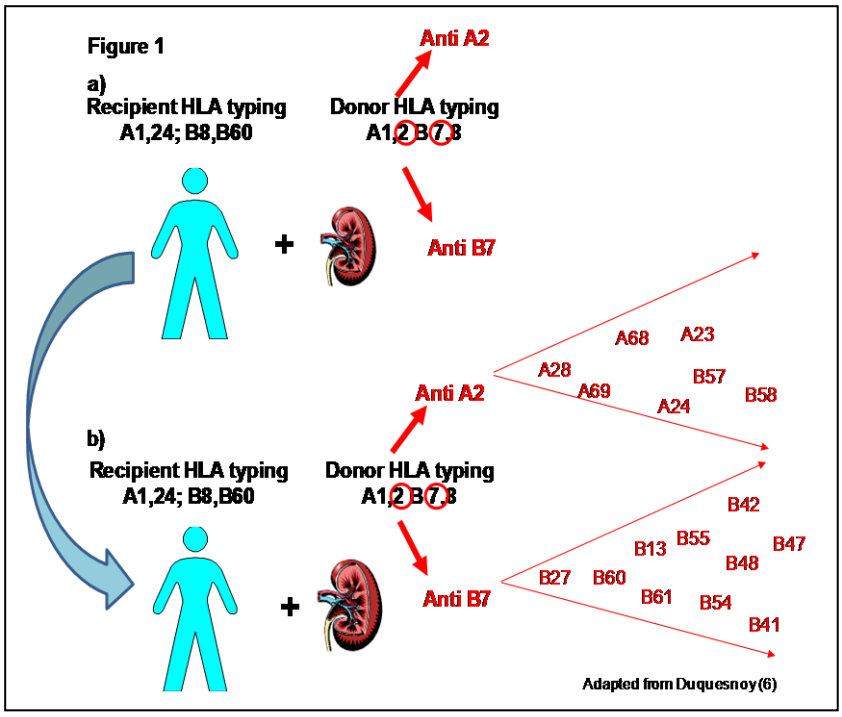


Figure 1: Development of DSA

a) After transplantation anti-HLA Abs against donor (DSA) may developed

b) Development of DSA but also of other anti-HLA Abs which have a broad affinity for HLA antigens of the donor. This phenomenon is due to shared epitopes between HLA antigens = EPLETS.

Of note, the aim to determine the presence of anti-HLA antibodies before transplantation and to monitor their subsequent progression was a prime concern of nephrologists with regard to their kidney-transplant recipients. Owing to a better understanding of the detrimental effect of these anti-HLA antibodies, particularly of DSA, in the context of rejection events and graft survival, transplant surgeons and clinicians have started to determine their role in other solid-organ transplants (SOT). Subsequently, the survival of islet or pancreas grafts was also shown to be affected by the presence of anti-HLA antibodies, since patients with high titers of anti-HLA antibodies proved to have a higher risk of organ rejection and loss of its function [10]. Like in heart transplantation, there is now a clear connection between anti-HLA antibodies and CAN [11] and with BOOP in lung transplantation [12].

For a long time, HLA mismatches in liver transplantation were not considered a problem, as graft survival of patients with 1 or 2 HLA-mismatches was similar to that of patients with 5 to 6 mismatches (Fig.2) [13]. This observation still applies, but the emergence of anti-HLA antibodies in liver transplant recipients is now also recognized as a potential cause of graft dysfunction since ductopenia and fibrosis appear to be associated with the appearance of anti-HLA antibodies [14].

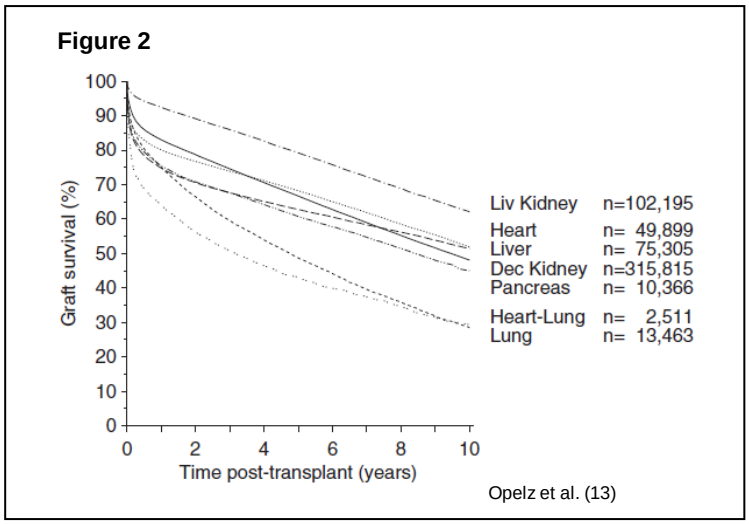


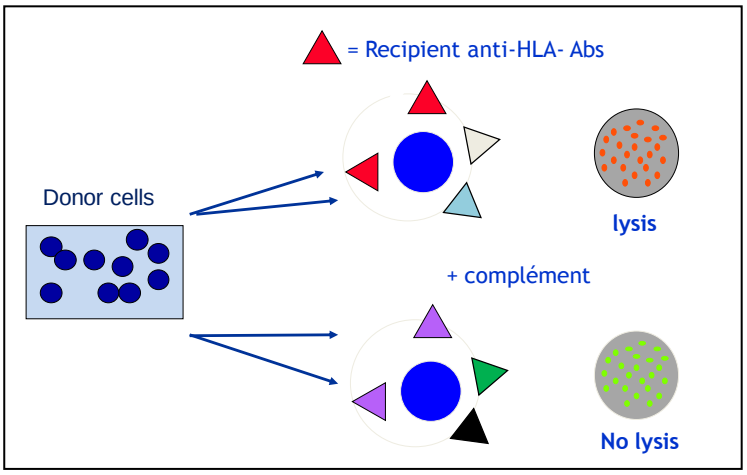
Figure 2: 10-year graft survival of solid organ transplants registered in Collaborative Transplant Study (CTS) (Kaplan Meier method).

This manuscript reviews the effect of anti-HLA antibodies on graft survival in SOT, thus revealing which anti-HLA antibodies are really detrimental and which technique/s should be performed to identify and characterize with precision the anti-HLA antibodies involved in rejection.

IV. Laboratory Tools & Techniques

a.Crossmatch

The complement-dependent cytotoxicity (CDC) crossmatch was the first method to be developed around 50 years ago [15]. It is based on the detection of circulating antibodies in the recipient's serum, which antibodies may bind to antigens on the cell surface of donor lymphocytes. In the presence of specific antibodies to lymphocytes, the classical complement pathway is activated, resulting in the lysis of donor cells by the membrane attack complex (MAC) (Fig.3a)**.** Even if technologies have evolved considerably, CDC crossmatch is still considered the "gold standard" by transplant centers, and so far, no better methods have been set up to detect harmful DSA during on call duties. Due to its higher sensitivity crossmatching by flow cytometry (FACS crossmatch) is also frequently used by centers, but similar to CDC crossmatch it is not specific for HLA antigens. If the patient's serum contains anti-HLA antibodies, these will bind to the donor's cell-surface antigens and by using a secondary anti-human-PE antibody, a positive reaction will be detected [16-18] .



b.CDC and lymphoscreen

Based on a method similar to the CDC assay, a lymphoscreen will reveal the percentage of "panel-reactive antibodies", or PRA, in the serum of an individual. A panel of 30 - 70 representative cells of all common HLA antigens observed in the population of potential donors in Switzerland is used as target cells. The percentage of wells (containing individual target cells) that show a positive reaction determines the percentage of "panel-reactive antibody", or % of PRA. This method can also be applied to determine the specificity of HLA allo-antibodies (e.g. anti-HLA A2). The advantage of the CDC assay is that it reveals complement-fixing antibodies (i.e. cytotoxic antibodies), but it is not specific for the donor's HLA. Indeed, other antibodies to non-HLA lymphocyte antigens or auto-antibodies can also bind to the complement and induce a positive reaction [19] .

c.Solid-phase assay

Thanks to the purification of HLA antigens from transfected cells and their binding to different supports, the solid-phase assay (SPA) was set up.

- ELISA (enzyme-linked immunosorbent assay): specific HLA antigens immobilized on a plastic surface are incubated with the serum of the patient. Patient's anti-HLA antibodies are revealed after addition of an enzyme-linked anti-human IgG antibody raised against the Fc fragment of the antibodies. The quantity of antibodies is determined by spectrophotometry using substrate-converting enzymes [20].
- Fluorescent microspheres (flow PRA® or Luminex®): specific HLA antigens immobilized on fluorescent microspheres are incubated with the serum of the patient. Binding of anti-HLA antibody binding is revealed by a second fluorescent anti-human IgG antibody detected by flow cytometry (FACS). Using specific microspheres that match the different HLA antigens, the repertoire of anti-HLA antibodies of a given serum can be assessed with great accuracy (Fig.3b) [21, 22] .

  The strength or the titer of an anti-HLA antibody can be determined through the mean fluorescence intensity (MFI) (or the absolute molecular equivalent of fluorescence intensity (MESH), more accurate but more complicated to set up.)

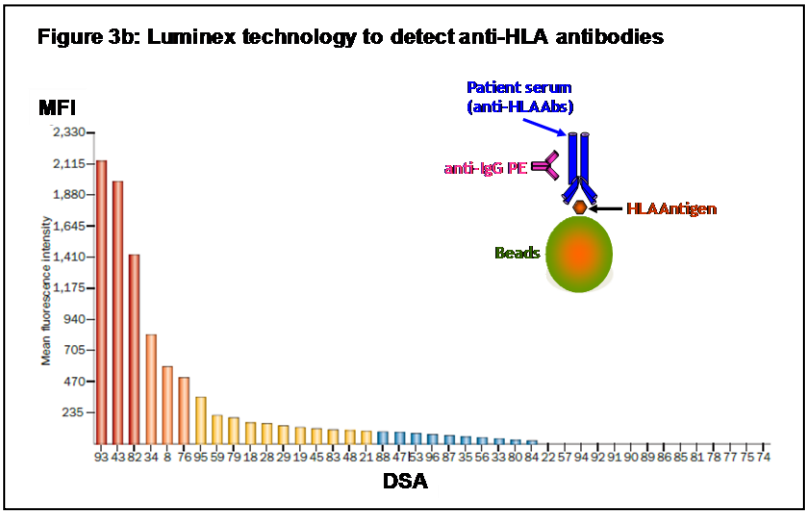


Figure 3b: Luminex technology to detect anti-HLA antibodies

d.Complement pathway assays

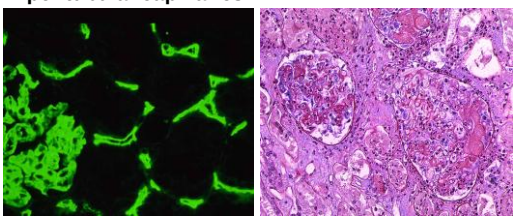
- **C4d**

The use of C4d staining on biopsies was introduced to detect deposition of antibodies on tissues (Fig.3c). The detection of C4d deposition within peritubular capillaries and the presence of anti-HLA antibodies detected by Luminex® confirmed the diagnosis of antibody mediated rejection (ABMR) [23]. A report on the detection of C4d-fixing HLA antibodies by solid phase assay were published recently [24]. Other assays of solid phase detection of complement-fixing antibodies are now routinely used (see below).

Figure 3c: Biopsy and histology of kidney

C4d staining in peritubular capillaries

Staining of glomeruli



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- C1q

To take advantage of the specific Luminex assay that detects specific anti-HLA antibodies and cytotoxic anti-HLA antibodies through the classical CDC crossmatch, the Luminex® C1q binding assay was recently developed**.** C1q is one of the first components of the classical activation pathway of the complement cascade (Fig.3d). This technique is designed to differentiate complement-binding anti-HLA antibodies (Fig.3e) from non-complement-binding anti-HLA antibodies, the former being the ones that are effective and detrimental. This technique may also help to avoid false positive results due to natural non-HLA antibodies linking to partially denatured antigens coating some beads [25, 26]. According to the most recent publications, C1q-binding DSA are associated with a worse graft survival than DSA not binding C1q . Although this approach appears to be promising after transplantation, current data fail to demonstrate that C1q-binding DSA in pre-transplant conditions can predict AMR and less favorable graft survival. [25, 27]. It therefore remains to be demonstrated that DSA binding C1q are a useful additional tool that can be integrated into the allocation algorithm to increase the clinical relevance of DSA and predict short- and long-term outcome.

Figure 3d: Complement pathway

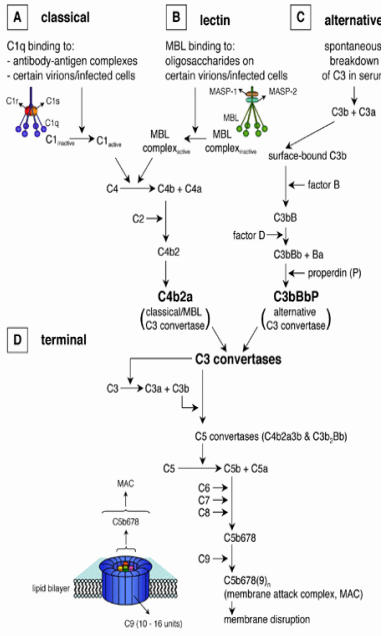
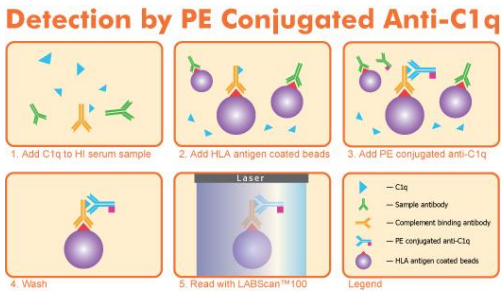


Figure 3e: Complement binding antibody (anti-C1q assay)



a. C3d

The different pathways of complement activation target the C3 convertase component which induces different products such as C3a, C5a or MAC. C3d is a cleavage product of C3 positioned downstream of the complement cascade. The C3d-binding antibody assay was developed - similar to the C1q assay - to help identify pathogenic anti-HLA antibodies. Sicard et al. [28] have demonstrated in two independent cohorts that DSA binding C3d are highly associated with AMR and an unfavorable outcome. In this study they also analyzed C4d deposition in graft biopsies and the C1q assay previously described. C3d-binding DSA was by far the most reliable indicator of an unfavorable graft outcome, unlike C4d deposition or the presence of C1q-positive DSA. The difference in performance between the C3d- and C1q-binding assays may be due to the fact that these two tests analyze different steps of the classical complement pathway. As already indicated, C1q is the first component of the classical complement pathway, and it is therefore not surprising that a C1q-binding assay should exhibit a lower sensitivity than a C3d-binding assay. Another plausible explanation could relate to the different mechanisms of inhibition which prevent uncontrolled activation of the complement cascade. By limiting the formation of C3 convertase - even when substantial amounts of C1q bind to antibodies - downstream inhibitory factors could reduce the clinical relevance of the C1q-binding assay. In contrast, the presence of C3d on DSA proves the efficient cleavage of C3 and is more closely associated with AMR. As far as we know, the use of C3d binding assay before transplantation has not yet been validated.

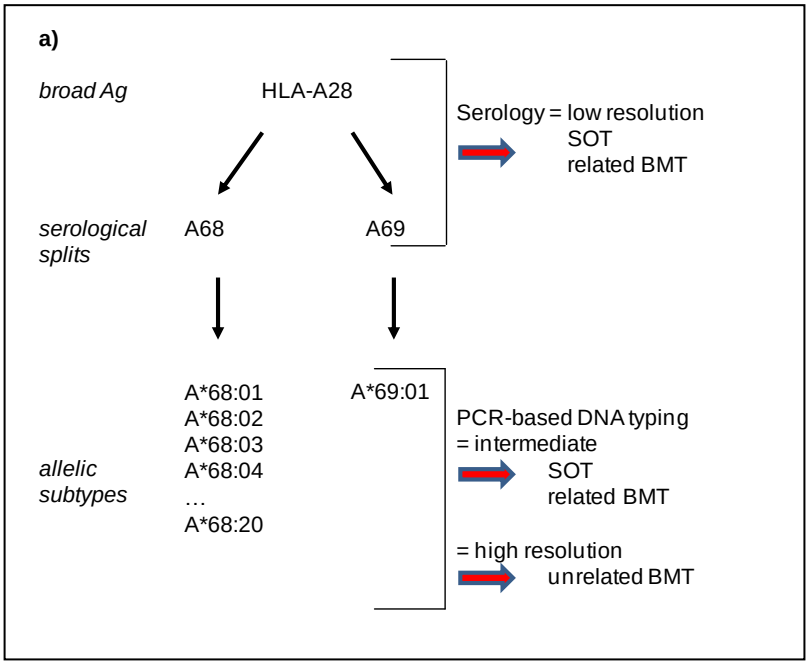
e.HLA typing

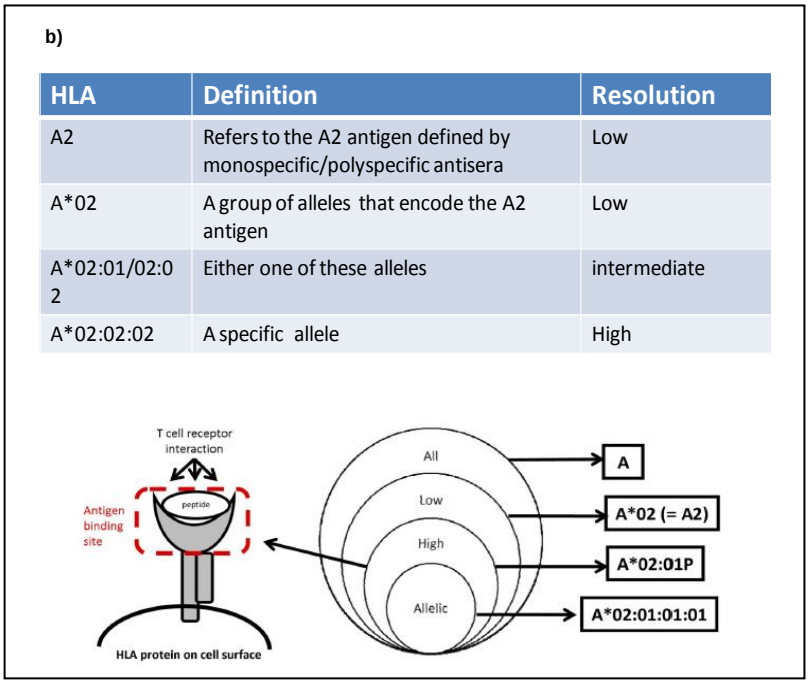
Passing from serology to molecular biology, typing of HLA genes has evolved tremendously since the 1960's. As to SOT, low-resolution typing by serology was the standard for several years. Interestingly, it was the development and accurate analysis of anti-HLA antibodies that led to intermediate- and to high-resolution typing! [29, 30]

As anti-HLA beads can resolve a four-digit allele (but ambiguities still remain of course), HLA typing had to shift from a two-digit to a four-digit resolution. Therefore, serology has almost disappeared from laboratory technology to give way to intermediate- and/or high-resolution PCR techniques, such as SSO-Luminex, or the SSP technique. Within the next 2 to 5 years, high-resolution typing based on sequencing will become the gold standard [31]. Soon the entire HLA gene typing will not only be instrumental in an improved characterization of

patients and donors but also in reducing any DSA in HLA-A,B,C,DR,DQ, and even DP loci as it leads to improve survival in unrelated HSCT (Fig.4) [32].

Figure 4: HLA typing: methods and resolution levels





a) For low resolution, serology is sufficient and is used for the 1st typing in SOT and for related BMT

For intermediate resolution, Luminex PCR or Linqsek HLA-typing are used for 2nd HLA typing in SOT and for related BMT

For high resolution typing, SSP PCR or sequencing are mandatory and used for unrelated BMT

b) Example of level of resolution. DNA nomenclature: the first field corresponding to the first 2 digits refer to serology (A2) and generic level (A*02). The second field (corresponding to high resolution) refers to the resolution of identical sequences in the peptide loading groove (A*02:01P). The polymorphism of the alleles is principally located in the antigen presenting groove. The third field defines substitution in the coding sequence (A*02:01:01), and the fourth field defines substitution in outside the coding region [33].

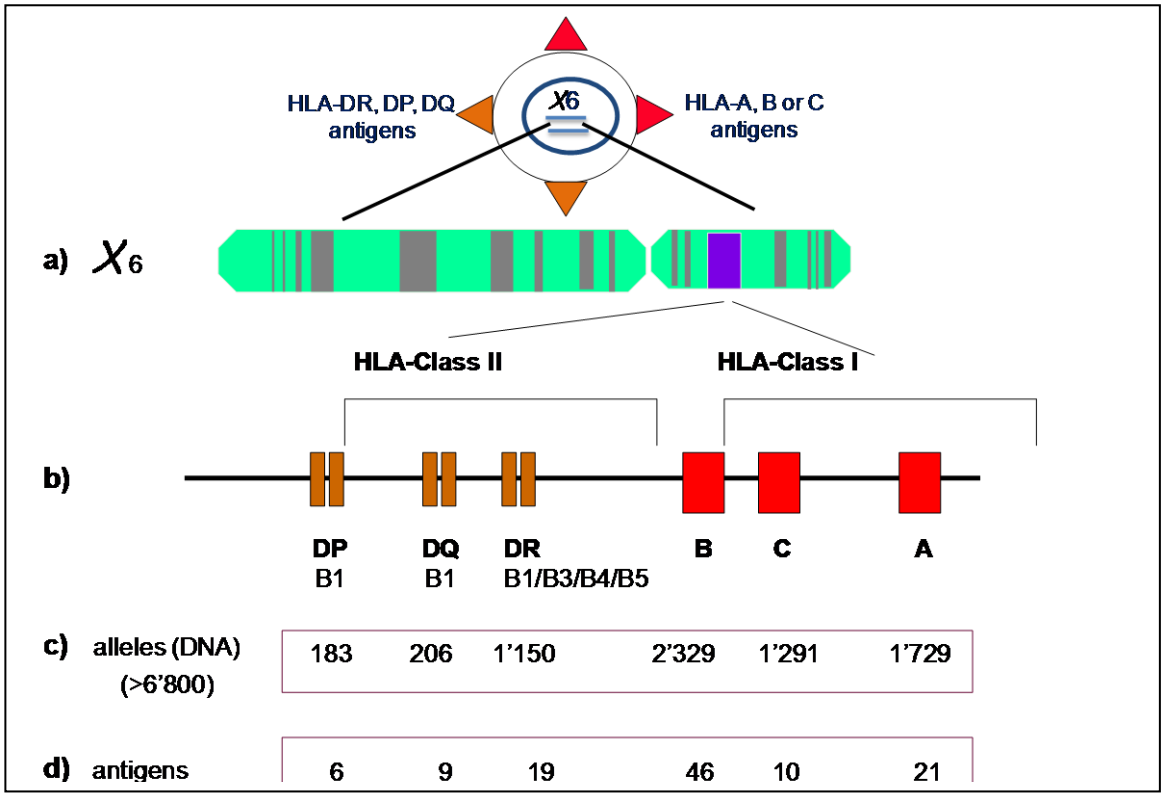
V. Graft Rejection

Human leukocyte antigens (HLA) encoded in the major histocompatibility complex (MHC) represent a major barrier to transplantation because the immune system unfailingly recognizes disparities between recipient and donor HLA [34]. An immune response to incompatible HLA antigens entails (a) an increased risk of post-transplantation complications such as rejection after solid organ transplantation (SOT) or graft-versus-host disease (GVHD) after HSCT or (b) a lower survival rate [35, 36].

HLA are mainly divided into two classes: HLA class I consists of the three major loci HLA-A, -B, and –C, and HLA class II of three major loci named HLA-DR, -DQ and –DP (Fig.5). In the context of SOT, HLA-A, -B, -DR and –DQ [37] are taken into account for matching and organ allocation, whilst HSCT specialists try to avoid mismatches by taking into account HLA-A, -B, -C –DR, –DQ and sometimes –DP [38].

Figure 5: MHC polymorphism

a) The MHC genes are located on the short arm of chromosome 6. The HLA region is composed of multiple genes that encode homologous cell surface proteins. These HLA loci are divided in two classes



b) Class I: HLA-A, -B and -C

Class II: HLA-DR, -DQ and –DP

c) These loci also have a high degree of allelic polymorphism, clearly defined by molecular cloning and sequencing. Each locus (A, DR, DQ….) has several alleles, numbers which are growing every day and their designations are based on the IMGT/HLA database, Version 3.20.0, April 2015 [39, 40] (WHO HLA Nomenclature Report (http://hla.alleles.org). These numerous alleles are the consequence of mutations as the MCH regions is a high polymorphic region

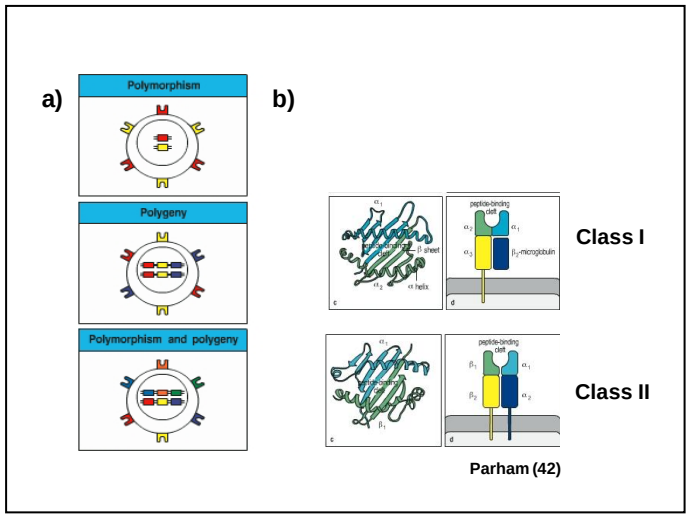
d) The transcription and traduction of these alleles give antigens that are detected and defined by serology. As this technique is less sensitive and specific, antigens are defined by groups and is sufficient for low resolution

HLA loci are highly polymorphic, which is crucial since the primordial role of HLA is to present foreign antigens - such as viral or bacterial peptides - to the immune system, triggering immune response and defense against microorganisms [41]. From the transplantation point of view, however, this high polymorphism represents a major barrier to successful transplantation since the recipient's immune system will react quickly to the foreign organism and try as hard as possible to eliminate it. The system is even more complex, MHC being not only highly polymorphic but also polygenic, i.e. all loci are expressed in each individual tissue or cell. Actually, HLA-class I are expressed by all the cells of the organism except red cells and HLA-class II are mainly expressed by antigen-presenting cells (APC) such as dendritic cells, macrophages and B cells (Fig.6) [42].

Figure 6: MHC complex

a) The MHC complex **is** polymorphic and polygenic

b) Class I molecules are expressed on most cells but red blood cells. They are composed of an α-chain and a β2 microglobulin (encoded on Χ15).Only the α-chain is polymorphic. Class II molecules are expressed on specific antigen presenting cells (APC) such as dendritic cells, macrophages and B cells. Upon inflammation, MCH class II can be expressed by any type of cells.

