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Epitopes of HLA antigens and their relevance for organ transplantation program

Epitopy HLA antigenů a jejich význam pro transplantační program orgánů

Diploma thesis

Supervisor: doc. MUDr. Antonij Slavčev, CSc.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její část nebyla předložena k získaní jiného nebo stejného akademické titulu.

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Abstract and Key words

This diploma thesis is focused on assessing the potential benefit of HLA epitopes for the prediction of de novo antibody production at kidney transplant recipients. The topic and patient selection criteria were selected in accordance with the 18th International HLA and Immunogenetics workshop (IHIWS), which is taking place in May 2022 in the Netherlands, where our data will also be contributed. Our aims were to compare HLA antigens mismatches (counted as total number of mismatched alleles) defined on the high-resolution level by NGS sequencing, HLA eplets mismatches defined by HLA matchmaker, and amino acid mismatches defined by HLA EMMA in their capacity to predict de novo antibody production and compare these results to other works by different authors from this field. We have identified N= 28 patients who developed de novo antibodies and N= 19 who didn't develop de novo antibodies in 5 years follow up their transplant. These two cohorts were compared based on all three approaches and correlation between number of mismatches and number of patients with de novo antibodies were made using ROC curves. Superiority of eplet mismatches over HLA antigen mismatches (total number of mismatched alleles) defined on high resolution was not detected. The HLA epitopes identified by the HLA matchmaker were further analyzed for their theoretical immunogenic potential. We managed to stratify each epitope defined by HLA matchmaker based on their theoretical immunogenicity value and created list of the 10 potentially most and least immunogenic epitopes.

Key words: HLA antigens, eplet, epitope, DSA, HLA Matchmaker, HLA EMMA

Abstrakt a klíčová slova

Cílem této diplomové práce je zhodnocení potenciálního benefitu HLA epitopů v predikci de novo protilátkové odpovědi u pacientů po transplantaci ledviny. Téma a selekční kritéria pacientů byli určeny ve shodě se selekčními kritérii pro 18. mezinárodní workshop pro HLA a Imunogenetiku, který se uskuteční v květnu 2022 v Nizozemsku, kterého součástí budou i data použity při vyhotovení této práce. Našim cílem bylo porovnaní neshod na úrovni HLA alel (definovaných ve vysokém rozlišení za pomoci NGS sekvenování), neshod v HLA epitopech (definovaných HLA Matchmakerem) a neshodných aminokyselin v sekvenci HLA molekuly (definované pomocí HLA EMMA). Podařilo se nám identifikovat N= 28 pacientů pozitivních na de novo protilátky a N= 19 pacientů. negativních na de novo protilátky po uplynutí 5 let od transplantace. Tyto dvě kohorty byly následně porovnávány na základě výše zmíněných metod a následně bylo množství neshod korelováno s počtem pacientů, kteří si vytvořili de novo protilátky, za použití ROC křivek. Nepodařilo se nám zaznamenat výhodu HLA epitopů v porovnání s HLA alelami ve vysokém rozlišení. HLA epitopy definované HLA Matchmakerem byly následně analyzovány samostatně. Ke každému epitopu byla přirazená teoretická imunogenicita na základě počtu neshod/DSA protilátek v konkrétním epitopu. Epitopy byli následně roztříděné dle imunogenicity a 10 potenciálně nejvíce imunogenních a 10 nejméně imunogenních epitopů bylo identifikováno.

Klíčová slova: HLA antigeny, eplet, epitope, DSA, HLA Matchmaker, HLA EMMA

Contents

Glossary

HLA- human leukocyte antigens

MHC- major histocompatibility complex

NK- natural killer cell

PCR- polymerase chain reaction

SSP- sequence specific primers

SSOP- sequence-specific oligonucleotide probes

CDC- complement dependent cytotoxicity

FACS- Fluorescent Activated Cell Sorting

DSA- donor specific antibody

TI- T independent

HAR- hyper acute rejection

AMR- antibody mediate rejection

BCR- B cell receptor

CDR- complementarity determining region

MFI- mean fluorescent intensity

ADCC- antibody dependent cell cytotoxicity

TNFA- tumor necrosis factor alpha

IG- immunoglobulin

IVIG- intravenous immunoglobulin

ROC- receiver operating characteristics

AUC- area under curve

Introduction

Solid-organ transplantation is currently the best possible treatment for patients suffering from end-stage kidney failure and a life-saving procedure for heart, lung, and liver organ failure patients (Black et al., 2018). According to the transplantation waiting list in the Czech Republic, by March 2021, more than 1000 patients were waiting for a kidney graft. The waiting times in different countries usually range from months to several years depending on the matching organ availability and patients' medical conditions (https://www.ikem.cz). The median survival rate of the kidney graft is currently determined to be 11.7 years for the organs from deceased donors and 19.2 years for living organ donors, based on the data from the USA (Poggio et al., 2020). The main barrier to successful transplantation and subsequent long-term organ survival is the recipient's adaptive immune system. As a result of tissue transplantation from a donor to an unrelated host, both humoral and cellular immunities are elicited to counter a foreign antigen challenge. The principal antigenic target in kidney allograft are human leukocyte antigens (HLA), as these molecules are highly polymorphic and present on nearly all nucleated cells (HLA class I) in the human body (Williams et al., 2016). A new generation of immunosuppressive drugs, such as calcineurin inhibitors, made a profound difference in the modulation of the cellular immune response and subsequently positively influenced graft survival and wellbeing of transplanted patients (Leas et al., 2016). Although the cellular immune response was successfully mitigated, alloreactive antibodies still remain a significant unresolved issue. The pathogenic role of alloreactive antibodies in transplantation is known since 1969 when recognized by Patel and Terasaki (Patel & Terasaki., 1969). The HLA-specific antibodies are produced either after transplantation in previously non-sensitized patients in response to the high load of HLA antigen mismatches or preformed due to previously failed grafts, blood transfusions, or pregnancies. Patients on the transplantation waiting lists with preformed HLA-specific antibodies are so-called sensitized patients. These patients are often disadvantaged and may be spending long time on the kidney transplantation waiting lists as the preformed antibodies are major limiting factor for transplantation (Uffing et al., 2019). Advances in tissue typing technologies have introduced a new possibility for optimal organ allocation and transplantation outcome prediction -HLA matching at the epitope level. Epitopes are small portions of polymorphic amino acid residues recognized by the antibody paratope and present on the HLA antigens. These epitopes can be recognized and predicted by specialized computer algorithms. These algorithms employ an extensive database of HLA antigen amino

acid sequences combined with 3D modeling of the epitope-paratope interface (Duquesnoy & Marrari., 2002). The resulting information of an HLA epitope mismatch load is assumed to be a more accurate predictor of the transplantation outcome than the standard assessment based on whole HLA molecules (Wiebe et al., 2013; Wiebe & Nickerson, 2016). Therefore, it is expected that better knowledge of the epitopes, their structure, and immunogenicity may be a solid foundation for a new, more personalized, and precise approach to the patient-donor HLA-based organ allocation (Kramer et al., 2018).

1 The Human leukocyte antigen complex

HLA molecules are vital components of the human immune responses. The genes encoding for the HLA give rise to thousands of alleles spread across the human population and expressed in the form of cell surface proteins. This extensive polymorphism is invaluable in defense against a tremendous variety of different antigens and in tumor immune surveillance (Kransdorf et al., 2017). However, the clinical relevance of HLA is also given by its involvement in autoimmune pathologies and its influence on the survival of solid organ transplants between two unrelated individuals (Madden & Chabot-Richards., 2019). In fact, the MHC (major histocompatibility complex) locus, responsible for the HLA proteins in humans, is the most disease-associated genetic region in the human body (Price et al., 1999).

1.1 Genomic organization and structure

The HLA molecules are encoded by the MHC genes located on the short arm of chromosome six in region 6p21.3. Its genetic information comprises approximately 200 genes or about 3600 kilobases of DNA (Beck & Trowsdale, 2000) organized into multiple gene clusters. Among these are clusters represented by HLA class I genes (HLA- A, B, and C), HLA class II genes (HLA- DR, DP, and DQ), and class III genes lying between class I and class II genetic regions. While class I and class II genes both code cell surface proteins, the HLA class III genes mainly code soluble factors involved in the immune response, including complement factors or cytokines. (Trowsdale & Knight, 2013). The HLA class I genes are further classified into two groups: classical and non-classical. The classical HLA class I one genes are translated into surface proteins designated HLA- A, B, C, which are a combination of a transmembrane polymorphic alpha heavy chain (comprised of three subunits: $\alpha 1$, $\alpha 2$, and $\alpha 3$) and a non-polymorphic light chain (β_2-microglobulin) bound non-covalently. (Bjorkman & Parham, 1990). The β_2-microglobulin is encoded outside the MHC region on chromosome 15 and helps anchor the heavy chain to the cytoplasmic membrane. MHC class I genetic locus is comprised of eight exons. Exons two and three code the $\alpha 1$ and $\alpha 2$ domains, respectively, which form the antigen-binding groove. Exon four codes $\alpha 3$ domain which is critical in forming the bond with β_2-microglobulin. A peptide has to be processed in proteasome and trimmed to a length of approximately ten amino acid residues in order to be presented. Fully completed HLA class I molecules are expressed in various numbers on every nucleated cell in the human body

(Bjorkman & Saper, 1987). HLA class II antigens include two polymorphic chains, alpha and beta, each comprising two extracellular domains designated α1 and α2, and β1 and β2 respectively. Both domains are encoded by separate genes inside the MHC region and bound non- covalently (Marsh et al., 2005). HLA class II alpha chain is encoded by HLA- DRA, DQA, and DPA genes, while the beta chain is encoded by HLA- DRB, DQB, and DPB. Different combination of alpha and beta chains makes for unique haplotypes offering an extra layer of polymorphism. Moreover, in HLA- DR, the β chain is significantly more polymorphic than the alpha chain, which is relatively conserved. To account for this polymorphism, HLA- DR involves additional three serological specificities termed HLA- DR51, 52, and 53. The resulting antigens are then a combination of alpha chain coded by one HLA-DRA1 gene and beta chain genes HLA- DRB1, 3, 4, or 5 (Kransdorf et al., 2017). Both alpha and beta (α1 and β1 domains, coded by exon 2) polymorphic chains participate in the peptide-binding, unlike in HLA class I antigens. Moreover, the peptide groove is more open and can accommodate longer peptides in their native form- up to 20 amino acid residues. Both HLA class I and class II antigens are bound to the cytoplasmic membrane by a short transmembrane peptide region. Given its structure, HLA class I is connected only through one terminal alpha subunit, whereas class II molecules are anchored from both alpha and beta subunits. Unlike HLA class I molecules, class II molecules can be found only on thymic epithelial cells and antigen-presenting cells, such as dendritic cells or macrophages or activated T cells (Brown et al., 1993). The structure of both molecules with a detailed view of their antigen-binding grooves can be seen in figure 1.

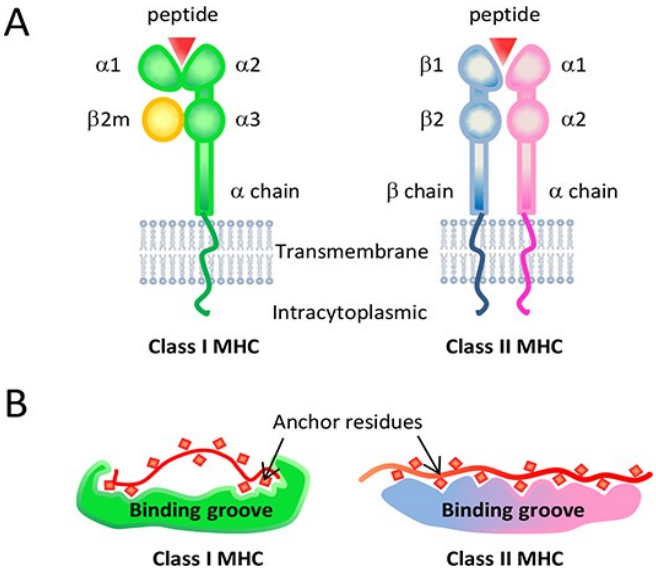


Figure 1: A structure of HLA. Schematic representation of HLA class I and class II molecules structure with bound peptide. The alpha, beta, and β2-microglobulin subunits can be distinguished along with transmembrane domains and cytoplasmatic regions.**B Binding groove structure.** Shows detail of the most variable region- binding groove with bound peptide and with marked anchor residues.
(Gutiérrez et al., 2017)

Haplotypes and inheritance

The alleles coded by the HLA genes are expressed on the cellular surface in a codominant fashion. The diversity of HLA alleles is overall given by the already mentioned genetic polymorphism and by amino acid substitutions within the regions of the peptide-binding groove, namely exons two and three for HLA I and exon two for HLA II. Alterations inside the peptide binding region increase allelic diversity of the HLA and subsequently modify the peptide (antigen) preference of a given HLA molecule. Moreover, many alleles differ in multiple single nucleotide substitutions, and this implies that segmental exchange of nucleotides between alleles from the same locus occurs (Klein & Sato., 2000). Variations in distributions and frequencies of the polymorphisms are population specific and vary significantly between ethnic groups due to specific selective pressures in the different geographic regions worldwide and are related to specific pathogenic load in different environments (Ameen et al., 2020). The HLA diversity may also be increased by genetic recombination, which is not uniform across the MHC but instead concentrated in so-called "hot spots" of recombination. The recombination occurs HLA- A and HLA- C, HLA- B and HLA DR/DQ and DR/DQ and DPA/DPB. Less often is recombination observed between HLA- C and HLA- B, while almost no recombination is observed between HLA- DR and HLA- DQ (Mehra, 2010). Up to date, we recognize 29 417 different HLA and associated alleles. This number, however, is estimated to be far from final as the database is regularly updated (figure 2), and new alleles are added every day (Robinson et al., 2015). The HLA alleles are not inherited separately but rather in the form of haplotypes (continuous segments of closely linked genes). Each haplotype is inherited in Mendelian fashion, and every individual inherits two copies of parental haplotypes (one from each parent). This inheritance pattern would imply that there can possibly be an immense number of combinations and massive potential for diversity of HLA haplotypes in the population. However, in some ethnicities, specific haplotypes are more common than others. This disparity is termed linkage disequilibrium, which means that alleles from different loci are not inherited at random as expected but instead reflect the history, mutation events, and genetic distance of the general and local population (Choo, 2007).

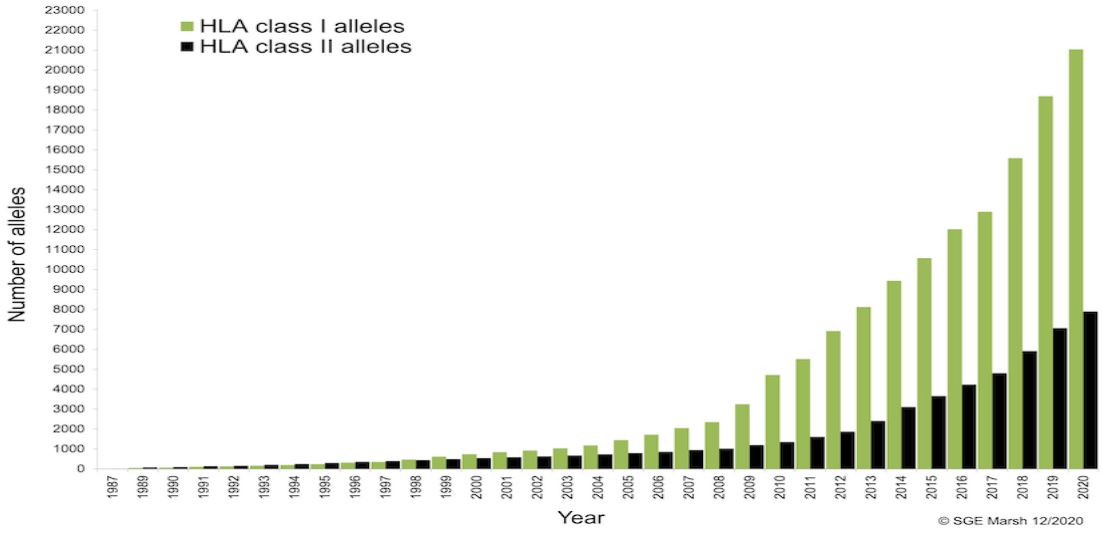


Figure 2 Numbers of HLA alleles Demonstration of the number of alleles discovered each year and added to the HLA allele database from 1989 to December 2020 (from the IPD and IMGT/HLA database website; Robinson et al., 2015).

