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HLA neshody u pacientů po opakované transplantaci ledviny a incidence akutní buněčné a protilátkami zprostředkované rejekce

HLA mismatches in patients after kidney retransplantation and incidence of acute cellular and antibody-mediated rejection

Diplomová práce

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Prohlášení:

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Abstract

Kidney transplantation is the most appropriate treatment for end-stage kidney failure. The risk of graft failure in retransplanted patients is generally higher than in first-transplant patients due to immunological and non-immunological reasons. An important risk factor to consider for retransplant patients is their sensitization, i.e. the presence of antibodies directed to HLA antigens of previous donor(s). For that reason, a project called Forbidden (Non-acceptable) Antigens was launched by IKEM with the aim of reducing the incidence of acute cellular and antibody-mediated rejection in retransplant patients. Work on the project was carried out between the years 2011-2013. Forbidden antigens were defined as mismatched HLA antigens of previous kidney donor(s) against which patients waiting for retransplantation produced antibodies. The aim of this diploma thesis is to evaluate whether the incidence of rejection is lower in patients with forbidden HLA antigens in comparison with a control cohort, where no forbidden antigens are defined. 234 patients (162 males and 72 females) were included in the study. Almost all tested patients were producing HLA antibodies (90.2%) and forbidden antigens were determined in 71.4% of patients. In a control group of 267 patients waiting for their first transplantation, the production of HLA antibodies was significantly lower (26.6%). 50 patients with forbidden antigens underwent retransplantation. In comparison with a similar cohort of 63 retransplanted patients without forbidden antigens, there was no significant difference in the incidence of cellular and antibody-mediated rejection between both groups.

Keywords: kidney retransplantation, forbidden (non-acceptable) antigens, donor-specific antibodies, HLA antibodies, Luminex, acute antibody-mediated rejection

Abstrakt

Transplantace ledviny je nejvhodnější léčbou konečného stadia selhání ledvin. Riziko selhání štěpu je obecně vyšší u retransplantovaných pacientů než u prvně transplantovaných pacientů, a to z důvodů imunologických i neimunologických. Důležitým rizikovým faktorem rejekce transplantátu u retransplantovaných pacientů je jejich senzibilizace, tzn. přítomnost protilátek namířených proti HLA antigenů předchozího dárce/dárců. Z tohoto důvodu projekt s názvem Zakázané (neakceptovatelné) antigeny byl spuštěn v IKEM v letech 2011-2013. Cílem bylo snížit výskyt akutní celulární a protilátkami zprostředkované rejekce u retransplantovaných pacientů. Zakázané antigeny byly definovány jako neshodné HLA antigeny předchozích dárcům ledviny, proti kterým pacient čekající na retransplantaci vytváří protilátky. Cílem diplomové práce bylo vyhodnotit, zdali byl výskyt rejekce nižší u pacientů se zakázanými antigeny ve srovnání s kontrolní skupinou, kde zakázané antigeny definovány nebyly. Do studie bylo zahrnuto 234 pacientů (162 mužů a 72 žen). Většina z nich produkovala protilátky (90,2%) a zakázané antigeny byly stanoveny u 71,4% pacientů. V kontrolní skupině 267 pacientů čekajících na první transplantaci byla produkce protilátek významně nižší (26,6%). 50 pacientů se stanovenými zakázanými antigeny podstoupilo retransplantaci. Ve srovnání s 63 retransplantovanými pacienty bez stanovených zakázaných antigenů nebyl zjištěný významný rozdíl ve výskytu akutní celulární a protilátkami zprostředkované rejekce.

Klíčová slova: retransplantace ledviny, zakázané (neakceptovatelné antigeny), donor specifické protilátky, HLA protilátky, Luminex, akutní protilátkami zprostředkovaná rejekce

Contents

List of Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AMR	Antibody-mediated rejection
APC	Antigen-presenting cell
BC	Borderline changes
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxic test
CHR	Chronic rejection
dATP	Deoxyadenine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DSA	Donor-specific antibody
DTH	Delayed-type hypersensitivity
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoSorbent assay
FCXM	Flow cytometry crossmatch
Fab	Fab part of immunoglobulin
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum
HLA	Human leukocyte antigen

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IKEM	Institute for Clinical and Experimental Medicine
IMDM	Iscove's modified Dulbecco's medium
LS MIX	LABScreen mixed
LS SA1	LABScreen single antigen HLA class I
LS SA2	LABScreen single antigen HLA class II
MAC	Membrane attack complex
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC Class I Chain Related
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
NC	Negative control
NK	Nature killer cell
PBS	Phosphate buffered saline
PC	Positive control
PC5	Phycoerythrin-cyanin 5,1
PCR	Polymerase chain reaction
PE	Phycoerythrin
PRA	Panel reactive antibodies
reTx	Retransplantation
SA	Single antigen