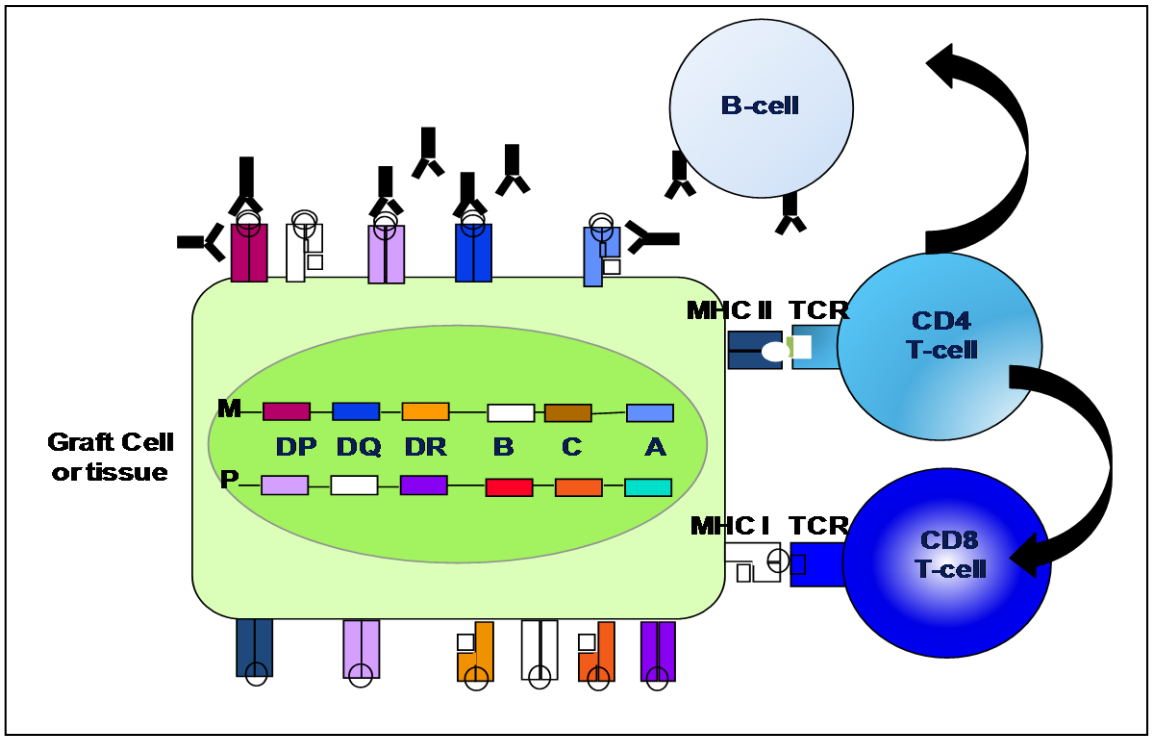


Class II are composed of two heterodimers α and β-chains. The α-chain presents less polymorphism than the β-chain.

However, HLA class II expression can also be induced and increased on any type of cells with the induction of cytokines, particularly IFN-γ [43].

The MHC complex is also highly sensitizing, leading to a rapid and robust production of anti-HLA antibodies [44, 45]. This is why allograft transplantations result in the violent acute response of the recipient's immune system, leading to a cascade of innate and adaptive immune responses along with its consequences: destruction, repair and fibrosis.

Figure 7: Allo-immune response to graft



The interaction of MHC expressed on the allograft and TCR expressed on T cells will activated CD4 as well as CD8 T lymphocytes. The activated T cells will produce cytokines and activating signals to help and activate each other as well as B lymphocytes which will produce antibodies( ).

Thanks to immunosuppressive drugs, this immune response can be controlled, at least to some extent, but it is never inactive, which implies that the recipient of SOT will need immunosuppressive treatment all his/her life. Several medications are currently available and the association of two to three drugs is prescribed, since - due to its inhibitory effect on immune cells - this association ensures a better control of the immune response. A summary of this aspect follows.

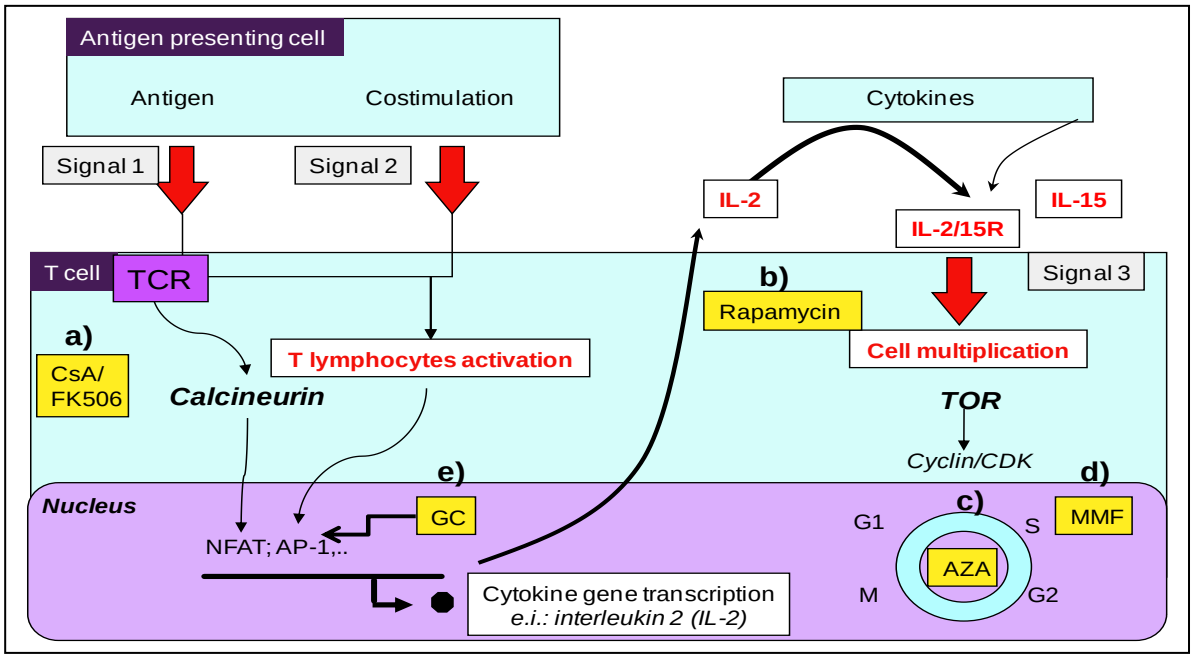
Three major categories of drugs exist that target essential pathways of T-lymphocyte activation and proliferation [46-49]:

1) Calcineurin inhibitors Inhibition of activation and proliferation of T lymphocytes by inhibition of TCR-dependent T-cell activation. CsA interfere with IL-2 synthesis AND IL-2R synthesis
2) Rapamycin = mTOR inhibitor: inhibition of the activating effect of IL-2

   These two first immunosuppressors are more specific of T lymphocytes
3) Azathioprine by interacting with sulfhydryl-containing compounds activates metabolites that will inhibit purine synthesis that is depletion of cellular purine. The final effect is the suppression of DNA and RNA synthesis.
4) Mycophenolate mofetil, the ester prodrug of mycophenolate acid (MPA), is the active metabolite. MPA is also a purine antagonist, acting downstream of the de novo purine synthesis pathway. By inhibition of the de novo synthesis of purine, a crucial step during the S phase of the cell cycle, DNA synthesis is blocked. As activated T and B lymphocytes need proliferation for an adequate immune response to antigenic stimulation, these cells are particularly shutdown.

   5) Glucocorticoids have potent anti-inflammatory and immunosuppressive effects. They affect multiple pathways and transcriptional regulators, but for our interest, one of their major effects is on inhibiting several transcription factors, as AP-1, NF-κB, NFAT and STAT families. The glucocorticoid inhibition of AP-1 and NF-κB will have major anti-inflammatory effects, as AP-1 and NF-κB play a central role on pro-inflammatory and T or B-cell targeting cytokines and acute phase proteins. Therefore Corticosteroids target proliferating cells (T cells, B cells) but also less dividing cells such as monocytes, fibroblasts, major players in the immune response and repairing phase [50].

Figure 8: Immunosuppressors:



a) CsA and FK506 = Calcineurin inhibitors

b) Rapamycin = mTOR inhibitor

c) AZA = Azathioprine suppression of DNA and RNA synthesis.

d) MMF = mycophenolate mofetil is also a purine antagonist

e) GC = Glucocorticoids inhibite several transcription factors, as AP-1, NF-κB, NFAT and STAT families.

These immunosuppressant drugs are often administered in a combination in order to target different pathways of immune cell activation, as well as to decrease their dosage and concomitant side effects [51, 52]. However, administration of immunosuppressors has to be controlled and their dosage adapted regularly to minimize side effects while keeping immune cells in check [53, 54].

Several other drugs are available, but they are mostly used to induce immunosuppression shortly after transplantation (during the first 3 to 5 days) or to treat a rejection episode.

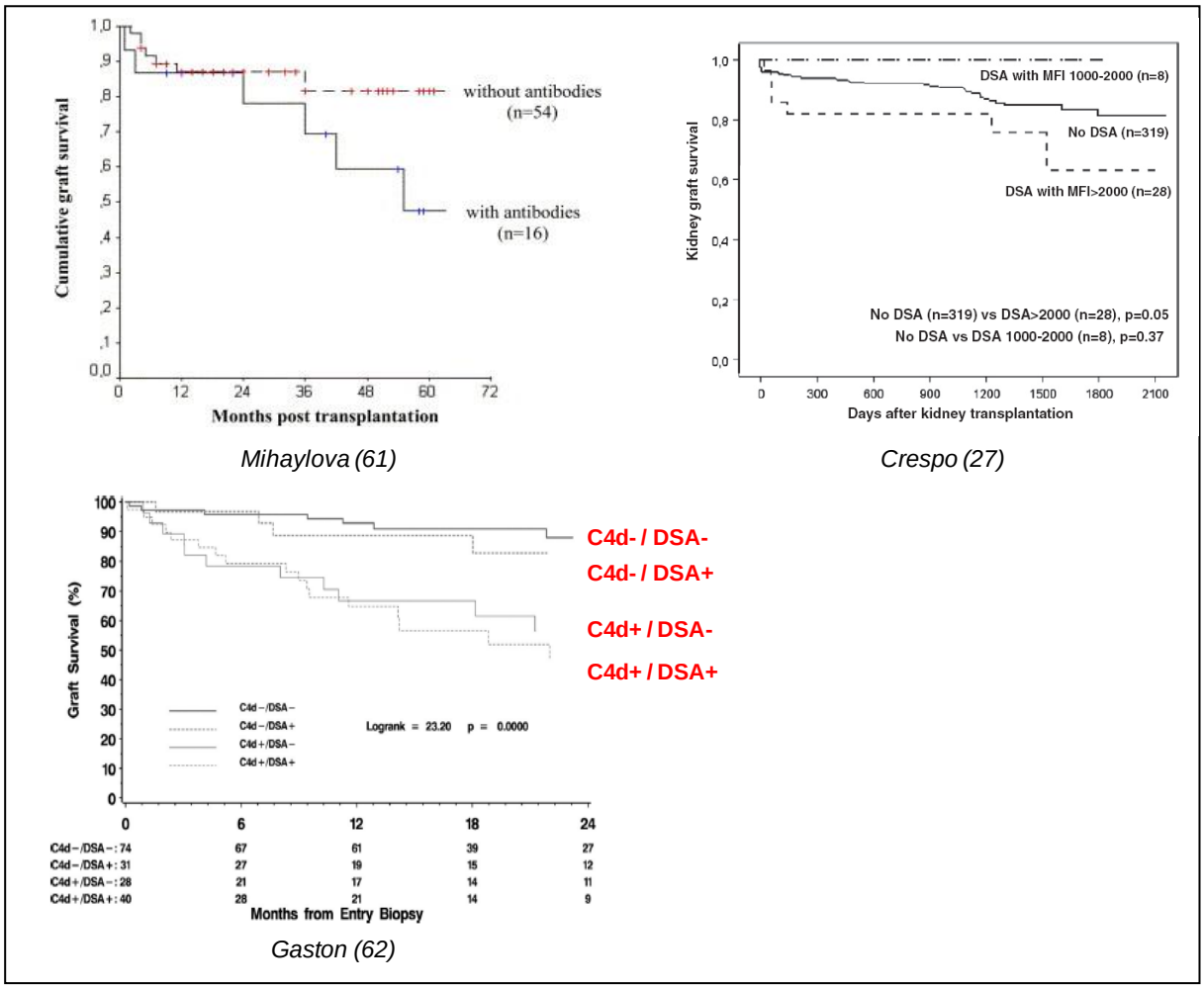
Despite all precautions, graft rejection can occur and is currently divided into three categories: hyperacute, acute and chronic graft rejection.

a. Hyperacute graft rejection

Hyperacute graft rejection is due to preformed circulating antibodies to antigens expressed at the surface of red blood cells and antibodies to HLA molecules expressed on the surface of transplanted tissue. Owing to the routinely performed identification of ABO blood group antigens as well as a laboratory crossmatch test (see above), this kind of graft rejection is rarely seen nowadays. The binding of preformed anti-A/B/AB antibodies or anti-HLA antibodies to their targets on grafted tissue induces the activation of the complement cascade and coagulation pathway that in turn results in the direct destruction of tissues. The consequence is that as soon as the transplanted organ is revascularized, hyperacute graft rejection sets in and leads to the immediate destruction and necrosis of the graft, leaving no chance to control it. Once this response was understood, it became possible to avoid it by proceeding with ABO-compatible transplantations and prospective negative crossmatches. But there are other ways to perform transplants across the blood barrier by resorting to circulating anti-HLA antibodies. However, ABO-incompatible transplantation is currently performed in numerous centers with great success, thanks to immunoadsorption. Applied 2 to 3 weeks before a planned living-donor kidney transplant, this technique helps to eliminate with great efficiency anti-A or anti-B antibodies [55, 56]. In our center, several living donor kidney transplants were performed without a single case of rejection and, to date, with a 100% survival rate for patients and grafts [57, 58].

As to preformed circulating anti-HLA antibodies, it was difficult before 2005 to determine whether a specific antibody to HLA was present on the organ to be transplanted. At that time, it was only by performing a crossmatch test before transplantation that - if positive - was evidence of the presence of such antibodies. Nowadays, the detection of specific anti-HLA antibodies is a routine test in all laboratories, and if a donor-specific antibody (DSA) is present at high density, transplantation will not be performed. As to ABO-incompatible transplantations, some clinical groups carry out kidney transplants despite the presence of DSA, using different methods to eliminate these DSA. However, the results are not satisfactory [2, 59, 60]. Indeed, even if hyperacute graft rejection is hardly ever observed, acute graft rejection does occur and it clearly decreases graft function and survival (Fig9) [27, 61, 62].

Figure 9: anti-HLA Abs are deleterious for graft survival.



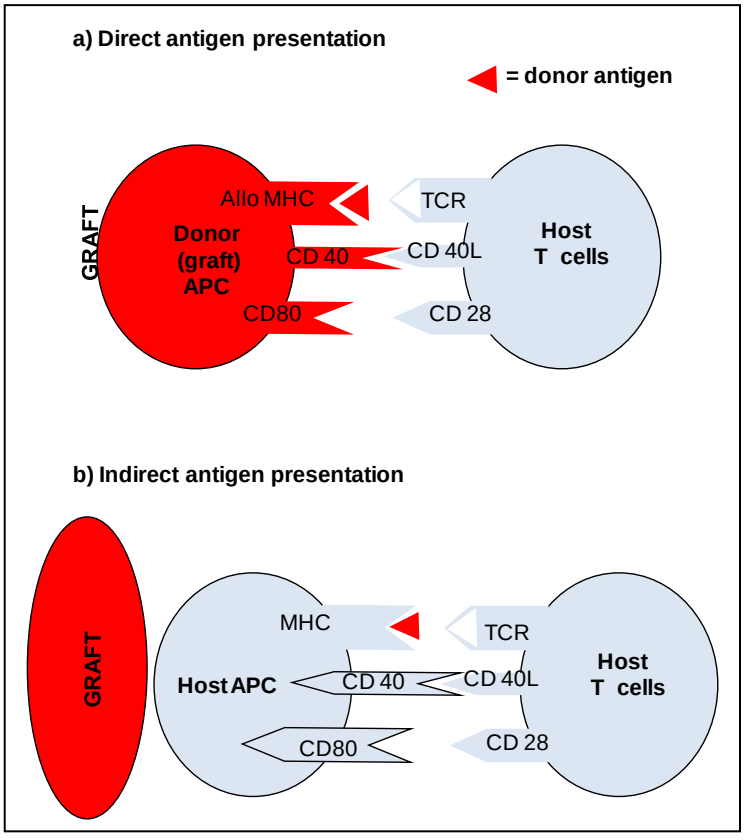
a) As presented by these figures the presence of DSA dramatically decrease allograft survival and is also dependent on the intensity (number) of DSA in serum

b) The presence of DSA (DSA^+) is deleterious for the graft but it is mainly the association of these DSA with episode(s) of rejection ($C4d^+$) that will lead to graft damage and lost

b. Acute graft rejection

Acute graft rejection sets in between three weeks and 6 months after transplantation - a consequence of the immune response to the foreign tissue. This process consists of cellular and humoral immune components. Since hyperacute graft rejection is dramatic but fortunately easy to avoid, acute graft rejection is more frequent and may occur despite adequate immunosuppression. First, foreign tissue, or more precisely foreign antigen presented by foreign APC, is recognized as non-self by host T lymphocytes in the local lymph nodes inducing a cellular immune response. This response is known as the direct alloimmune response. Simultaneously, CD4 helper T lymphocytes trigger a humoral immune response leading to anti-HLA antibody production. Second, host APC migrating in the graft capture and present foreign antigens to host T lymphocytes. This response is known as the indirect alloimmune response. Both responses end in the destruction of the graft (Fig.10).

Figure 10: Direct and Indirect antigen presentation



a) **Direct:** the donor APC (red) is presenting its own antigen (◀) to host T lymphocytes (blue). This occurs soon after transplantation.

b) **Indirect:** The host APC (blue) is presenting donor antigen (◀) to host T lymphocytes (blue). These donor antigens are coming for apoptotic donor cells. The indirect presentation occurs when recipient activated immune cells migrate to the "inflammatory site" (=transplantation site). At that point, recipient APC will catch recipient donor antigen and present them to recipient T lymphocytes.

Clinicians suspects acute graft rejection when the patient's graft function deteriorates progressively (i.e. in case of increased levels of creatinin or liver enzymes). A biopsy of the graft will provide specific criteria required for the diagnosis of acute graft rejection.

Immunohistology reveals whether the graft rejection is due to cellular and/or humoral processes. In the case of acute humoral graft rejection, the appearance of donor-specific anti-HLA antibodies (DSA) is the third crucial component to confirm the diagnosis [23, 63].

Due to their experience and to the conscientious and efficient follow-up of their patients, clinicians are able to recognize rapidly an episode of acute graft rejection, various parameters and laboratory tools being now available to help diagnosis. Thus, acute graft rejection can be treated promptly, and the consequences on the transplanted tissue tend to be minor. Therefore, major challenges nowadays lie in avoiding and treating chronic graft rejection.

Figure 11: Revised (BANFF 2013) classification of antibody-mediated rejection (ABMR) in renal allografts (adapted from Haas et al [23])

Acute/active ABMR; all 3 features must be present for diagnosis

1. Histologic evidence of acute tissue injury:
   microvascular inflammation or
   intimal or transmural arteritis or
   acute thrombotic microangiopathy or
   acute tubular injury
2. Evidence of current/recent antibody interaction with vascular endothelium: linear C4d staning in peritubular capillaries, (moderate) microvascular inflammation, increased expression of gene transcripts in the biopsy tissue indicative of endothelium injury (!?)
3. Serologic evidence of DSAs (HLA or other antigens)

Chronic, active ABMR; all 3 features must be present for diagnosis

1. Morphologic evidence of chronic tissue injury:
   Transplant glomerulopathy or
   severe peritubular capillary basement membrane multilayering or
   arterial intimal fibrosis of new onset
2. Evidence of current/recent antibody interaction with vascular endothelium: linear C4d staning in peritubular capillaries, (moderate) microvascular inflammation, increased expression of gene transcripts in the biopsy tissue indicative of endothelium injury (!?)
3. Serologic evidence of DSAs (HLA or other antigens)

C4d staining without evidence of rejection; all 3 features must be present for diagnosis

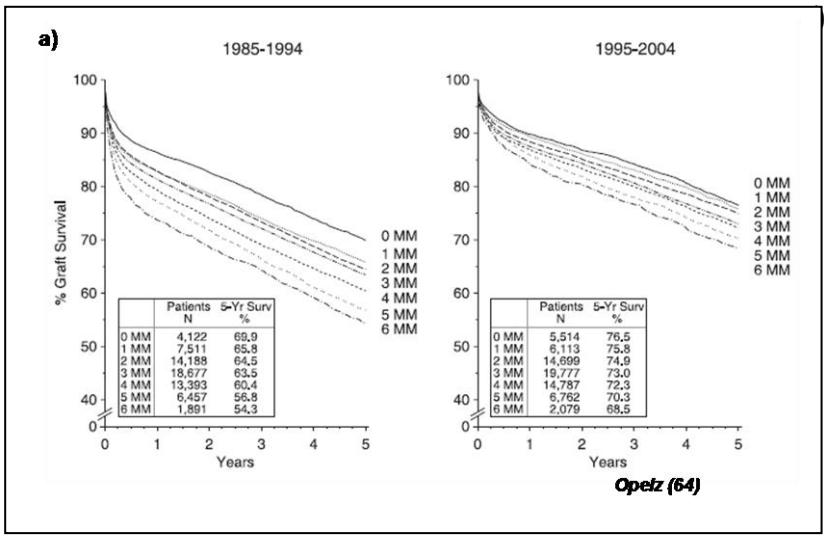
1. linear C4d staning in peritubular capillaries
2. No histologic evidence of acute tissue injury
3. No acute cell-mediated rejection (Banff 97 type 1A or greater) or borderline changes

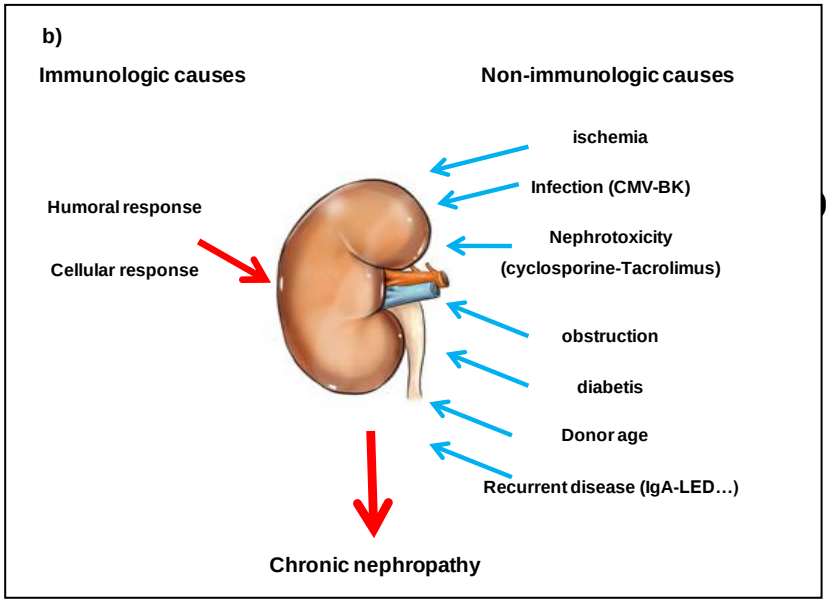
Banff criteria are frequently updated [63]. Last updated on 2014 [23].

c. Chronic graft rejection

Chronic graft rejection sets in more than 6 months after transplantation. It is often difficult to diagnose since the decrease in graft function appears slowly and progressively and is the consequence of various causes and triggers. Several processes are involved in chronic graft rejection [13, 64], the immune response being one of them. At first glance, recurrent infections and drug toxicity on tissue are deleterious, major side effects of immunosuppressors. Recurrence of the underlying disease (diabetes, autoimmune disease, IgA nephropathy) also occurs with time [65-67]. Therefore, late allo-immune response plays a part and insidiously creates irreversible damage to the transplanted tissue.

Figure 12: Long term graft survival





a) Kidney graft survival is dependent of the number of HLA mismatches (MM) (HLA-A, -B, -DR) between recipient and donor graft. Left panel = graft survival between 1985 and 1994. Right panel = graft survival between 1995 and 2004. Graft survival has increased over years but is still related to the number of HLA MM. Graft lost is mainly due to *de novo* DSA.

b) Lon-term graft survival is dependent on numerous factors: immunologic and non-immunologic.

The main challenge in chronic graft rejection is to recognize the beginning of the process as early as possible so that it can be stopped, thus minimizing the destruction and pathological repair that it entails. The key precaution consists in a regular follow-up of transplanted patients and watching out for any clinical changes that can hint at chronic graft rejection. Here again, determining anti-HLA antibodies and testing the presence of DSA need to be part of the follow-up.

VI. anti-HLA antibodies and graft rejection

a. Kidneys

Hyperacute and acute kidney rejection

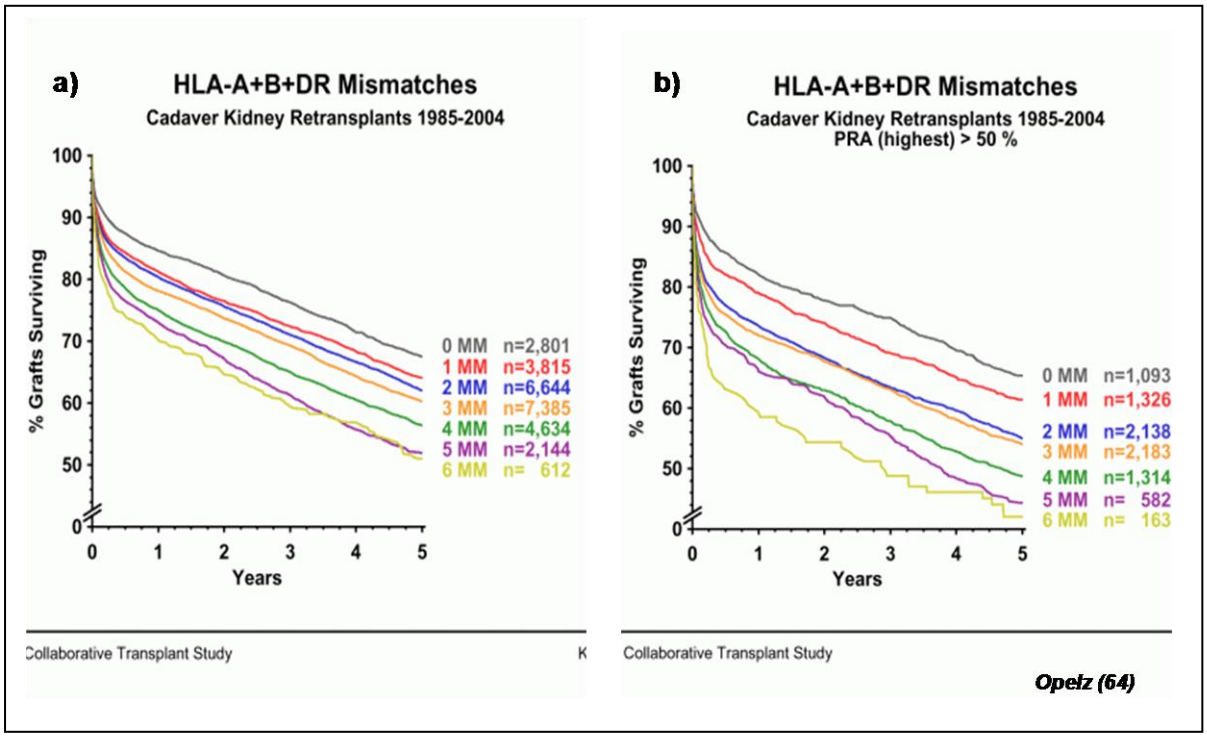
In the field of solid organ transplantation, the allocation of a kidney from a deceased donor is strictly subject to HLA-A, -B, -DR matching. Recently, the Collaborative Transplant Study demonstrated that HLA-C matching also impacts on kidney graft survival [68]. The presence before transplantation of anti-HLA antibodies - in particular donor-specific antibodies (DSA) - may induce antibody-mediated rejection and mechanisms leading to chronic graft nephropathy.