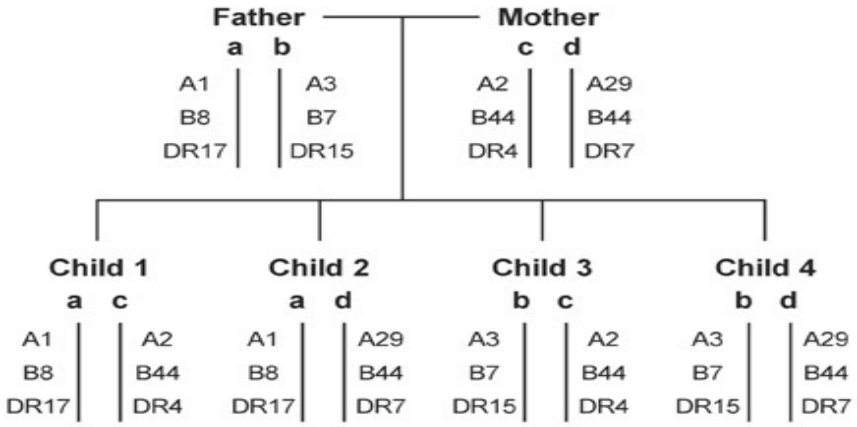


Figure 3 Inheritance of haplotypes. Parental haplotypes (a,b,c, and d) are passed from parents to offspring in Mendelian fashion. Every child carries one haplotype from each parent; both haplotypes are co-dominantly expressed (Choo, 2007).

HLA nomenclature

Due to the significant number of different HLA alleles, the critical requirement to understand and apply current knowledge of HLA polymorphism in clinical practice is standardized nomenclature (Robinson et al., 2015). Every HLA molecule is firstly identified by the prefix "HLA," referring to the MHC gene complex on chromosome six, followed by a letter

designating a specific locus on the chromosome (A, B, C in the HLA class I or DR, DP, DQ in HLA class II). As HLA class II molecules comprise two different subunits, additional information to identify the specific polypeptide chain in question is necessary. This discrimination is provided by the letters of the Greek alphabet alpha and beta (i.e., HLA-DRβ or HLA-DQα). The HLA class I includes only the alpha polypeptide chain complemented by β2- macroglobulin. The latter is not represented in the name. Further information describing the HLA molecule is portrayed by four-digit numeric fields representing DNA sequencing results, as shown in Figure 4. Molecular typing-based description begins with the number indicating a specific amino acid sequence of the locus. Then followed respectively by the number of specific synonymous polymorphism present and terminated by two numbers related to differences and mutations in non-coding regions. In some cases, the allele name may be ended with a letter suffix, which can be either L or N, and describes changes in the level of expression (L represents low expression and N null expression). The numeric fields are separated from the HLA locus designation by an asterisk indicating results of molecular typing. HLA molecular typing methods are distinguished based on their ability to describe minor differences in allele sequences and may be executed on three levels. The basic HLA description is provided by low resolution or two-digit typing, which is similar to serological typing and was usually sufficient for solid organ transplantation. Low-resolution typing is represented in the nomenclature by the HLA prefix and the respective amino acid sequence number (i.e., HLA-A*01). The most extensive information is from high-resolution typing or four-digit typing, where all four numerical fields are described (i.e., HLA*01:01). This kind of detailed representation is vital in bone marrow transplantation and some diseases associated with HLA.

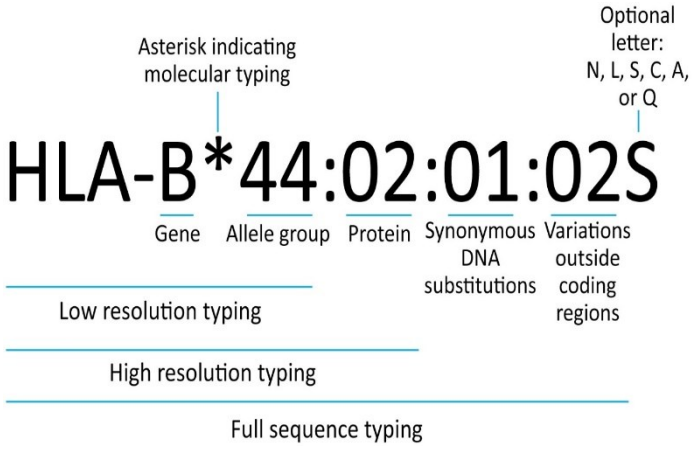


Figure 4 HLA classification. From left to right, respectively: specific HLA gene classification, asterisk, numbers representing a corresponding allelic group, protein, mutation in the coding region, mutation in the non-coding region. A letter at the end of the sequence represents a level of expression.

(http://www.hla.alleles.org/nomenclature/naming.html)

Alternatively, an intermediate resolution typing can be utilized to decipher some amino acid sequences, but not all, as the results usually depend on the specific allele in question and the method utilized. HLA typing is described based on the information provided by the Anthony Nolan Research Institute database (2019).

Physiological functions

HLA molecules are interacting with the immune system on multiple levels. Most notably, as indicated above, they can bind antigen and present the respective antigenic peptides to the adaptive immune system. The HLA is present at the early stages of T cell development in the thymus to modulate T cell responses and help determine which antigens are recognized as self and non-self (Xing & Hogquist, 2012). HLA molecules also present antigenic peptides to mature T cells, and if the antigen is recognized as a danger (i.e., pathogenic origin or altered self), the T cells induce appropriate cellular and humoral response. The HLA class I molecules present cytosolic peptides previously degraded in the proteasome. The peptides are then coupled with the compatible HLA I glycoprotein in the endoplasmic reticulum and transported to the cellular surface to be recognized by CD8+T cells. At the same time, HLA II glycoproteins are coupled with peptides of extracellular origin derived by endocytosis. These peptides are degraded in the lysosome and transported to the cellular surface, where they are recognized by CD4+ T cells (Mosaad., 2015). Apart from activating the adaptive immune response, HLA molecules also interact with the innate immune system. Some HLA class I self- molecules are recognized as ligands by KIR (killer cell immunoglobin-like) receptor present on Natural Killer (NK) cells providing inhibitory signals. When a cell is stressed (i.e., infected or transformed), the number of HLA expressed on its surface may decrease due to changes in cell metabolism. Without inhibitory signals from HLA molecules, the NK cells will activate lysis of the particular cell (Norman & Parham., 2005).

1.5 HLA in transplantation

Despite recent advances in immunosuppressive therapy, the HLA molecules still play pivotal role in transplantation medicine. The positive effect is prominent, especially in kidney, heart and pancreas allografts, where HLA matched organs have significantly better long-term survival rates. For solid organ transplantation in general, two or more HLA antigen mismatches are common, which is in contrast to bone marrow transplantation, where usually only one

mismatch in HLA A, B, or DR loci is permitted. Methods of serological HLA typing are now almost entirely replaced by molecular typing based on polymerase chain reaction (PCR). This method allows for increased precision, flexibility, and reproducibility compared to classical serological procedures. In the PCR method, the HLA is identified using sequence-specific primers (PCR- SSP) or sequence-specific oligonucleotide probes (PCR- SSOP). In clinical practice are usually these two methods (SSP and SSOP) combined together in order to obtain the best possible results. Ambiguities from PCR based typing results are usually resolved by molecular sequencing using sequence-based techniques providing high-resolution typing. The HLA are also tested in complement dependent cytotoxic (CDC) test used for HLA crossmatch. In this test, lymphocytes from a potential donor are incubated with sera from a potential recipient and rabbit complement. The reaction is positive when allospecific antibodies for recipients' sera activate complement and lyse the lymphocytes. Alternatively, flow cytometry based FACS crossmatch or solid phase-based method Luminex may be used to detect alloantibodies. (Kieslichova et al., 2015). The presence of HLA-specific antibodies in recipients' sera may indicate antibody-mediated graft rejection and subsequent graft loss and therefore have significant prognostic value (Loupy & Lefaucheur., 2018).

2 Alloantibodies

Surface antigens from the graft are often targeted by the humoral immune response represented by the IgG or IgM antibodies. These donor-specific antibodies (DSA) are recognized as potential triggers of rejection and tissue injuries resulting in a poor transplantation outcome. Human leukocyte antigens and ABO blood group antigens are among the most prominent antibody targets (Montgomery et al., 2011).

2.1 Origin of antibodies

The cause of antibody generation varies in nature. Some are part of natural defense and are physiologically applied in defense against foreign agents (natural antibodies). Others may be created as a byproduct of some medical treatments or pregnancies (preformed antibodies) or direct response to challenge by the mismatched antigens from the graft (de novo antibodies). The difference in the antibody origin may influence severity and progression of AMR. For example, preexisting antibodies can launch the rejection most likely shortly after the transplantation, while de novo antibodies get involved months later (6+ months). On the other hand, the de novo antibodies often incorporate B cell and T cell-mediated rejection, while preformed antibodies generally offer little evidence of effector T cell interplay. This fact correlates with the significantly worse prognosis for patients diagnosed with de novo DSA. The target of the antibodies differs depending on the origin of the antibodies. According to the data available, de novo antibodies are more often specified for HLA class II antigen (mostly HLA DQ). In contrast, preexisting antibodies usually comprise a mixture of specificities against HLA class I and HLA class II alike (Montgomery et al., 2011).

2.1.1 Natural antibodies

Naïve B cell and helper T cell interplay is usually required for the initiation of production of graft-specific alloantibodies (DSA). However, with natural antibodies, this is not the case. Natural antibodies are produced by the innate B cells, equipped to recognize non-self and altered self. Their presence in the human sera is T helper independent, and they are produced constantly from a very young age. Their specificity is directed against xenoantigens, bacterial components, or incompatible ABO blood group antigens (T independent antigens (TI)). TI

antigens originating from bacterial components are termed TI1 and are bound to B cell surfaces through other than B cell receptors (BCR) such as toll-like receptors. The second category, or TI2, elicits antibody production by crosslinking immunoglobulin receptors on BCR (Zorn and See., 2016). Among the most significant TI2 antigens are A and B blood groups antigens, presented on nearly all tissues in the human body. Although diverging only in glycosyltransferase enzyme, A and B blood group antigens are potent triggers of hyperacute AMR. The natural antibodies levels are often elevated in AMR patients and contribute to graft damage. As for the effector functions, they are usually of IgG1 or IgG3 subclasses and can activate complement cascade (Dalmasso et al. 1991).

2.1.2 Preformed antibodies

Preformed antibodies may be present in the patient's sera long before transplantation due to previous sensitization events (as indicated above). In general, preformed antibodies are created in high titers as a byproduct of previous insults such as blood transfusion, mechanical support implantation, pregnancies, or as a result of previous transplantations (Gloor et al., 2010). Main antigenic targets in these cases are usually HLA or endothelial tissue antigens. If the presence of such antibodies is not recognized before the transplantation, the graft is lost within 24 hours in so-called hyperacute rejection (HAR). HAR is a complex cascade started by alloantibodies and carried out by the complement cascade, innate immune cells, and coagulation cascade. The most damaged areas are endothelial microvasculature of kidney allografts, where the primary endothelial injury caused by the innate immune cells and the complement components triggers coagulation leading to thrombosis and tissue necrosis. Progression of HAR to graft failure at this point is fast and hardly preventable (Game et al., 2001). Unique situations arise when the preformed antibodies are present in the low titers. In this case, accelerated AMR can occur, usually, within the first seven days post-transplantation, carried out by the low-level preformed antibodies combined with a boost provided by the immune systems' anamnestic response. Typical conditions for accelerated AMR are in case of high immunologic risk transplantations or HLA, ABO-incompatible kidney recipients who were subjected to pretransplant plasmapheresis (Gloor et al., 2010). With similar conditions in play, a milder phenotype of accelerated AMR was also documented. In this case, the first clinical manifestations of rejection start slowly within the first thirty days after transplantation and less severely. The result is usually also less severe. When treated in time, the progress to graft failure may be sufficiently averted (Gloor et al., 2007).

2.1.3 De-novo antibodies

Exposure to allograft antigens may trigger, in some cases, DSA antibody production in previously non-sensitized patients. Patients with de novo DSA created shortly after transplantation are reported to have a similar course of rejection as patients with the anamnestic humoral response. However, the onset time may vary significantly between individuals ranging from days to months, possibly years after successful transplantation. Late production of de novo antibodies is usually developing more leniently, contributing slowly to graft injury, and leading to complete graft failure. In connection with kidney allografts, the only warning sign is a slow but steady rise in creatinine levels, one of the kidney filtration capability markers (Lee et al., 2009). Immunosuppression dosage may play a significant role in progression to chronic AMR, as de novo DSA production is caused by naïve B cells, and their proper activation would not be possible without the help of CD4+ T cells activity. Even today's modern immunosuppressive drugs are connected to numerous adverse effects from the long-term perspective, such as malignancies, chronic infections, and nephrotoxicity. Therefore, it is common practice to attempt dosage reduction, if possible. This may, in turn, lead to de novo antibody production when the dose becomes too low. De novo DSA are usually specific for both classes of HLA (class I and class II) alike, although some studies suggest greater importance of HLA class II targeting antibodies (Issa et al., 2008). The rise in creatinine is one of the reliable markers of DSA presence. Nevertheless, the higher levels of creatine are often detected late, and at this point, the rise is already accompanied by extensive glomerulopathy and graft damage (Kraus et al., 2009). Regular screening for DSA in the sera or protocol biopsies might be helpful in early diagnostic, although they might not always be feasible for various reasons.

2.2 Antigen presentation and co-stimulation

Naïve B cells, unlike naïve T cells, recognize antigen in its native form through its B cell receptor (BCR). BCR are membrane-bound antibodies of the IgM or IgD isotypes in association with other transmembrane signal conducting molecules comprising activation motives responsible for further downstream signaling (Reth, 1989). However, BCR cannot initiate downstream signaling only based on its recognition of compatible soluble peptides. Help from T CD4 + helper cell is also required. In order to elicit T helper cell signaling, antigen has to be processed by B cell, internalized, and presented bound on the B cells surface MHC II molecules to be recognized by the respective T cell. If the antigen is recognized, the T cell become

activated and starts cytokine production and costimulatory molecule expression. Co-stimulation through the CD40 ligand is essential to provide additional signaling that enables full B cell activation, differentiation, and proliferation. Activated B cells then proceed to class switching and affinity maturation to deliver antibodies required in sufficient affinity and specificity. Cytokines originating from the activated helper T cells orchestrate all the necessary morphologic changes to guide B cells into fully activated antibody-producing stage called plasma cell or long-lived memory cell required for further immunological memory. Plasma cells are, from that moment, ready to produce antibodies without any further stimulation by helper cells for an extended period (Karahan et al., 2017).

2.3 Antibody binding

After an antigenic challenge, antibodies are secreted by differentiated B cells. These antibodies recognize their respective targets through antibody paratope. The paratope is a highly variable terminal region of an antibody comprising heavy and light chains. Moreover, both of these chains have complementary determining regions (three light CDR and three heavy CDR) on their surface that allow for potentially immense binding specificity repertoire even within antibodies of the same class. Upon antigen recognition, the antibody associates with the antigenic peptide and creates an antibody-antigen complex. The binding force that holds antibodies connected to antigens involves multiple noncovalent bonds (hydrogen, ionic, hydrophobic, and van der Waals forces). Distance between antigen molecule and antibody also affect the strength of their interaction. In general, every noncovalent bond is weak, but the overall strength is defined by the additive strength of all formed bonds. Although, describing antibody-antigen interaction may be complicated as it is a dynamic structure, shifting and moving while creating and breaking bonds in the process (Reverberi & Reverberi., 2007).