SAPE	Streptavidin, phycoerythrin conjugated
SNP	Single nucleotide polymorphism
SSOP	Sequence-specific oligonucleotide probes
SSP	Sequence-specific primers
TCMR	T cell-mediated rejection
TCR	T cell receptor
TNF	Tumor necrosis factor
Tx	Transplantation
UV	Ultraviolet light
xMAP	x-multianalyte profiling

1. Literature review

1.1. Introduction

Kidney transplantation is the best treatment for end-stage kidney failure because it improves the quality for life of patients and reduces mortality compared to dialysis treatment (Viklický et al., 2008). In the Czech Republic the program of kidney transplantation is carried out in several transplant centers (the Institute for Clinical and Experimental Medicine (IKEM) in Prague, the Centre of Cardiovascular and Transplant Surgery in Brno, the Transplant Centre of the University Hospital Motol in Prague, in Ostrava, Olomouc, Plzeň and Hradec Králové). The first successful transplantation was performed in Hradec Králové in 1961. However, the transplantation program was started a few years later in 1966 in IKEM. Overall, 1252 kidney transplantations were performed from both living and deceased donors during the years 2011-2013. Currently, more than half of kidney transplantations in the Czech Republic are performed in IKEM (The Czech Transplantation Coordinating Center).

Highly pre-sensitized potential kidney recipients are less likely to receive a donor kidney because of pre-formed HLA specific antibodies which are the main obstacle for receiving a graft. Pre-sensitization is also associated with inferior graft outcomes (Süsal et al., 2009). This may be linked to the presence of donor-specific antibodies (DSA), however, non-donor specific antibodies have been reported to be also associated with a higher incidence of acute rejection and worse graft survival (Hourmant et al., 2005). Pre-transplant antibodies directed to mismatched donor antigens have been associated with a higher incidence of immunological complications after transplantation (Lefaucher et al., 2010).

For that reason, IKEM launched a project called Forbidden (Non-acceptable) Antigens in patients awaiting retransplantation. This project took place between the years 2011-2013. The aim was to determine whether forbidding mismatched HLA antigens of previous kidney donor(s), against which patients produce HLA antibodies

would lead to a decrease of rejection episodes and better graft survival after transplantation.

1.2. Immunobiology of Kidney Rejection

1.2.1. HLA Complex

The genetic system of the Human Leukocyte Antigen complex (HLA) is the human version of the Major Histocompability complex (MHC). It is located on the short arm of chromosome 6 (Fig. 1). It is the most polymorphic region of the human genome and thus the most extensively studied. Various HLA genes are associated with susceptibility or resistance to autoimmune, infectious and inflammatory diseases and they also in have a significant influence on transplantation. The HLA complex contains about 200 genes and is approximately 7.6 megabasepairs large (Thorsby and Lie, 2005).

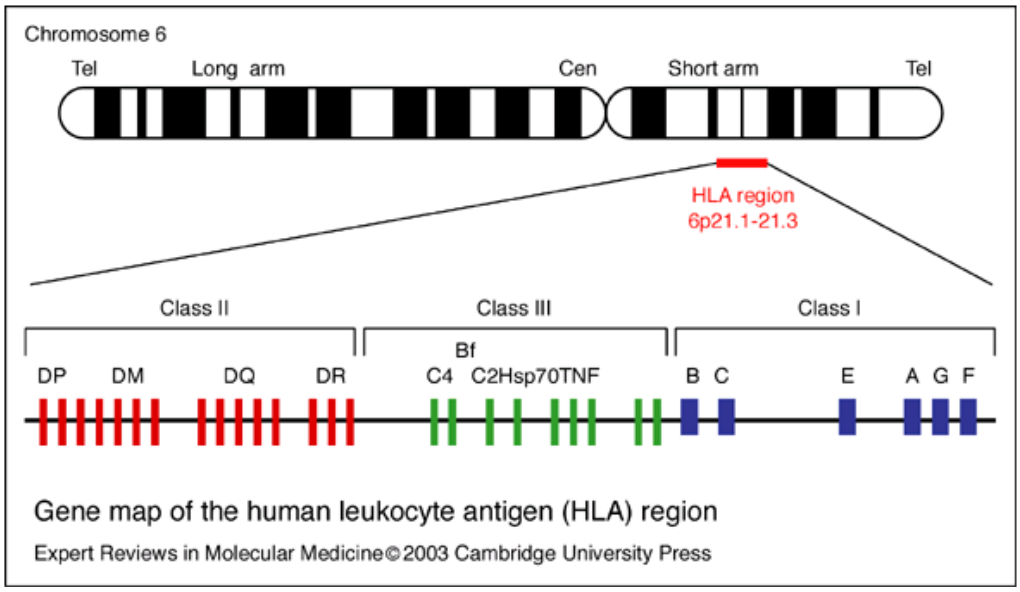

Figure 1: Gene map of the human leukocyte antigen complex.