Thanks to prospective CDC crossmatching, hyperacute rejection is rare, but acute rejection is still observed - mainly because of preformed anti-HLA antibodies that were not detected at the time of transplantation, or due to de novo DSA surfacing in case of insufficient immunosuppression [61]. Anti-HLA antibodies show up in patients having received blood transfusions, after pregnancy and obviously after transplantation [69-71]. The presence of these circulating preformed anti-HLA may cause acute rejection as the antibodies are already present and may quickly reach the transplanted organ.

The diagnosis of acute rejection after kidney transplantation is based on three parameters: 1) decreased renal function, 2) histopathological signs of rejection, 3) the presence of DSA [63]. Fortunately, nowadays, acute rejection if rapidly diagnosed has a good prognosis, and a rapid modification of immunosuppression will help to attenuate inflammation and destruction of the transplanted tissue. Owing to a large panel of immunosuppressive drugs and the considerable advances in the field help physicians to rapidly cut short any episodes of rejection. Nevertheless, acute rejection tends to lead to inadequate tissue repair and fibrosis, resulting to some extent in the loss of some of the kidney function [10, 72].

Patient survival curves, as well as graft survival curves have dramatically improved within the past 5 to 10 years (see below), owing to several factors such as better management of patients, a better understanding of immunosuppressive drugs and an appropriate use, detection and elimination of DSA. But sensitized patients are still a challenge as their survival is decreased compared to non-immunized patients Fig 13).

Figure 13: HLA mismatches (MM) are deleterious for long term graft survival



a) Kidney graft survival decreased with number of HLA MM between recipient and donor

b) This phenomenon is worsen when recipient have anti-HLA Abs

It would therefore appear that now the principal challenge is late or chronic rejection as long-term survival has barely improved in the past 10 years.

Chronic kidney rejection

The fine tuning of immunosuppressive regimens as well as the monitoring of anti-HLA antibodies have improved the management of acute antibody mediated-rejection (AMR). However, long-term graft outcome may be affected unfavorably because of a delay in the diagnosis.

The diagnosis of chronic rejection applies if rejection sets in 6 to 12 months after transplantation. Chronic rejection may be due to several factors such as recurrent infections, side-effects of the immune suppression and cellular or humoral processes [65-67]. The early diagnosis of humoral rejection and its treatment may drastically affect graft outcome even if the episode manifests years after transplantation [62, 73]. To forecast the outcome of patients

suffering from chronic rejection in our institution, we recently analyzed our cohort of kidney-transplant recipients [74].

In our cohort of 850 kidney transplant recipients followed between 1983 and 2013 we observed de novo anti-HLA antibodies to be present in 31 patients (3.6%) out of these 31 patients 27 presented de novo DSA (87%). Of the 27 patients, 4 had anti-HLA class I only, 17 had class II only and 6 had class I and class II

.

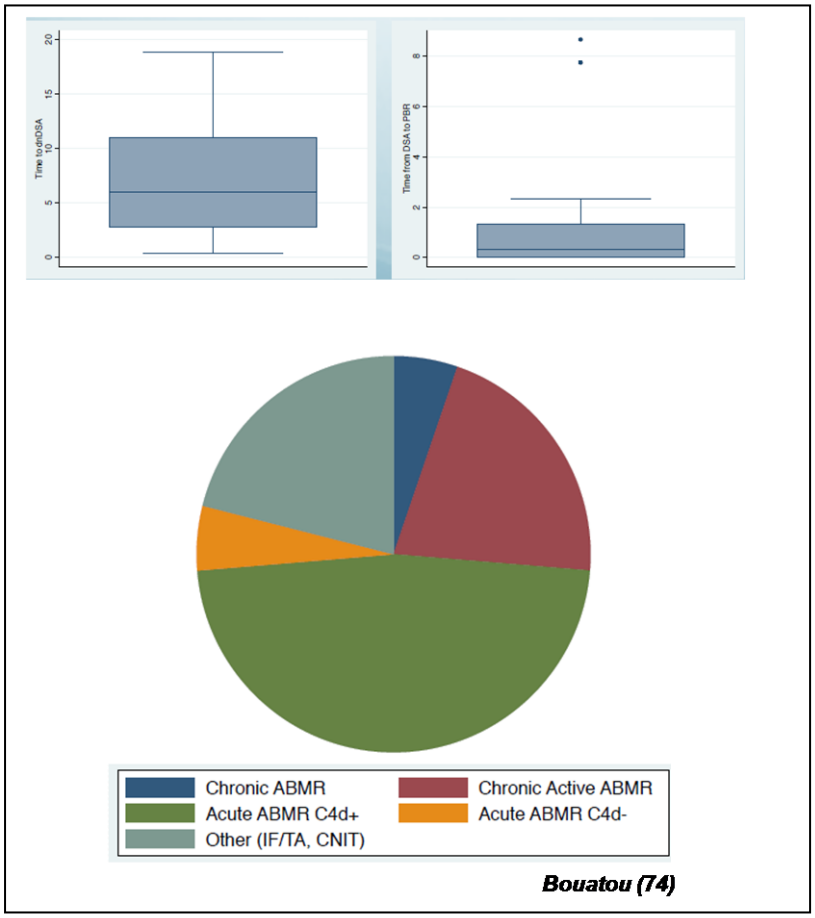
Figure 14: Characteristics of patients analyzed in our cohort of long-term kidney transplanted patients with *de novo* DSA (*dn*DSA)

Patients Caracteristics	Patients with *dn*DSA (19)
Age on the day of transplantation (years)	41 (19-65)
Female/male (n)	9/10
Class I (n,%)	9 (47)
Class II (n,%)	16 (84)
Class I + II (n,%)	6 (31)
Acute humoral rejection (< 1 an)	0
Acute cellular rejection (n,%)	4 (21)
Renal dysfunction at time of biopsy in patients with *dn*DSA (n,%)	7 (36)

Bouatou (74)

12 were therefore diagnosed with late ABMR (> 1 year post-transplant), 8 with early ABMR (< 6 month post-transplant) and 2 with early followed by late ABMR. The rate of patient and graft survival after 15 and 30 years was 100%. A graft biopsy was performed whenever DSA appeared regardless estimated glomerular filtration rate. The graft biopsy showed no rejection in 10 patients, ABMR in 16 patients, T-cell mediated rejection in 6 patients, and mixed rejection in one patient.

Figure 15: *de novo* DSA in long term kidney transplanted patients (> 1 year)



a) Left panel represents the interval between transplantation and the apparition of *de novo* DSA. Right panel determine the interval between the discovery of *de novo* DSA and the biopsy

b) Histological classification of kidney after discovery of *de novo* DSA. As shown, even in long term graft outcome, acute ABMR and chronic active ABMR are the major lesions observed.

Depending on the type of rejection, treatment consisted of rituximab, plasmapheresis and/or methylprednisolone pulses. At last follow-up, on December the 31st 2013, no graft was lost due to ABMR. This single center observation favors the need for systematic therapeutic management of subclinical ABMR in order to avoid chronic transplantation glomerulopathy. DSA validation in a randomized controlled study as well as molecular microscopy will probably help the clinician to improve disease reclassification and lead to enhanced treatment tailoring.

b. Islets-pancreas

To treat type I diabetes mellitus, whether complicated or not by end-stage-renal failure, the transplantation of either kidney or pancreas or both simultaneously, is an approach that is now widely accepted for selected patients. Pancreatic islet transplantation is an alternative used successfully to achieve insulin independence or improve glycemic control for patients with "brittle" type I diabetes. Pancreatic islet transplantation frequently requires several infusions from different donors. Both procedures expose the recipient to multiple HLA antigens and *a*

fortiori to multiple mismatches. As in kidney transplantation, DSA are associated with antibody-mediated rejection (ABMR) and reduced long-term graft survival [10, 75].Reports of isolated or simultaneous kidney-pancreas transplantation and islet transplantation mention that the development of anti-HLA antibodies is common and that it constitutes a risk factor for graft dysfunction and for survival [76]. It has been established that there is a correlation between the presence of anti-HLA antibodies and poor graft survival in pancreas transplant recipients. In islet transplantation, anti-HLA sensitization has a significant impact on islet-graft function when compared to non-sensitized patients. Other reports have also demonstrated an association between DSA and islet graft deterioration and failure [77]. More recently, the collaborative Islet Transplant Registry published an analysis of the largest cohort of 303 recipients of islet transplantation. In this study, HLA class I sensitization defined by the panel-reactive antibodies (% of PRA) was associated with significantly poorer islet graft function when compared to non-sensitized patients [78]. However, other reports indicate that DSA were not deleterious for islet function [79]. We previously published our cohort study in 2008 [80]. In this initial publication we concluded that the addition of islets does not represent a risk factor for the development of anti-HLA antibodies when combined with a kidney transplant.

Recently, we analyzed an updated cohort of patients that had undergone pancreatic islet or pancreas transplantation [81]. The goal of this study was to characterize the anti-HLA antibody repertoire of the recipients before and after transplantation and to assess the specific risk of anti-HLA sensitization in islet and pancreas transplantation. Eighteen patients received islet transplants and 27 patients received a pancreas transplant. The presence of anti-HLA antibodies was determined in 11 out of 18 islet transplant recipients (61.1%), but only in 12 out of 27 pancreas recipients (44.4%). Six patients (33.3%) developed donor-specific antibodies (DSA) to HLA antigens in the islets, and 10 patients (37%) developed DSA antibodies to HLA antigens in the pancreas. We did not find any factors that could have predicted the development of anti-HLA antibody after transplantation. Development of de novo DSA did not influence graft survival as estimated by insulin independence. These current data confirm our first publication [80] and we did not observe significant differences between islet and pancreas transplantation (Fig 16 and 17)

Figure 16: Risk of anti-HLA sensitization after islet or pancreas transplantation.

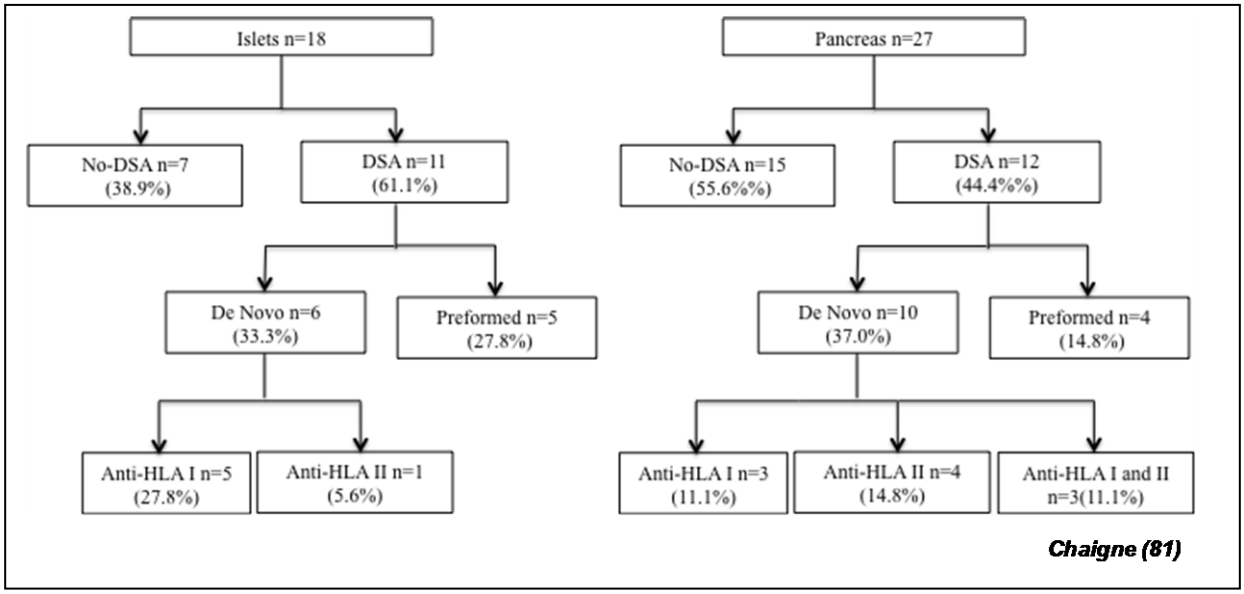
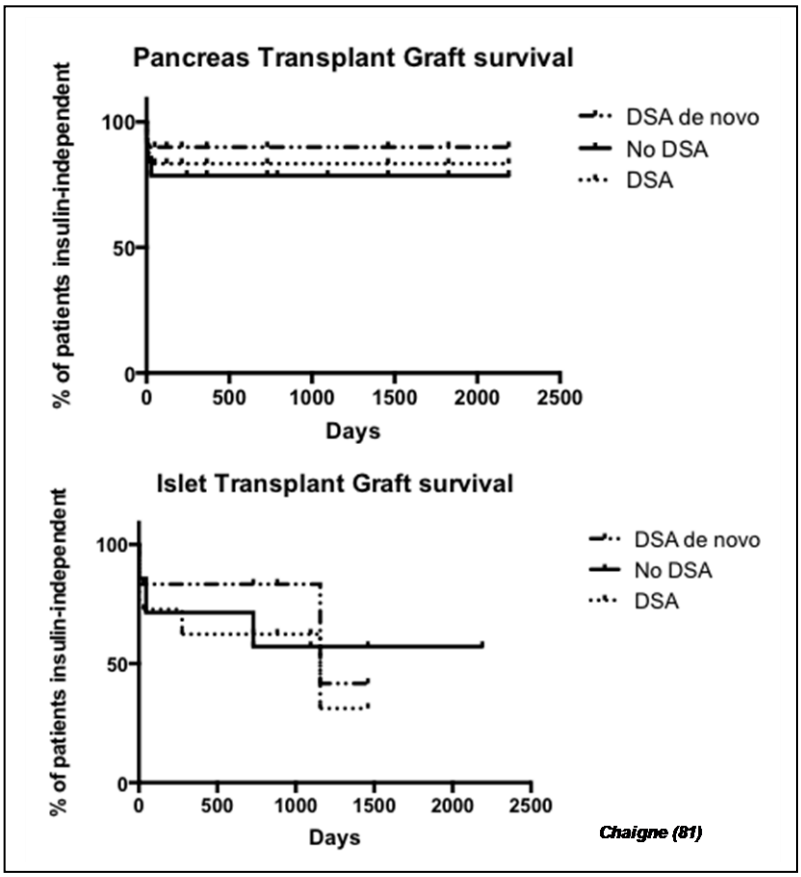


Figure 17: Pancreas and islet graft survival among patients with and without DSA.

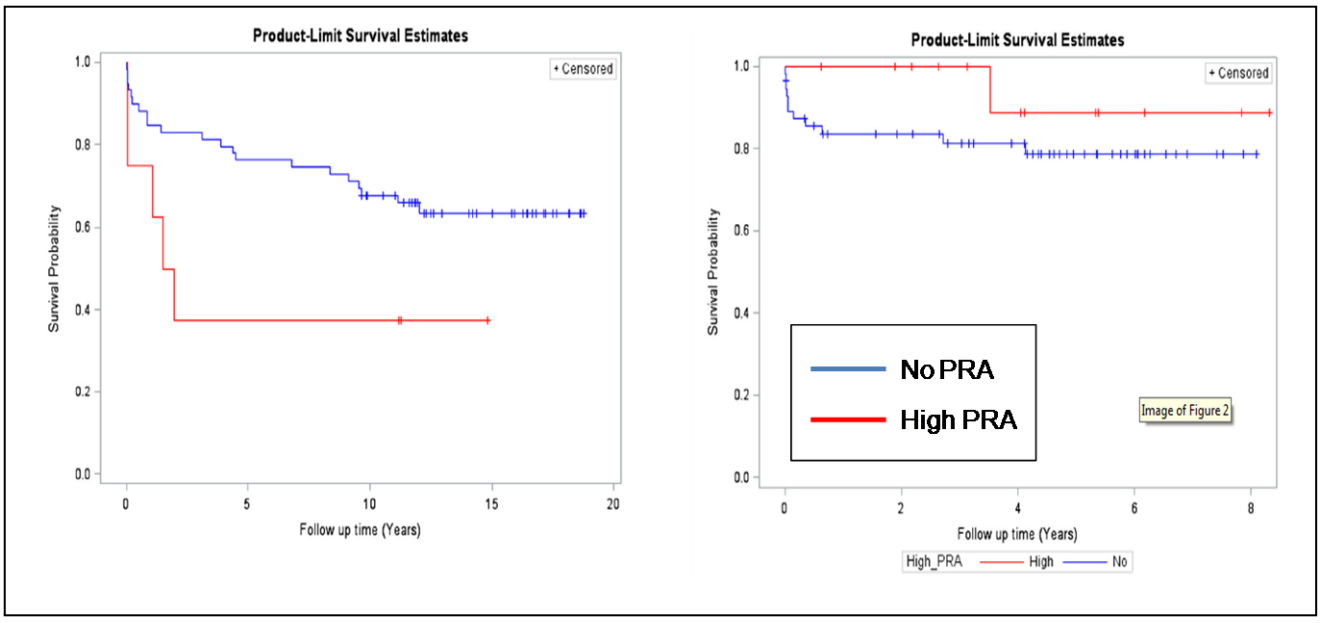


Our data indicate that DSA develop at similar rates in recipients of pancreas and islet transplants, and are not associated with reduced graft survival and reduced graft function (as estimated by insulin independence). These results contradict other reports describing increased HLA sensitization in patients with failed islet grafts [77] and poorer graft survival in pancreas transplantation [76, 82]. We assume that the immunosuppressive treatment of our cohort tended to be more intense than that in other centers, whose studies were reported previously. This could explain why we did not find statistical differences in terms of insulin dependence in the follow-up of our patients.

c. Heart and Lung

Adverse effects of DSA in other organ transplants such as heart [11] and lung [12] have been reported. In heart transplantations preformed anti-HLA antibodies are frequently detected that are due - in addition to other sensitizing events - to ventricular assist devices (VAD) [83]. As acute rejection after heart transplantation may jeopardize cardiovascular hemodynamics, screening for anti-HLA antibodies before transplantation is of prime importance. Considering the high sensitivity of SAB and the high percentage of anti-HLA antibodies detected in patients waiting for heart transplants it would appear that the detection of anti-HLA antibodies by SAB warrants predicting AMR and short-term graft survival (Fig.18) [11, 84, 85]. Therefore, SAB alone does not provide sufficient criteria to predict cardiac allograft rejection and graft survival, as VAD induces the development of numerous anti-HLA antibody specificities that are probably not toxic [86].

Figure 18: Pediatric heart graft survival among patients with and without anti-HLA Abs (Asante-Korang et al [85])



a)Kaplan–Meier survival plot comparing sensitized patients with non-sensitized patients before 2005. **b)** Kaplan–Meier survival plot comparing sensitized patients with non-sensitized patients after 2005.

After lung transplantation, the presence of de novo DSA may also induce AMR associated with bronchiolitis obliterans syndrome (BOS) and cystic fibrosis. Several reports are in agreement that anti-HLA antibodies and early DSA development are associated with poor

lung graft outcome and high risk of mortality [12, 87-89]. As illustrated below, determination of anti-HLA antibodies will dictate the acceptance or refusal of an organ offer [90].

Figure 19: Lung graft survival among patients with and without anti-HLA.

Organ offer assessment algorithm.

Status	Prospective CDC XM	Transplant
No DSA, cPRA <5 0%	NO	YES
No DSA, cPRA >5 0%	YES	If negative CDC XM
DSA present	YES	If negative CDC XM

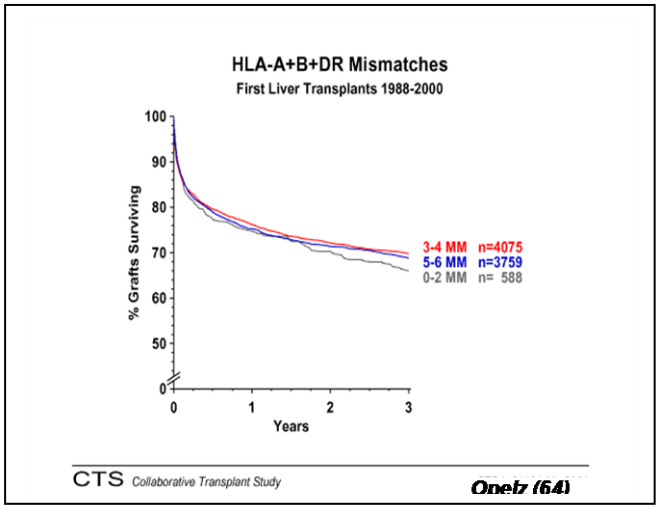
Adapeted from Kim et al (90)

Therefore, the close follow-up of these patients is crucial in order to detect de novo DSA and rapidly increase the dosage of immunosuppressant, thus avoiding AMR and its disastrous complications.

d. Liver

For many years, it was thought that HLA matching had no influence on liver transplant survival as illustrated:

Figure 20: Graft survival in liver transplantation.



In liver transplantation, graft survival is independent of HLA MM.

For this reason gastroenterologists took little notice of anti-HLA antibodies in their patients. Indeed, on the one hand, the complexity of liver transplant surgery along with its post-transplantation complications and, on the other hand, the immune privilege of the liver, the

allo-immune response and its role in post-transplant complications was not in the forefront of concerns.

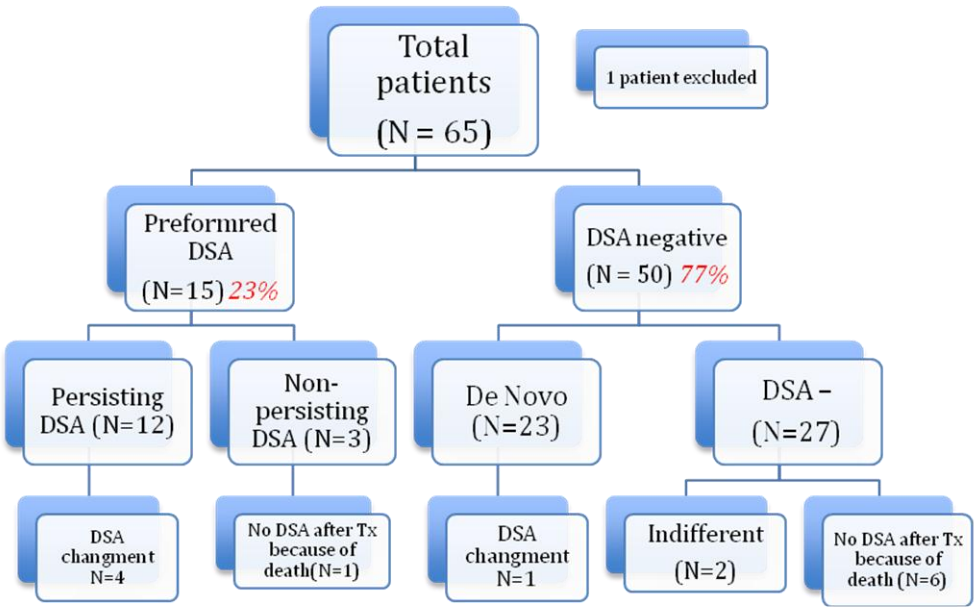
The role of the allo-immune response in liver transplantation has been debated, but recent literature suggests that anti-HLA antibodies - as in other organs - may have a major impact on liver transplants. To date, most of the reports refer to adult liver transplantation recipients, investigating the role of anti-HLA antibodies on events like acute and/or chronic rejection after liver transplantation [91, 92]. According to these reports, the impact of DSA on liver allograft function is as follows: 1) 20 to 40% of patients have DSA before and after transplantation [93]; 2) the presence of these DSA is associated with late chronic post-transplant complications such as ductopenia, biliary strictures and fibrosis [94, 95]; 3) the direct involvement of DSA in these liver complications may be complicated by tangled images of humoral and/or cellular mechanisms of rejection [96]. As no clear and systematic determination of anti-HLA antibodies and DSA took place in the liver transplant population, conflicting results are still being reported. Nevertheless, to understand long-term graft outcome, it seems clear that extensive prospective studies including the determination of DSA and histopathology data are needed for a more efficient approach to humoral and cellular rejection [97].

Reports dealing with pediatric patients also suggest that DSA may play a part in the long-term survival of liver grafts [98]. Here in Geneva, we also started to study liver transplant patients. We analyzed the presence of anti-HLA antibodies in our pediatric cohort of young liver recipients to determine whether DSA predict or not acute cellular rejection of pediatric liver transplants [99]. Of the 64 liver transplantations performed between 2005 to 2013, 23 patients (43%) were sensitized by the presence of preformed anti-HLA antibodies, of whom 13 had preformed DSA (20%). After transplantation, 25 patients developed de novo DSA (39%). Acute allograft rejection occurred in 23 (43%) transplanted children, 4 of which (6%) had preformed DSA, while 10 of the patients (16%) with rejection presented de novo DSA after transplantation. Nine of the patients with rejection did not have HLA antibodies (14%) (Fig.21). During this study we were surprised to observe that (1) 43% of this young population was already sensitized before transplantation, a fact that may have a negative effect on transplant outcome and (2) that the presence of these anti-HLA antibodies - in fact DSA - had no impact on either rejection episodes or patient and graft survival. According to our results, preformed DSA or de novo DSA do not seem to predict acute cellular rejection in pediatric liver transplants. However, it cannot be ruled out that chronic inflammation may

lead to other specific hepato-biliary damage as suggested by Miyagawa-Hayashino and others [98, 100]. Consequently, we will continue this study and compare histopathological liver lesions with the presence of anti-HLA antibodies.

Figure 21: Development of DSA in pediatric liver transplantation

a)



b)

Rejection (Banff)	Preformed DSA	De novo DSA	No DSA	Total patients
Light	1	1	0	2
Moderate	3	7	6	16
Severe	0	2	3	5
Total rejection	**4**	**10**	**9**	**23**
No rejection	9	15	17	41
Total	**13**	**25**	**26**	**64**

Schluckebier (99)

a) Even if more than 50% of the children have preformed DSA or developed de novo DSA, b) we did not observe an increased risk of rejection episodes in our cohort.

e. Bone marrow

Transplantation of hematopoietic stem cells (HSC) from related or unrelated donors is becoming increasingly common for treating patients with certain hematological diseases such as leukemia and lymphoproliferative syndromes [101-103]. The HLA system being highly polymorphic [36], it is difficult to identify a 'perfectly matched' donor. Consequently, an increasing number of patients are transplanted with HSC from unrelated volunteer donors registered in the Bone Marrow Donor Worldwide (BMDW) international database, which donors are often HLA-mismatched, at least at one locus [30, 104-107]. Although transplant centers agree that HLA incompatibilities entail an increased risk of post-transplant complications and mortality, no consensus on acceptable mismatches has yet been reached [108]. It is still unknown why some patients are successfully transplanted with HLA-mismatched HSC while others are not. At present, no official criteria exist for selecting the optimal donor from several HLA-incompatible donors. One option would be to select donors based on permissive antigens, i.e. antigens to which the recipient does not have antibodies!