2.4 Alloantibody detection

Multiplex, solid-phase assays, comprising approximately 200 known and the most common HLA antigens bound to fluorescently stained beads, are used to detect patient's alloantibody repertoire (figure 5). This method makes possible to capture the targeted antigen, and every specific antibody bound to its surface is described by the mean fluorescent intensity value (MFI) (Vlad et al., 2009). Higher MFI detected usually means a higher chance for rejection and graft damage. Specifically, when there is an MFI of over 10 000, there is a considerable possibility

for hyperacute rejection, while when the MFI value is around 3500, the rejection over a more extended period could be indicated (Orandi et al., 2015). Solid-phase assays are reliable standard methods widespread among clinical laboratories, and although quite specific, they might sometimes fail to accurately describe the potential of detected DSA. Therefore, some additional procedures may be introduced to examine the effectiveness and damage potential of DSA. Namely, in vitro assessment of complement binding capability (testing for C1q binding) (Chin et al., 2011), or depositions of complement cascade proteins C3d (Sicard et al., 2015) and C4d (Smith et al., 2007). Information about complement activity may provide insight into the complement binding capability of anti-HLA antibodies. However, complement activity-based prognosis worth is controversial because it was proved that complement binding activity is not constant but changes with antibody titer (Loupy et al., 2013). Another supplementary method with slightly better reliability is IgG subclasses assessment. It is known that IgG1 and IgG3 are potent complement activators, and their presence may imply potential for complement-related damage to the graft (Arnold et al., 2014). Despite all the clinical procedures in use, it is still challenging to predict the actual transplantation outcome accurately. Sometimes patients may be tested positive for DSA but in reality, may never proceed to AMR (Sicard et al., 2014). Another specific case is for patients who are sensitized through memory B cells, although they are not exhibiting any DSA at the time of transplantation. Further studies are required to decipher how to approach alloreactive memory B cells in patients lacking antibodies in the circulation (Lúcia et al., 2015).

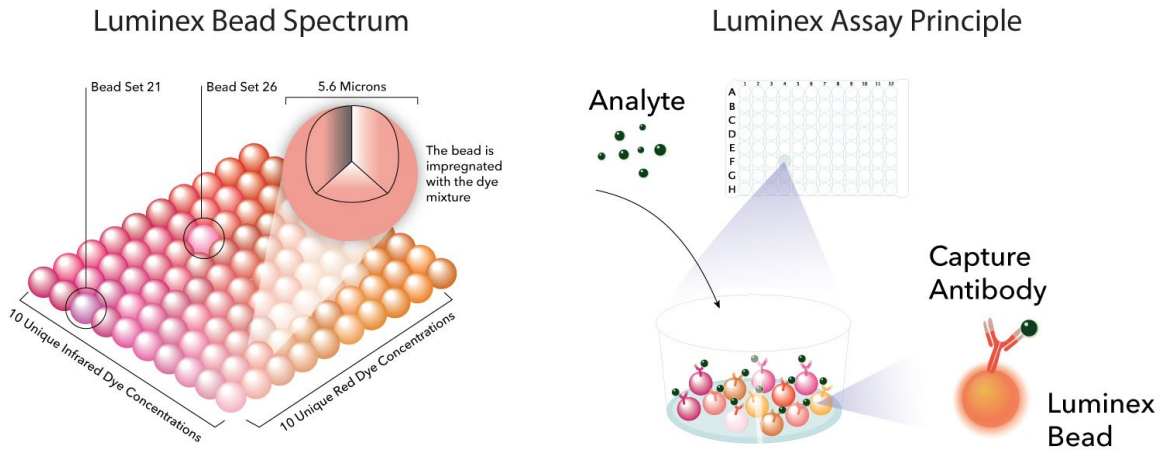


Figure 5 Solid-phase alloantibody detection principle. Antigens representing HLA molecules are first immobilized on the bead surface and then mixed with patients' sera. The analyte is treated with fluorescently marked antibodies, and positive beads are identified (https://www.rndsystems.com/what-luminex-assay)

2.5 Mechanisms of rejection

Specific antibodies of their respective classes are secreted from the B cells to the circulation to carry out their effector functions. Either in defense against infective agents or foreign antigens originating from the graft, they first bind and opsonize their targets. Opsonized viruses might be unable to bind to their receptors, but the graft is not directly harmed by the mere presence of donor-specific antibodies on its surface. However, following antibody binding, other effector molecules and cells of the immune system participate, mainly through the so-called classical activation pathway of the complement and antibody-mediated cell cytotoxicity (ADCC) (Rocha et al., 2003).

2.5.1 Complement dependent rejection

Since it was already established that antibodies are not the negative factor alone, not every donor-specific antibody present in the patient's sera inevitably means organ rejection and graft failure. In most cases, the actual harm is done when the complement system is activated through the classical complement activating pathway. This scenario starts with recognizing the HLA molecules on the surface of the endothelial cells by the DSA. When the antibody is successfully bound to its antigen, the C1 complex of soluble molecules is activated. The C1 then sets in motion a cascade of reactions culminating in the creation of the membrane attacking complex (MAC), resulting in NF-kB activation within the cells and production of pro-inflammatory cytokines IL-1b and IL-8 (Nakashima et al., 2002). Byproducts of complement cascade such as C3a, C5a promote inflammation by attracting neutrophils and monocytes to the site and contributing further to the tissue damage and multiply the inflammation (Verschoor et al., 2016). Another protein byproduct of the complement cascade C4d can spontaneously bind to the cell collagen or endothelia and activate complement cascade in a non-classical way. In addition to direct injury to the graft, complement can also facilitate and enhance B cell response. Deposition of complement on the graft tissue surface may occur as a result of ischemic/reperfusion injury. The C3d fragment bound to the cell can then signal through the CR2 complement receptor on the B cell and enhance its response to the T cell-dependent antigens. Although, it is not fully elucidated whether the increased activity of B cell is the sole result of complement signaling or instead due to cytokine gradient generated downstream of the complement injury (Fang et al., 1998). Complement-dependent rejection is represented in figure 6.

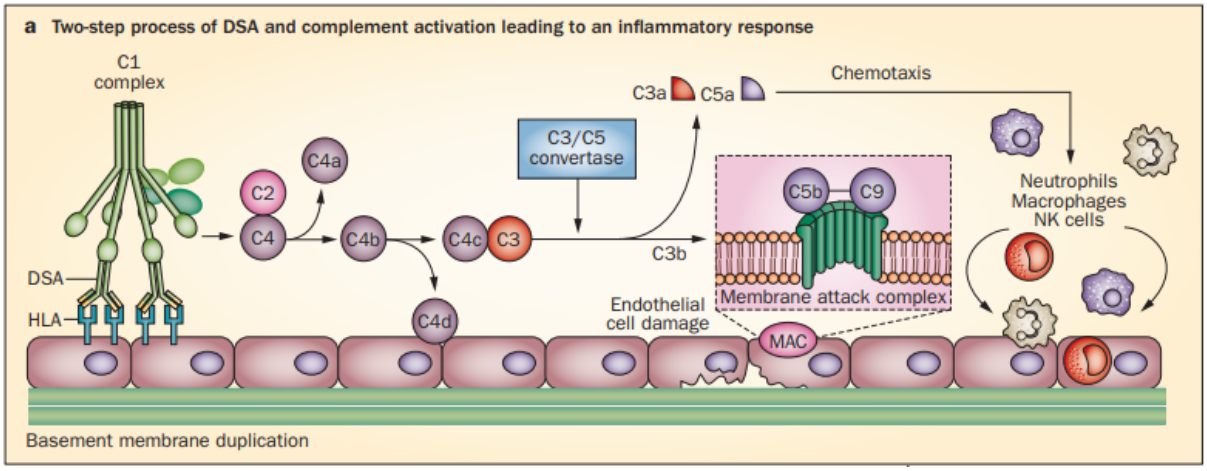


Figure 6 Complement activation. The illustration of complement activation steps from antibody binding to the endothelial surface (left) to the creation of MAC complex and tissue destruction (right) (Stegal et al., 2012)

2.5.2 Complement independent rejection

Another pathway for antibody-mediated damage is through antibody-dependent cell cytotoxicity (ADCC), where antibody-endothelial cell complexes are recognized by macrophages or natural killer (NK) cells. The NK cells represent an essential part of the innate immune system and are potent producers of tumor necrosis factor-alpha (TNFA), interferon-gamma (INFG), and are also capable of cell lysis. Through the expression of Fc receptors such as FcgRIIIA (CD16a) on the surface of NK cells, they recognize cell-bound antibodies and initiate cell death through the Fas ligand and perforin/ granzyme mechanisms (Hidalgo et al., 2010).

2.6 Treatment of AMR

When dealing with AMR, it is vital to remove circulating DSA first and then undermine further antibody production. Plasmapheresis combined with doses of intravenous immunoglobulin (IVIG) is the method of choice. Plasmapheresis allows for active antibodies to be removed from plasma, and IVIG is reported in the in vitro setting to inhibit B cell responses through the binding on CD22 receptors on B cells, inducing their apoptosis. However, in a clinical setting, the main beneficial effects were reported and documented only from a short-term perspective. From the long-term perspective, the results are more ambiguous, and efficacy of IVIG treatment decreases (Roberts et al., 2012). Other options in AMR treatment involve mitigation of

antibody-mediated damage. As described earlier, complement is a significant contributor to AMR. The main goal of this procedure is to stop downstream damage of complement to the allograft. To this end, monoclonal antibody eculizumab may be used. Eculizumab blocks the terminal activation of MAC by targeting the C5 subunit (Cornell et al., 2015). Some other proximal inhibitors are also clinically tested involving C1 esterase inhibitors (Viglietti et al., 2016). In some cases, splenectomy may be effective if performed fast after the onset of AMR (Locke et al., 2007). Bortezomib (proteasome inhibitor) (Ejaz et al., 2014), cyclophosphamide (Waiser et al., 2017), or interleukin six inhibitors (tocilizumab) (Choi et al., 2017) are also suggested as possibly helpful. However, none of these strategies was proven effective from the long-term perspective (Schinstock et al., 2019).

3 HLA epitopes

The central premise of the humoral theory of transplantation states that mismatched HLA antigens (along with other polymorphic systems) may elicit production of donor-specific antibodies after transplantation (Terasaki, 2003). In reality, however, it is not the entire HLA molecule that the immune system recognizes but rather a small portion of its surface, responsible for initiation of production and subsequent reaction with the antibody. Combining advanced amino acid sequencing techniques and three-dimensional modeling of HLA molecules, it is now possible to recognize specific these polymorphic segments accessible to antibody binding. These segments of amino acid residues are commonly called HLA epitopes as mentioned above (El-Awar et al., 2017).

3.1 Definition of epitope

An epitope is a minimal determinant for the structural composition of an antigen binding site that is recognized by an antibody. It is not an intrinsic property of a given protein, as an epitope may be defined only in its relationship to a complementary paratope (Van Regenmortel, 2009). As defined by X-ray crystallographic studies, an epitope's particular structure involves 15 to 25 amino acid residues in antibody accessible position (Duquesnoy, 2012). These residues may be continuous or brought together after the protein folding. Given the small size of these residues, one large protein usually comprises multiple distinct epitopes (Stave & Lindpaintner, 2013). Furthermore, the epitope comprises structural and functional epitopes. The structural epitope spans an area of 700 to 900 A^2 within a radius of 15Å. In contrast, the functional epitope is a much smaller region at the center of the epitope comprising only one or several polymorphic amino acids within the radius of 3 Å. The functional epitope is alternatively called an eplet. The eplet is not necessarily continuous. Interaction of an eplet with the paratope involves recognition of central mismatched polymorphic amino acid in the eplet by the third, most variable, CDR in a paratope. The remaining 5 CDRs of a paratope help to stabilize the synapse. Nomenclature of epitopes involves the number of the central amino acid (eplet) in the sequence, and this number is followed by the name of the respective ammino acid from the given position. For most epitopes, names are based on their functional epitope only, although some require additional pairing with other amino acids within the structural epitope region. These additional ammino acid residues outside the functional epitope may be positioned either on the surface of

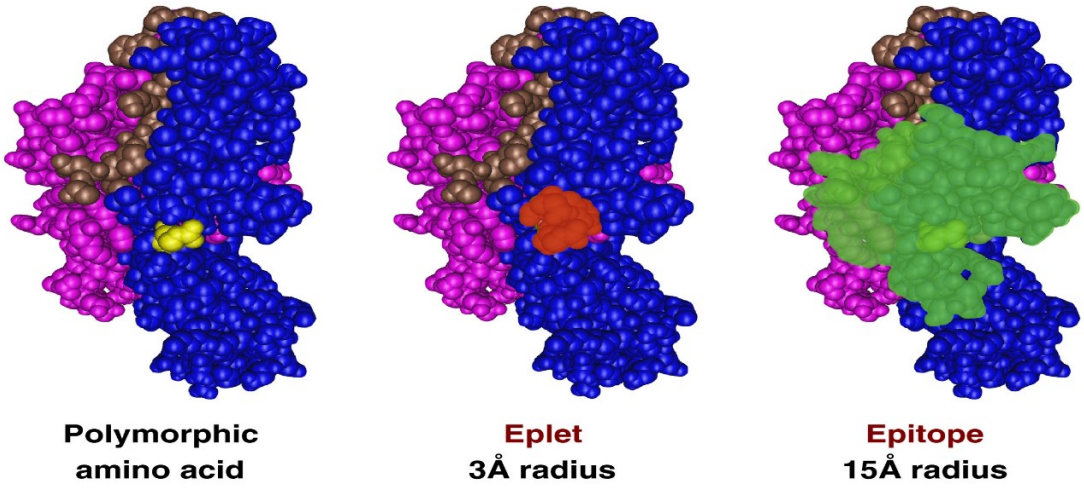


Figure 7 Polymorphic determinants of antibody binding. Polymorphic amino acid (yellow) is the most basic unit of mismatch. Eplet (orange) is polymorphic acid or small area of polymorphic acids 3 angstrom radius recognized by third complementary determining region of an antibody. The epitope (green) is a sequence of amino acids in a radius of 15 angstrom mirroring the whole sequence recognized by an antibody paratope (Wiebe et al., 2018).

HLA or may be confined within its structure. Surface residues usually influence pairing with the CDRs on the antibody while confined residues are usually inside the peptide groove where they influence configuration of the functional epitope. Amino acids for functional and structural epitopes are divided by the "+" sign in their name (Duquesnoy, 2006). The difference between epitopes and eplets can be seen in figure 7. HLA epitopes may be further described as private or public epitopes based on their presence on HLA antigens. Private epitopes are restricted to one antigen, whereas public epitopes are present on multiple antigens and responsible for antibody cross-reaction. These public epitopes are also known as cross-reactive groups or CREGs and may be responsible for producing a broad range of seemingly nondonor-specific anti-HLA antibodies after solid organ allotransplantation (Tambur et al., 2014).

3.2 Recognition of epitopes

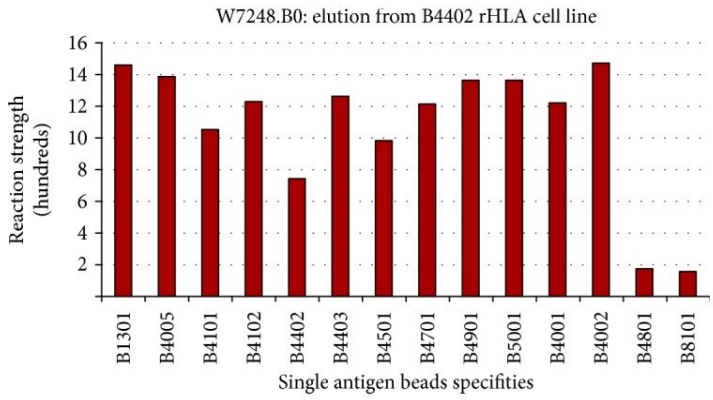
To successfully implement the epitope concept into clinical practice, it is crucial to identify all reactive epitopes present on the HLA molecules. Two main approaches of epitope identification were introduced: (1) analysis of epitope specificity for a broad range of anti-HLA monoclonal antibodies based on their reactivity patterns and (2) in silico prediction of epitopes based on the amino acid sequence and structural data for HLA in combination with known epitope parameters and subsequent verification by assigning predicted epitopes to detected antibodies.

The first approach was successfully implemented by Terasakis's group, using either mouse monoclonal antibodies or human alloantibodies (absorbed and eluted for single-antigen cell lines) tested in combination with single antigen beads. As a result, amino acid sequences were identified that were shared by all reactive beads based on sequences of all HLA amino acids from the database. Epitopes defined by this method are named TerEps and share common properties: range from 1 to 4 amino acids within the area of 750 A^2, present on the molecular surface in antibody accessible position, and shared by all the reactive alleles from a single antigen bead (El Awar et al., 2007). In contrast, one of the in silico approach involves HLA Matchmaker, a computer program developed by Duqeusnoy, capable of predicting epitopes based on the known polymorphic amino acids on the surface of the HLA with a radius of 3A (eplets) (Duquesnoy, 2006). It was shown that there is a strong correlation (90% in HLA class I) between TerEps and eplets defined by HLA Matchmaker (Duquesnoy & Marrari, 2009; Mararri & Duquesnoy, 2009). However, many more eplets are predicted by the program, waiting for antibody verification. Efforts in the elucidation of HLA epitopes lead to the creation of an epitope database accessible at http://www.epregistry.com.br website. The epitopes in the database are sorted into five separate registers: HLA ABC, HLA DRB, HLA DQ, HLA DP, and MICA (Duquesnoy et al., 2014).

3.3 Immunogenicity and antigenicity of epitopes

It is well established in organ allotransplantation that the significance of mismatched HLA epitopes in connection to DSA production may range from acceptable to completely prohibited (Doxiadis et al., 1996). Moreover, graft recipients usually develop DSA only to a portion of mismatched epitopes from a certain donor, and other recipients may not develop any reaction at all despite a significant number of mismatches (Duquesnoy et al., 1990). What factors determine antigenicity and immunogenicity of the certain epitope is not completely clarified. One important contributing factor is the HLA class II phenotype of respective recipients. HLA class II molecules are essential in facilitating the interaction between CD4+T cells and B cells, as they present portions of donors' peptides to the T cells and determine the repertoire of antigens that can be indirectly recognized (Otten et al., 2013). It was also hypothesized that a similarity between HLA antigens of the antibody producer and HLA antigens of the organ donor has to exist for epitopes to be recognized. To account for this theory, the concept of HLA epitope relying on mere amino acid string differences was extended and divided into a structural epitope and functional epitope resulting in the nonself-self paradigm. The cornerstone of this

hypothesis is the physiological presence of low-affinity immunoglobulin (IG) receptors recognizing self-HLA molecules. These IG receptors are essentially harmless against self-antigens but elicit a strong response when exposed to the minor mismatches in the HLA sequence of donor antigens (Marrari et al., 2010). A similar process is utilized in human B cell maturation, and development as receptor editing following positive selection shapes the repertoire of alloreactive and autoreactive B cells (Cancro & Kearney., 2004). This phenomenon can also be explained from an evolutionary perspective as the immune system has to recognize peptides on self-cells presenting a small nonself antigen in the context of own antigens (self). These targets deviate from self-molecules only by small modifications. The relative immunogenicity of epitope mismatches mentioned above is complemented by other factors determining the strength of antibody response. Indeed, the mere presence of after transplantation is considered a biomarker of predicting graft injury and possible graft failure (Zhang, 2017), but no universal guidelines for assessing the potential risk for specific DSA have been introduced. The antibody pathogenicity level may be measured either as mean fluorescent intensity (MFI) value or in the actual titer of the antibody, even though there is no reliable way to determine antibody pathogenicity. Clinical laboratories currently rely on MFI provided by single antigen flow bed techniques. The MFI is semi quantitative value describing the relative concentration of the anti-HLA antibodies. However, differences between laboratories in the Luminex solid-phase assay, such as varying density of beads, the difference in preparation, the possibility of false-negative and false-positive results, and shared epitopes, renders MFI value not entirely reliable (Taylor et al., 2009; Reed et al., 2013), as seen in figure 8. Likewise, the subclass of the detected antibodies and their ability to bind complement should be considered as it has an effect on the extent of possible damage caused to the graft (Lowe et al., 2013). These difficulties are making it a complex task to define some universal MFI threshold in addition to the fact that not every epitope is of the same importance to induction of the immune system response. It was demonstrated that numbers of continuous and discontinuous mismatched epitopes between recipients and donors could be not only associated with the production of DSA but also with their binding strength. Nevertheless, this alone is not sufficient to explain significant heterogeneity in antibody binding strength. Another work by the Kosmoliaptsis group was able to show that differences in antibody binding strength are linked with the number and distribution of polar and charged amino acid side chains in the region outside of the conventional epitope site. These ammino acid chain differences may influence the folding, and structural composition of HLA antigens expressed (Kosmoliaptsis et al., 2011). Moreover, the ability to bind epitopes by antibodies is influenced by a specific



Antigen	Allele B0702	aa & position 41 A
B13	B1301	T
B4005	B4005	T
B41	B4101	T
B41	B4102	T
B44	B4402	T
B44	B4403	T
B45	B4501	T
B47	B4701	T
B49	B4901	T
B50	B5001	T
B60	B4001	T
B61	B4002	T
B48	B4801	A
B81	B8101	A

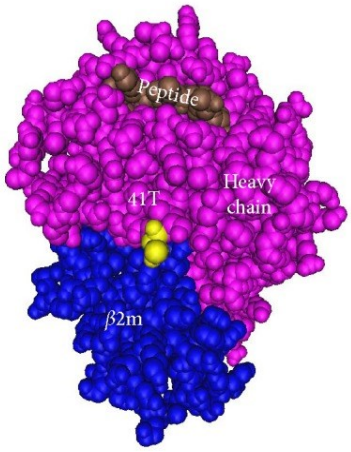


Figure 8 Changing reactivity of a public epitope. Reactivity of multiple diverse antigens is facilitated through one identical epitope 41T. In single antigen assay this reactivity without epitope consideration can be interpreted as presence of multiple anti-HLA antibodies where in reality all reactivity goes through one mismatched epitope and MFI value is spread through multiple solid assay beads and therefore falsely lower. Interestingly, one amino acid change from 41T to 41A renders antibody nonreactive. (El-Awar et al., 2017)

peptide presented within the particular HLA molecule. The correct epitope assessment may also be difficult due to the technical limitations of the current most popular single antigen solid assays. The most prominent being detection of antibodies targeting denaturized targets or the so-called Prozone effect. Nevertheless, it would be overall beneficial to stratify all the known epitopes based on their ability to bind antibodies and identify potentially high and low immunogenic epitopes or possibly non-immunogenic epitopes (Haarberg & Tambur, 2014).

3.5 HLA epitope matching software

As a rule, the vast majority of organ allografts have HLA mismatch (Kieslichova et al., 2015). The HLA epitope matching software aims to introduce immunogenetic and bioinformatic progress into pre-transplant screening for better risk stratification and prediction of graft compatibility. Up to this day, many software algorithms describing B-cell epitopes, using slightly different approaches, were created, such as HLA Matchmaker, HLA EMMA, or

Cambridge HLA immunogenicity algorithm. For the purposes of this thesis, HLA Matchmaker and HLA EMMA will be discussed in greater detail.

3.4.1 HLA Matchmaker

HLA Matchmaker is a computer algorithm introduced to identify polymorphic amino acid residues in linear HLA sequences termed triplets. The triplets were considered essential parts of the structural epitope involving polymorphic amino acid conformations in antibody-accessible positions. This version of HLA matchmaker, while simplistic, was proven to be clinically relevant in kidney transplantation or platelet transfusion even though it was providing a distorted image of the original epitope repertoire. However, recognizing the fact that antibodies also recognize discontinuous and longer polymorphic sequences in addition to linear triplets, more extensive epitope criteria were introduced. Therefore, a new version of HLA software was created. The updated version, which is currently available, no longer considers triplets for the analyses but eplets in 3 Å radius involving at least one non-self residue. Eplets can sometimes be identical to triplets, but they are also in discontinuous positions ranging from 3 to 3,5 Å apart and correspond well to serologically defined determinants. The eplet version of HLA matchmaker can be downloaded free from the epitopes.net website. MS Excel provides the working interface, and the application is, therefore, necessary for software to run. It is divided into four independent matching algorithms for HLA matching: 2 for mismatched epitope analysis (1 for HLA ABC and 1 for HLA DRDQDP) and 2 for antibody specificity analysis (1 for HLA ABC and 1 for HLA DRDQDP). Both experimentally verified and theoretically predicted epitopes are incorporated and distinguished in the analysis. Epitope database of included epitopes can be accessed on www.epregistry.com.br, and Matchmaker software is regularly updated. Data input required is high-resolution typing of HLA antigens in case of epitope matching and combination of high-resolution typing and serological analysis of antibody reactivity by flow cytometry (LUMINEX assay) (HLA Matchmaker: http://www.epitopes.net/index.html). An example of the HLA Matchmaker working environment is represented in figure 9.

9A

Numbe	Nr	Nr	Descriptions of	Descriptions of
itcorII Eple	AbVEp	OthEp	AbVer Eplets	Other Eplets
- 25	16	9	56R44KM44RT62QE65RNA73AN76ANT80K80TLR82LR131S144KR138MI156DA163RG166DG	73ID66N71TD76ED77N77NGT156WA245AS275EL
- 0	0	0		
- 21	10	11	56R44KM144KR144K156DA180E163RG166DG267QE193PL	12M73ID76VDT77D105S147L149AH152A156R177DT275EL
- 19	14	5	44RT62EE65GK71TTS73AN76ESN80I80N90D144KR138MI163LW166DG219W	14W69RA77S156QA275K
- 17	12	5	44RT44RMA62EE65GK71TTS73AN80I90D144KR144QL163EW166DG	14W69RA156QA156WA275K
- 23	15	8	44RMA62EE62LQ65GK73TVS76ANT79GT127K144KR144K150AAH173K163LW166DG219W	77NGT91R80TL105S149AH152A156QA193LV
- 16	12	4	62QE71TTS73TVS76ESN76VRN80N144KR143S161D173K180E163LW	77S147L177DK275EL
- 17	12	5	21H41T44RMA45KE62GE73TVS76ANT107W127K144TKH145KHA173K	43R69RT77NGT91R105S
- 21	15	6	21H44KM62GE73TVS76ANT107W127K143S144TKH145KHA173K163LW163RG166DG219W	69RT77NGT156R177DK184A193AV
- 16	12	4	21H62EE65GK65QIA71TTS73AN76ESI76ESN127K163EW166DG219W	16S14W45EE275K
- 18	12	6	62EE65GK65QIA69AA71ATD80I80K80TLR82LR127K163EW166DG	16S45EE71TN76ED76EN77N
- 18	11	7	21H41T45KE73TVS76VRN80I143S158T173K180E163EW	45EE69RT71TN73AS76EN147L177DK
- 23	15	8	21H62EE62GE65GK73TVS80I82LR107W127K144TKH145KHA173K219W193PV248M	69RT76EN91R152V156QA184A184H193AV
- 16	9	7	44KM44RT62QE80I82LR90D144KR163RG166DG	69RA71TN73AS76EN152A170RH275EL

Figure 9 HLA matchmaker interface 9A Example for eplet matching. The numbers of mismatched eplets and their position with respective amino acids are counted based on high-resolution typing data.

9B

7-Apr-21	Pt Id	x	x	SerumDate	x	Method	x	Imm	Reactive Eps	All Eps	Imm	Reactive Eps	All Eps
	1st A	2nd A	1st B	2nd B	1st C	2nd C	AbVer Epl	A*01:01			B*37:01		
Patient	A*01:01	A*23:01	B*37:01	B*44:03	C*04:01	C*06:02	HiElliProEps	A*01:01			B*37:01		
Type OK?							LoElliProEps	A*01:01			B*37:01		
Immunizer	A*01:01	A*02:17	B*37:01	B*52:01	C*06:02	C*12:02	AbVer Epl	A*02:17		62GK62GE107W142TKH144KHA1	B*52:01		163LW
Type OK?							HiElliProEps	A*02:17		65RK76VDT150AH150AHV184A	B*52:01		170RH
							LoElliProEps	A*02:17		207S	B*52:01		95W97T113HN
				mean+3sd	15416								
				sd	3926			CutOff					
				Average self count	3637			1000					

Source	Lot#	Nr	QC	Allele	Self	Imm	Score	MFI	Comments	Ab Ver Eplets	Hi ElliPRo Eps	Low ElliPro Eps	
LC	3008213 3	103	x	A*01:01	329	Imm	NEG	329	-				
LC	3008213 3	104	x	A*02:01			NEG	373	-				
LC	3008213 3	106	x	A*02:02				3806	-		43R		
LC	3008213 3	107	x	A*02:03			NEG	381	-				
LC	3008213 3	108	x	A*02:05				8836	-		43R		
LC	3008213 3	109	x	A*03:01				2187	-	161D			
LC	3008213 3	110	x	A*11:01			NEG	436	-				
LC	3008213 3	111	x	A*11:02			NEG	462	-				
LC	3008213 3	112	x	A*23:01	9869			9869	-				
LC	3008213 3	113	x	A*24:02				8514	-				
LC	3008213 3	114	x	A*24:03				10787	-				
LC	3008213 3	115	x	A*25:01				15101	-	76ESI	71STS		
LC	3008213 3	116	x	A*26:01			NEG	386	-				
LC	3008213 3	117	x	A*29:01			NEG	479	-				
LC	3008213 3	118	x	A*29:02			NEG	555	-				
LC	3008213 3	119	x	A*30:01			NEG	902	-				
LC	3008213 3	120	x	A*31:01			NEG	912	-				
LC	3008213 3	121	x	A*32:01				14688	-	76ESI	71STS		
LC	3008213 3	122	x	A*33:01			NEG	827	-				
LC	3008213 3	123	x	A*33:03			NEG	554	-				

9B represents an example of antibody matching in HLA class I. Input data involves high-resolution typing of patient and donor on the top left side and flow cytometry MFI values in the middle row (cut off 1000 for HLA class I). Suggested epitopes are computed on the right side

3.4.2 HLA EMMA

HLA EMMA is computer software designed for performing HLA class I and HLA class II analysis based on comparing amino acid sequences of patient and donor. The sequence of every locus is compared with the corresponding locus on donor HLA, and mismatches are

highlighted. Additional description of mismatches provided by the software includes information on whether the mismatched amino acid is buried within the molecule or exposed on the molecular surface and amino acid chemical properties (neg/pos, polar/apolar, aromatic). A combination of prediction tools (NetSurfP2.0 and Porter Pale4.0) and known structures of HLA antigens are behind amino acid definition by HLA EMMA. The amino acid position is recognized as solvent/ exposed when at least one prediction tool discloses a threshold of equal or higher than 25% of relative solvent accessibility in at least one HLA structure. High-resolution typing of HLA molecules from patients and donors is standard input required, but the analysis is also feasible with minimal information such as low-resolution typing. When only the HLA locus and the allelic group are known, the software will automatically predict the most likely protein number based on the preset population. Conversion from low resolution to high resolution is currently possible for three populations: Netherland -Leiden, European Caucasian, and Thailand. HLA EMMA is free to download from the hla-emma.com website. Results are in the form of a specific number of mismatched molecules (conventional mismatches) and a specific number of mismatched amino acids for every HLA antigen. The amino acids are either counted together or may be accessed in detailed view as a string of all amino acids from individual HLA molecules with mismatches highlighted. An example of an EMMA working environment can be seen in figure 10 (Kramer et al., 2020).

10A

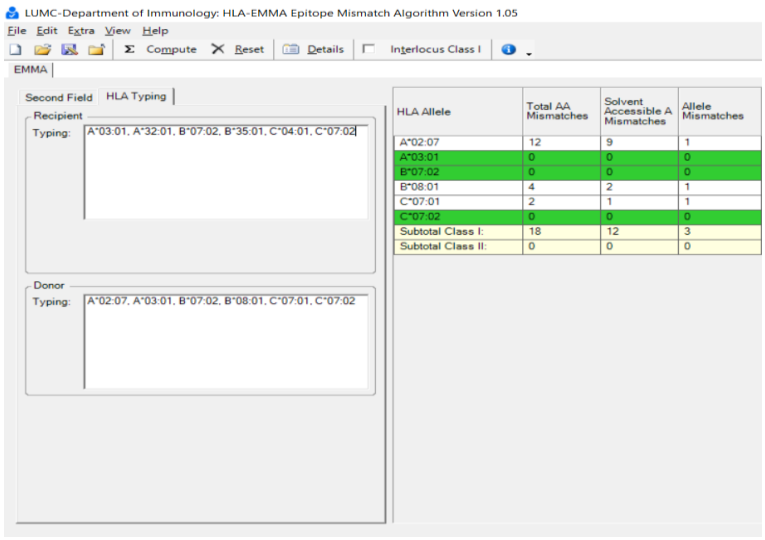
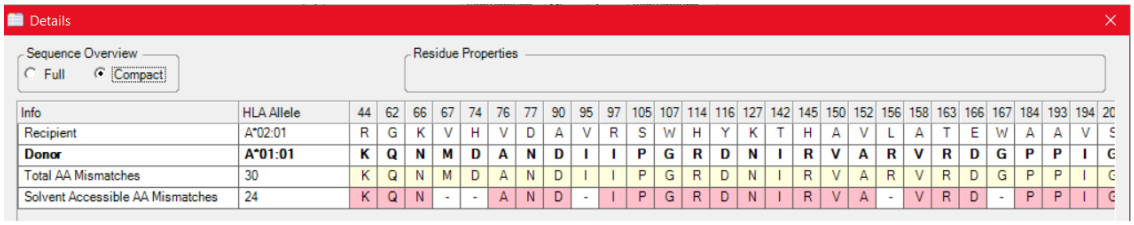


Figure 10 HLA EMMA software Example of HLA class I matching in HLA EMMA environment. **10A** On the right side is field to insert high resolution typing. On the left side are already computed results. The number of allele and amino acid mismatches is main output. Completely matched alleles are highlighted in green.