However, when no suitable unrelated donor can be identified, the transplantation of mismatched umbilical cord blood (UCB) is becoming an increasingly common alternative. The drawback of UCB transplantation is that hematopoietic recovery is delayed and the risk of graft failure increased, as compared to HSCT from related or unrelated volunteer donors [109-111]. This is partly due to the fact that concentrations of nucleated cells and CD34+ cells are lower in the UCB grafts than in marrow grafts [109, 112]; another reason is mainly poor HLA matching of donor and recipient cells [113].

Early studies have highlighted the role of anti-HLA antibodies in graft failure after HLA-mismatched related HSCT [114, 115], positive cytotoxic crossmatch being an independent risk factor for graft failure [116]. Having analyzed anti-HLA antibodies soon after HSCT, Lapierre et al. [117] reported that peripheral blood HSCT mobilized by granulocyte-colony-stimulating factor (G-CSF) resulted in an increased incidence of circulating anti-HLA antibodies, as compared to bone marrow transplants. In HLA-mismatched HSCT, however, serum crossmatching was considered to be of good predictive value for graft failure [116]. Because of lower cell concentrations and higher HLA mismatching rates, UCBT is associated with a higher risk of graft failure [118].

When multiple UCB units are transplanted, patients with failed donor engraftment showed increased host T-cell proliferation in mixed lymphocyte reaction assays [119]. A Japanese study of 250 UCB recipients showed an incidence of anti-HLA antibodies of 16.4% [120]. Whilst the success of engraftment was similar in antibody-positive and antibody-negative patients, it was significantly lower in the 8 patients with DSA (93% vs. 58%, p=0.017) [120]. In two patients in need of SCT, the microbead assay was used to identify DSA and to select the CBUs accordingly [121]. According to the only published study on the use of virtual crossmatch in monitoring alloimmunization in UCB transplant patients, 24% of patients in a cohort of 46 patients [122] had anti-HLA antibodies. Although MICA antibodies have been associated with lower kidney graft survival [123] , very few studies have been published in allogeneic stem cell transplantation [120, 124].

Our hypothesis that UCB engraftment failure might correlate with the presence of DSA as determined by solid-phase assay techniques prompted us to study a cohort of 70 pediatric UCB transplant recipients. All cord blood transplant patients were tested for alloantibodies to HLA and to MICA on pre-transplant and post-transplant plasma samples. Based on two previous cohorts of UCB transplant patients, a 16-24% rate of anti-HLA antibodies is expected [120, 122]. Regarding pediatric patients awaiting donation of a liver, we were also surprised to see that 44 % of the children were sensitized before transplantation [99, 125].
The presence of anti-HLA class II antibodies was associated with reduced overall survival and event-free survival and is in relation with the strength of antibody titers in terms of mean fluorescence intensity. The presence of anti-MICA antibodies is significantly associated with a reduced platelet recovery rate after transplantation [125] .

VII. Articles

a) The role of anti-HLA Abs and particularly of DSA in graft outcome has become a pivotal concern in transplantation and has to be taken into account before carrying on with the transplant procedure. Since the various groups report different DSA intensities, we studied 155 living-donor kidney transplant patients from the areas around Geneva (42 patients) and Zurich (113 patients) between January 2005 and June 2008, and determined which anti-HLA intensities would be most appropriate for our patients on the waiting list.

The value of donor-specific antibody strength for predicting antibody-mediated rejection in sensitized kidney allograft recipients

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Transplantation, 90(2). 2010

Donor-Specific Antibody Levels and Three Generations of Crossmatches to Predict Antibody-Mediated Rejection in Kidney Transplantation

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Background. This study evaluated the prognostic impact of pretransplant donor-specific anti-human leukocyte antigen antibodies (DSA) detected by single-antigen beads and compared the three generations of crossmatch (XM) tests in kidney transplantation.

Methods. Thirty-seven T-cell complement-dependent cytotoxicity crossmatch (CXM) negative living donor kidney recipients with a retrospectively positive antihuman leukocyte antigen antibody screening assay were included. A single-antigen bead test, a flow cytometry XM, and a Luminex XM (LXM) were retrospectively performed, and the results were correlated with the occurrence of antibody-mediated rejections (AMRs) and graft function.

Results. We found that (1) pretransplant DSA against class I (DSA-I), but not against class II, are predictive for AMR, resulting in a sensitivity of 75% and a specificity of 90% at a level of 900 mean fluorescence intensity (MFI); (2) with increasing strength of DSA-I, the sensitivity for AMR is decreasing to 50% and the specificity is increasing to 100% at 5200 MFI; (3) the LXM for class I, but not for class II, provides a higher accuracy than the flow cytometry XM and the B-cell CXM. The specificity of all XMs is increased greatly in combination with DSA-I values more than or equal to 900 MFI.

Conclusions. In sensitized recipients, the best prediction of AMR and consecutively reduced graft function is delivered by DSA-I alone at high strength or by DSA-I at low strength in combination with the LXM or CXM.

Keywords: Kidney transplantation, Luminex crossmatch, Solid-phase assay, Anti-HLA antibodies, Donor-specific antibodies.

(*Transplantation* 2010;90: 160–167)

Humoral sensitization to human leukocyte antigens (HLA) is a barrier for solid organ transplantation, which can occur because of pregnancy, blood transfusion, previous transplants, or sensitization to cross-reactive epitopes found on microorganisms and ingested proteins (*1*). The presence of donor-specific anti-HLA antibodies (DSA) is associated with all forms of antibody (Ab)-mediated rejection (AMR) (*2*). Screening for preformed DSA has evolved from complement-dependent cytotoxicity crossmatch (CXM) (*3*) to the more sensitive flow cytometry crossmatch (FXM) (*4, 5*). In contrast to these "cell-based" or "membrane-dependent" assays, in which the DSA target is the HLA expressed on the intact lymphocytes, advances in HLA purification technology have facilitated the development of "solid-phase assays" (SPA), whereby the isolated (membrane-independent) HLA are bound to a solid matrix. Assays with both mixed and single HLA antigens directly linked to fluorescent-labeled beads are available (*6–8*), which detect complement-binding and noncomplement-binding anti-HLA Abs using fluorescent anti-human immunoglobulin G as a secondary Ab. Recently, a crossmatch (XM) test using the solid-phase technology has been introduced, namely the Luminex crossmatch (LXM; LIFECODES DSA, Tepnel Lifecodes, Stamford, CT) (*9, 10*).

Preexisting or de novo DSA correlate with a higher risk of graft failure (*11–14*). However, the relevance of DSA de-

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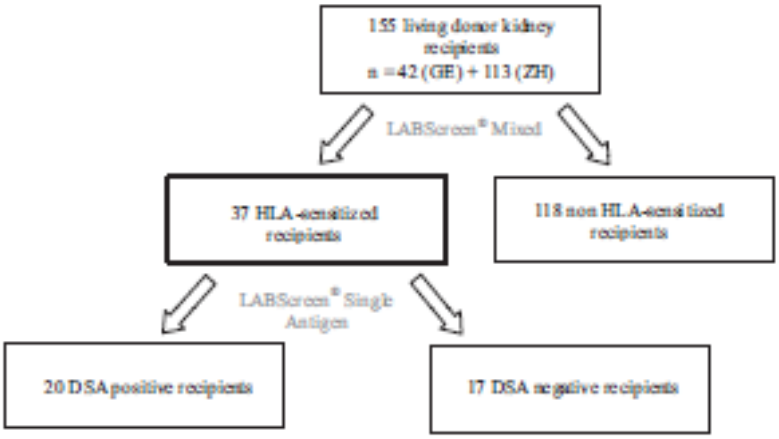

FIGURE 1. Study design. From two transplant centers, 155 living donor kidney recipients were screened for the presence of pretransplant anti-human leukocyte antigen (HLA) antibodies by LABScreen Mixed assay. Among those, 37 were found to be positive and represent our target population for evaluation of the single-antigen bead assay and three different crossmatch tests. DSA, donor-specific antihuman leukocyte antigen antibodies.

fined solely by SPA (i.e., with a negative XM) is less clear (*15–20*). The high sensitivity of this technology might detect DSA that are clinically irrelevant, whereas false-negative reactions could be the result of difficulties in adherence of the solubilized product from recombinant cells to the beads and HLA denaturation during the purification process (*8*).

The aim of this study was to evaluate the prognostic impact of DSA class and strength expressed as a mean fluorescence intensity (MFI) detected by single-antigen beads (SAB) and to compare the three generations of XMs in sensitized, but T-cell CXM (T-CXM)-negative recipients.

PATIENTS AND METHODS

Patients

In the University Hospitals of Zurich (ZH) and Geneva (GE), 155 (ZH: 113; GE: 42) living donor kidney transplantations with a negative T-CXM were performed between January 2005 and June 2008. Thirty-seven patients (ZH: 30; GE: 7) had a positive anti-HLA Ab screening assay (LABScreen Mixed, OneLambda Inc., Canoga Park, CA) on the day of transplantation and were included in this retrospective study (study design, see Fig. 1). In addition to the anti-HLA Ab screening assay, our procedure included panel reactive antibody (PRA), LABScreen Single Antigen assay, and three different XMs in the same serum. All recipients and donors were typed for HLA-A, -B, and -DR by serology and polymerase chain reaction with sequence-specific primer and for HLA-DQ by serology only. Follow-up was evaluated until 1 year posttransplant.

Standard immunosuppression in all patients consisted of prednisone, mycophenolate, and—according to physician's judgment—cyclosporine or tacrolimus. Ab induction therapy including rituximab, basiliximab, or Thymoglobuline (Genzyme, Cambridge, MA) was used if one or more of the following conditions were fulfilled: B-cell CXM (B-CXM) positive, current or peak PRA more than 10%, retransplantation, and six HLA-mismatches. In addition to these parameters and the T-CXM, there was no additional information about the risk of AMR available at the time of transplantation.

Panel Reactive Antibodies

All serum samples were tested in a microlymphocytotoxicity assay against a T-lymphocyte panel of 30 donors. Cells were incubated with serum for 30 min at 21°C and then with complement for 30 min.

Anti-HLA Antibody Screening Assays

Patients were screened with LABScreen Mixed (LSM12, OneLambda Inc., Canoga Park, CA). This assay contains a panel of color-coded microbeads coated with multiple HLA antigens to identify class I or II anti-HLA IgG Abs and was performed according to the manufacturer's instructions (*21*). Test interpretation was performed using HLA Visual software (OneLambda Inc.) on the LABScan100 flow cytometer (Luminex Inc., Austin, TX), and the positive cutoff was at 3.0.

CXM and FXM

CXM was performed by the classical complement-dependent cytotoxicity method. Patient sera were tested with and without dithiothreitol according to the European Federation for Immunogenetics standards. XMs turning negative after dithiothreitol treatment were considered negative for further analysis.

FXM was retrospectively performed for all patients in one center (GE) with a two-color fluorescence technique as described previously (*22, 23*). Cell staining was analyzed using FACSCalibur and Cell Quest softwares (BD PharMingen, San Jose, CA). T- and B-cell flow XM (T-FXM and B-FXM, respectively; 1024-channel log scale) were reported positive, if median channel shifts with a value of more than 3 standard deviations of control serum values were observed.

Luminex Crossmatch

Donor lymphocytes (30×10^6) were lyzed with lysis buffer according to the manufacturer's instruction (LIFECODES DSA, Tepnel Lifecodes). Eight microliters of donor lysates were incubated for 30 min with 5 μl of anti-HLA class I- or II-specific beads, which capture donor HLA antigens. Fifty-five microliters of these donor HLA antigen-loaded beads were added to 96-well plates and washed three times. Patient sera were cleaned with SeraClean (Tepnel Lifecodes) before incubation for 30 min with donor HLA antigen-loaded beads. Revelation was performed with 5 μl of anti-human phycoerythrin-conjugated IgG. Detection of the patient anti-HLA IgG Abs and result interpretation were performed using LifeMatch software (Tepnel Lifecodes) on the LABScan100 flow cytometer (Luminex). Positivity was defined according to the manufacturer's rules: the raw MFI value of each capture bead is compared with three cutoff values (background adjustment factors [BAFs]). The three BAF values are calculated from the background measured on the three negative control beads (CON) in each test well. Each CON has a separate and lot-specific equation for calculating the BAF values. The BAF calculated for a CON is subtracted from the raw MFI value of the capture bead. The process is repeated for each of the remaining two CONs to obtain three adjusted MFI values. A sample is considered to be positive for DSA if at least two adjusted MFI values are positive.

Single-Antigen Bead Assay

To identify the specificity of anti-HLA IgG Abs, we retrospectively performed a high-definition LABScreen Single Antigen (OneLambda) class I assay in LABScreen Mixed class I positive individuals and a class II assay in LABScreen Mixed class II positive individuals (*7, 24*). Twenty microliters of

serum were added to 5 μl of class I panel (LS1A01, LAT1-HD) or class II panel (LS2A01, LAT2-HD) microbeads, and the mixture was processed according to the manufacturer's instructions. Result interpretation was performed using LABScan 100 software (OneLambda) on the LABScan100 flow cytometer (Luminex). A positive result was defined as more than 500 MFI, which was calculated as (fluorescence of beads coated with HLA and incubated with patient serum)−(fluorescence of beads without HLA and incubated with patient serum)−(fluorescence of beads with and without HLA and incubated with negative control serum).

Outcome Parameters

The primary outcome parameter was AMR during the first year posttransplant. All clinically suspected rejections were confirmed by needle core biopsy. Biopsy specimens were evaluated by light microscopy and immunofluorescence including C4d staining. The histologic classification followed the Banff'97 classification with its updates (25, 26). One case with moderate transplant glomerulitis and peritubular capillaritis without C4d positivity was also assumed as AMR.

Secondary outcome parameters were the incidence of T-cell–mediated rejections (TMR) and allograft function, measured as absolute and relative decline of the creatinine clearance (CrCl) according to Cockcroft-Gault (27). The decline was defined as the difference between the best graft function in the outpatient setting during the first 3 months posttransplant and the graft function 1 year posttransplant.

Statistics

For comparison of continuous variables, Student's *t* test and Mann-Whitney *U* test were used as appropriate. Categorical data and differences among proportions were compared with Fisher's exact test. All *P* values were two tailed. Receiver operator characteristic (ROC) curves, along with the area under the curve, were computed for DSA values and their sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) (28). Binary logistic regression model was used to identify independent predictors (recipient's age, sex, number of transplants, HLA mismatches, and DSA values) of rejection.

RESULTS

Patient Characteristics, Type, and Frequency of Rejections

Patient characteristics are given in Table 1. Diagnoses leading to need of transplantation were glomerulonephritis (27% patients), pyelonephritis or interstitial nephritis (24%), polycystic kidney disease (16%), vascular and hypertensive nephropathy (5%), diabetic nephropathy (5%), and "other" (22%). There were no differences between the DSA± and AMR± groups. Nineteen patients received any Ab induction (nine Thymoglobuline, nine basiliximab, and one rituximab). The DSA+ group received more Ab induction compared with the DSA− group (14 vs. 5 patients, Table 1). No difference was observed with regards to the type of Ab induction between the DSA± and AMR± groups.

Of the 37 sensitized living donor kidney recipients, 20 had DSA (nine against class I only, seven against class II only, and four against classes I and II). Of all patients, 18 (49%) had at least one rejection episode during the first year posttransplant, and

TABLE 1. General characteristics of all patients, those with and without AMR and with and without DSA

Parameter	All (n=37)	AMR			DSA		
		No AMR (n=29)	AMR (n=8)	*P*	DSA− (n=17)	DSA+ (n=20)	*P*
Recipient age (yr), mean (SD)	38 (±17)	37 (±19)	40 (±9)	0.738	35 (±17)	40 (±17)	0.392
Male sex	21 (57%)	16 (55%)	5 (62%)	0.806	9 (53%)	12 (60%)	0.746
Donor age (yr), mean (SD)	49 (±9)	50 (±10)	46 (±6)	0.246	50 (±10)	48 (±9)	0.498
No. transplants, n (%)							
1	24 (65)	21 (72)	3 (38)	0.100	14 (82)	10 (50)	0.082
2 or 3	13 (35)	8 (28)	5 (62)		3 (18)	10 (50)	
HLA mismatch (%)							
A: 0/1/2	13/66/21	14/65/21	11/67/22	0.447	18/70/12	5/65/30	0.177
B: 0/1/2	8/50/42	10/48/42	0/56/44	0.510	6/70/24	10/30/60	0.110
DR: 0/1/2	24/50/26	24/48/28	22/56/22	0.905	35/47/18	15/50/35	0.149
IS, n (%)							
Prednisone	37 (100)	29 (100)	8 (100)		17 (100)	20 (100)	
CyA/tacrolimus	37 (100)	29 (100)	8 (100)	0.683	17 (100)	20 (100)	0.745
MPA	37 (100)	29 (100)	8 (100)		17 (100)	20 (100)	
Antibody induction[a]	18 (49)	15 (52)	4 (50)	1.000	5 (29)	14 (70)	0.042
CrCl (mL/min) at 1 yr, mean (SD)	64 (±19)	64 (±20)	64 (±14)		66 (±21)	63 (±17)	0.607
CrCl (mL/min) decline after 1 yr, mean (SD)							
Absolute	11 (±11)	8 (±8)	24 (±13)	0.027	8 (±7)	14 (±13)	0.133
Relative	14%	11%	26%		11%	16%	

[a] Any antibody induction therapy (rituximab or basiliximab or Thymoglobuline).

AMR, antibody-mediated rejection; DSA, donor-specific anti-human leukocyte antigen antibody; SD, standard deviation; CrCl, creatinine clearance; IS, immunosuppression; CyA, cyclosporine A; MPA, mycophenolate.

TABLE 2. Frequency of AMR and TMR according to anti-HLA antibody and DSA status

Rejection type	Anti-HLA ab− [a] (n=118)	Anti-HLA ab+ [a] (n=37)	P	DSA− (n=17)	DSA+ (n=20)	P
AMR	0	8 (22%)	<0.001	1 (6%)	7 (35%)	0.048
TMR	24 (20%)	15 (40%)	0.018	7 (41%)	8 (40%)	1.0

[a] Screened with LABScreen Mixed assay; our study population consisted only of the 37 screen positive patients but for comparative purposes, the results for the screen negative group are also listed here.

AMR, antibody-mediated rejection; TMR, T-cell–mediated rejection; HLA, human leukocyte antigen; DSA, donor-specific anti-human leukocyte antigen antibody; ab, antibody.

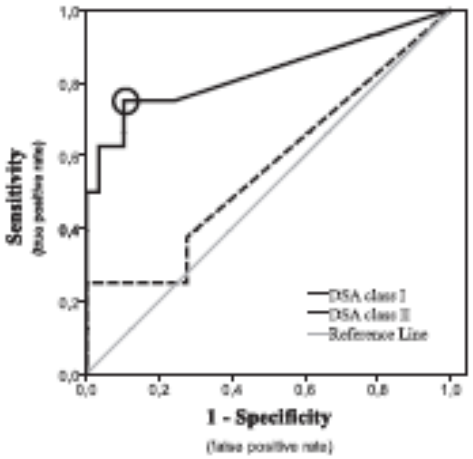

FIGURE 2. Receiver operator characteristic (ROC) curve analysis for prediction of antibody-mediated rejection (AMR) by single-antigen bead (SAB). The ROC curve for the predictability of AMR by SAB using varying cutoff levels based on mean fluorescence intensity (MFI) shows a significant result only for donor-specific antihuman leukocyte antigen antibody (DSA) against class I (DSA-I; area under the curve 0.828, $P=0.005$), whereas the result for DSA against class II (DSA-II) was not significant. The circle marks the point on the curve, where sensitivity and specificity are equally weighted and corresponds to a DSA value of 900 MFI. (DSA-I: n=13; DSA-II: n=11).

two patients had two episodes. Eight patients had AMR (44% of the patients with rejection; 22% of all patients), of which two of them were hyperacute and three were chronic. Only one patient had an isolated chronic AMR, and in two other patients, chronic AMR was accompanied by acute AMR and TMR. Fifteen patients had acute TMR, of which five of them had AMR at the same time. Only 7 of 37 patients had a time-of-transplant PRA class I value more than 0% (3%, 7%, 27%, 63%, 70%, 82%, and 93%), and two of them had AMR (the patient with 27% PRA had hyperacute AMR, and the patient with 70% PRA had acute and chronic AMR and acute TMR).

AMR and DSA Analysis by SAB

The incidence of AMR was significantly higher in patients with DSA (7 of 20; 35%) than in patients without DSA (1 of 17; 6%; $P=0.048$, Table 2). Thus, in terms of the predictability of AMR, just the presence or absence of DSA measured by SAB reached a sensitivity of 87.5% and a specificity of 55.2%. In our population, it results in a PPV of 35.0% and a NPV of 94.1%. TMR was not predicted by any humoral sensitization test. Interestingly, during the first year posttransplant, none of the 118 recipients without anti-HLA Abs experienced AMR, and the rate of TMR was much lower (Table 2).

The ROC curve analysis for prediction of AMR by SAB yielded a significant result only for DSA against class I (DSA-I), whereas the result for DSA against class II (DSA-II) was not significant (Fig. 2). Therefore, we evaluated DSA-I separately. For every patient, only the DSA with the highest MFI was used to compute sensitivity and specificity. The cumulative MFI values for all DSA-I or DSA-I and DSA-II yielded no better results (data not shown). Table 3 summarizes sensitivity, specificity, PPV, and NPV of DSA-I at different levels in terms of AMR. If sensitivity and specificity are equally weighted, the cutoff value has to be set at 900 MFI, as revealed by ROC curve analysis. Further cutoff values are 500 MFI (i.e., whether DSAs are present or not) and 5200 MFI, where specificity reaches 100%.

We observed only one AMR positive for DSA-II but negative for DSA-I. This was a chronic AMR combined with acute AMR and Banff IIa TMR. The patient's serum contained a donor-specific anti-DR1 Ab (672 MFI). The one patient with isolated chronic AMR revealed a high-level anti-DQ2 Ab (14,759 MFI) together with an anti-A11 Ab (1022 MFI), both of which were donor-specific.

Three patients had graft loss. One patient (with DSA-II only and no rejection episode) lost his graft after 7 months because of renal vein thrombosis. The other two patients had hyperacute AMR within 24 hr after reperfusion and had pretransplant DSA as follows (MFI in brackets): patient 1 showed activity against A2 (9795), A24 (9420), and B51 (5296); patient 2 against B44 (12791), A1 (12651), DR7 (12927), DR17 (5828), and DQ2 (2901).

Binary logistic regression analysis revealed that only the presence of DSA was an independent risk factor for AMR, but not the recipient's age, sex, number of transplants, and HLA mismatches (data not shown).

Luminex Crossmatch

In a next step, we compared SAB results with three different XM methods. The correlation between several DSA levels (classes I and II) and the three generations of XMs are presented in Table 4. Then, the prediction of AMR was determined with regards to different XMs alone and in combination with DSA class I (Table 5). Of special interest, there was the newly developed XM with Luminex technique (LXM) because it uses the same bead-based fluorescent technique similar to the SAB assay. The LXM for class I (LXM-I) achieved the highest values for both test characteristics and signifi-

TABLE 3. Test characteristics for prediction of AMR by SAB-derived DSA at different strengths (n=37)

DSA class I (MFI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	P
≥500	75	76	46	92	76	0.013
≥900	75	90	67	93	86	0.001
≥5200	50	100	100	88	89	0.001

AMR, antibody-mediated rejection; SAB, single-antigen bead; DSA, donor-specific anti-human leukocyte antigen antibody; MFI, mean fluorescence intensity; PPV, positive predictive value; NPV, negative predictive value.

TABLE 4. DSA class I/II levels and three generations of crossmatches in the 37 recipients with a positive screening assay and a negative T-CXM

DSA (MFI)	B-CXM	T-FXM	B-FXM	LXM I	LXM II
DSA− (n=17)	3 (17)	1 (6)	2 (12)	1 (6)	5 (29)
DSA ≥500 (n=20)	4 (20)	6 (30)	11 (55)	7 (35)	6 (30)
DSA ≥900 (n=17)	4 (24)	5 (29)	10 (59)	6 (35)	6 (35)
DSA ≥5,000 (n=8)	4 (50)	4 (50)	7 (87)	5 (63)	2 (25)

Here DSA classes I and II are given, in contrast to tables 3 and 5, where only DSA class I are considered. Values are presented n (%).

DSA, donor-specific anti-human leukocyte antigen antibody; MFI, mean fluorescence intensity; T-CXM, T-cell complement-dependent cytotoxicity crossmatch; B-CXM, B-cell complement-dependent cytotoxicity crossmatch; T-FXM, T-cell flow crossmatch; B-FXM, B-cell flow crossmatch; LXM I, Luminex crossmatch for class I; LXM I, Luminex crossmatch for class II.

cance. In contrast, the LXM for class II (LXM-II) was not predictive for AMR. The highest accuracy was achieved by combination of LXM-I and DSA-I more than or equal to 900 MFI. Patients with a positive LXM-I and DSA-I more than or equal to 900 MFI experienced AMR with a PPV of 80% by a test specificity of 96%.

FXM and CXM

The FXM achieved no significant test results in our study. By trend, the sensitivity was higher and the specificity was lower compared with B-CXM (Table 5). The combination of FXM and DSA-I with a cutoff value of 900 MFI was of borderline significance, but predictability of AMR was weak compared with the other two XMs combined with DSA values.

In 7 of 37 patients, the transplant was performed with a positive B-CXM. Four of these patients had DSA-I or DSA-I and -II, which led to hyperacute AMR in two cases and to chronic AMR in one case. By trend, the B-CXM had a low sensitivity of 38% but a high specificity of 86%. In combination with DSA-I more than or equal to 900 MFI, specificity and PPV increased to 100% ($P=0.007$), that is, all three patients having this combination experienced AMR, and two of them experienced hyperacute AMR.

Graft Function

The mean CrCl decline after 1 year was 11 mL/min in all patients, 24 mL/min ($P=0.027$) in patients with AMR, 13 mL/min in patients with TMR, and 7 mL/min in patients without any rejection episode. Patients with DSA-I more than 500 MFI had a CrCl decline of 15 mL/min, patients with DSA-II more than 500 MFI had a CrCl decline of 12 mL/min, and patients with DSA-I more than or equal to 900 MFI had a significant stronger CrCl decline of 21 mL/min ($P=0.025$). The three cases with graft loss because of hyperacute AMR and renal vein thrombosis were excluded from CrCl decline analysis.

DISCUSSION

AMR because of preformed DSA is responsible for a large proportion of renal allograft losses and above average CrCl decline within the first year posttransplant (*17, 18, 29–31*). Thus, in this study, we analyzed the outcomes of 37 HLA-sensitized living donor kidney recipients to define the predictability of AMR by cell-based assays and by Luminex SPAs performed with day-of-transplant sera. The main findings were as follows: (1) pretransplant DSA-I, but not DSA-II, are predictive for AMR; (2) with increasing strength of DSA-I, sensitivity is decreasing and specificity is increasing; (3) LXM-I, but not LXM-II, provides a higher accuracy than FXM and B-CXM (in the context of a negative T-CXM), and the specificity of all XMs is greatly increased in combination with DSA-I values above a particular cutoff.

We would like to stress that these MFI values are only valid for LABScreen Single Antigen assays (OneLambda) measured on a LABScan100 flow cytometer platform. In the recent Australian National Association of Testing Authorities quality assurance program, the performances of SAB assays were compared among laboratories and between the two vendors. There was a good correlation of the MFI values among laboratories using products from the same vendor. However, when comparing the test parameters between the two vendors, there were important differences in MFI (*32*). Furthermore, PPV and NPV are only valid for our HLA-sensitized population.

In the past 2 years, several studies investigated the problem of low-strength pretransplant DSA detected by SAB. The results are conflicting. In one study of 64 living donor kidney recipients with 12 DSA-positive patients, no difference in rejection and graft function was observed (*16*). Gupta et al. (*15*) found DSA not to be relevant for rejection, but for long-term graft failure in CXM-negative patients. However, AMR and TMR were not differentiated, and there was no accurate information about DSA levels. Nevertheless, a trend for more rejections in the DSA-positive group was described (32% vs. 23%). Several authors found that DSA went along with an increased risk for AMR but not for long-term graft function (*19, 20, 33*), and others found DSA to be relevant for both AMR and graft failure (*17, 18, 30, 31*). Unfortunately, there are no standardized populations. In most studies, DSA detection by SPA was performed in the context of a negative T- and B-CXM (*18–20*) or even a negative T- and B-FXM (*33*), but in some studies, a positive B-CXM was accepted (*30, 31*). In addition to us, one other group found a positive correlation

TABLE 5. Test characteristics for prediction of AMR by different XMs alone and in combination with DSA class I ≥900 MFI (n=37 per method)

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	P
LXM I	57	85	50	89	79	0.037
LXM II	14	63	9	74	53	0.384
LXM I and DSA ≥900	57	96	80	90	88	0.003
T-FXM	38	85	43	82	74	0.312
B-FXM	63	70	39	86	69	0.116
T/B-FXM	63	63	33	85	63	0.246
T/B-FXM and DSA ≥900	50	85	50	85	77	0.060
B-CXM	38	86	43	83	76	0.156
B-CXM and DSA ≥900	38	100	100	85	86	0.007

AMR, antibody-mediated rejection; XM, crossmatch; DSA, donor-specific anti-human lenkocyte antigen antibody; MFI, mean fluorescence intensity; B-CXM, B-cell complement-dependent cytotoxicity crossmatch; T-FXM, T-cell flow crossmatch; B-FXM, B-cell flow crossmatch; LXM I, Luminex crossmatch for class I; LXM II, Luminex crossmatch for class II; PPV, positive predictive value; NPV, negative predictive value.

between the strength of pretransplant peak DSA and AMR so far. In the study by Lefaucheur et al. (*17*), including only day-of-transplant CXM-negative patients, 35% of patients with pretransplant DSA experienced AMR, which is similar to our study. The risk for AMR was significantly increased in patients with strongly positive (semiquantitative) peak DSA compared with those with weakly positive peak DSA. The difficulty to draw definitive conclusions came from the absence of standardization of the studies with regard to immunosuppressive therapy and definition of rejection. We believe that the current evidence from the literature argues for a certain degree of relevance of low-strength pretransplant DSA for AMR and presumably also for graft failure. However, we also agree that the CXM remains an invaluable tool in clinical decision making (*34*).

With our cohort of HLA-sensitized patients, we were able to establish a clear relationship between day-of-transplant DSA-I strength and the probability of AMR. By ROC curve analysis, two MFI cutoff values were defined, which provide highest accuracy. The DSA-I cutoff at 900 MFI provides highest equally weighted sensitivity and specificity, whereas the cutoff at 5200 MFI leads to a specificity and, therefore, a PPV of 100%. We advise special caution for MFI values more than 10,000: both patients in our study having such values have rejected hyperacutely.

It might well be that inclusion of B-CXM positive patients in our study and historic B-CXM positive patients in the study by Lefaucheur et al. have a stake in gaining significant results. However, even when performing the analysis without the seven B-CXM positive patients, the results of AMR prediction by DSA-I with more than or equal to 900 MFI were still significant, as were the results of the LXM.

The 7 patients with DSA-II only and the 11 patients with DSA-II or DSA-I and II did not show a higher risk for AMR than patients without DSA-II. These findings are consistent with the results reported by Ho et al., (*31*) where patients with DSA-II only showed no increased AMR rate or decreased graft survival, and by Eng et al., (*30*) where graft survival in B-CXM positive patients with DSA-I only was significantly poorer than in patients with DSA-II only and in patients with a negative B-CXM. Other studies reported that DSA-II were correlated with the development of chronic rejection such as transplant glomerulopathy (*35–37*). Because of the small number of DSA-II-positive patients, the short follow-up time, and the lack of protocol biopsies, our study failed to show such an effect. The clinical relevance of DSA-II may be related to the HLA-locus and expression level of HLA class II antigens in kidney endothelium, and the latter is in turn dependent from the inflammation status (*38, 39*). Thus, when determining the risk associated with DSA-II, it could be useful to take into consideration the specificity and strength of DSA-II and the inflammation status of the patient.

The LXM assay uses "capture beads," which are coated with monoclonal Abs targeted against a fragment shared by almost all HLA antigens. Beads with captured HLA molecules from lyzed donor cells are incubated with patient serum. Because LXM uses the same technology as SAB, it could be a more relevant XM compared with CXM and FXM, when correlation with SAB is performed. Billen et al. (*9*) demonstrated the validity of the LXM-I (HLA A, B, and C), but not for LXM-II, because most of the HLA-DQ and -DP molecules were not captured by the LXM beads, as also attested by the manufacturer. LXM has several advantages compared with CXM in that (1) no viable donor lymphocytes are necessary and lysate can be stored at minus 80°C for up to 6 months, which is particularly useful for posttransplant monitoring; (2) it only detects DSA of IgG isotype, so it prevents false-positive results because of irrelevant Abs; (3) a clear differentiation between class I and II is guaranteed; and (4) it may detect Abs against rare donor HLA antigens, because the sensitivity does not rely on a bead panel. Unlike CXM, LXM also detects noncomplement-binding Abs but misses non-HLA Abs.

Our study tested for the first time the association between LXM and AMR. We found for LXM-I a sensitivity of 57% and a specificity of 85%. Hence, the specificity is in the same range as the B-CXM and T-FXM, but the sensitivity is higher. In contrast, the LXM-II was not predictive for AMR, similar to that in the studies of Billen et al. (*9, 10*). Until now FXM was the most sensitive technique, but despite some centers strongly support a broad use of FXM (*29*), the complexity of the test and a persistent debate about the relevance of positive FXM for graft survival has prevented its larger utilization (*40, 41*). We did not use pronase in our FXMs, which has been demonstrated to increase the quality of test results (*42*);

therefore, the interpretation of B-FXM results should be done with caution.

A great advancement is achieved by combining the LXM and the SAB results because of reduction of false-positive results. Patients with a positive LXM-I and DSA-I more than or equal to 900 MFI experienced AMR with a PPV of 80% by a test specificity of 96%. This is important to avoid inappropriate exclusion of patients from transplantation. Consistent with our results, one recent study showed that 45% of patients with DSA did not experience clinical or subclinical AMR and had no reduced 5-year graft survival (*18*), emphasizing the clinical importance of a high PPV. Thus, DSA-I at low strength (≥900 MFI) in combination with a positive LXM-I or DSA-I alone at high strength (>5200 MFI) are the best predictors of AMR.

To the strengths of our study belong that (1) confounding induction therapies were distributed equally in patients with and without AMR, although more DSA-positive patients received Ab induction therapy, making our results even stronger (Table 1); (2) we included only living donor kidney recipients with a short ischemia time, thereby reducing the possible influence of different inflammation levels on the HLA expression and therewith the probability of early AMR (*38, 39, 43, 44*); (3) we examined both classes of DSA separately; and (4) we included in the analysis DSA levels in addition to three different XM techniques. However, our study also has shortcomings, because we included relatively few patients, who were followed up only for 1 year, and we only investigated DSA against HLA-A, -B, -DR, and -DQ, but not against -C and -DP.

In conclusion, we believe that the best prediction of AMR and consecutively graft function in a T-CXM–negative population is delivered by the SAB test alone or by SAB in combination with LXM or B-CXM. Both the SAB test and the LXM yield only significant results for anti-HLA class I Abs. The decision to transplant or not should not be based solely on a binary XM result, but the clinician should use SPAs, which provide greater sensitivity for detecting DSA and a semiquantitative analysis to enhance the interpretation of XM results. These tests together with patient's clinical data must be integrated into the decision algorithms for performing a given transplant or not and for guiding immunosuppressive treatment and strategies for desensitization.

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Conclusion

Based on this study, we were able to define the appropriate cut-off for anti-HLA antibodies. Until then, each laboratory in Switzerland chose a mean fluorescence intensity (MFI) of anti-HLA Abs according to their own experience or feeling (!). After this report, all HLA laboratories in Switzerland agreed on a consensus defined as followed:

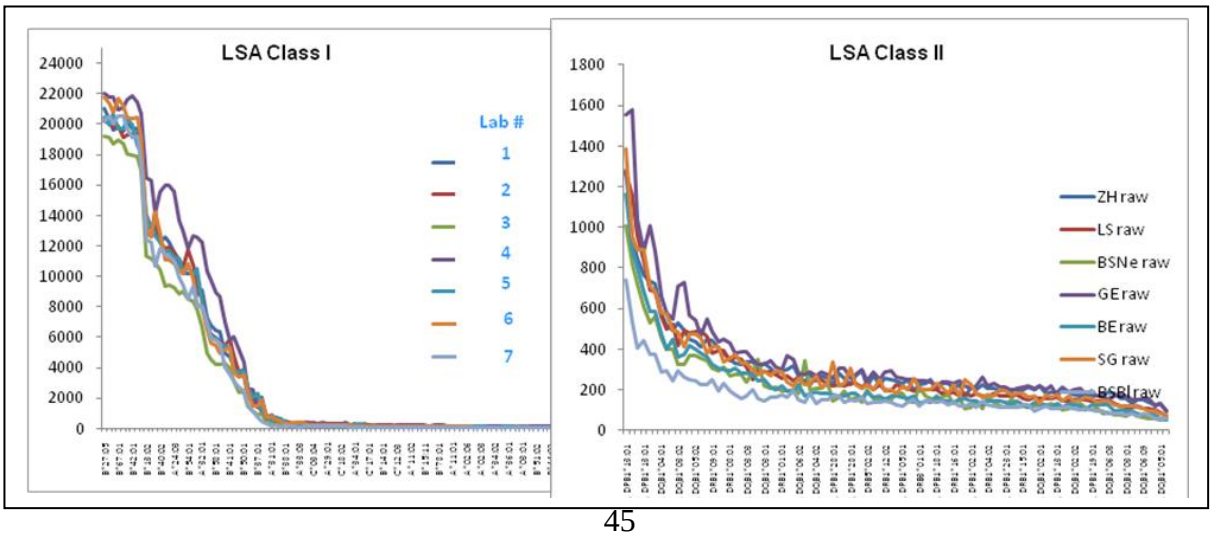
Figure 22: Pratical standpoint with deceased donor

DSA (MFI)	XM	Transplantation	Treatment
>10'000		NO offer	
>1'000 - <10'000	Negative	No contra-indication. Case by case decision	Induction with ATG-plasmapheresis-Rituximab
>1'000 - <10'000	T positive B negative	No transplantation	
>1'000 - <10'000	T negative B positive	No contra-indication. Case by case decision	Induction with ATG-plasmapheresis-Rituximab
<1'000		YES	standard

According to the intensity of DSA and the results of the CDC crossmatch performed before transplantation, the decision to go ahead or to refuse the graft will be taken in accordance with clinicians and surgeons.

We also agreed to perform a Swiss quality check, organized by Geneva, in order to ensure that all HLA laboratories obtain identical data and intensities for given sera.

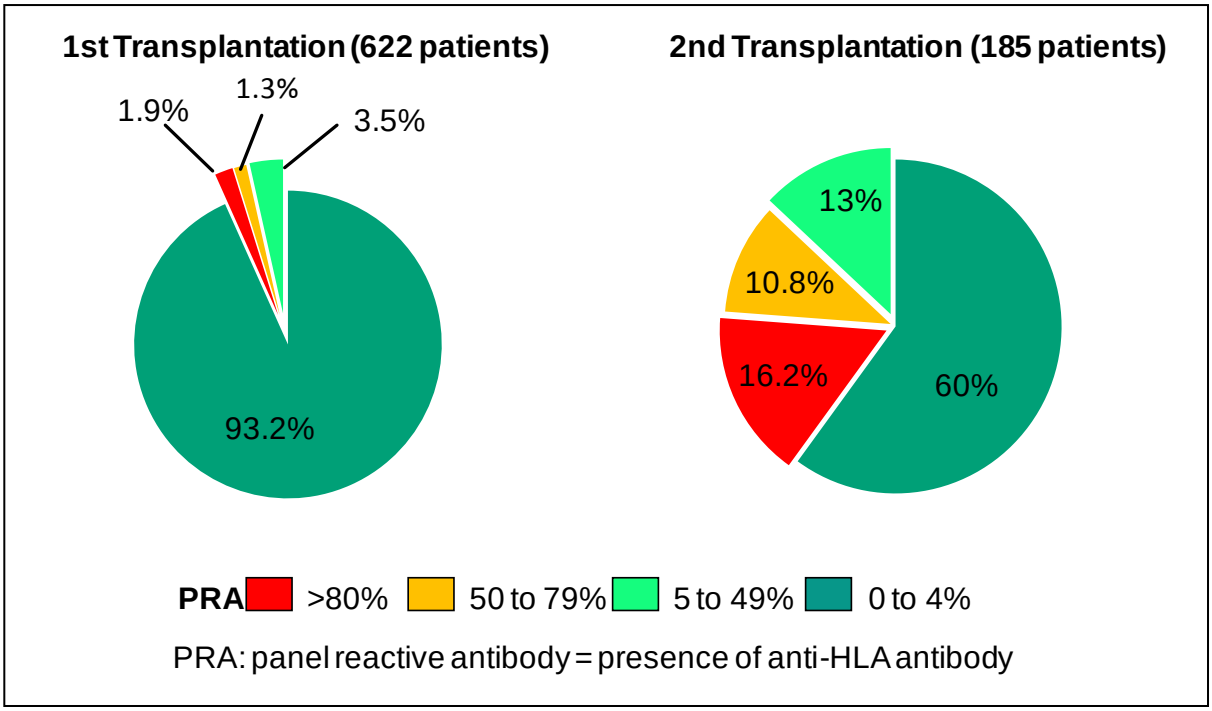
Figure 23: Comparative Luminex LSA data between Swiss HLA Laboratory



We therefore have to provide a list of anti-HLA Abs list and intensities in the SOAS (Swiss Organ Allocation System) program for each sensitized patient on the waiting list to ensure that the appropriate criteria are met and to avoid transplantations with high intensity DSA.

This becomes even more important as more and more patients are waiting for a second or third transplant.

Figure 24: Kidney transplantation: Waiting list in Switzerland (1.1. 2014)



On the left panel, 93% of the patients on the waiting list do not have anti-HLA antibodies. 7% are sensitized (with a certain % of circulating anti-HLA antibodies, as represented by PRA (panel reactive antibody).

Moreover, even though the prospective crossmatch remains the gold standard test to guarantee a successful transplantation procedure, other crossmatch techniques exist. We therefore considered it appropriate to validate our choice of methods and establish a consensus for transplant centers and HLA laboratories in Switzerland. We also agreed that CDC-XM associated with LSA data is still the best predictor of graft function. Luminex XM was a promising new methodology, but unfortunately it did not yield satisfactory results for anti-

HLA class II since the detection of anti-HLA-DQ antibodies was not possible. This method is no longer used for clinical purposes.
For organs donated from living donors most laboratories also use Facs-XM, which technique is more sensitive than CDC-XM (63% versus 38%).

In conclusion, to arrive at both the right decision and process for transplantation, clinicians should take several test results (XM CDC and/or Facs, LSA, PRA) and patient's clinical data into account.

b) As specified in the previous manuscript, detection of anti-HLA Abs has become a major criterion in decision-making before and after transplantation. But it is also a major tool for following up transplanted patients and for diagnosing antibody-mediated rejection, considering that the development of DSA is part of the diagnostic components and will dictate the adequate treatment and/its potential adjustments.

However, the Luminex® technology for detecting anti-HLA Abs is so sensitive that an increasing number of patients are shown to have pre-formed anti-HLA antibodies, which may obviate their access to an organ. Luminex® also detects de novo anti-HLA Abs or DSA in a high number of patients! For this reason we have to develop strategies for quantifying these anti-HLA Abs and determine those that are relevant and those that are not.

Detection of anti-HLA antibodies by solid-phase assay in kidney transplantation: friend or foe?

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REVIEW ARTICLE

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Key words
anti-human leukocyte antigen antibody; crossmatch; kidney transplantation; rejection; solid-phase assay

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Abstract

Pre-formed and *de novo* anti-human leukocyte antigen (HLA) antibodies induce antibody-mediated rejection and are also involved in mechanisms leading to chronic graft nephropathy. The detection of anti-HLA antibodies by solid-phase assay (SPA) has revolutionized the management of immunized patients before and after kidney transplantation. Characterized by high sensitivity and specificity, the clinical relevance of anti-HLA antibodies by SPA has to be clarified. The presence of donor-specific antibody at the epitope level, their titer, and the use of different crossmatch technologies could help to determine which of the anti-HLA antibodies are friends and which are foes in kidney transplantation. In this review, we summarize the current state of the art on this debated topic, and give clinical guidelines for the management of antibody detection pre- and post-transplantation, based on these evidences and our own clinical expertise.

Introduction

Solid-organ transplantation (SOT) encounters various obstacles of genetic and immunological nature, e.g. ABO blood groups, major histocompatibility complex [MHC/human leukocyte antigen (HLA)], and minor histocompatibility antigens. In the majority of cases, ABO-compatible transplantation remains the rule, the main purpose being to prevent hyperacute humoral rejection even though living donor ABO-incompatible transplantation is possible with acceptable long-term results (1–3). Although HLA matching between donor and recipient is associated with improved long-term graft survival, the HLA barrier is no longer an impediment to successful SOT, thanks to immunosuppressive drugs.

Patient exposure to HLA molecules from a genetically unrelated individual can lead to the development of anti-HLA antibodies which occurs in three situations: blood transfusion, pregnancy, and previous transplantation (4, 5). Anti-HLA antibodies present before transplantation are referred to as 'pre-formed anti-HLA antibodies' and anti-HLA antibodies arising after transplantation as '*de novo* anti-HLA antibodies'. In kidney transplantation, the presence of pre-formed anti-HLA antibodies directed against the donor (donor-specific antibody, DSA) can induce hyperacute rejection or delay antibody-mediated rejection (AMR) (6, 7). In addition to AMR, anti-HLA antibodies play a crucial role in the pathophysiology of chronic rejection, leading to graft dysfunction (5). For decades, the method of detection based on complement-dependent cytotoxicity (CDC) was the only available technology to detect anti-HLA antibody (5, 8), and it allowed to prevent disastrous hyperacute rejections (8). However, this assay is neither particularly sensitive nor specific and the identification of specific antibodies to HLAs in highly sensitized patients was therefore a difficult task. Thanks to enzyme-linked immunosorbent assay (ELISA) and flow cytometry using fluorescent microspheres (Luminex® technology, Austin, TX), the detection of specific anti-HLA antibodies by solid-phase assay (SPA) is now more sensitive, accurate, and quite easy to perform in standardized protocols, in accordance with the requirements of the laboratory quality assurance system. However, the significance of the presence and strength of pre-formed or *de novo* anti-HLA antibodies detected by SPA is still debated in terms of their possible role in rejection, long-term graft survival, and with regard to the most useful therapeutic approach for their elimination. In this review, we will discuss the role and clinical relevance in

kidney transplantation of pre-formed and *de novo* HLA antibodies detected by SPA, as well as their relevance according to crossmatch techniques, based on the current state of the art literature and our own clinical experience.

Diagnosis and mechanisms of antibody-mediated and humoral rejection

Endothelial cells expressing MHC class I constitutively but MHC class II in response to inflammation are the targets of anti-HLA antibodies (9–11). These antibodies have direct and indirect cytotoxic effects mediated by the membrane attack complex of complement. This complex attracts inflammatory cells and activates phagocytosis. The damaged endothelial cells secrete von Willebrand factor and the exposed basal membrane induces aggregation and adhesion of platelets, leading to thrombosis and vascular occlusion. During hyperacute rejection, anti-ABO or anti-HLA antibodies in high concentrations lead to irreversible ischemic damage of the transplanted organ. The role of anti-HLA antibodies in acute and chronic rejections is a repetitive 'damage-repair-damage' process characterized by inflammation and proliferation of both endothelial and smooth muscle cells. Three factors influence the development of this process: (1) concentration of antibodies, (2) repair capacity of the tissue, and (3) intensity of the immunosuppressive therapy (4, 5, 12).

The current criteria for the diagnosis of kidney allograft rejection mediated by HLA antibodies (AMR), established at the Banff Conference in 1997 and reviewed in 2001 and 2007 (13, 14) are the following:

- On biopsy, morphologic evidence of acute tissue damage, such as (1) acute tubular damage, (2) presence of neutrophils and/or mononuclear cells in the peritubular capillaries and/or glomerules and/or capillary thrombosis, or (3) intimal arteritis/fibrinoid necrosis/transmural inflammation of arteries.
- Immunopathological evidence (immunohistochemistry) of antibody effects such as (1) C4d deposits and/or (in rare cases) immunoglobulins at the basal membranes of the peritubular capillaries or (2) presence of immunoglobulins and complement in arterial fibrinoid necrosis.
- Serological evidence of circulating antibodies directed against the donor (DSA) HLAs or against other endothelial antigens of the donor.

All three criteria must be met to justify the diagnosis of AMR. If only two of these criteria are met, the correct diagnosis is suspicion of AMR.

Methods for detecting anti-HLA antibodies

It is important to determine the repertoire of specific anti-HLA antibodies before transplantation, as the presence of DSA has a direct impact on the management of organ allocation. In addition, the development of DSA after transplantation should prompt modification of the immunosuppressive treatment. An overview of the current techniques in use follows (for further details refer to the review by Tait et al. (15)):

- CDC assay: Historically, the first method for detecting anti-HLA antibodies was the CDC assay. It consists of testing the serum of the recipient for specific antibodies to lymphocytes. If antibodies are present, the classical complement pathway is activated, resulting in the lysis of donor cells by the membrane attack complex. A panel of 30–70 representative cells expressing common HLAs observed in the local population of potential donors are used as target cells. The percentage of wells (containing individual target cells) showing a positive reaction determines the percentage of 'panel-reactive antibody' (PRA). This method can also be applied to determine specificity of HLA alloantibodies (e.g. anti-HLA-A2). The advantage of the CDC assay is that it shows complement-fixing antibodies (i.e. cytotoxic antibodies), but it is not specific for the donor's HLA, as other antibodies to non-HLA lymphocyte antigens or auto-antibodies can also bind to complement and induce a positive reaction.

SPAs were developed thanks to purification of HLAs from transfected cells and their binding to different supports:

- ELISA: Specific HLAs immobilized on a plastic surface are incubated with the serum of the patient. Anti-HLA antibodies are showed after addition of an enzyme-linked anti-human IgG antibody directed against the Fc fragment of the antibodies. The amount of antibody is determined by spectrophotometry using substrate-converting enzymes (16).
- Fluorescent microspheres (flow PRA® or Luminex, One Lambda, CA): Specific HLAs immobilized on fluorescent microspheres are incubated with the serum of the patient. Anti-HLA antibody binding is showed by a second fluorescent anti-human IgG detected by flow cytometry (FACS). Using specific microspheres that match the different HLAs, the repertoire of anti-HLA antibodies of a given serum can be assessed with great accuracy (17, 18) (Figure 1A).

The strength or the titer of an anti-HLA antibody can be determined through the mean fluorescence intensity (MFI) (or the absolute molecular equivalent of fluorescence intensity, more accurate but more complicated to set up) (Figure 1B).

- Crossmatching methods: in addition to SPA, crossmatching methods continue to be useful in the detection of harmful DSA. As suggested by Taylor et al. (19), this allows differentiation between high and intermediate levels of immunological risk. From the CDC crossmatch developed 50 years ago (20) – still considered

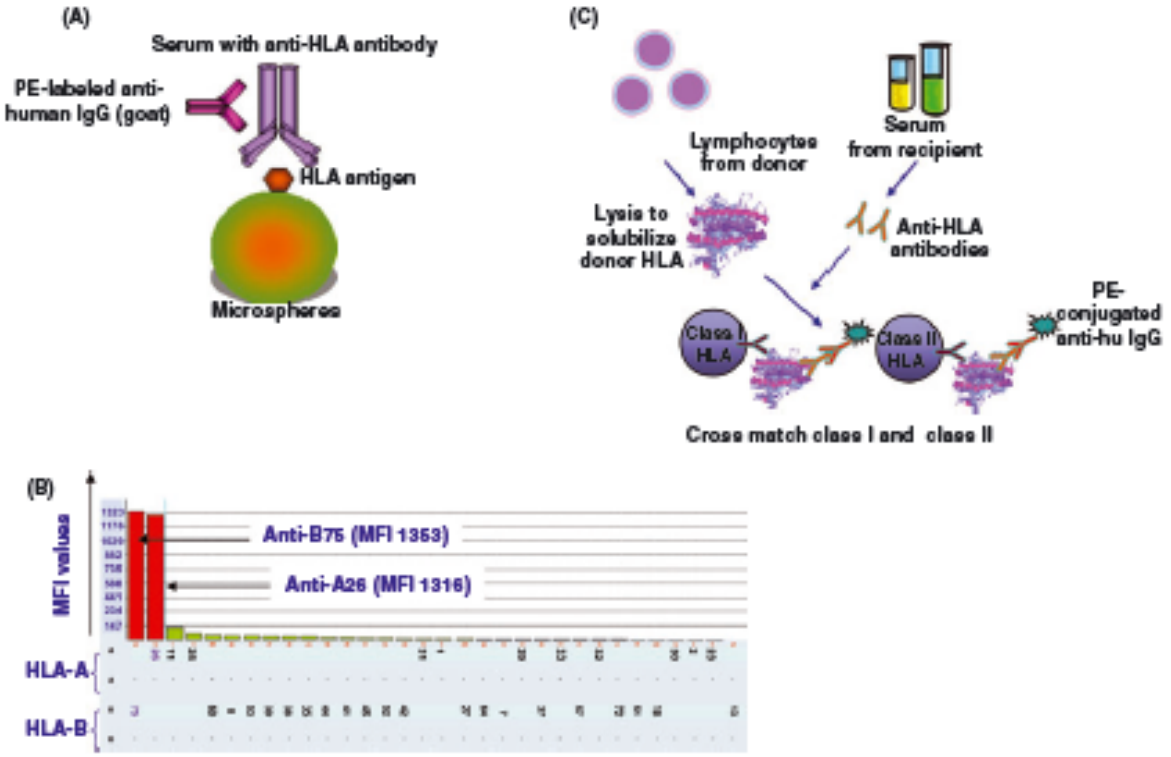

Figure 1 Luminex technology. (A) Patient serum is incubated with fluorescent beads coated with specific purified HLAs. Anti-HLA antibodies bound to individual HLA beads are targeted by goat anti-human IgG-PE. The first laser of the fluorometer (Luminex) excites the beads which are classified according to their fluorescence intensity, and the positive beads are also showed by IgG-PE staining. (B) Serum of a recipient positive for HLA-A26 and -B75 with MFI. (C) Schematic view of a crossmatch by solid-phase assay technology. Donor lymphocytes are lysed with lysis buffer and then incubated with anti-HLA class I or II specific beads, which capture donor HLAs. These donor HLA-loaded beads are then incubated with patient sera and anti-human PE-conjugated IgG is finally added for revelation (26). HLA, human leukocyte antigen; MFI, mean fluorescence intensity.

the 'gold standard' by most centers – technology has evolved to crossmatch by flow cytometry (FACS crossmatch), which is more sensitive but not specific for HLAs (21, 22). More recently, the SPA crossmatch (Luminex crossmatch) has provided interesting information (23–26). In this latter approach, donor cells are solubilized to extract cell membranes that express HLA, which are then captured by beads specific for anti-HLA class I or class II. The serum of the patient containing DSA binds to the cell membranes and the positive reaction is showed by a secondary anti-human-PE antibody (Figure 1C). This method has the advantage of involving similar technologies to that of SPA used for the detection and identification of anti-HLA antibody, but its clinical relevance needs to be assessed.