10B



Info	HLA Allele	44	62	66	67	74	76	77	90	95	97	105	107	114	116	127	142	145	150	152	156	158	163	166	167	184	193	194	20
Recipient	A*02:01	R	G	K	V	H	V	D	A	V	R	S	W	H	Y	K	T	H	A	V	L	A	T	E	W	A	A	V	S
Donor	**A*01:01**	**K**	**Q**	**N**	**M**	**D**	**A**	**N**	**D**	**I**	**I**	**P**	**G**	**R**	**D**	**N**	**I**	**R**	**V**	**A**	**R**	**V**	**R**	**D**	**G**	**P**	**P**	**I**	**G**
Total AA Mismatches	30	K	Q	N	M	D	A	N	D	I	I	P	G	R	D	N	I	R	V	A	R	V	R	D	G	P	P	I	G
Solvent Accessible AA Mismatches	24	K	Q	N	-	-	A	N	D	-	I	P	G	R	D	N	I	R	V	A	-	V	R	D	-	P	P	I	G

10B Detailed view of amino acid mismatches between two alleles. All positions of the HLA sequence are displayed, and amino acid is assessed as a match (white) mismatch (yellow) or solvent accessible mismatch (red).

mismatches) and a specific number of mismatched amino acids for every HLA antigen. The amino acids are either counted together or may be accessed in detailed view as a string of all amino acids from individual HLA molecules with mismatches highlighted. An example of an EMMA working environment can be seen in figure 10 (Kramer et al., 2020).

3.5 Potential for clinical application

Integration of epitope matching into standard clinical practice of solid organ transplantation is considered on three primary levels. First, it can be applied before organ transplantation for more precise allocation of donor organs as epitope mismatches are predicted to be superior to conventional HLA mismatches (Wiebe & Nickerson, 2016). This can be demonstrated in figure 8, where are conventional HLA mismatches compared to the epitope mismatches. One conventional mismatch in HLA class II DR or DQ as defined by low-resolution molecular typing is composed of variable numbers of mismatched epitopes raging from zero to more than 50. The meaning of this diversity is further explained on figure 11, where donor with specific HLA*B51 with all its polymorphic residues highlighted is compared to potential recipients with three different phenotypes. The number of mismatched residues between phenotypes varies considerably despite the fact that the number of conventional mismatches remains the same. Allocation of the recipient-donor pairs on epitope level is supposed to promote better survival of allocated organs and reduce sensitization risk after re-transplantation (Kausman et al., 2016). The second important application is so-called virtual crossmatching. This approach benefits group of highly sensitized patients by recognizing unacceptable HLA mismatches.

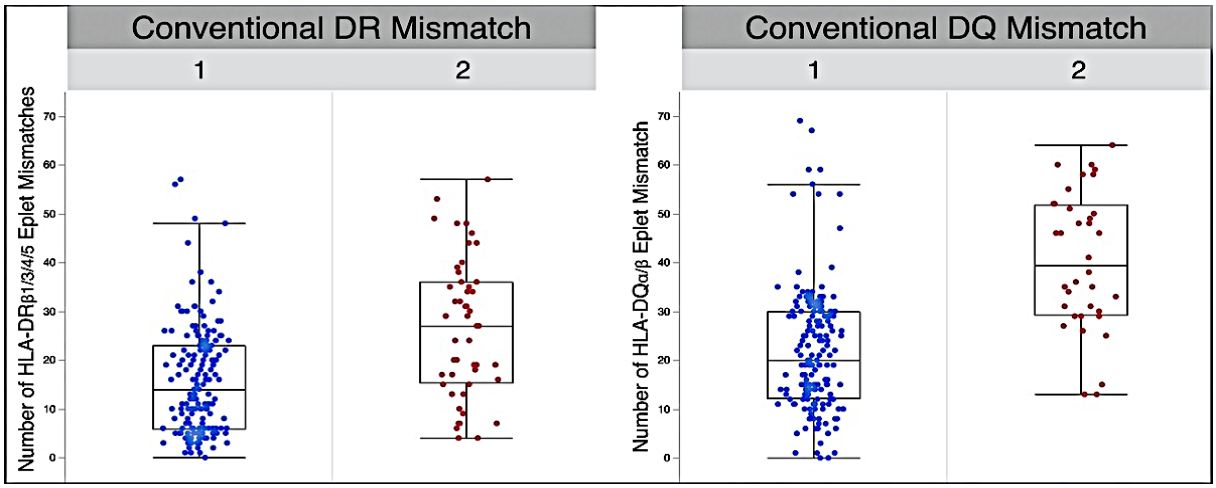


Figure 11 Comparison of conventional and epitope mismatches. Example how conventional HLA mismatch can be misleading in some cases. Each dot represents an epitope mismatch compared to a conventional mismatch for donor-recipient pairs on the HLA locus (Wiebe & Nickerson, 2016).

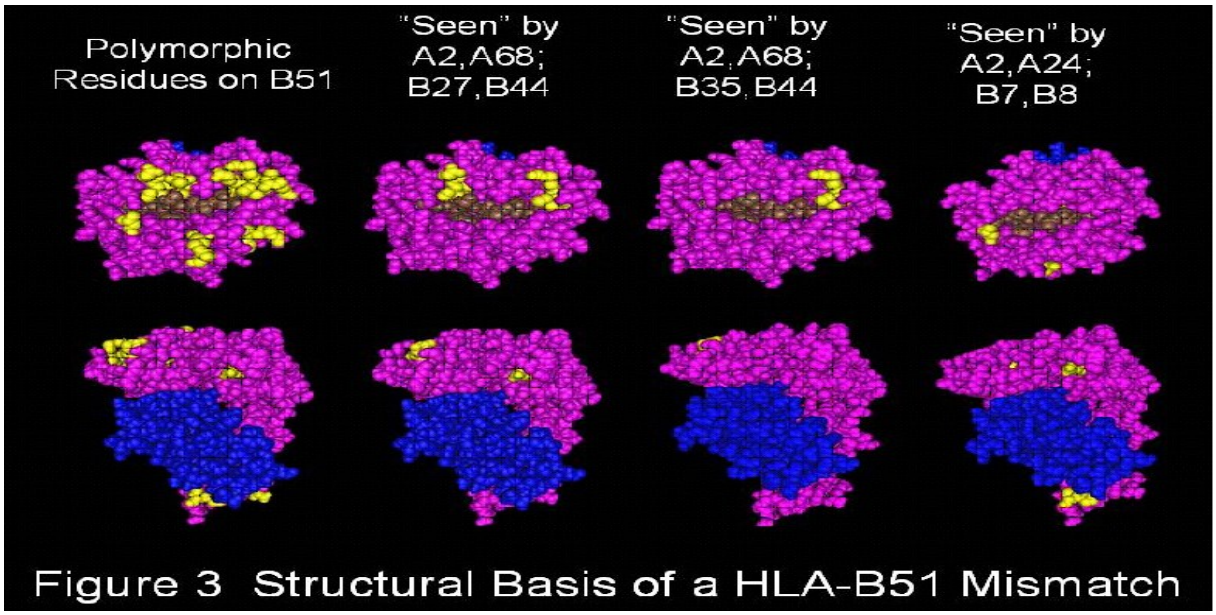


Figure 12 Difference in reaction patterns. Comparisons of mismatched HLA antigen B51 to different HLA phenotypes. There is the same number of classical mismatches; however, the number of epitope mismatches (in yellow) varies greatly, and the most compatible antigens are easily recognized **(**http://www.epitopes.net/img/hla/Fig3Matching.jpg**).**

The Eurotransplant group already adopted the Acceptable Mismatch Program (AM). They determine AMs by negative reactions in antibody detection assays or by using the HLA Matchmaker algorithm (Claas et al., 2009). As a result of this program, waiting times of sensitized patients were significantly reduced, and their long-term survival was comparable to non-sensitized patients (Heidt et al., 2015; Heidt et al., 2018). The third significant benefit of

epitope matching is a more accurate assessment of current immunological risk. Even though the data remain contradictory about which of the antibody determining strategies is the most beneficial, multiple studies suggested that higher epitope load has a significant connection to de novo DSA development and inferior graft survival (Silva et al., 2010; Nguyen et al., 2016; Wiebe et al., 2018). This may imply a potential for better risk stratification of organ recipients in order to optimize post-transplant follow up strategies and personalize immunosuppressive therapy as proposed by Wiebe's group (Wiebe et al., 2018).

4 Aims

To determine whether a significant difference in the number of HLA antigen and eplet mismatches exists between 1) non-sensitized patients who developed anti-HLA antibodies post-transplantation and 2) non-sensitized patients who did not develop anti-HLA antibodies for 5 years follow up post-transplantation. Mismatches are determined by three different approaches: HLA antigen mismatches, HLA Matchmaker score, HLA EMMA score.

To assess correlation between the number of mismatches (HLA molecular mismatches, HLA Matchmaker score, HLA EMMA score,) and number of patients with de novo antibodies.

To analyze 1) specific mismatched eplet positions as determined by HLA matchmaker eplet matching tool and 2) correlate them to number of different DSA specificities (DSA as determined by HLA Matchmaker antibody analysis tool) against the mismatched positions respectively to determine 1) whether some eplet positions are more/less immunogenic than others and 2) if possible to stratify eplets based on their immunogenicity.

5 Material and methods

Retrospective analysis of 894 data files from kidney recipients transplanted in IKEM between the years 2012- 2019 based on strict selection criteria (as explained further) was conducted. Specific anti-HLA antibodies were detected by SAB Luminex Labscreen technology from recipients' pre-transplant and post-transplant sera. High-resolution typing from both donors and recipients was obtained by Next Generation Sequencing from their stored DNA samples. The resulting data from high-resolution typing and antibody analysis were applied in HLA epitopes analysis by HLA Matchmaker and HLA EMMA computer software.

5.1 Patient selection

Patients were selected based on the following criteria:

1. First kidney recipients.
2. Recipients non-sensitized at the time of transplantation (based on Single Antigen Bead analysis
3. Recipients who either:
    a. Developed de novo antibodies after transplantation and were either (I.) on immunosuppressive therapy or (II.) suffered graft loss due to chronic antibody-mediated rejection,
    b. Or recipients who did not develop de novo antibodies after transplantation despite minimal five-year clinical follow-up.

Patients' history was verified using Orpheus, HLA fusion, and Zlatokop hospital databases. Recipients with a history of pregnancy, repeated transplantation, and recipients who received combined renal and non-renal transplantation were excluded based on the data from the databases. Pre-transplant sera were tested by Luminex MIX assay for recipients without Luminex data before transplantation. Post-transplant serum samples are not stored, and therefore blood samples were obtained for a portion of recipients without Luminex data after transplantation in cooperation with the Clinic of Nephrology, IKEM. Patients' sera derived from blood samples were tested by Luminex using Single Antigen Beads. Recipients without Luminex data before or after transplantation were excluded.

Table 1 Patient characteristics (positive group, HLA class I N= 12, HLA class II N= 22)

Parameter	**average (range/ percentage)**
Age	51 (21-79)
Living donor	10 (35,7%)
HLA allelic Mismatches in HLA- A, B, C[1]	5 (2- 6)
HLA allelic Mismatches in HLA- DR, DQ, DP[2]	7 (4- 10)
HLA Matchmaker score in HLA- A, B, C[1]	14.5 (10- 29)
HLA Matchmaker score in HLA- DR, DQ, DP[2]	26 (8- 48)
HLA EMMA score in HLA- A, B, C[1]	28 (18- 42)
HLA EMMA score HLA- DR, DQ, DP[2]	39.5 (9- 120)

[1] for patients who developed anti-HLA antibodies against HLA class I

[2] for patients who developed anti-HLA antibodies against HLA class II

Table 2 Patient characteristics (negative group, N= 19)

Parameter	**average (range/ percentage)**
Age	46 (25-70)
Living donor	6 (31,6%)
HLA allelic Mismatches in HLA- A, B, C	3 (0- 6)
HLA allelic Mismatches HLA- DR, DQ, DP	5 (1- 10)
HLA Matchmaker score in HLA- A, B, C	13 (4- 36)
HLA Matchmaker score HLA- DR, DQ, DP	17 (0- 38)
HLA EMMA score in HLA- A, B, C	21 (6- 48)
HLA EMMA score HLA- DR, DQ, DP	23 (0- 103)

5.2 Detection of HLA antibodies by Luminex assay

LABScreen™ Luminex mixed assay by ONE LAMBDA Inc. was used for the initial determination of positive and negative sera. It is a bead-based immunoassay for the qualitative detection of anti-HLA IgG antibodies. In mixed assay presence of class I and class II anti-HLA

IgG antibodies are being determined. Sera positive for either HLA class were further tested separately. LIFECODES LSA^{TM} Luminex screening assay by Immucor was used to detect IgG antibodies for specific HLA class I and HLA class II molecules.

5.2.1 Main principle

A small volume of the recipient's serum samples is mixed with beads aliquot and incubated. Unbound antibodies are washed from the sensitized beads. Conjugate of an antihuman IgG antibody and phycoerythrin is then added to the test sample, followed by second incubation. After the incubation, the test sample is diluted and analyzed on the Luminex instrument. Signal intensity from every bead is compared to signal intensity of the lowest ranked specific bead included in the bead preparation to distinguish if the bead is positive or negative for bound alloantibody. The main principles are the same for both producers (Immucor and One Lambda)

5.2.2 Testing procedure[4]

1. Recipients' sera were thawed, and centrifuged (10 min/ 8000g).
2. Wells for each sample, including one negative and one positive control, were assigned on 96 well plate.
3. Wells to be used for analysis were pre-wet with 200 µl of distilled water (water was removed after 2 minutes by flicking).
4. Vial containing the LSA beads were vortexed for 1 min to resuspend the beads
5. 40 µl of LSA beads was added to each test well, followed by 10 µl of test serum/ control serum for LSA1 or 20 µl of test serum/ control serum for LSA2.
6. The plate was covered by adhesive foil and incubated for 30 minutes at room temperature on the rotating platform in the dark.
7. After incubation 100 µl of wash buffer was pipetted to each well.
8. The plate was centrifuged at 1300g for 5 minutes with no or low break. When centrifugation was finished, the wash buffer was removed by flicking once.
9. To wash the samples: the plate was vortexed for 10s, 250 µl of wash buffer was added to each well, the plate was centrifuged for 1300g/ 5 min, and flicked.
10. Wash step 9 was repeated two more times for a total of three washes.

11. During the last centrifugation from step 10, the conjugate was prepared. Conjugate was diluted with Wash Buffer 10 times (5 µl of the conjugate to 45 µl of Wash Buffer per sample).
12. 50 µl of the conjugate was added to each well with the test sample, the plate was incubated for 30 min at room temperature on a rotating platform in the dark.
13. In the final step, 150 µl of Wash Buffer was added.
14. The plate was analyzed using Luminex FLEXMAP 3D® System

[4] protocol followed in Luminex Single antigen assay by Immucor described in detail as this protocol was modified in accordance with our laboratory equipment. Luminex MIX assay by One Lambda was conducted following the protocol described in the official application manual accessible on: https://www.ctotstudies.org/HLA-antibody_luminex_sop_OneLambda.pdf website.

5.3 High resolution typing- NGS sequencing

High-resolution typing of HLA molecules was conducted using NGS assay by Ion Torrent S5 sequencing system (One Lambda- Thermo Fisher Scientific).