As these highly specific technologies are also very sensitive, the clinical relevance of anti-HLA antibodies with low and even with intermediate or high MFI is a matter of intense debate in the transplantation community (27, 28). The presence of such anti-HLA antibodies could result in denying a transplant to a patient on the basis of DSA that are clinically not relevant or, after transplantation, in over-treating a recipient who develops DSA.

Donor-specific and non-donor-specific anti-HLA antibodies

Donor-specific and non-donor-specific anti-HLA antibody (DSA and NDSA, respectively) are critical when considering clinical relevance of anti-HLA antibodies. DSA are deleterious while NDSA should not be. However, the situation is complicated by the fact that the high polymorphism of the MHC system is also characterized by sharing of epitopes between alleles not only of the same locus but also of different loci (29). Humoral sensitization is generally associated with anti-HLA antibodies specific for epitopes rather than antigens. A distinction between HLA epitopes and antigens is important for a better understanding of the humoral immune response to the sensitizing HLA mismatch, and even more importantly also for the determination of mismatch acceptability

of sensitized recipients. Epitopes can be defined as the physical area of an antigenic molecule that an antibody binds to in a specific way. In the case of proteins, epitopes are defined by the tertiary conformation of amino acid sequences. This means that the primary sequence of amino acids, i.e. the consecutive linear sequence of amino acids in the primary structure of a protein, does not necessarily define an epitope. Amino acids distant from each other in the primary structure can be close in the tertiary structure and may define amino acid epitopes. HLA epitopes can be structurally defined by HLAMatchmaker, an algorithm that considers eplets as critical elements of epitopes recognized by alloantibodies (30). An eplet represents a patch of amino acid residues within a radius of about 3 Å from a polymorphic residue on the HLA molecular surface (http//www.HLAMatchmaker.net). The concept of epitope matching should be considered when classifying a given anti-HLA antibody as DSA or NDSA, because antibodies NDSA at the antigen level can be DSA at the epitope level. The clinical relevance of the HLAMatchmaker approach is by now well established and should be used in clinical practice, as it allows optimization of organ allocation based on a functional algorithm. In this context, an important asset is represented by SPA which allows for the detection of a wide range of anti-HLA antibodies with high sensitivity, even in case of exposure to a limited number of HLAs (31, 32).

A second element that should be taken into consideration is the difference between the antigenicity of epitopes (i.e. the reactivity with anti-HLA antibody) and the immunogenicity of epitopes (i.e. the capacity of inducing anti-HLA antibody). Better characterization and understanding of epitope immunogenicity will be critical for designing new strategies to define permissible mismatches for sensitized and non-sensitized recipients.

Of note, false-positive results are also a matter of concern, mainly due to the partial denaturation of antigens coating some beads and that might be recognized by natural non-HLA antibodies (33).

Clinical relevance of pre-formed anti-HLA antibodies detected by SPA

Hyperacute rejection by high titers of pre-formed anti-HLA antibodies may be prevented by selecting a donor with a negative CDC crossmatch. Due to the high sensitivity of SPA, anti-HLA antibodies detected by this technique often fail to be associated with a positive pre-transplant CDC crossmatch, but could nevertheless contribute to AMR and long-term complications. A better understanding of the clinical relevance of pre-formed DSA is warranted to improve the criteria for kidney allocation. Several recent studies have addressed this crucial issue.

Gibney et al. (34) has compared retrospectively the results of anti-HLA antibody detection by CDC-PRA and by SPA (Luminex) in kidney transplant patients with negative CDC crossmatch. The study consisted of 136 patients with 55 patients presenting a CDC-PRA >15%, of whom 20 had DSA. At 6 months after transplantation, kidney dysfunction was manifest in 12/20 (60%) of PRA^+/DSA^+ patients, as compared to 9/35 (26%) of the PRA^+/DSA^- patients. Thus, two subgroups were described, one of high-risk patients (PRA^+/DSA^+) with a 25% rate of acute rejection and one of low-risk patients (PRA^+/DSA^-) whose rate of acute rejection was as low as 3%, with very good graft survival at 6 months (Table 1).

The clinical relevance of DSA detected by SPA has also been analyzed in subclinical AMR (SAMR). Loupy et al. (35) assessed SAMR in a cohort of 54 DSA-positive kidney transplant recipients from a deceased donor without pre-transplant desensitization treatment. SAMR is a frequent finding in patients with pre-formed DSA. This study showed that in addition to DSA, C4d deposition and typical histology are associated with poor clinical outcome. In this group, MHC class II DSA with high MFI has the worst outcome. This study highlights the importance to correlate DSA and histology status (35).

Amico et al. (36) analyzed the long-term survival of grafts and patients in a cohort of 67 with preformed DSA. The salient observation was that 37/67 patients with DSA detected by Luminex at the time of transplantation experienced clinical/SAMR (DSA^+ AMR^+) with 20% lower death-censored allograft survival at 5 years post-transplantation. However, the remaining 30/67 patients with DSA were free from AMR (DSA^+ AMR^-) and allograft survival rate was equal to that of patients without DSA. Interestingly, neither the number of DSA, HLA-classes (HLA-class I *vs* HLA-class II), and MFI nor the sensitizing events proved predictive for AMR. These results suggest that almost 50% of DSA as defined by SPA are clinically irrelevant and do not lead to AMR, emphasizing the difficulty of predicting their impact on graft survival (36).

In contrast to the above study, Lefaucheur et al. (37) found a correlation between the risk of AMR and graft loss with peak DSA titer (MFI). This study analyzed 402 kidney transplant patients and focused on 118 patients with DSA. They found that 8-year graft survival was significantly worse (61%) among patients with preexisting DSA at high MFI (>3000) compared with sensitized patients without DSA (93%) or with non-sensitized patients (84%). The peak HLA-DSA Luminex MFI (DSA-MFI) predicted AMR better than DSA-MFI in the current (at transplant) serum sample. The peak DSA-MFI was inversely correlated with graft survival and directly correlated with the risk of AMR. Patients with MFI >3000 had a more than 100-fold higher risk of AMR than patients with MFI <465.

Aubert et al. (38) analyzed a cohort of 113 patients of whom 11/113 (11%) had DSA, and reported that DSA with MFI below 2000 was not associated with short-term rejection events (cellular and humoral).

Table 1 Literature review over pre-formed anti-HLA antibodies, AMR, and kidney graft survival

Article	Technique studied	Sample size DSA (%)	Follow-up (days)[a]	AMR[b]	Graft survival[c] (months)
Gloney et al. (34)	CDC X-match T and B	136 patients	180	DSA^+ = 25%	DSA^+ = 75%
	SPA	20 DSA^+ (14)		DSA^- = 3%	DSA^- = 91% (6)
Loupy et al. (35)	CDC X-match T and B	137 patients	365	DSA^+ = 10%	DSA^+ = 66.2%
	SPA	54 DSA^+ (39)		DSA^- = 0%	DSA^- = 95.2% NBI
Amico et al. (36)	Current and historical	334 patients	200	DSA^+ = 55%	DSA^+ AMR^+ = 68%
	CDC X-match T and B	67 DSA^+ (20)		DSA^- = 6%	DSA^+ AMR^- = 87%
	SPA				DSA^- = 89% (60)
Lefaucheur et al. (37)	CDC X-match T and B	402 patients	365	DSA < 465 = 0.9%	DSA < 465 = 82.5%
	SPA	118 DSA^+ (30)		DSA < 3000 = 18.7%	DSA <3000 = 78%
				DSA > 3000 = 35%	DSA >3000 = 60.6% (66)
Aubert et al. (38)	CDC X-match T and B	113 patients	365	DSA^+ = 9%	Not reported
	SPA	11 DSA^+ (9)		DSA^- = 0%	
Reinsmoen et al. (39)	CDC X-match T and B	155 patients	365	DSA^+ = 35%	DSA^+ = 65%
	FACS X-match T and B. Luminex X-match T and B	20 DSA^+ (13)		DSA^- = 6%	DSA^- = 100% (12)
	SPA				
Gloor et al. (40)	CDC X-match T and B	189 patients	540	DSA^+ = 41%	DSA^+ > 5000 = 80%
	FACS X-match T and B	119 DSA^+ (61)		DSA^- = 1%	DSA^+ > 10, 000 = 50%
	SPA				DSA^- = 100% (72)

AMR, antibody-mediated rejection; CDC X-match, crossmatch by complement-dependent cytotoxicity; DSA, donor-specific antibody; HLA, human leukocyte antigen; SPA, solid-phase assay.
[a]Follow-up of the study with regard to AMR.
[b]Percent of AMR with regard to the presence or absence of DSA.
[c]Long-term graft survival reported with regard to the presence or absence of DSA.

These studies suggest that DSA detected by SPA alone are not sufficient to predict the risk of clinical events but that DSA with low MFI and those not correlated with positive CDC-PRA, positive CDC crossmatch, or FACS crossmatch could have a limited effect on graft survival in presence of sufficient immunosuppression.

Recently, we assessed the prognostic value of pre-transplant DSA detected by Luminex in association with crossmatch tests (26). Three generations of crossmatch tests were performed on 37 living donor kidney recipients that tested positive according to the anti-HLA antibody screening assay (20 patients DSA^+), but negative for T-cell CDC crossmatch. In 100% of DSA-positive patients – with MFI above 5000 – AMR occurred in the first year of transplantation. When the B-cell CDC crossmatch and, more importantly, the Luminex crossmatch for class I were positive, the prediction of AMR was associated with DSA at lower MFI (≥900 MFI). Three patients with high levels of DSA (MFI >10,000) had major or irreversible AMR in the weeks following transplantation. Therefore, for sensitized recipients with a T-cell-negative CDC crossmatch, the most reliable prediction for AMR and consecutive graft function is provided either by DSA-class I alone at high strength or by DSA-class I at low strength added to a positive Luminex or CDC crossmatch.

Zachary et al. (39) have also reviewed the correlation between DSA defined by SPA and crossmatches. They showed that high MFI threshold values for DSA correlate with positive FACS (MFI > 6000) and CDC crossmatches (MFI >10,000), but the study did not establish a correlation with the clinical outcome.

In a large cohort of recipients with DSA determined by single antigen beads (Luminex), Gloor et al. (40) assessed the risk of AMR in recipients with positive (119/189) or negative (70/189) crossmatch, in correlation with MFI. In this study, two generation of crossmatches, CDC and FACS, with two different channel shifts were performed. The level of DSA defined by MFI was compared to a positive AHG-CDC crossmatch, to a positive FACS crossmatch with channel shift >300 or <300, and to a negative crossmatch. The authors observed a significant correlation between DSA levels and sensitivity of the various crossmatches. Of note, every patient with a high level of DSA (MFI > 10,000) had a positive AHG-CDC crossmatch.

Together, these studies suggest that pre-formed DSA at high levels (MFI > 10,000) should be considered a contraindication to transplantation. DSA with low MFI (<1000 or <2000) are unlikely to have a deleterious effect on the graft (at least in the short term). For DSA with MFI between 2000 and 10,000, additional results of FACS crossmatch and/or Luminex crossmatch could help to consolidate a reasonable prediction of graft rejection events after transplantation. Table 1 summarizes the most relevant studies on pre-formed anti-HLA antibody analysis by SPA with regard to AMR and graft survival.

Clinical relevance of *de novo* anti-HLA antibodies arising post-transplantation

The development of anti-HLA antibodies after kidney transplantation, as consequence of an immune response to allo-HLAs, can be observed in 10%–50% of the recipients (41, 42). The considerable variation in the reported percentages of this event is mainly due to different sensitivity of the methods used to detect anti-HLA antibodies (41, 42). According to previous work (34), the incidence of anti-HLA antibodies developing in patients 6 months after transplantation is roughly the same as after 10 years. In a prospective study, Terasaki et al. (43) assessed the survival of kidney grafts from living and deceased donors based on the presence or absence of anti-HLA antibodies. Anti-HLA antibodies were determined by CDC or SPA before transplantation and 6, 12, and 24 months post-transplantation. Of the 2231 patients analyzed, 478 (21.4%) were positive for anti-HLA antibodies 1 year post-transplantation. Of these, 6.2% underwent graft failure, in contrast to only 2.8% patients without anti-HLA antibodies ($P < 0.01$). Two years after transplantation, graft loss occurred in 15.1% of the patients with anti-HLA antibodies, whereas in recipients without anti-HLA antibodies the percentage was as low as 6.8% ($P < 0.02$). When the authors compared the patients who produced anti-HLA antibodies *de novo* after transplantation (233 patients) with those who had never developed anti-HLA antibodies (1331 patients), the rates of graft failure were 16.7% and 6.5% ($P < 0.01$), respectively (Table 2). This study clearly points out that the development of *de novo* anti-HLA antibodies increases the risk of graft failure (43). However, if these data are stratified according to the methods of anti-HLA antibody detection (CDC *vs* SPA), the predictive value of CDC for graft failure was higher than that of SPA.

A second study (44) consisted of 72 patients who had received their first kidney transplant from deceased donors. These 72 patients tested negative for anti-HLA antibodies by CDC and SPA before transplantation. All of them had undergone triple immunosuppressive therapy and were followed for at least 4 years. Sixteen of the 72 patients (22.2%) became positive for anti-HLA antibodies after transplantation, and in 12 of these 16 (75%) the detected antibodies were DSA. Ten of the 12 had acute or chronic rejection.

A statistically significant correlation was established between the appearance of anti-HLA antibodies and delayed graft function, acute rejection, chronic rejection, and graft loss due to immunological causes (44). Patients with high titers of *de novo* anti-HLA antibodies experienced more severe acute rejection and early graft loss, whereas those with lower titers tended to develop chronic rejection. This suggests that titration and monitoring of alloantibodies could be useful in evaluating the risk of rejection (44).

Lachmann et al. (45) showed that in a cohort of 1014 kidney transplant recipients from deceased donors, monitored in a cross-sectional manner for the development of anti-HLA

antibodies using SPA (Luminex), 29% of the recipients tested positive. DSAs were found in 31% of these antibody-positive patients (9% of the 1014 patients). The presence of DSA was associated with a significantly lower graft survival of 49% compared to 83% in the anti-HLA antibody-negative subjects. Interestingly, at late stage of graft failure (low GFR), the presence of anti-HLA antibody without donor specificity (NDSA) also had an adverse effect on graft survival (70%), similar to DSA. Concomitant DSA absorbed by the graft or NDSA related to mismatched can be explained by this intriguing finding. Shared epitope analysis was not performed in this study.

In a prospective 5-year longitudinal analysis of 54 patients without anti-HLA antibodies, graft survival was 86% as opposed to 14% in patients who developed *de novo* anti-HLA antibodies in the 5 years after the first testing ($P = 0.05$). Interestingly, those with anti-HLA antibodies not directed against the donor (NDSA) also had an impaired graft survival (53%). No information as to antibody titer was provided by the authors (46).

In summary, the presence of *de novo* DSA is clearly deleterious to the graft. It leads to AMR and/or contributes to chronic graft nephropathy that in turn reduces long-term graft survival. The development of DSA *de novo* could be viewed as a condition of insufficient immunosuppression, and we therefore believe that any reduction in the dosage of immunosuppressant should be followed by the repeated determination of anti-HLA antibody in the subsequent weeks. Table 2 summarizes the most relevant studies on *de novo* anti-HLA antibody analysis by SPA with regard to AMR and graft survival.

Management of DSA before and after transplantation

The management of patients with anti-HLA antibodies requires a multidisciplinary approach involving close collaboration between HLA laboratory and clinicians.

Before transplantation, careful evaluation of the anti-HLA antibody repertoire with SPA is strongly recommended. Due to cost-effectiveness considerations, anti-HLA determination by SPA should be repeated once a year, except in the case of an immunization event. Indeed, the relevance of the 'natural' fluctuation of anti-HLA levels (MFI) is not known. Virtual PRA (or calculated PRA) and virtual crossmatch approaches (47, 48) or the acceptable mismatch system (49) will be more and more based on anti-HLA antibodies detected by SPA only. Therefore, there is a risk that transplant candidates with anti-HLA antibodies based on DSA identified by SPA only could be discriminated in that they are denied a transplant.

The combination of DSA detected by SPA and crossmatch (by FACS and Luminex) with high sensitivity seems to be an interesting option. Easy to implement in living donor transplantation, where it can be part of the patient/donor evaluation before transplantation, it would probably be more difficult in cases of deceased donor transplants, especially for the HLA laboratory, which should be able to perform FACS and/or Luminex crossmatches on a 24-h basis.

Schemes of risk assessment according to the presence of anti-HLA antibody and crossmatch should help the discussion between laboratory and clinicians and could also be of great interest to develop a consensus between laboratories from different centers (19).

Figure 2 illustrates the current strategy in our institution to stratify the risk of AMR with regard to the different technologies of anti-HLA antibody detection. We recommend that the determination of anti-HLA antibody should be carried out by CDC and SPA before transplantation. The CDC crossmatch remains the gold standard but the FACS and the Luminex crossmatch should be performed whenever possible. In living donation, FACS and Luminex crossmatches can be easily implemented before transplantation to assess the risk of AMR and early graft loss. For cadaveric donation, FACS and Luminex crossmatch are not performed on a routine basis before transplantation since it can be carried out in the next 24–48 h.

The decision to transplantation will be taken according to this risk, knowing that in presence of DSA, a CDC-positive T-cell crossmatch T remains an absolute counter-indication to transplantation. The other options leave space for desensitization protocols in selected cases but in principle a positive FACS crossmatch and high MFI (> 10,000) DSA are also considered a counter-indication to transplantation. Further studies with regard to the relevance of DSA by SPA will fine-tune the decision.

Before transplantation, the presence of anti-HLA should be extensively studied by both CDC and SPA methods (Figure 3A).

After transplantation, the development of anti-HLA antibody by SPA (or CDC) should be carefully followed and lead to early kidney biopsy (Figure 3B). Such a strategy needs to be validated by prospective studies.

To ensure successful transplantation, several approaches of desensitization (50) to remove anti-HLA antibodies have been reported on a limited number of patients with significant success in terms of short- and long-term graft survival (6, 51, 52). The long-term graft survival remains to be determined but the main deficiencies of such approaches are due to the limited number of control studies. However, we believe that such approaches should be intended for patients with very limited chances of receiving a transplant without DSA, i.e. those with large numbers of anti-HLA antibodies (Figure 3).

Acute AMR requires rapid lowering of circulating DSA by plasmapheresis in addition to the standard treatment consisting of steroids, anti-thymoglobulin, and anti-CD20 antibody (53–56) (Figure 3B). However, despite the reversal

Table 2 Literature review over de novo anti-HLA antibodies and kidney graft survival

Article	Technique studied	Sample size Abs (%)	Follow-up (years)[a]	AMR[b]	Graft failure[c] (months)
Terasaki et al. (43)	CDC X-match T and B	1564 patients	2	—	*De novo* Abs = 16.7%
	ELISA	*De novo* Abs (15)			Abs^- = 6.5%
	SPA				241
Mitterbua et al. (44)	CDC X-match T and B	72 patients	1–5	*De novo* Abs = 37.5%	*De novo* Abs = 50%
	FACS X-match T and B	*De novo* Abs (22.2)		Abs^- = 3.6%	Abs^- = 88.2%
	SPA				601
Lachmann et al. (45)	CDC X-match T and B	1014 patients	Mean 5.5	—	DSA^+ = 40%
	ELISA	*De novo* Abs (29)			$NDSA^+$ = 70%
	LSM	DSA^+ = 9%			DSA^- = 83%
	SPA	$NDSA^+$ = 20%			66)
Mao et al. (46)	CDC X-match T and B	54 patients	5	—	DSA^+ = 86%
	LSM	*De novo* Abs (50)			$NDSA^+$ = 4.7%
	SPA	DSA^+ = 28%			DSA^- = 14%
		$NDSA^+$ = 31%			601

Abs, de novo antibodies development; AMR, antibody-mediated rejection; CDC X-match, crossmatch by complement dependant cytotoxicity; DSA, donor-specific antibody; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; LSM, LABScreen Mixed; NDSA, non-DSA; SPA, solid-phase assay.

[a]Follow-up of the study after transplantation.

[b]Percent of AMR with regard to the presence or absence of DSA.

[c]Graft failure reported with regard to the presence or absence of de novo Abs or DSA.

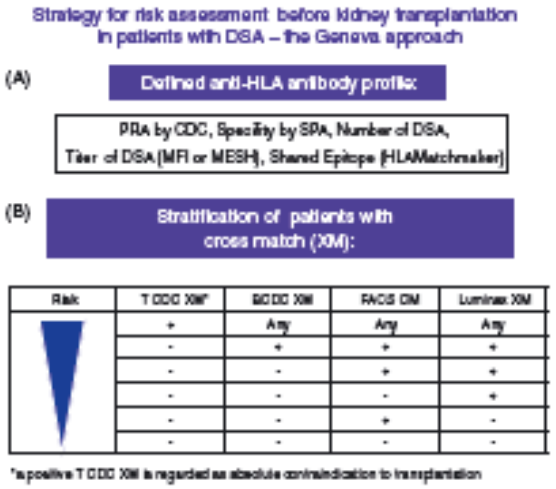

Figure 2 Strategy for risk assessment before kidney transplantation in patients with DSA – the Geneva approach. The determination of the presence of anti-human leukocyte antigen antibodies is performed by CDC and SPA (A). Crossmatch performed by CDC, FACS, and Luminex is used to stratify the risk of AMR before transplantation, and decision to transplantation is based on these results (B). AMR, antibody-mediated rejection; CDC, complement-dependent cytotoxicity; DSA, donor-specific antibody; SPA, solid-phase assay.

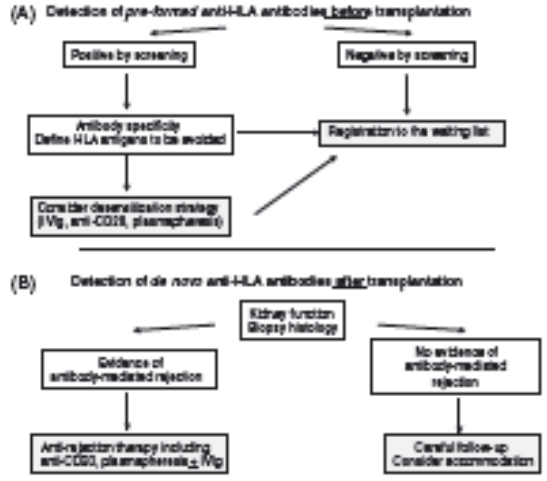

Figure 3 Clinical strategies according to the patient anti-human leukocyte antigen (HLA) antibody status before or after transplantation. Possible HLA antibody testing results (white boxes) and resulting clinical strategies (gray boxes) before transplantation (A). After transplantation, clinical strategies (gray boxes) after detection of *de novo* anti-HLA antibodies should be based on biopsy results (white boxes) (B).

of acute renal dysfunction, this treatment does not deplete antibody-secreting plasma cells in spleen and bone marrow, and circulating DSA commonly remains detectable in peripheral blood. DSA induce microvascular endothelial lesions that can lead to chronic AMR. Long-term exposure to anti-HLA antibodies is also associated with shortened allograft survival and transplant glomerulopathy, even in the absence of documented acute AMR. Although effective treatment is available for acute AMR, allografts remain at risk for chronic AMR and shortened survival (57).

Conclusion

New methods for detecting anti-HLA antibodies such as SPA provide a valuable tool in anticipating AMR or graft dysfunction before transplantation. These methods have proved a 'friend' to physicians in charge of immunized patients before and after transplantation. Results of anti-HLA antibody detection by SPA should be integrated into the global decision of organ allocation, ensuring both short- and long-term benefits to the selected recipient. We also believe that before transplantation, CDC crossmatch should remain mandatory for patients with anti-HLA antibodies. FACS crossmatch or the new SPA crossmatch method (Luminex crossmatch) may also be an excellent means of improving the decision algorithm and should be implemented in HLA laboratories. More studies are necessary to optimize SPA results for anti-HLA antibody titers, such as their predictive value (sensitivity and specificity) for the risk of AMR, as well as short- and long-term graft survival. A better interpretation of these SPA results would increase the values of this technique, its widespread use, and the resulting benefits for the patients.

Conflict of interest

The authors have declared no conflicting interests.

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Conclusion

Quantify patient at risk according to cross match (XM):

RISK	XM CDC	XM Facs	DSA	Transplantation
	positive	positive	positive	NO
	negative	positive	positive	NO
	negative	negative	positive	YES if MFI<5'000
	negative	positive	negative	YES
	negative	negative	negative	YES

The purpose of this study was to pinpoint (a) those anti-HLA antibodies that may be deleterious for the graft, (b) those that should be taken into account for preventing graft rejection, and (c) those that are not deleterious according to (1) the techniques used, (2) the interpretation of the combined results and (3) the type and intensity of the anti-HLA antibodies detected (Fig.25)

Figure 25: Pratical standpoint with living donor

With living donation, several tests and decisions can be made time before transplantation. Here again, according to the intensity of DSA and the results of the CDC crossmatch and/or Facs, the decision to go ahead or to refuse the donation will be taken in accordance with clinicians and surgeons.

As described in the manuscript, we established stratification of patients at risk according to the 3 criteria mentioned in Figures 2A and B, and we hope that these strategies for risk assessment - before (Figure 3A) and after (Figure 3B) transplantation - will help clinicians to make appropriate decisions with regard to the intervention and, if necessary, the adjustment of the immunosuppressive treatment if needed!

c) Isolated or simultaneous kidney-pancreas transplantation is an accepted procedure for treating certain patients suffering from type I diabetes mellitus with or without end-stage-renal failure. Pancreatic islet transplantation is an alternative, more recent, procedure designed to achieve insulin independence or to improve glycemic control in patients with "brittle" type I diabetes. Pancreatic islet transplantation tended to require several infusions from different donors, therefore exposing the recipient to multiple HLA mismatches, which may favor development of anti-HLA antibodies. The presence of antibodies to HLA before transplantation is associated with acute antibody-mediated rejection that could lead to rapid graft loss or to reduced long-term graft survival [4, 126]. After transplantation, *de novo* anti-HLA Abs indicate insufficient immunosuppression and are also associated with reduced graft survival. Whether referring to isolated or simultaneous kidney-pancreas transplantations or to islet transplantations, several reports demonstrate that anti-HLA antibody is a risk factor for graft function and graft survival [77, 79].

In our cohort of islet infusion recipients, we characterized the anti-HLA repertoire before and after transplantation to assess the specific risk of anti-HLA sensitization.

Brief report:

Low risk of anti-HLA antibody sensitization after combined kidney and islet transplantation

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Low Risk of Anti-Human Leukocyte Antigen Antibody Sensitization After Combined Kidney and Islet Transplantation

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Anti-human leukocyte antigen (HLA) antibody could lead to humoral rejection and a decrease in graft survival after kidney transplantation. A recent report has suggested that islet transplantation alone is associated with a high rate of sensitization. The withdrawal of the immunosuppressive therapy because of the progressive nonfunction of the islets could explain the high rate of sensitization. Because the specific risk of immunization of multiple islet infusions remains unknown, we studied the immunization rate in our cohort of multiple islet infusions transplant recipients. De novo anti-HLA antibodies were analyzed in 37 patients after islets alone (n=8), islet-after-kidney (n=13), and simultaneous islet-kidney (n=16) transplantation by solid phase assays over time. The rate of immunization was 10.8% that is comparable with the risk of immunization after kidney transplantation alone. Multiple islet infusions do not represent a specific risk for the development of anti-HLA antibodies after combined kidney-islets transplantation.

Keywords: Islet transplantation, Kidney transplantation, Anti-HLA antibody, Sensitization.

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Islet of Langerhans transplantation is emerging as a promising therapy for type 1 diabetes mellitus. The procedure can be performed as a simultaneous islet-kidney (SIK) or a sequential islet-after-kidney (IAK) transplant in cases of end-stage diabetic nephropathy (*1*, *2*), or as an islet transplant alone (ITA) procedure for the treatment of brittle diabetes (*3*). Thanks to improvements in the islet isolation and transplantation procedures and in the refinement of immunosuppressive protocols, transplantation of islets alone or combined to kidney leads to a significant improvement of glycemic control, to a reduction in insulin requirements, and even to long-term insulin independence in some patients. Although this procedure is safe in experimented centers more than one donor is usually required to achieve metabolic success, exposing patients to multiple allogenic human leukocyte antigens (HLA) and therefore to the risk of sensitization (*4*). Indeed, two recent studies reported a higher risk of anti-HLA sensitization in ITA recipients (*5*, *6*). However, the high frequency of anti-HLA antibody development reported in both studies is observed mostly as a consequence of immunosuppression withdrawal in patients who experience loss of islet graft function (*5*, *6*). Therefore, as the specific risk of anti-HLA sensitization after multiple donor islet infusions remains unknown, we analyzed our cohort of islet transplant recipients, which includes a majority of combined kidney and islet transplants.

Herein, we present the results on the risk of immunization in 37 islet-transplanted patients who were tested negative for the presence of anti-HLA class I and class II antibodies before the first islet infusion by complement dependent cytotoxicity (CDC) and ELISA. Sixteen patients received an SIK, 13 patients an IAK, and 8 patients an ITA. The first 16 patients received immunosuppression based on cyclosporine, mycophenolate mofetil or azathioprine and prednisone, and the last 21 patients received a steroid-free "Edmonton regimen" based on sirolimus and tacrolimus (*7*). All patients were transplanted with a negative CDC crossmatch. Sera of the patients were analyzed for the presence of anti-HLA class I and class II antibodies by solid phase assays ELISA (One Lambda), and all positive results were tested for anti-HLA class I and class II specific antibodies by Luminex single antigen (One Lambda).

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We analyzed 37 transplantations in 34 patients who represents a total of 59 islet cell infusions. The mean quantity of islets infused was 385,859 ± 121,839 islet equivalents. Three patients received a second islet transplant more than 3 years after a first infusion and a complete loss of islet graft function (C-peptide negative), and were considered as newly transplanted patients. Patient characteristics are shown in Table 1. Patient serums were tested at 3 months, 6 months, 1 year after transplantation, and yearly thereafter. Four of 37 patients (10.8%) developed anti-HLA antibodies after transplantation: 1 of 16 patients in the SIK group (5%), 2 of 13 patients in the IAK group (15%), and 1 of 8 patients of the ITA (12.5%). Two patients developed anti-HLA antibody at 12 months, one at 24 months, and the last at 36 months. Sensitization never appeared earlier after the islet transplantation. In all four cases, the islets had class I and class II mismatches with respects to the patients and the kidney HLA typing.

Donor-specific antibodies directed against HLA antigens of the islets were detected in only two of the four sensitized patients by Luminex; patient 2 (ITA) and patient 3 (IAK; Table 2). Anti-HLA antibodies developed by patient 3 who received the islets after a kidney transplantation, were islet-specific only. Those developed by patient 4 (SIK) were specific to the kidney only and those develop by patient 1 (ITA) were related to blood transfusions (Table 2). Serum of patients 2 and 3 only were also positive by CDC–PRA (20% and 25% PRA, respectively).

TABLE 1. Descriptive statistics

Factor	N (%)
No. patients	37
Age (range)	49 (29–64)
Gender	
Male	22 (59%)
Female	15 (41%)
Off Immunosuppression	3
Type of Tx	
SIK	16 (43%)
IAK	13 (35%)
ITA	8 (21%)
Autoantibodies	
Anti-Islets[a]	0/30 (<20 U IDS)
Anti-GAD[b] (range)	4/26 (0–5670 U/mL)
Anti-Insulin[c] (range)	3/24 (0–8.5%)
Nb of Islet Infusions mean (range)	1.7 (1–3)
Nb of IEQ/Infusion (mean ± SD)	385,859 ± 121,839
Rejection[d] (%)	9 (31%)
Insulin Independence[e]	17 (46%)
Fasting C-peptide (pmol/L)	588 (1.2–2317)

[a] Anti-Islets (IF, U IDS), positive results on 30 patients.
[b] GAD (Elisa, U/mL), positive results on 26 patients.
[c] Anti-Insulin(RIA, % of binding), positive results on 24 patients.
[d] Nb of Kidney rejection episodes (on 16 SIK and 13 IAK patients).
[e] Nb of transplantation with insulin independence for at least 2 months.
SIK, simultaneous islet and kidney transplantation; IAK, islet after kidney transplantation; ITA, islet transplantation alone; IEQ, islet equivalent number.

TABLE 2. Development of islet donor-specific antibody (DSA) after transplantation

Patient	Type of transplantation	DSA anti-class I by luminex	DSA anti-class II by luminex
1	ITA	—	—
2[a]	ITA	—	DR11, 4, 13, 14, DQ5, 6
3[a]	IAK	B35, B38	—
4	SIK	—	—

[a] Patients who developed anti-HLA antibodies after withdrawal of the immunosuppressive therapy.
IAK, islet after kidney transplantation; ITA, islet transplantation alone; SIK, simultaneous islet and kidney transplantation.

Two patients were off immunosuppression and the two others received a steroid-free "Edmonton regimen" when de novo antibodies were detected. Patient 1 was still insulin independent 4 years after the transplantation. None of the three other patients reached a state of insulin independence.

The frequency of anti-HLA antibodies detected after kidney transplantation is extremely variable (*8*) but our study showed that the rate of sensitization (10.8%) after islet transplantation is similar to the rate of sensitization after kidney transplantation alone tested with a similar approach (*9*). Our results contrast with a recent report of a large cohort of ITA that has shown a rate of 34% sensitization. In this study, a large number of the sensitized patients developed anti-HLA antibodies after withdrawal or reduction of immunosuppression (*5*). The rate of sensitization was even higher in a second study that has shown the development of donor-specific antibodies in 75% of ITA recipients after discontinuation of immunosuppression (*6*). In our study, three patients were progressively withdrawn from the immunosuppressive therapy and two of them developed anti-HLA antibodies. Eight patients received two to three islet infusions alone (group ITA), and two of them (20%) developed anti-HLA antibodies.

The development of de novo anti-HLA antibodies is associated with humoral rejection and worse long-term graft survival in renal transplantation (*8*) and can also preclude further kidney transplantation. Several mechanisms can explain the loss of islet function including humoral rejection, but a direct role of preformed or de novo anti-HLA antibodies on the islet function in not known and is difficult to assess. Islet graft loss coincidental to development of donor-specific anti-HLA antibodies has been reported only once to our knowledge (*10*). De novo development of anti-HLA antibodies are harmful to the transplanted kidney (*11, 12*), but preformed anti-HLA antibodies detected by sensitive technology are also associated with a reduced graft survival after kidney (*13*) and islet (*14*) transplantation. In addition, the reexposition to mismatched HLA represents a significant risk factor for graft loss in kidney transplantation (*15*). Our data suggest that multiple infusions of islets with multiple HLA mismatch do not increase the risk of sensitization. We can speculate that islet transplants may have a certain degree of protection in the liver that is considered to be an immune-privileged site (*16*) or alternatively that islets may be less immunogenic in this environment. Indeed in vitro cellular assay suggested that the

T-cell reactivity is low or absent in response to HLA islets allograft mismatches after islets transplantation in the liver (*17*). Although the short-term success of ITA is good, only 10% of the patients are still insulin free by 5 years (*18*), and progressive reduction of the immunosuppression seems logical even if the risk of sensitization can be problematic for further transplantation. The situation in SIK and IAK is different, even if islets have lost function, immunosuppression is maintained for the kidney. In addition, because of the deleterious role of anti-HLA antibodies for the renal allograft, immunological testing, and modification of the immunosuppressive therapy are performed on a routine basis after kidney transplantation. We may have slightly underestimated the rate of sensitization by using ELISA that is less sensitive for low levels of anti-HLA antibodies compared with the Luminex or with the flowPRA methods (*19*). In addition we cannot exclude the presence of undetectable anti-HLA antibodies trapped in the transplanted kidney but the high sensitivity of solid phase assay makes this hypothesis less relevant (*20*). Finally, by ELISA or Luminex, only anti-HLA antibodies of IgG isotypes, considered deleterious for the graft, are detected, and not IgM isotypes that have been shown to be detectable after islet transplantation (*21*).

In conclusion, our data suggest that the addition of islets does not represent a risk factor for the development of anti-HLA antibodies when combined with kidney transplantation. Our results should be confirmed on a larger cohort of patients but progresses in the efficacy of islet isolation and strategies to decrease cell loss should continue to be made, to reduce the number of infusions, as multiple transplantation of any tissue or organ-HLA incompatible with the recipients remain a risk factor for anti-HLA immunization.

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Conclusion

In this study, we compared the extent of sensitization in our cohort of 37 recipients of islets alone, islet-after-kidney, and simultaneous islet-kidney transplantation. Our data indicate that the development of DSA after transplantation was similar in islet infusion recipients and kidney transplant recipients (10.8%), and that the addition of islets does not represent a risk factor for the development of anti-HLA antibodies when combined with kidney transplantation. These results contrast with those of others [77, 79] who report high percentages of sensitization, i.e. 34% and 75%, respectively. Since in our study immunosuppression was progressively withdrawn from 3 out of 4 patients developing anti-HLA Abs, we concluded that - even with unsuccessful islet infusions - immunosuppressants at low dosage should be maintained in order to avoid sensitization as remaining non-functional tissue is still in the body.

d) We hypothesized that failure of umbilical cord blood (UCB) engraftment might correlate with the presence of DSA. We studied a total of 70 UCB pediatric transplant patients. All of them were tested for alloantibodies to HLA and MICA by microsphere arrays on the Luminex platform [127]. Pre-transplant and post-transplant plasma samples were available for the study, and before conducting the analysis we ascertained that antibody screening on plasma yielded results comparable to those obtained with serum samples, as described previously [128]. Samples were first tested by the Luminex® LabScreen for the detection of antibodies to HLA class I, class II and to MICA. Positive screening results revealed the specificities of anti-HLA and anti-MICA antibodies determined by Luminex® LSA. Both assays are routinely used in the clinical laboratory for testing patients on the kidney waiting list and patients in need of platelet transfusions. Based on two previous cohorts of UCB transplant patients, a 16-24% rate of anti-HLA antibodies is expected [120, 122].

The clinical relevance of pre-formed anti-HLA and anti-MICA antibodies after cord-blood transplantation in children

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The Clinical Relevance of Pre-Formed Anti-HLA and Anti-MICA Antibodies after Cord Blood Transplantation in Children

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Abstract

Preformed anti-HLA antibodies (AHA) are known to be associated with delayed engraftment and reduced overall survival after adult hematopoietic stem cell transplantation. However, limited data is available in pediatric patients. In this study, we explored the role of AHA on clinical outcomes in 70 pediatric patients who received a single unit of HLA mismatch cord blood for hematologic malignancies, immunodeficiencies or metabolic diseases. The presence of AHA was detected in 44% (31/70) of the patients. Preformed class I AHA was associated with an increased occurrence of grade 1–4 acute graft-versus host disease ($p<0.05$). The presence of anti- major-histocompatibility-complex class I-related chain A antigens (MICA) antibodies was significantly associated with a reduced platelet recovery after transplantation ($p<0.05$). AHA of class II with the strength of antibody titer measured as the mean fluorescence intensity above 2000 was associated with reduced event-free survival ($p<0.05$). A reduction of high titer of AHA and anti-MICA antibodies might have to be considered before cord blood transplantation in pediatric patients for better outcomes.

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Introduction

The presence of preformed anti-HLA antibodies (AHA) is a risk factor for antibody mediated rejection and is associated with reduced clinical outcomes in solid organ transplantation, especially in kidney transplantation [1–6]. Therefore, detection and determination of specific anti-HLA are a part of the preparatory tests performed by the laboratory before kidney transplantation [1].

Investigating the role of AHA in determining clinical outcomes of hematopoietic stem cell transplantation (HSCT) has recently gained interest. Although the objective of HSCT is to select a complete HLA matched donor, currently more transplants are being performed with partially matched donors with the availability of cell sources from umbilical cord blood, unrelated donors from the worldwide registry and haploidentical donors. In adults, the presence of AHA directed against HLA-mismatched donors has been associated with graft failure, delayed neutrophil, platelet recoveries, and graft versus host disease (GVHD), leading to reduced overall survival (OS) [7–16]. In children, cord blood unit transplantations became more frequent and are preferred in situations of HLA mismatch. To the best of our knowledge clinical relevance of preformed AHA in pediatric transplantations with cord blood as the only source has not been reported until now. Similar to the reports from adult HSCT patients available in the literature, the presence of preformed AHA, arising mainly due to blood transfusions, could also be deleterious in clinical outcomes of HSCT in children. In addition to AHA, preformed antibodies against major-histocompatibility-complex class I-related chain A antigens (anti-MICA antibodies) might also be detrimental in HSCT outcomes.

In this explorative study, we had the opportunity to analyze the presence of AHA and anti-MICA antibodies in a cohort of 70 children receiving single cord blood transplantation. Using the Luminex technology, we investigated the presence of AHA of class I, II, and anti-MICA antibodies prior to HSCT, and correlated them with clinical outcomes.

Materials and Methods

Umbilical Cord Blood Transplantation

This study comprise 70 children, 53 of them underwent allogeneic HSCT at CHU Sainte-Justine, Montreal between May 2000 and August 2010 and 17 children underwent allogeneic HSCT at the Hospital for Sick Children, Toronto, between July 2008 and October 2009.

The patients received a busulfan (Bu) based conditioning regimen either myeloablative (94.3%, n = 66) or non-myeloablative (5.7%, n = 6) (Table 1). Intravenous (iv) Bu (Busulfex®, Otsuka Pharmaceuticals) first dose was based on age of the patient and a pharmacokinetics guided dose adjustment was subsequently performed [17,18]. The majority of the patients received iv cyclophosphamide (200 mg/kg total dose; table 1) following Bu administration. GVHD prophylaxis was provided with cyclosporine to all the patients, with the addition of either, methotrexate, mycophenolic acid, steroids and mycophenolic acid combinations to 92.5% (49), 5.6% (3), 1.2% (1), and 1.2% (1) of the patients, respectively (data available for 53 patients). Sixty-four (94.3%) patients received anti-thymocyte globulin. Granulocyte colony-stimulating factor (G-CSF) was given to all CHU Sainte-Justine patients after each cord blood infusion but not to patients from the SickKids Hospital. Prophylaxes against fungal, viral, *Pneumocystis jiroveci* infections were administered as per institutional standards (fluconazole, acyclovir and trimethoprim/sulfamethoxazole) and ursodeoxycholic acid was given as a veno-occlusive disease (VOD) prophylaxis only to CHU St-Justine patients. Seizure prophylaxis was provided with phenytoin (26.9%), midazolam (19.4%) or lorazepam (53.7%). All patients received a single umbilical cord blood (UCB) unit. The HLA matching between the UCB unit and the recipient was at least 4 out of 6 for the majority of cases (>97%) at an antigen level of HLA-A, B and allelic level of DRB1 (see table 1).

GVHD grading was based on the 1994 Consensus Conference on Acute GVHD [19]. Neutrophil recovery was defined as the first of 3 consecutive days with an absolute neutrophil count of 0.5×10^9/L or higher. Platelet recovery was defined as the first of 7 consecutive days with platelet counts higher than 50×10^9/L without transfusion. Primary graft failure was defined by persistent pancytopenia with no evidence of hematologic recovery of donor cells beyond 28 days after transplantation and secondary graft failure by a rapid decrease in neutrophil count after successful engraftment. Event-free survival (EFS) was calculated from time of transplant until death, relapse or graft failure, whatever occurred first. Overall survival (OS) was the time between transplantation and death due to any cause.

Anti-HLA Antibody Assays

Plasma samples collected prior to the conditioning regimen were tested with the screening assay LAB Screen® Mixed (One Lambda Inc., Canoga Park, CA) according to manufacturer's instructions. Test interpretation was performed using HLA Visual® software (One Lambda) on the LABScan100™ flow cytometer (Luminex Inc., Austin, TX) with a positive cut off of 3.0. Anti-MICA antibodies were also determined by the same technology and are included in the LABScreen® Mixed kit.

To identify the specificity of AHA, high definition LAB Screen® Single Antigen (One Lambda) class I or class II assays were performed with plasma of recipients who tested positive for the LAB Screen® Mixed assay. Results were interpreted using the LABScan™ 100 software (One Lambda) on the LABScan 100™ flow cytometer (Luminex). A positive result was defined when a mean fluorescence intensity (MFI) was above 1000 and calculated as follows: (fluorescence of beads coated with HLA and incubated with patient plasma) – (fluorescence of beads without HLA and incubated with patient plasma) – (fluorescence of beads with and without HLA and incubated with negative control plasma).

Statistical Analysis

The distribution of demographic characteristics between the groups with and without antibodies was compared using Chi-square test for categorical variables and non-parametric tests (Mann-Whitney and Kruskal –Wallis rank tests) for continuous variables. Cumulative incidences of neutrophil and platelet recoveries were estimated using the cumulative incidence (CI) function, with death as a competitive event. Cumulative incidences in the presence of the competing risk were compared between the groups using the Gray's test in a univariate analysis, and Fine and Gray multivariate regression model [20,21]. Probabilities of survival i.e. OS and EFS were estimated using Kaplan-Meier curves and log-rank test by comparing the differences between groups in a univariate analysis. Hazards ratios (HR) with 95% confidence intervals (CI) were estimated using univariate cox-regression and impact of antibody status was assessed in multivariate cox-proportional hazards regression analysis. In multivariate analyses co-variates were included as categorical models such as diagnosis (malignant, non-malignant), number of nucleated cells infused (above and below the median value), number of $CD34^+$ cells infused (above and below the median value), G-CSF receiving status (received versus not received) and conditioning regimen (myeloablative, non-myeloablative). For aGVHD (grade 1–4) cumulative incidence, in addition to the above co-variates, GVHD prophylaxis (cyclosporine and steroids versus other prophylaxis), serotherapy (received versus not received) and HLA matching status (100% match versus less than 100% match) were included in the multivariate analysis. Two sided p-values are presented and a probability less than 0.05 was considered as significant. Analyses were performed using the IBM® SPSS® statistical package (version 19, SPSS Inc, NY) and R statistical software (http://r-project.org).

Results

Patient Characteristics

Seventy patients from two study centers were included in the analysis. The patient demographic characteristics are given in table 1. Most of the patients (47/70) were transplanted for hematopoietic malignancies, 1 patient for a neuroblastoma and the others for immunodeficiencies (11/70), metabolic diseases (5), genetic related hemophagocytic syndrome (3) or hemoglobinopathy (1). Among the leukemic patients (36), 25.7% (18), 17.1% (12) and 2.9% (2) were in first, second and third complete remission, respectively. Three patients were in first relapse and 1 patient was in acute phase of the disease. HLA mismatch profiles are depicted in table 1. The mean (median) number of infused total nucleated cells was 2.1×10^8/kg (1.4×10^8/kg) and for 64 patients where there was data on the number of $CD34^+$ cells infused the mean (median) calculated to 0.02×10^8 cells/kg (0.004×10^8/kg).

The incidences of overall OS, EFS, neutrophil, and platelet recoveries were 62.9%, 45.7%, 84.3%, and 65.7%, respectively. Median time to neutrophil engraftment was 20 days (mean = 22.9 days), and median time to platelet recovery was 58.5 days (mean = 60.2 days). VOD, hemorrhagic cystitis, acute GVHD (grade 1–4) and lung toxicity incidences were 7.1%, 28.6%, 24.3%, and 7.1%, respectively. Eight patients had primary and three had secondary graft failures. The median follow up of the cohort was 26.8 months (mean = 35.2 months). A total of 26 patients died with 3 patients before neutrophil engraftment, and 6 patients before platelet recovery.

Preformed anti-HLA and anti-MICA Antibodies

To validate the assay using plasma (instead of serum, according to the classical assay from the manufacturer), 10 serum samples and 10 plasma samples were tested from immunized patients for the presence of AHA and anti-MICA antibodies. In line with

Table 1. Demographic characteristics of whole cohort.

Demographic characteristics		Whole cohort
Number of patients n (%)		70 (100)
Age (Years)	Mean (median)(range	6.5 (5.0) 0.1–19.9
Body weight (Kg)	Mean (median)(range	27.0 (20.8) 4.3–95.6
Gender n (%)	Male	41(58.6)
	Females	29 (41.4)
Diagnosis n (%)	AML	26 (37.1)
	MDS	12 (17.1)
	Immunodeficiency	11 (15.7)
	ALL	9 (12.9)
	Metabolic disease	5 (7.1)
	Hemophagocytic syndrome	5 (7.1)
	Hemoglobinopathies	1 (1.4)
	Neuroblastoma	1 (1.4)
Conditioning regimen n (%)	Bu+Cy	53 (75.7)
	Bu+Cy+Mel	3 (4.3)
	Bu+Cy+VP 16	7 (10)
	Bu+Flu	4 (5.7)
	Bu+Mel	3 (4.3)
Disease status in malignancies n (%)	First acute phase of the disease	1(2.7)
	First CR	18(50.0)
	second CR	12(33.3)
	third or more CR	2(5.5)
	First Relapse	3(8.3)
HLA Matching n (%)	3/6	2 (2.9)
	4/6	20 (28.6)
	5/6	28 (40)
	6/6	16 (22.9)
	7/8	1 (1.4)
	8/8	2 (2.9)
	7/10	1 (1.4)
HLA match compatibility n (%)	MRD	2(2.8)
	MUD	16 (22.9)
	MMU	52 (74.3)
Days after transplant	Mean (median)	1064.4 (815.0)
Nucleated cells injected (10^8/kg)	Mean (median)(range	2.1 (1.4) 0.16–18.0
$CD34^+$ cells injected (10^6/kg) n=64	Mean (median)(range	0.02 (0.004) 0.0005–0.14

Abbreviations: ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; ATG: anti-thymocyte globulin; Bu: busulfan; Cy: cyclophosphamide; CR: Complete remission; Flu: fludarabine; MDS: myelodisplastic syndrome; Mel: melphalan; MMU: mismatch unrelated; MRD: matched related donor; MTX: methotrexate; MUD: matched unrelated donor; VP16: etoposide.

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previously reported data [22], our results confirmed an excellent concordance between the two types of samples (data not shown).

AHA were detected in 44.3% of the patients among which 38.1% were positive for antibodies against either HLA class I, or class II AHA and 41.9% were positive for both class I and II (Table 2). Donor specific antibodies (DSA) were detected in 12 individuals (4 for class I, 5 for class II and 3 for both classes). Anti-MICA antibodies were detected in 11 patients; in 8 patients, anti-MICA antibodies status was undetermined (borderline to the limit of detection). We did not observe any statistical differences with regards to the presence of pre-formed AHA or anti-MICA antibodies and demographics, or any clinical parameter of the patients before transplantation like age, gender, conditioning regimen or type of disease (data not shown), except that patients detected for AHA for class I were infused with a lower number of $CD34^+$ cells compared to the remaining patients ($p<0.05$).

Effect of Anti-HLA Antibodies and anti-MICA Antibodies on Hematologic Recovery and other Clinical Outcomes

Patients detected for AHA of both classes had a trend of lower incidence of neutrophil recovery (69.3%, 9/13 engrafted, 0/13 dead before recovery) compared to patients negative for both

Table 2. Patient demographic characteristics distribution among groups based on anti-HLA antibodies, DSA and MICA detection status.