5.3.1 Main principle

Sample DNA after amplification and purification is loaded on a semiconductor chip with microwells. Each microwell contains copies of single-stranded DNA template and DNA polymerase. The wells are then periodically flooded by known unmodified dNTPs. If dNTP is complementary, it is incorporated by DNA polymerase to create a complementary strain, and hydrogen ion is released into the solution, changing its pH. Change in the pH is detected by an ion-sensitive field-effect transistor (ISFET). If dNTP is not complementary, no reaction occurs, and nucleotide is washed away before every new cycle is started. (https://www.thermofisher.com/cz/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-technology.html)

5.3.2 Testing procedure[5]

1. Patients DNA samples were amplified, and amplicons were purified
2. Purified amplicons were quantified using Qubit Fluorometer, diluted, and pooled

3. Amplicons present in the amplicon pool were fragmented
4. Adaptors were ligated to fragmented amplicons while simultaneously nick- repair processes were carried out
5. Size selection of the adaptor-ligated product was performed, followed by secondary amplification of the size selected product and final purification
6. Template preparation and Sequencing preparation process was automatically run using Ion Chef™
7. DNA sequencing was conducted using S5™ system

4 general steps are outlined. The detailed description of the protocol followed can be accessed on: NXT-CHEF-PI-EN-00.pdf (veritastk.co.jp) website as the official application manual.

5.4 HLA sequence mismatch analysis

Eplet mismatches for HLA class I (A, B, C) and HLA class II (DRB1, DRB3,4,5, DQA1/DQB1, DPA1/DPB1) were determined using HLA matchmaker software (ABC eplet matching program V4.0 and DRDQDP eplet matching program V3.1) based on 4-digit high-resolution typing. Both "antibody verified" and "other" eplets were included in the analysis. Anti-HLA antibodies against HLA class I (A, B, C) and HLA class II (DRB1, DRB3,4,5, DQA1/DQB1, DPA1/DPB1) were determined using HLA Matchmaker software (ABC antibody analysis program V3.1 and DRDQDP antibody analysis program V3.1) based on 4-digit HLA typing and MFI values from Luminex single antigen assay. DSAs are considered as "reactive eplets" identified by HLA matchmaker sorting, including "antibody verified" and "other" eplets. Number of mismatched polymorphic amino acid residues was analyzed using HLA EMMA software V1.05 based on 4-digit high-resolution typing. Only amino acids in solvent accessible positions were included for further analysis. Interlocus positions in HLA class I were not included. Antigen mismatches were counted as a sum of mismatched HLA alleles defined on high resolution by NGS sequencing.

5.5 Statistical analysis

The difference between number of mismatches in the positive and the negative cohort for HLA ABC, HLA DRDQDP, HLA DQ (Defined by molecular mismatches, HLA eplets, or HLA amino acid mismatches) were analyzed using Student's T-test. P-value below 0.05 was

considered significant. To test correlation between the number of mismatches as determined by various approaches, we used receiver operating characteristics (ROC) curves. ROC curves are described as the area under the curve (AUC) with 95% confidence interval. The AUC value depends on the ability of the model to distinguish between outcomes. AUC value of 1 implies perfect discrimination, while the value of 0.5 implies random discrimination. Eplet positions were statistically tested by Fisher exact test and P value was calculated. P-value below 0.05 was considered significant.

6 Results

As indicated in the Materials and Methods section, the study cohort was comprised of two groups of renal transplant patients. The first cohort (N= 28) included non-sensitized patients with average age of 51 years who developed anti-HLA antibodies at some time point after transplantation. The second cohort (N= 19) included non-sensitized patients with average age of 46 years with no antibody production detected during 5 years follow-up. The first cohort was further divided into three groups: patients who developed antibodies against HLA class I (N= 6), HLA class II (N= 16), and patients who developed antibodies against HLA class I and HLA class II simultaneously (N= 6). Patients who produced antibodies against both HLA classes were evaluated separately for each HLA class respectively. Predominant occurrence of de novo anti-HLA antibodies in HLA class II was detected in HLA DQ as these antibodies were present in 81,8% of cases, followed by HLA DR antibodies (59.1%) and HLA DP (45.5%). HLA DR antibodies without HLA DQ antibodies were present in 13% of cases, HLA DP antibodies were detected without simultaneous sensitization to HLA DQ or HLA DR in one patient only. Mean cumulative mismatch for recipients in HLA A, B, Cw was 5 HLA allelic mismatches corresponding to 14.5 HLA matchmaker score or 28 HLA EMMA score for the positive cohort and 3 allelic mismatches corresponding to 13 HLA matchmaker score and 21 HLA EMMA score for the negative cohort. As for HLA class II the mean cumulative mismatch in HLA DR, DQ, DP was 7 HLA allelic mismatches corresponding to 26 HLA matchmaker score or 39.5 HLA EMMMA score for the positive cohort and 5 HLA allelic mismatches corresponding to 17 HLA matchmaker score or 23 HLA EMMA score in the negative cohort. Patient cohort parameters are summarized in Table 1 and Table 2. Full high- resolution HLA typing for patient- donor pairs in both cohorts and respective numbers of mismatches for each pair can be found in the supplement 1.

The positive and negative cohorts were first compared based on the total number of mismatches in HLA class I, HLA class II, and HLA DR, DQ, and DP separately (figure 13) for all the three approaches, and the P-value was calculated. The number of HLA allelic mismatches was significantly higher in the positive cohort for HLA class I (P= 0.049) and HLA class II (P= 0.012) over the respective negative cohort and positive cohort was also elevated significantly for HLA DR (P= 0.026) and DQ (P= 0.029) over the respective negative cohort when the loci

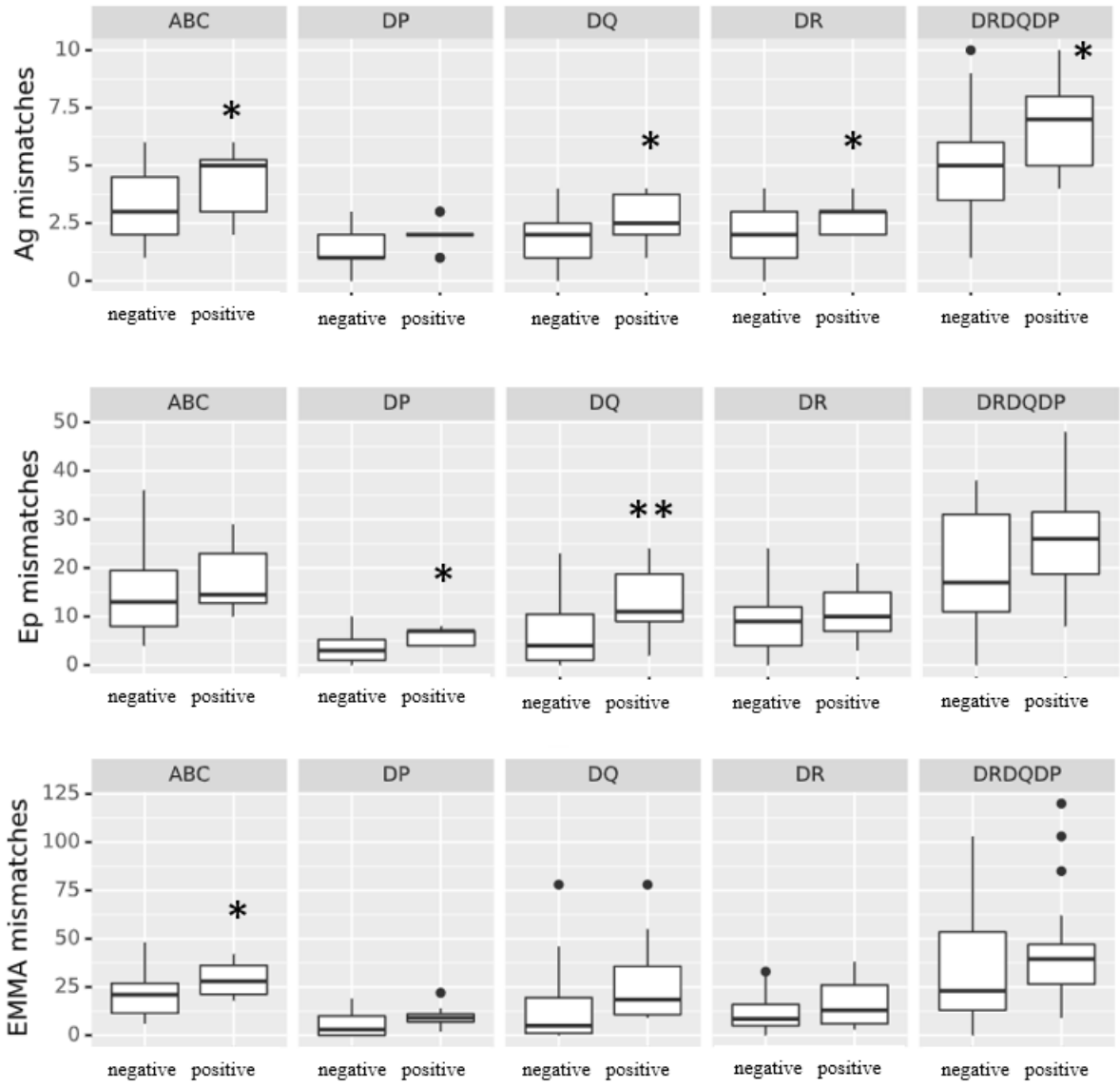
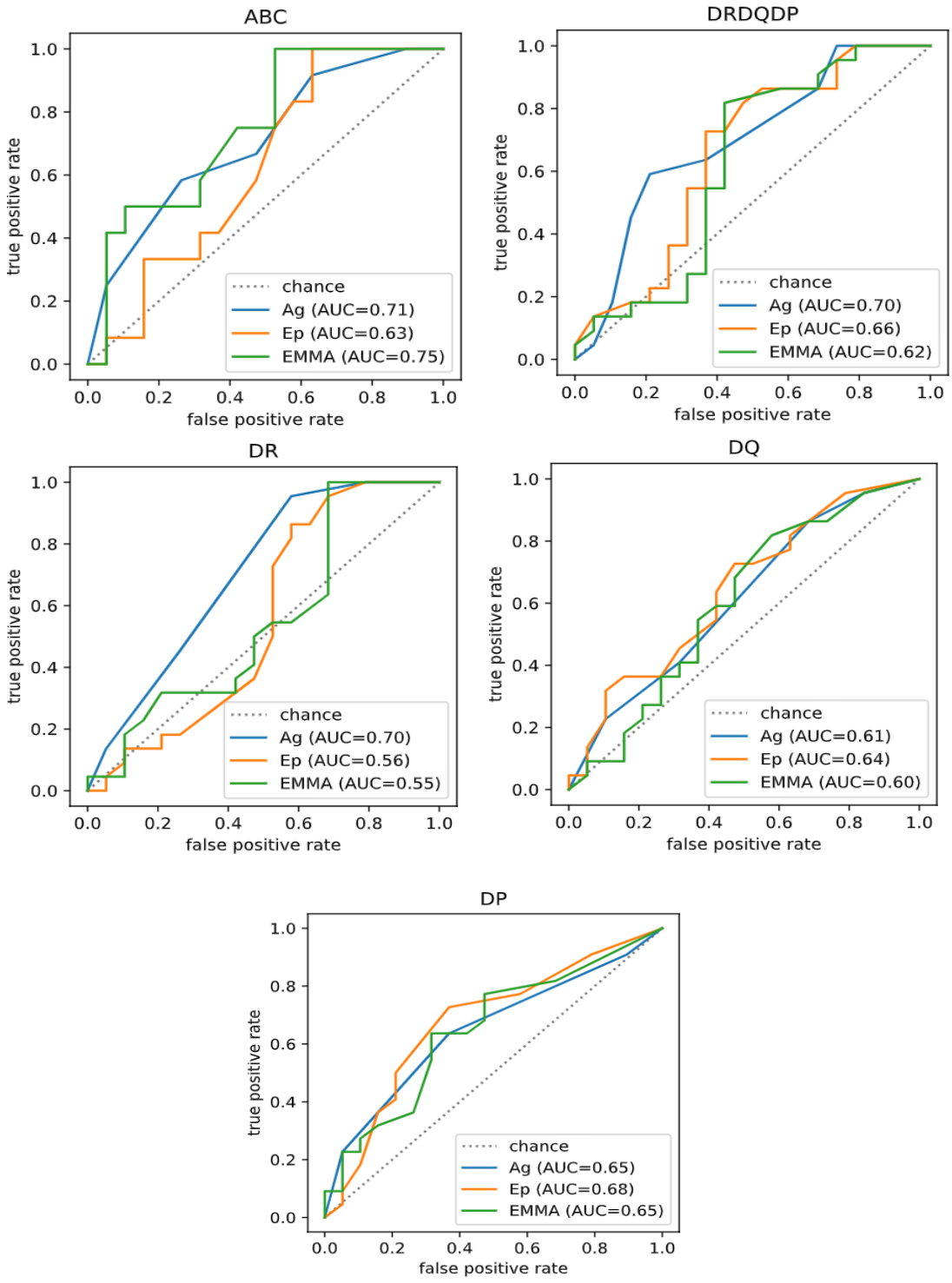


Figure 13 Comparison in total number of mismatches between the positive and the negative cohort in HLA ABC (HLA class I) and HLA DR DQ DP (HLA class II) based on HLA allelic mismatches (Ag mm), HLA Matchmaker score (Ep mm), and HLA EMMA score (EMMA mm). HLA class II loci, the HLA DR, DQ, and DP loci mismatches were also considered separately. Statistical significance is indicated by the asterisks (* $P \leq 0.05$, ** $P \leq 0.01$)

were considered separately. No significant difference was detected in DP (P= 0.086) allelic mismatches. When comparing the positive and negative cohorts based on the eplet mismatches as defined by the HLA Matchmaker score, no significant results were obtained either in HLA class I (P= 0.41) or HLA class II (P= 0.072),although the numbers of mismatches were elevated for the positive cohort in both cases. However, when considering each HLA class II loci separately, we observed significantly higher numbers of eplet mismatches in the positive cohort for DQ (P= 0.0019) and DP (P= 0.032) loci, while no significant difference in DR (P= 0.21).



14 Correlation of HLA mismatches in HLA ABC (MHC I), HLA DR DQ DP (MHC II), and HLA DR, DQ and DP loci based on HLA allelic mismatches (Ag) , HLA matchmaker score (Ep), and HLA EMMA score (EMMA) in relation to the number of patients with de novo antibodies. The ROC curves indicate connection between the method of mismatch detection and either true positive or false negative rate represented by corresponding AUC value.

The number of amino acid mismatches as defined by HLA EMMA was significantly elevated in the positive cohort in MHC I (P= 0.020) and slightly higher in the positive cohort in MHC II (P= 0.28) over the corresponding negative cohorts.No significant results were obtained in either of separate HLA loci (DR P= 0.15, DQ P= 0.067, DP P= 0.079). The correlation between the number of HLA mismatches in HLA class I (ABC), HLA class II (DRDQDP), and the production of de novo antibodies was assessed using ROC curves in figure 14 (HLA DR, DQ, and DP loci were also considered individually). According to the ROC curves, the most accurate predictors for anti-HLA antibody production in HLA class I is the HLA EMMA score (AUC= 0.75) followed by HLA allelic mismatches (AUC= 0.71) and eplet mismatches (AUC= 0.63). For MHC II were the best predictors mismatched HLA alleles (AUC= 0.70) followed by HLA Matchmaker eplets (AUC= 0.66) and by HLA EMMA score (AUC= 0.64). When considering HLA class II loci separately, the predictive potential of mismatched HLA alleles was determined 0.70 (AUC) for DR, 0.61 (AUC) for DQ, and 0.65 AUC for DP. The HLA Matchmaker score results were 0.56 (AUC) in DR, 0.64 (AUC) in DQ, and 0.68 (AUC) in DP. In comparison, the HLA EMMA score were 0.55 AUC, 0.60 AUC, 0.65 AUC for HLA DR, DQ, and DP, respectively. Together 396 different mismatched eplets were detected by HLA Matchmaker. 28% of these eplets were targeted by de novo donor specific antibody (DSA) (Figure 15). Likelihood of de novo DSA production against certain eplet was observed to be independent from number of patients with this mismatched eplet. Therefore, immunogenicity of each epitope was assessed individually, ranking them from the most immunogenic to the least immunogenic compared to average immunogenicity for our dataset with P values calculated. The most and least immunogenic positions are described in Tables 3 and 4, respectively. 6 eplets were significantly more immunogenic as compared to the average. Namely 30H, 76ESI, 86V, 55LL, 135G and 48Q with P value ranging from 0.001981 to 0,040054. Due to the large portions of eplets that were not targeted by DSA (immunogenicity= 0.0) as the least immunogenic eplets are considered those with the highest mismatch rate (highest number of patients with this eplet not producing de novo antibodies).

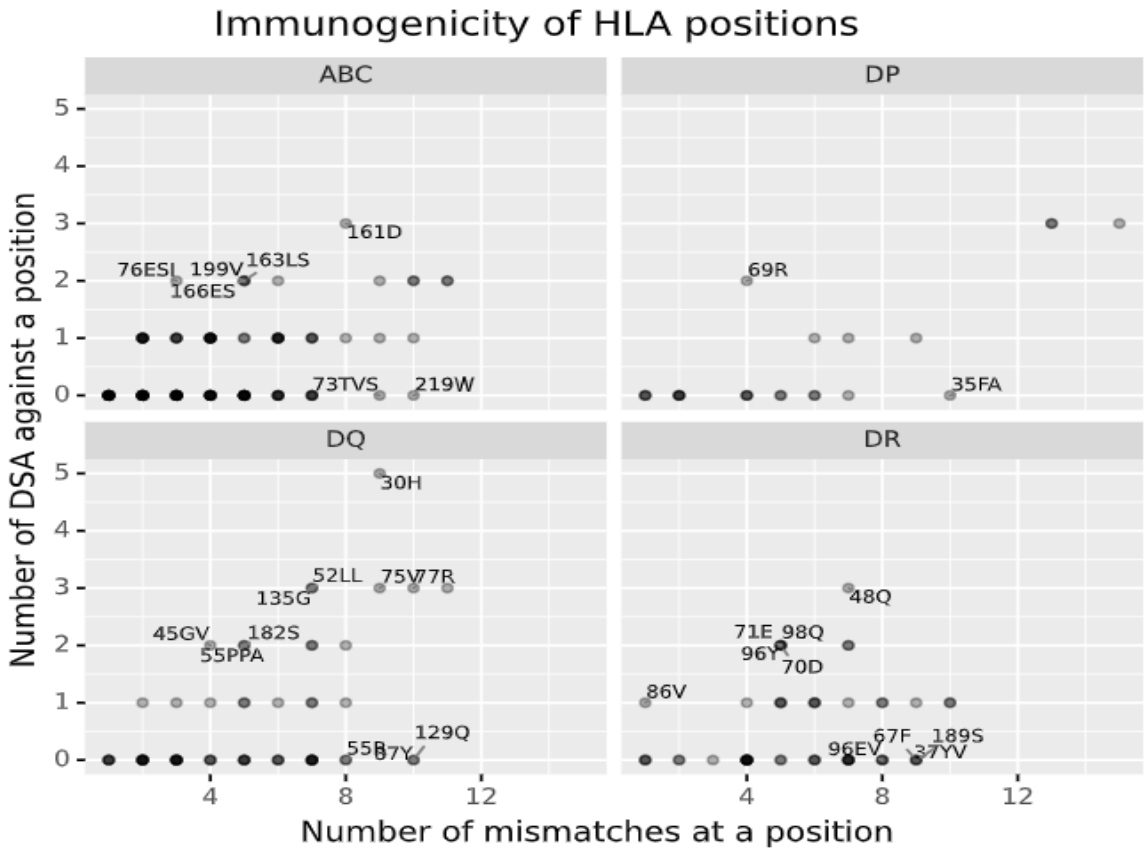


Figure 15 Number of mismatches and DSA. Screening for specific mismatched eplet positions in the positive and negative cohorts and number of patients with DSA against the eplet positions. The overall 10 most and 10 least immunogenic positions are indicated by number and respective ammino acid as defined by HLA Matchmaker.

Table 3 The 10 most immunogenic positions. The positions are sorted based on their immunogenicity as compared to the average immunogenicity of all eplet positions, and P-value was calculated. Each eplet is described based on its locus and HLA Matchmaker eplet name. The number of patients with DSA against the eplet and total number of patients with the mismatched eplet (MM) are indicated.

locus	eplet	DSA	MM	immunogenicity	P_values
DQ	30H	5	9	0.555556	0.001981
ABC	76ESI	2	3	0.666667	0.038495
DR	86V	1	1	1.000000	0.117696
DQ	52LL	3	7	0.428571	0.040054
DQ	135G	3	7	0.428571	0.040054
DR	48Q	3	7	0.428571	0.040054
DQ	45GV	2	4	0.500000	0.070943
DP	69R	2	4	0.500000	0.070943
ABC	161D	3	8	0.375000	0.058493
DQ	75V	3	9	0.333333	0.080144

Table 4 The 10 least immunogenic positions. The positions are sorted based on their immunogenicity as compared to the average immunogenicity of all eplet positions, and P-value was calculated. Each eplet is described based on its locus and HLA Matchmaker eplet name. The number of patients with DSA against the eplet and the total number of patients with the mismatched eplet (MM) are indicated

locus	position	DSA	MM	immunogenicity	P_values
DQ	55R	0	8	0.0	0.607083
DR	96EV	0	8	0.0	0.607083
DR	37YV	0	9	0.0	0.609283
ABC	73TVS	0	9	0.0	0.609283
DR	189S	0	9	0.0	0.609283
DR	67F	0	9	0.0	0.609283
DP	35FA	0	10	0.0	0.616224
ABC	219W	0	10	0.0	0.616224
DQ	129Q	0	10	0.0	0.616224
DQ	87Y	0	10	0.0	0.616224

7 Discussion

In order to determine recipient/ donor compatibility in solid organ transplantation, most allocation schemes rely on HLA antigen comparisons (defined at the serological level). However, due to HLA amino acid sequence elucidation advancements combined with the high specificity of HLA antibody analyses, the attention is shifting to HLA matching on the structural level. The polymorphic amino acid configurations (epitopes) pose as the direct targets for antibody paratope binding, and therefore matching on amino acid level may theoretically reduce formation of the de novo DSA. Several studies showed that a reduced number of epitope mismatches as defined by the HLA Matchmaker is associated with a reduced possibility of de novo antibody production (Tafulo et al., 2019; Wiebe et al., 2017). Furthermore, it was postulated that HLA epitope matching is superior to HLA matching on the antigenic level in predicting the possibility of de novo antibody formation (Wiebe et al. 2013, Daniëls et al., 2018). Although none of their data were confirmed by the study on a large cohort of patients, as in the case of HLA antigens (Wiliams et al., 2016) and therefore more data on this subject is still needed. To assess the antibody inducing potential of the HLA mismatches, we have selected two cohorts of kidney allografts recipients: 28 patients with de novo HLA targeting antibodies created after the transplantation (positive group) and 19 patients without de novo antibody production in 5 years of follow up monitoring after the transplantation (negative group). To focus specifically on the HLA mismatches and their effect on de novo antibody induction, we have excluded all patients with preformed antibodies or women with pregnancy history. Patients from the positive group were further divided based on their antibody specificity (HLA class I or HLA class II). Patients with de novo antibodies against both HLA classes were considered separately in HLA class I and HLA class II. The HLA mismatches were described on three levels: total number of mismatched HLA alleles (defined on higher-resolution typing), HLA eplets (defined by HLA matchmaker), and polymorphic amino acid residues in the HLA sequence (defined by HLA EMMA). We first focused on determining whether there is a significant difference in the number of HLA mismatches (defined by all methods mentioned above) between the positive and negative groups. High-resolution defined HLA antigen-based matching (allelic mismatches) was the only technique significant for both HLA class I ($P= 0.049$) and HLA class II ($P= 0.012$). From HLA structure-based mismatch determining methods (HLA EMMA and HLA matchmaker score), the solvent accessible amino acid mismatches identified by HLA EMMA were significantly elevated for the positive group in HLA class I

(P= 0.020) and not significantly elevated in HLA class II (P=0.28). At the same time, the HLA matchmaker score, altough elevated in both positive groups, was not significant for either HLA class I (P= 0.41) or HLA class II (P= 0.072). This discrepancy between HLA EMMA class I, class II and HLA matchmaker may be caused by the difference in HLA EMMA matching algorithm for HLA class I. Default setting in HLA EMMA is intralocus comparison for HLA class I and interlocus comparison for HLA class II, while HLA Matchmaker is using interlocus comparison for both of the HLA classes. In practice this mean that HLA EMMA is comparing HLA mismatches only between same loci (eg. locus A to locus A) in the HLA class I ,while the HLA matchmaker is comparing mismatched eplets from one locus to mismatches on all the loci from given class (eg. locus A to the A, B, C loci) (Kramer et al, 2020). Given the fact that HLA EMMA appears to be better predictor in HLA class I as compared to HLA Matchmaker, it seems that the interlocus comparison used by HLA matchmaker might be too restrictive and whole epitopes not only eplets are important for the analysis. We have also considered HLA DR, DP, and DQ loci separately. For the patients who developed de novo antibodies targeting DR antigens, only HLA allelic mismatches (P= 0.0094) were significantly elevated compared to the negative cohort. For the DQ antigen, mismatched HLA alleles (P= 0.029) and HLA matchmaker (P= 0.0019) were significantly elevated in the positive cohort as compared to the negative cohort. Interestingly, HLA DQ appears to be the most important of HLA class II loci in terms of the humoral immune response, as most DSA and non-DSA antibodies were directed towards this locus in our study. The antibodies towards DQ were present in 81.8% of HLA class II positivity cases. This locus is usually not considered by matching algorithms that rely on HLA A, B, and DR loci. These findings are consistent with the work of Daniëls et al. (2018), where they identified HLA DQ as the most targeted locus for their cohort of patients. Willicombe et al. (2018) and Béland et al. (2017) also recognized the HLA DQ as frequently associated with AMR. Moreover, the DSA against HLA DQ was suggested to be better rejection predictors than the HLA class I DSA in the same study. For patients with anti-DP locus de novo antibodies, only the number of eplets (HLA matchmaker, P= 0.029) was significantly elevated compared to the negative group. Based on our data, HLA allelic mismatches (defined on high resolution) appear to be superior to HLA Matchmaker eplets for determining de novo antibody production potential for both HLA classes. This in accordance with study conducted by Kosmoliaptsis et al (2016) reporting that they were not able to find any benefit of eplet matching or amino acid-based sequence matching over HLA antigen matching in HLA class I or HLA class II, when simply enumerating the total number of mismatches. However, these results are in contrast to the majority of studies published on this

topic so far. The work published by Silva et al. (2010) conducted on 62 patient cohort concluded that HLA eplet matching associate better with anti HLA antibody production than matching on antigen level in HLA B locus but reported no significant difference for locus A. However, their results were based only on serologically defined low-resolution HLA antigen mismatches excluding locus C and, therefore, might not be entirely accurate. In case of HLA class II, the Wiebe et al reports data on HLA class II DR and DQ antigens. In their study (Wiebe et al., 2013), HLA eplet mismatches defined by HLA matchmaker were determined to be superior to HLA antigen-based matching for both high resolution and low-resolution defined antigens. They are even stating that there is no added benefit for high-resolution typing over low-resolution typing. However, they included only DR and DQ antigens in their study. An important conclusion from their work is that DR and DQ linkage disequilibrium is not strong enough to justify excluding HLA DQ in traditional HLA A B DR matching, and antibodies against HLA DQ are more common than antibodies against DR. This is in accordance with our own results.

An important issue with the determination of superiority/ inferiority for HLA structure-based matching over traditionally used HLA antigen matching is that HLA epitopes correlate with traditional HLA mismatches and show collinearity. The HLA epitope matching, and HLA antigen matching are both based on genetic differences between patients and donors in the HLA region on chromosome 6 (Sahin et al., 2020). Due to this fact, the HLA epitope matching should be associated with de novo antibody production as this was already demonstrated for HLA antigen matching in the past (Wiliams et al., 2016). This was confirmed by Nguyen et al. (2016) where he showed a high degree of correlation between HLA matchmaker eplets and HLA antigens for class I (0.80) and class II (0.84). For their analysis, they used estimated 4- digit typing of HLA antigens. Another complication for comparing HLA antigen and HLA structure-based mismatches is associated with a different range of values, where antigenic mismatches in traditional ABDR HLA antigen matching range from 0 to 6 as compared to eplet/amino acid mismatches ranging from 0 to few hundreds (Wiebe et al., 2017).

Due to the issues stated above, it is not the total numbers of mismatches but rather their predictive value in terms of de novo antibody production that should matter.

Therefore, in the second part of our analysis, we have focused on the correlation between different numbers of mismatches as defined by the three approaches mentioned above and de

novo antibody production. We detected a significant association between the number of patients with the de novo antibodies and mismatches described by all approaches. In HLA class I, the HLA EMMA score was the most likely to predict de novo antibody production, followed by mismatched HLA alleles and HLA Matchmaker score. In HLA class II, the de novo antibody production was most significantly associated with mismatched HLA alleles. However, when considered separately, only HLA DR targeting antibodies were most significantly associated with allelic mismatches. The HLA DQ and DP targeting antibodies were better associated with the HLA Matchmaker score. When inspecting ROC curves in HLA class I more closely we can see wide difference between HLA matchmaker curve and HLA EMMA curve this may be caused by already mentioned difference in the HLA EMMA algorithm (interlocus vs intralocus comparison) and indicate that interlocus comparison might be better approach for HLA class I mismatch identification. Furthermore, for HLA DR, we detected a discrepancy in AUC value between HLA allelic score AUC= 0.70 and HLA Matchmaker score AUC= 0.56 and HLA EMMA score AUC= 0.55. The curve for mismatched HLA alleles is steadily rising with the rising number of mismatches. However, the curve for eplets and amino acid mismatches at some point falls into the negative predictive values. Despite being less extreme, the same trend is repeated for the entire HLA class II. This may by influenced by still limited number of HLA DR structures available that are included in HLA matching software (Kramer et al, 2020). Nevertheless, based on our correlation studies HLA EMMA appears to be the best option when considering HLA class I mismatches for predicting de novo production of DSA antibodies while resulting poorly in the HLA class II. Overall, the HLA alleles (high-resolution) seem to be scoring the best for predicting HLA class I and HLA class II de novo antibody production, although there is no statistical test that is capable of comparing different ROC curves. Mismatched HLA alleles appear better in displaying antibody-producing potential for patients with lower numbers of mismatches in HLA class II which is in accordance with Kosmoliaptsis et al 2016 who concluded that only high mismatched eplet load is significant predicting factor for de novo antibody development. However, our results are in contrast to Daniëls et al. (2018), who reports based on their own ROC analysis that HLA eplets as defined by HLA matchmaker are best predictors (over HLA antigens) for de novo antibody production in both HLA class I and HLA class II. Our AUC values are relatively low in eplet mismatches as compared to the Daniëls et al. This may be caused by the limited size of the graft recipients meeting the strict criteria of sample selection for our cohorts or other factors correlating to de novo antibody production. These factors may include non-adherence with immunosuppression, cyclosporine-based immunosuppression regiments, or early T cell-mediated rejection (Wiebe et al., 2018).

The different immunogenic potentials of each epitope should also be considered (Kramer et al., 2019).