Demographic characteristics		Anti-HLA antibodies detection status							
		Not detected	Class I AHA	Class II AHA	Both	DSA negative	DSA positive	MICA negative	MICA positive
Number of patients		39	22	22	13	58	12	51	11
Age in years, Mean (median)		7.4 (5.2)	5.2 (3.2)	5.7 (5.9)	5.9 (6.2)	6.5 (4.6)	6.6 (5.9)	6.4 (5.0)	6.3 (4.4)
Body weight, Kg, Mean (median)		29.8 (23.2)	23.2 (15.8)	25.1 (24.1)	25.8 (26.9)	26.9 (18.8)	27.3 (25.1)	26.7 (21.5)	28.3 (20.1)
Gender (n)	Male Females	23 16	11 11	12 10	5 8	36 22	5 7	30 21	8 3
Diagnosis(n)	AML	15	9	7	5	21	5	16	7
	MDS	7	1	5	1	10	2	11	1
	Immunodeficiency	4	6	5	4	8	3	6	2
	ALL	7	0	2	0	7	2	9	0
	Metabolic disease	2	3	2	2	5	0	3	1
	Hemophagocytic syndrome	2	3	1	1	5	0	5	0
	Hemoglobinopathies	1	0	0	0	1	0	0	0
	Neuroblastoma	1	0	0	0	1	0	1	0
Nucleated cells injected X10⁸/kg) Mean (median) range		2.1 (1.4) 0.16–18.0	1.8 (1.1) 0.29–9.0	2.8 (1.4) 0.29–14.00	2.5 (1.3) 0.29–9.0	1.9 (1.4) 0.16–18.0	3.2 (1.3) 0.29–14.00	2.3 (1.4) 0.16–18.0	1.3 (0.9) 0.33–3.15
CD34⁺ cells injected (X10⁶/kg) N = 64 Mean (median) range		0.02 (0.005) 0.0004–0.13	0.005 (0.002) 0.0005–0.046	0.02 (0.004) 0.0007–0.14	0.007 (0.002) 0.0007–0.05	0.01(0.004) 0.0004–0.14	0.02 (0.002) 0.0005–0.11	0.02 (0.004) 0.0007–0.14	0.007 (0.003) 0.0004–0.041

Abbreviations: AHA : anti-HLA antibodies; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; ATG: anti-thymocyte globulin; Bu: busulfan; Cy: cyclophosphamide; DSA : Donor-specific anti-HLA antibody; Flu: fludarabine; MDS: myelodysplastic syndrome; Mel: melphalan; MICA : major-histocompatibility-complex class I–related chain A antigens MM: mismatch; MRD: matched related donor; MTX: methotrexate; MUD: matched unrelated donor; VP 16: etoposide. AHA of either classes were detected in 31 patients. Class I AHA and class 2 AHA alone were detected in 9 patients, and both in 13 patients, thus 22 (9+13) patients were positive for class I AHA and class II AHA. MICA antibody data was available for only 62 patients out of 70. Data for infused number of CD34⁺ cells was available for only 64 patients out of 70.

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classes of AHA (89.3%, 50/57 engrafted, 3/57 dead before recovery, p = 0.21; HR:1.6 (0.8-3.3), Figure 1A). In a multivariate regression analysis including AHA status, $CD34^+$cell number infused, G-CSF receiving status, age and gender, only $CD34^+$ cell number above the median was associated with low neutrophil recovery (p = 0.023). AHA status showed a trend of association (p = 0.15). No significant differences in neutrophil engraftment was observed between patients who received G-CSF (84% engraftment) compared to those not received (82% engraftment). The presence of preformed anti-MICA antibodies was significantly associated with a lower incidence of platelet recovery (26.5%) compared to those who were negative (80.5%) for anti-MICA (p = 0.04; HR: 4.2 (1.02–17.08), Figure 1B). Patients diagnosed for malignant condition also had lower incidences of platelet recovery (63.6%) compared to those with non-malignant disease (90%) (p<0.0001, HR: 3.5 (1.9–6.6)). In a multivariate regression analysis including MICA status, AHA status, nucleated cell number and diagnosis, MICA positive status showed a trend of association with lower platelet recovery (p = 0.06), whereas diagnosis was significantly associated with lower platelet recovery (p = 0.003). Dead patients were considered as a competing risk in univariate and multivariate analysis for neutrophil and platelet recoveries, when they died before recovery.

Influence of anti-HLA Antibodies and their Titers on aGVHD, Event Free Survival or Overall Survival

Patients detected for class I AHA had a higher incidence of acute GVHD compared to the remaining patients (36.4% Vs 18.7%; p = 0.03, HR: 2.7 (1.03–7.4); Figure 2A). In univariate analysis, $CD34^+$ cell number above the median, myeloablative conditioning regimen and serotherapy showed trends of association with higher incidence of aGVHD. No aGVHD was seen in patients receiving non-myeloablative conditioning regimens. Interestingly, HLA match showed (categorical) no association with aGVHD. A higher incidence of aGVHD was seen in patients who did not received serotherapy (50% Vs 23%, p = 0.06). In a multivariate analysis including GVHD prophylaxis, serotherapy, $CD34^+$ cell number, conditioning regimen and disease, class I AHA remained independently associated with the occurrence of aGVHD1-4 (p = 0.024).

The presence of preformed class II AHA was associated with reduced EFS. The presence of class II AHA with MFI > 2000 was associated with a lower EFS compared to those negative for class II AHA or positive but with MFI <2000 (12.5% vs. 50% p = 0.01, HR:2.8 (1.3–6.4); Figure 2 B). Lower EFS (HR: 3.6 (1.2–10.2)) was seen in individuals detected for both class I and II AHA at MFI > 2000 compared to the remaining patients (0% vs. 49% p = 0.01, Figure 2 C).

A trend for lower OS (25% vs. 50%; p = 0.085; Figure 2D) was observed in patients positive for DSA (n = 12), compared to patients with no DSA (n = 58). When analyzed for impact of AHA and DSA status on OS, we observed 66.7% OS in patients with no AHA detected; 68.4% OS in patients with AHA detected but not donor specific whereas patients detected with DSA had 41.7% OS (log-rank p value = 0.2).

Among other factors known to influence OS and EFS patients diagnosed with a malignant disease demonstrated a lower EFS (p = 0.007), whereas nucleated cell number and CD34+ cell number showed only a trend of association in a univariate analysis (p = 0.2–0.4). In a multivariate analysis only diagnosis showed a significant association with EFS, whereas class II AHA with MFI > 2000 showed only a trend.

Discussion

In this retrospective analysis, we have demonstrated the interest of detecting preformed AHA and anti-MICA antibodies in a

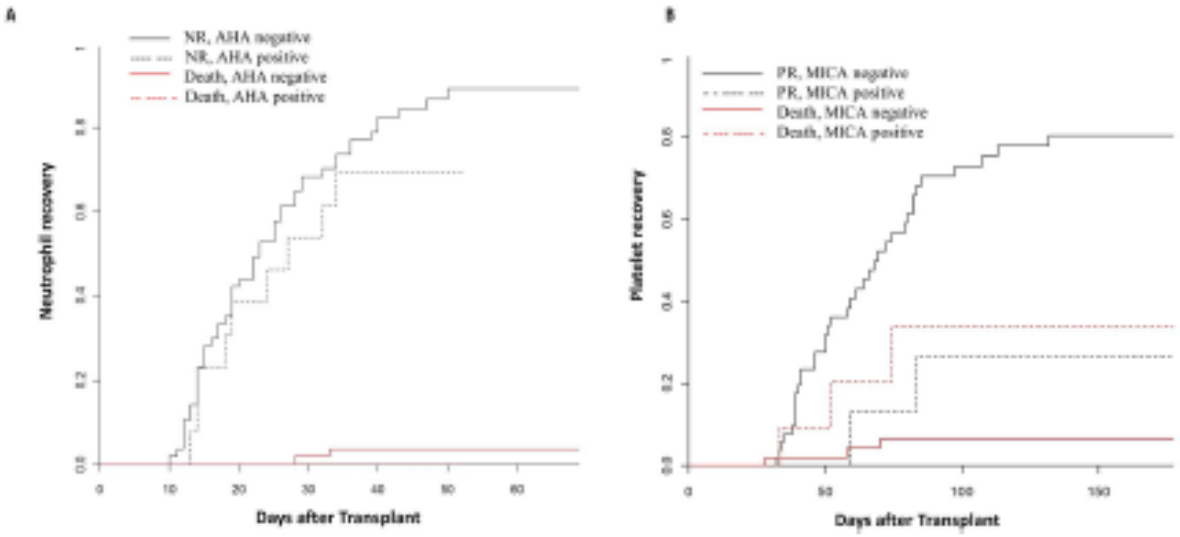

Figure 1. Cumulative incidence of neutrophil and platelets recovery. A) 69.3% (95% CI: 58.49-80.11%) of patients positive for both class I and II anti HLA antibodies (AHA) had neutrophil recovery (NR) compared to 89.3% of negative patients (95% CI: 82.06–96.54%). NR did not differed in both groups, Gray's test p-value = 0.21, hazards ratio is 1.6 (0.8–3.3). No patients detected for both class AHA were dead before NR. B) Patient's positive for anti major-histocompatibility-complex class I–related chain A antigen (MICA) antibodies had lower cumulative incidences of platelet recovery (PR, 26.5% (95% CI: 15.51–37.49%) compared to negative patients (80.5%; 95% CI: 70.64–90.36%).Total number of patients included in analysis was 62; death before PR was used as competing event, and for 8 patients we do not have conclusive MICA status. Gray's test p value = 0.04, hazards ratio is 4.3 (1.02–17.8).
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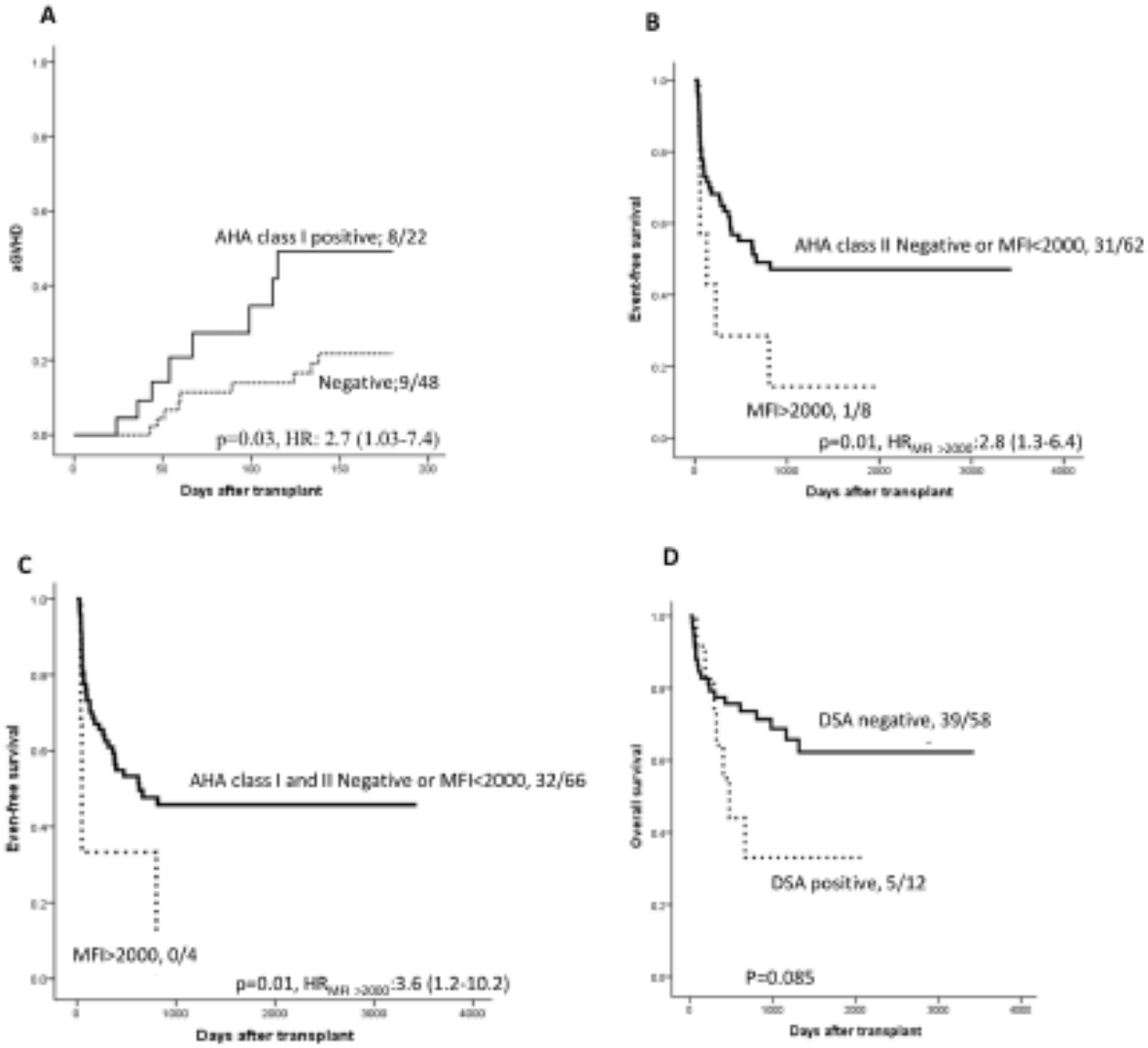

Figure 2. Cumulative incidence of acute GVHD (grade 1–4), event-free survival and overal survival. The number of patients with aGVHD occurrence or with no events or survived/total number of patients in each group, hazards ratios with 95% confidence interval is presented on the plot. A) Patients positive for class I AHA had higher incidence of aGVHD (36.4%) compared to those negative for class I AHA (18.7%). B) Patients positive for class II AHA with mean florescence intensity (MFI) above 2000 had lower event free survival (12.5%) compared to those negative or positive with MFI less than 2000 (50%). C) Patients detected for both class I and II AHA had lower EFS (0%) compared to those negative or positive with MFI below 2000 (48.5%). D) Patients with DSA against the HLA mismatch of the cord blood had a trend of lower overall survival (41.6%) compared to patients without DSA (67.3%).
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pediatric cohort receiving cord blood unit transplantation. Our data shows the importance of preformed anti-MICA antibodies, which appears to be associated with lower cumulative incidences and prolonged times to platelet recovery. The presence of class I AHA showed an associated with acute GVHD incidence. Preformed AHA of class II was also associated with reduced EFS when MFI is above 2000. These observations highlight the importance of AHA in determining EFS in cord blood transplants which has been shown to result in similar disease free survival as that of bone marrow transplant in children [23]. The data presented here also highlights that the delay in engraftment with cord blood transplant will be further augmented by the presence of AHA.

The analysis included all patients with both myeloablative and non-myeloablative (n=4) conditioning regimens. The statistical significance was not altered when analyzed with patients receiving myeloablative-conditioning regimen only.

The major limitation of our study is that the sample size is small, which reduces statistical power. The power of the study was 50 to 60%, calculated for the observed incidences of neutrophil recovery and EFS in the study sample at a probability (alpha) of 0.05. Due to the small sample size, we were only able to pick up trends in a

few of the associations described. These associations and trends observed in this study must be confirmed in a larger cohort to establish a real association. However, we believe that our results are of interest and specific to a pediatric population, transplanted with a single cord blood unit, since this is the largest cohort ever investigated for this topic in the best of our knowledge.

The level of immunization in our pediatric cohort was quite high (44%), compared to existing data on adults [11]. The cohort is mostly comprised of patients with malignant condition, especially leukemia which usually necessitate transfusions. This consequently might result in alloimmunization and adverse outcomes. Unfortunately, we do not have the transfusion data from these patients. Since malignant disease status itself was independently associated with less survival, we did not include stage of leukemia's in the multivariate analysis owing to a low number of patients. However, it would be interesting to prospectively evaluate the degree of alloimmunization in relation to the disease status and the level of transfusions within a large homogeneous cohort before transplantation.

The presence of anti-MICA antibodies was associated with reduced platelet recovery. MICA encodes the highly polymorphic MHC (HLA) class I chain-related gene A. Although unlike classical MHC class I molecules MICA does not seem to be associate with beta-2-microglobulin [24] with functions related to innate immunity [25–27]. MICA antigens are expressed on the surface of endothelial cells, dendritic cells, fibroblasts, epithelial cells, activated $CD4^+$ T, $CD8^+$ T cells and as well as in many tumors. MICA functions as a stress-induced antigen that is broadly recognized by Natural killer (NK) cells, NKT cells, and T cells. MICA acts as a ligand for the NK cell activating receptor NKG2D [25–27]. Solid organ transplantation, pregnancy, blood transfusion [28], and other mechanism like cross reactivity with substances from the environment [29] could explain the development of anti- MICA antibodies that can be detected by different technologies like ELISA or Luminex [30]. In renal transplantation, the presence of anti-MICA antibodies is associated with renal allograft rejection and reduced graft survival [31,32]. In HSCT, the data for the role of anti-MICA antibodies is limited [33,34], but in adult, Parmar *et al.*, demonstrated an association between the presence of anti-MICA antibodies and acute GVHD [33]. In our study, the influence of anti-MICA antibodies on platelet recovery was independent of the presence of AHA of class I and/or class II. Typing the platelets of donors for MICA antigens could not be performed because donor DNA was not available, hence formal proof of donor specificity could not be obtained. To the best of our knowledge, there is no data on the expression of MICA by platelets available in literature.

We observed a higher incidence of acute GVHD (grade 1–4) in patients positive for class I AHA. Overall there is a trend of higher incidence of acute GVHD in patients positive for AHA irrespective of their class and titers. Though the incidences and severity of acute GVHD are lower in cord blood transplant compared to bone marrow transplant, our data demonstrates that this could be further lowered in patients negative for class I AHA [23]. The distribution of prophylaxis for GVHD was similar in groups positive and negative for AHA with most of them receiving cyclosporine and steroid based prophylaxis (data available for 53 patients only). Association of AHA with GVHD has not been found in previous studies conducted in adults or mixed cohorts [13,15]. AHA can participate in the inflammation of the tissue of the recipients by a direct binding or by an indirect mechanism and play a role in the GVHD process. It is interesting to note that patients detected for AHA of class I received a significantly lower number of $CD34^+$ cells compared to those who were negative for class I AHA. We did not find an independent association of AHA class I status and engraftment but $CD34^+$ cell numbers below the median was independently associated with a delayed engraftment, irrespective of AHA status. Moreover, AHA class I status was independently associated with aGVHD, but not $CD34^+$ cell number (showed only a trend). Engraftment was delayed in patients detected for both classes of AHA compared to those who are positive for either one class or negative for AHA, suggesting an important role of class II AHA.

Our data indicates that the presence of AHA with high MFI are more deleterious with regards to EFS in cord blood transplant patients. This is important because this suggests that a strategy could be implemented to keeping or lowering the level of AHA below MFI = 2000, using the antibody titer. The clinical utility of antibodies titer defined by MFI is a subject of debate in solid organ transplantation [1,35,36]. Although the test is only certified by the regulatory agencies for qualitative results, there is increasing evidence in the literature that MFI allows stratification for the risk of clinical event [1,15,36].

Our study suggests that the reduced EFS was mainly linked to AHA of class II. In contrast to MHC class I, MHC class II is not expressed constitutively on any cell type. Only specialized cells express MHC class II, like antigen presenting cells, B cells or thymic epithelial cells. However, MHC class II can be induced by pro-inflammatory cytokines like γ-interferon or TNFα. The expression of MHC class II on any tissue reflects a state of inflammation. Therefore we hypothesize that in addition to the specific and direct effect of donor specific antibodies, global inflammation related to conditioning regimen before transplantation and any event after transplantation (like infection, GVHD, mucositis, hemorrhagic cystitis) can induce the expression of MHC class II. This indirectly reflects a state of inflammation associated with the occurrence of clinical events such as rejection, relapse and infections and finally reduced EFS.

Patients either detected for AHA which are not donor specific or not detected for AHA had similar OS, whereas patients detected for AHA which are donor specific had lower OS, but this observed difference is not statistically significant. As mentioned above, this could be explained by the limited number of patients in the cohort, but is also due to the fact that some of the DSA have a low antibody titer (MFI<2'000). Of note, DSA against HLA-Cw, HLA-DQ and HLA-DP were not taken into consideration because HLA typing for these loci were not available for every patient. This observation is of importance, because it is likely that our ability to assess DSA is not completely accurate and some antibodies deemed DSA-negative in fact might have had reactivity against the donor. This is also consistent with the observation of multiple epitope sharing among HLA-II molecules [37].

In conclusion, our data strongly suggests that in children the presence of preformed high titer of AHA and anti-MICA antibodies are associated with reduced hematologic recovery and reduced event-free survival. The presence of class I AHA irrespective of its titer is an important risk factor for developing acute GVHD. Presence of AHA below MFI 2000 is probably not deleterious. Blood transfusion policy should be carefully evaluated before transplantation and therapeutic strategy to reduce the high titer of AHA before transplantation by plasma exchange could be implemented to reduce the risk of events due to the presence of preformed AHA and anti-MICA antibodies. However, due to the lack of sufficient power, the trends and the significant findings of this study should be validated in a larger cohort of children.

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Author Contributions

Conceived and designed the experiments: MA CRSU MK JV. Performed the experiments: MA CRSU SFL JV. Analyzed the data: MA CRSU SFL JV MK. Contributed reagents/materials/analysis tools: SFL HB FGP YC JMT TS AG JD LD MD. Wrote the paper: MA CRSU MK JV.

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Conclusion

This was one of the first studies to demonstrate that preformed antibodies to HLA and to MICA have a deleterious effect on the hematological recovery of children transplanted with UCB.

On the basis of this observation, we were able to confirm that analysis of anti-HLA Abs is essential before and after transplantation to guarantee optimal care and follow-up of these young patients. Therefore, determination of anti-HLA Abs by SPA has become a routine test in hematological care units, the presence of anti-HLA Abs being associated with reduced hematopoietic graft survival [129]. This becomes even more accurate as haplo-identical HSC appears again to be an option in HSC transplantation. Therefore, the determination of anti-HLA antibodies in all patients awaiting a HSC transplant is essential to avoid DSA again the potential allo/haplotype donor, and it is now recommended that before any HSC transplantation, patients should be screened for HLA antibodies [130].

VIII. Conclusion and Perspectives

Transplant immunology was slowly "dying" due to better knowledge and the improved management of immunosuppressive drugs. HLA mismatches ceased to be a problem in transplantation.

With the introduction in 2005 of the Luminex technology along with the identification of anti-HLA antibodies and their specificities, came a complete change of techniques, tests and approaches in HLA laboratories. Consequently, follow-up of patients on the waiting list as well as transplanted patients also had to change and new guidelines had to be established for physicians and technicians alike.

At the same time, determination of anti-HLA antibodies with these sensitive technologies (SPA) led to new areas of interests and developments:

1) With the clear identification of anti-HLA antibodies at an intermediate allelic level, HLA typing of donors and recipients also had to evolve, and intermediate to high HLA-typing has replaced low-level HLA typing.
2) Results of crossmatches and SPA have to be analyzed in parallel in terms of practicalities so that a better assessment of the risks of an allograft can be made (Fig 22 and 25).
3) The sensitive determination of specific anti-HLA Abs gives rise to the question as to which intensities are they clinically relevant and which anti-HLA Abs are detrimental.
4) Can we consider SPA as granted or should we also - or even exclusively - take complement-binding Abs into account? Work in this area is still in progress.
5) The panel of anti-HLA Ab specificities revealed for a given patient is now an additional tool to guide our decisions regarding patients on the waiting list. With the new SOAS (Swiss Organ Allocation System) program, the specific anti-HLA Abs of each patient are listed, and anti-HLA Abs with MFI >1,000 are avoided (Fig 22). This led to the development of a program called virtual crossmatch, i.e. an organ is offered for donation only in case of permissive antigens, DSA being categorically ruled out [1, 131] .
6) Owing to this program, organ allocations are more accurate and the organs offered are a better match, resulting in a decrease of inadequate crossmatches and thus the length of time to the final process becomes shorter.

7) We know that antibodies to HLA class I (HLA-A and –B) [132] and to HLA class II (HLA-DR and –DQ) [133, 134] are risk factors for acute and late rejection. However, SPA reveals antibodies to HLA-C, and –DP, which justifies an extensive HLA-typing of donors and recipients by new methods. The main question was: what to do about these anti-HLA-C or –DP Abs? Are they clinically relevant? Several reports have confirmed a major role of anti-DQ antibodies [135], anti-HLA-C Abs having also been described as detrimental for kidney graft survival [6, 68, 136, 137] A recent survey of 1,069 patients on the waiting list for organ transplantation has revealed a lower frequency of anti-C antibodies as compared to anti-A, -B, -DR and -DQ antibodies, and lower median mean fluorescence intensity (MFI) values for anti-HLA-C antibodies [137]. Eight patients received kidney transplants with DSA specific for HLA-C, but these antibodies did not appear to affect graft survival and acute rejection [137]. So far, only case reports have demonstrated the harmful effect of anti-HLA-DP Abs on the survival of kidney allograft [138, 139]. Larger cohort studies are needed before a conclusion can be reached.
8) Nevertheless, taking into account all these additional anti-HLA Abs, and therefore the HLA-C and –DP for allocation, will also give rise to additional difficulties when deciding as to the allocation of an organ to a particular recipient, given the small number of deceased donors available in Switzerland.

   Therefore, the identification of these anti-HLA Abs revealed that pre-transplantation immunological risk is an increased problem and that the best donor-recipient combination is becoming a real challenge.

Moreover, to counteract the major problem of the shortage of deceased donor, leading to long waiting time for patients on the waiting list, different programs were developed and extended in Geneva:

1) Living donation: Nowadays, 50% of our kidney donations are coming from living donors.
2) ABO-incompatible kidney transplantation: As described in paragraph V.a, ABO-incompatible living kidney transplantations have been performed with great success in Geneva, and so far the graft survival rate has been 100% [57, 58].
3) Crossover transplantation: when living kidney donation cannot be performed due to an immunological incompatibility between the donor and the recipient, a crossover transplantation could be performed between 2 couples in the same situation. We have

performed this kind of kidney transplantation since 2010, and we are lucky to have 13 happy crossover couples, also with a graft survival rate of 100%. According to Dr Karine Hadaya (Division of nephrology and transplantation at Geneva) report, a 6 and 12 month post-surgeries'psychological evaluation of the 13 donors and recipients reports no regret [140].

These excellent results are due to very accurate analyses of the donor's and recipient's parameters, including thorough analysis of their immunological status, DSA having to be avoided at all costs.

The development of de novo anti-HLA antibodies is a sign of non optimal immunosuppression, and as their presence have major clinical relevance, the follow up of patients should be taking into a very careful attention. Appearance of de novo anti-HLA antibodies should lead to appropriate investigations such as graft biopsy. Acute ABMR responds to therapy such as plasma exchange and anti-B depletion. As soon as ABMR is in a chronic phase any therapeutic strategy is less efficient. A better characterisation of ABMR as proposed by the new Banff classification is certainly of great help and leads to new clinical studies with innovative therapeutical strategies [23]. The clinical relevance of preformed anti-HLA antibodies remains a challenge for clinicians and laboratories, and their role post-transplantation is variable and often unpredictable. To date studies are performed to better determine antibody class, specificity, strength and ability to bind complement in order to better stratify the risk of ABMR and organ loss [141].

The solution could come from the development of new therapies focusing on antibodies and B cells. B cells may act as potent APCs to activate CD4 T cells, they differentiate into antibody producing plasma cells, and recently it has been reported that sub-populations of B cells may act as immunoregulatory cells and are contributors to transplant tolerance [142-144]. A better understanding of B cell activation and function through variation in DNA sequences, co-receptors expression, protein biomarkers and cytokine production will help in the characterisation of alloimmune response and therefore in its interference (see for review [145]).

Thanks to classical immunosuppressive drugs targeting the T cells, cellular rejection is not a problem anymore. In the near future, we hope that ABMR will also become a rare phenomenon, as the development of new targets involved in the B cells activation and complement inhibition will hopefully significantly regulate harmful alloimmune response.

To conclude, our major goals and perspective are to improve tools to help physicians and patients for better care and follow up. We also need to be accurate in the tests “needed” to be performed, their timing before and after transplantation and keep in mind that any result could considerably influence/modify treatment and intervention on a given patient. For this reason interaction of physicians on the bench should always stay in close contact with physicians on the bedside, and vice versa. This is essential for the good care of patients and I think that we pretty much reached that goal in our hospital.

IX. References

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