We focused on the difference in the reactivity of epitopes as the final part of our study. Eplets are vital components of HLA epitopes and are potential targets for donor-specific antibodies in the transplantation setting. There are some indications that mismatched HLA alleles defined on the high resolution may benefit overall comparison and de novo antibody prediction in solid organ transplantation from our results. However, eplets are not staying too far behind this method in our correlation studies. Moreover, considering total numbers of eplets may not be entirely accurate as eplets are predicted to be of different immunogenic properties. This means that in theory, not all the detected mismatched epitopes participate equally in antibody response, and rather participation of one or few highly immunogenic epitopes is sufficient in order to elicit an immune response. Therefore, the total number of eplet mismatches for a patient- donor pair might be hard to correlate with the risk of the antibody production reliably. We have identified 396 different eplet mismatches together for both HLA class I and HLA class II in our cohort. Each eplet is identified based on its position and corresponding amino acid residues. We have considered antibody verified and theoretically predicted epitopes alike as defined by the HLA matchmaker software. Out of this number, only approximately 28% of mismatched epitopes detected were targeted by the DSA antibody. This follows the presumption that 3D elements of HLA molecules possess a different immunogenic potential and therefore should be considered individually. In our study, we have associated each eplet with a numerical value relating to its theoretical immunogenicity. The immunogenicity is assigned to each eplet as compared to the average immunogenicity of all the eplets in our dataset. Based on this value, we created a list of the 10 supposedly most and least immunogenic eplets and counted the P-value for each of them. The positions of the most and least immunogenic eplet from our study are also visualized in figure 16 on the respective HLA 3D molecules. Antibody verified and theoretically predicted eplets were almost equally represented in our most immunogenic and least immunogenic positions (40% verified in the reactive group, 50% verified in the non-reactive group). Altough, there is currently limited data on antibody verified and nonverified eplets contribution to de novo antibody production. Altough Sapir-Pichadze et al. (2020) argue that there is no effect of non-antibody verified eplet mismatches on graft rejection except for HLA-DRB1. Some particular antibody verified eplets (55R, 96EV, 73TVS, 219W, 87Y) in our study did not induce antibody production at all. Based on the position of eplets on 3D HLA molecule eplet position 30H and 86V identified by our study as the most immunogenic looks

like embedded inside the structure with no possible antibody access. The Eplet registry database (http://www.epregistry.com.br) defines eplet 30H as antibody verified, while eplet 86V is only theoretically predicted. From the least reactive group of eplets, eplet positions 37YV and 87Y seem to be located inside the HLA molecule. Eplet 87Y is antibody verified, while eplet 37VY is just theoretically predicted. As the antibody verified eplet position ability to bind antibody was verified multiple times it is unlikely that this position will be embedded inside the structure as shown by the 3D models. This may suggest possible inaccuracies in *in sillico* predicted 3D models used by the HLA matchmaker and HLA EMMA software.

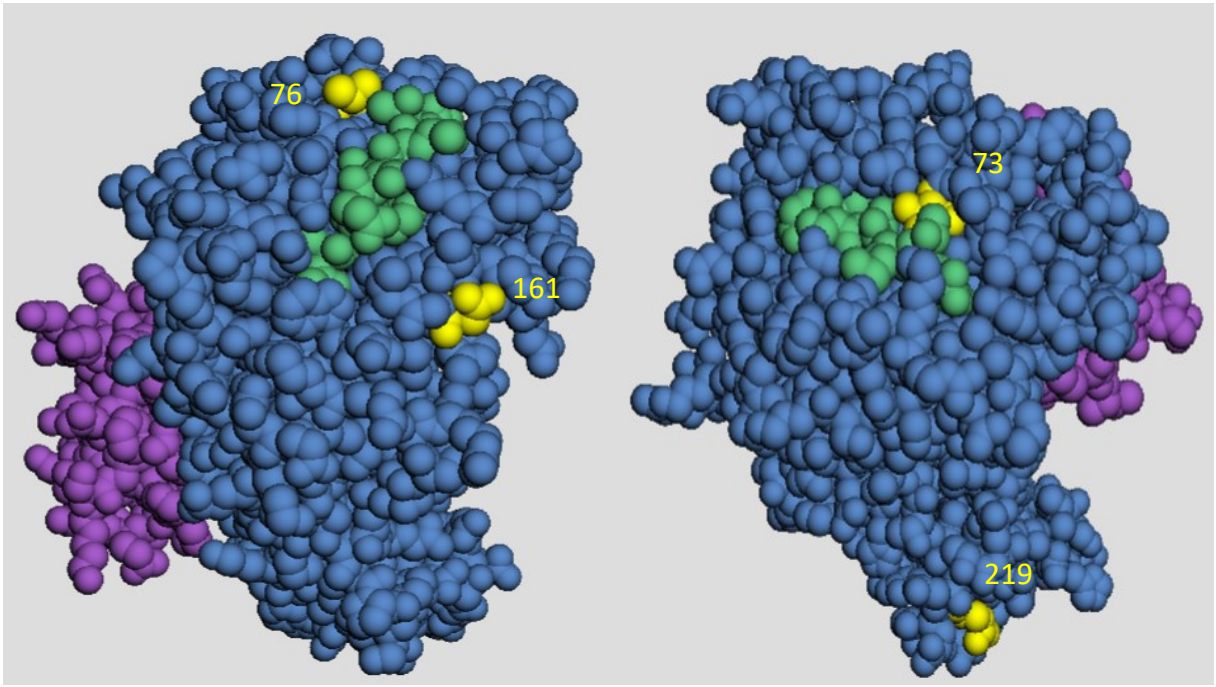
Similar work in identifying reactivity of eplet positions was conducted by Hönger et al. (2020), where most and least reactive eplets were identified for HLA class I based on a cohort of 159 women giving first full-time birth. Their study implicates eplet 161D as potentially highly immunogenic with the immunogenicity of 0.214, although underrepresented (only 14 patients) in their study. In accordance with their finding, we have identified the same eplet as one of the most immunogenic in our cohort, with an immunogenicity of 0.375 (P= 0.058). However, in the same study, they identified eplet 76ESI as potentially nonimmunogenic, altough again underrepresented in their study with 11 mismatches, and no antibody was produced targeting this particular eplet. Our analysis placed eplet 76ESI as one of the most immunogenic positions with an immunogenicity of 0.67 (P= 0.038). This discrepancy may be caused by the relatively low number of mismatches for this eplet in our cohort. Despite probably not being one of the most immunogenic, it should not be ruled out as non-reactive either. The limitation in our study is that de novo antibodies are analyzed from the first positive patient's sera, and detection time may significantly vary between the patients. Different immunogenic properties of HLA epitopes can also be determined based on the topographic properties of eplets. Reactive epitopes in the proximity of the plasma membrane such as 135G may be more effective in inducing membrane attacking complex of the complement. Additional variables may be the B cell and T cell repertoire of the respective recipients (Tambur et al., 2019).

The HLA matchmaker often showed a discrepancy between its two eplet/ antibody analyzing programs. Reactive eplets sorted by HLA matchmaker antibody analysis were not identical to DSA antibodies, as a number of reactive eplets were not indicated as mismatched by the HLA eplet sorting program. Moreover, some of the eplets showed as reactive or targets for DSA seem to be embedded inside the structure of the HLA molecule and, therefore, not accessible to donor-specific antibodies (figure 16). Two eplets identified by the HLA matchmaker could

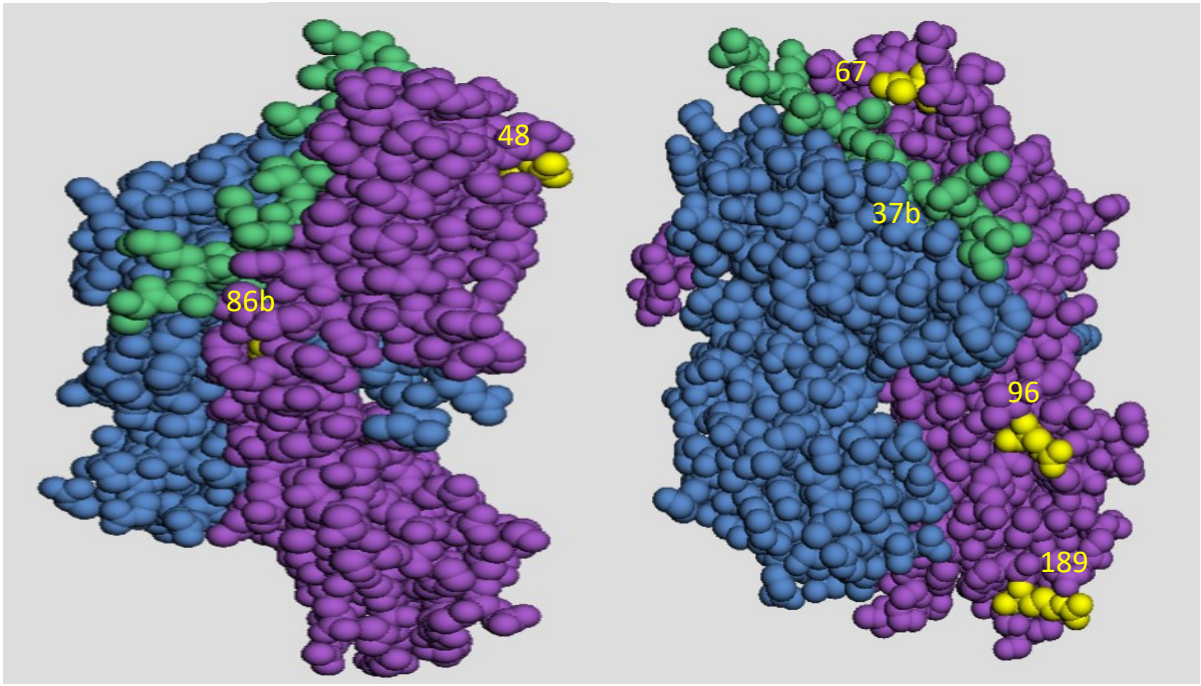
not be found in the international eplet registry (http://www.epregistry.com.br). These problems suggests that despite considerable advances since the creation of HLA matchmaker it might still not be ready for clinical practice in its current version.

Despite IKEM being one of the largest transplantation centers in Europe, we collected only a limited number of data that would meet strict selection criteria. Additional studies with large cohorts of patients are required to reliably assess the overall benefit of epitope-based matching for solid organ transplantation with a focus on epitope antigenicity and immunogenicity. Therefore, our data will be in the future part of the 18th International HLA and immunogenic workshop (IHIWS) in the Netherlands.

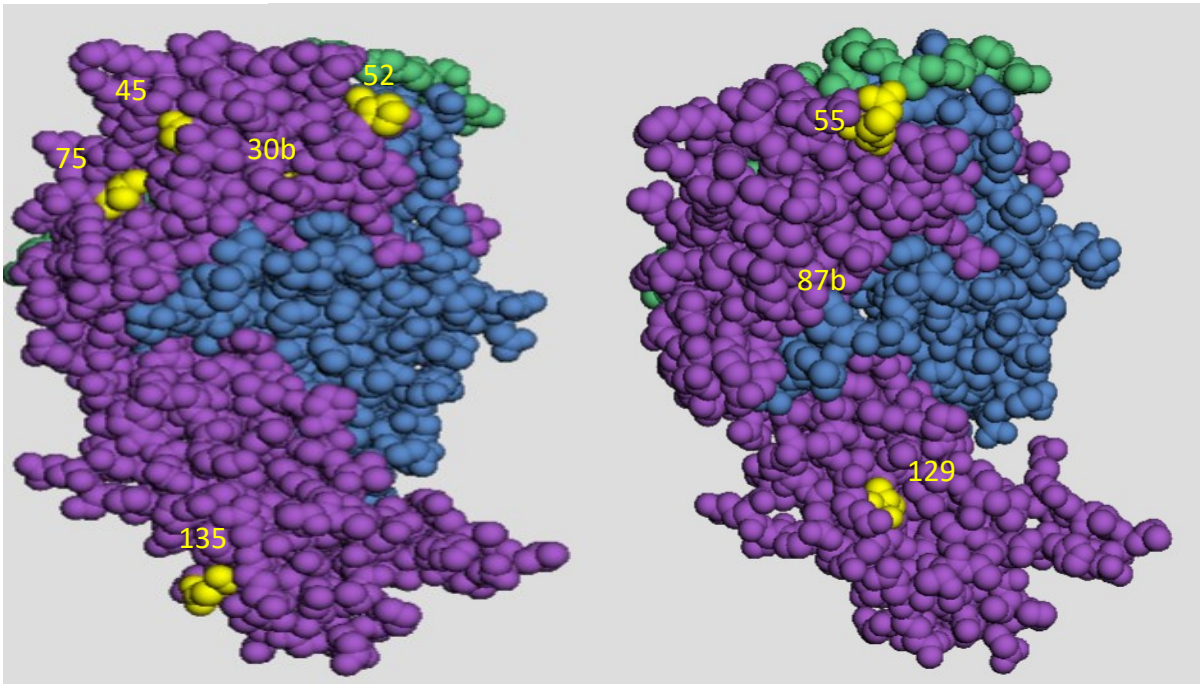
HLA A*03:01



HLA DRB1*01:01



HLA DQB1*05:01



HLA DPB1*01:01

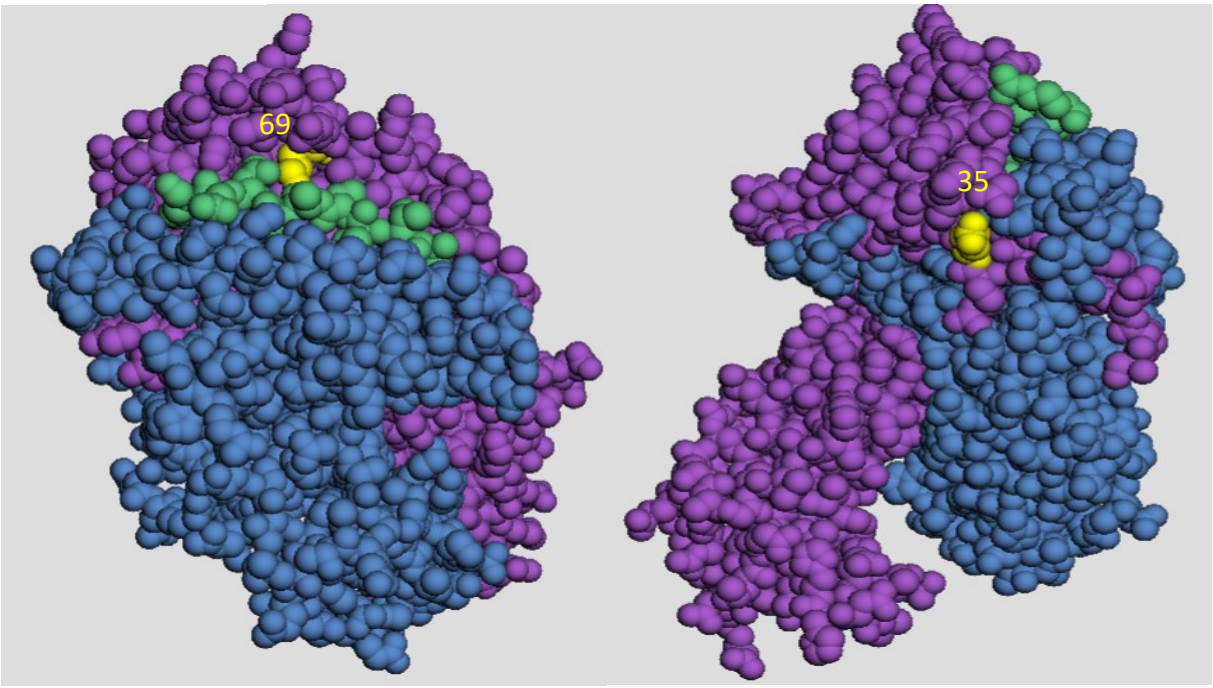


Figure 16 Visualization of eplets on the 3D structure. The most reactive (left) and least reactive (right) eplet positions from our cohort are schematically visualized on representative HLA molecules. For HLA class I: blue color = alpha chain, purple= ß2-microglobulin, green= peptide. For HLA class II: blue color = alpha chain, purple= beta chain, green= peptide. b= eplet position is buried in the structure and not visible. (Based on pHLA3D database, Menezes Teles e Oliveira et al, 2019)

8 Conclusion

In conclusion our findings demonstrate clear relationship between HLA epitope and amino acid mismatches and de novo antibody production in HLA class I and HLA class II. However, despite some promising results for HLA EMMA in HLA class I and for HLA Matchmaker in HLA DQ and DP, we failed to show superiority of HLA structure-based approach for calculating HLA compatibility when compared to compatibility calculated as mere number of mismatches defined on high resolution level. This was also demonstrated by the correlation study where mismatches in high-resolution HLA alleles were more strongly correlated with number of patients with de novo antibodies than number of mismatches in HLA epitopes in HLA class I and HLA class II.

The HLA DQ appears to be the most important locus in HLA class II as this was the most targeted antigen by the de novo antibodies in our study (81%).

It was possible to stratify each epitope based on their different reactivity and estimate the theoretical immunogenicity of each epitope which resulted in creating a list of the ten most immunogenic and ten least immunogenic epitopes. This may indicate that more sophisticated approach taking into consideration individual immunogenicity and antigenicity of each epitope is needed in order to implement epitope matching into clinical practice.

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