(Adapted from http://journals.cambridge.org/fulltext_content/ERM/ERM5_07/S1462399403005957sup002.htm)

The HLA genes which take part in immune responses are divided into two categories: class I and class II (Fig. 2). They encode proteins which differ both structurally and functionally. HLA class I antigens are transmembrane proteins consisting of an α-polypeptide chain which is associated with β_2-microglobulin (encoded on chromosome 15). There are about 20 genes in the class I region; the so-called classical genes are HLA-A, HLA-B and HLA-C. HLA class I antigens are expressed on all nucleated cells and platelets. The non-classic genes are HLA-E, HLA-F and HLA-G whose polymorphism is limited and which are expressed only on certain cell types. The HLA class II transmembrane proteins consist of non-covalently associated α and β polypeptide chains. The HLA-DR, HLA-DP and HLA-DQ loci (genes) are located in the class II region. They are expressed by subgroups of immune cells – APC (antigen presenting cells) and thymic epithelial cells (Klein and Sato, 2000). However, HLA class II expression can be induced by IFN-γ and other stimuli in other cells (non-APCs), including mesenchymal stromal cells, fibroblasts and endothelial cells (Neefjes et al., 2011).

The HLA antigens are transmembrane glycoproteins whose main function is to present short peptide fragments (own or foreign) to the immune system, the so-called phenomenon of MHC restriction (Zinkernagel and Doherty, 1997). Differences in HLA antigens (mismatches) between patient and donor have a significant effect on the immune response against the transplanted organ. They play a primary role in graft rejection and graft survival because the immune response is mostly directed to donor HLA antigens.

The HLA complex also includes the class III region of the HLA complex (Fig. 1). However, these genes are not functionally related to the class I and class II genes. They encode genes for complement, cytokine (e.g. TNF) or heat-shock proteins.

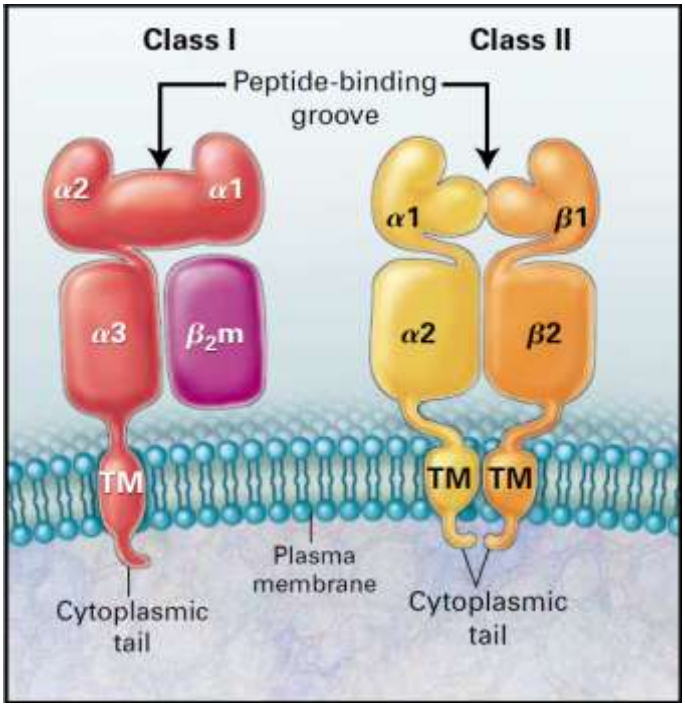

Figure 2: Structure of Class I and II transmembrane proteins.

TM = transmembrane region; β2m = β2-microglobulin

(Adapted from Klein and Sato, 2000)

1.2.2. Mechanism of recognition of HLA antigens

The HLA mismatch between the recipient and the donor leads to the initiation of a transplant immune reaction. The alloreactive immune response is mediated by T cells which recognize the alloantigens of the graft by a direct, indirect or semidirect pathway (Fig. 3). In organ transplantation this reaction is called the host-versus-graft response (Geneugelijk et al., 2014).

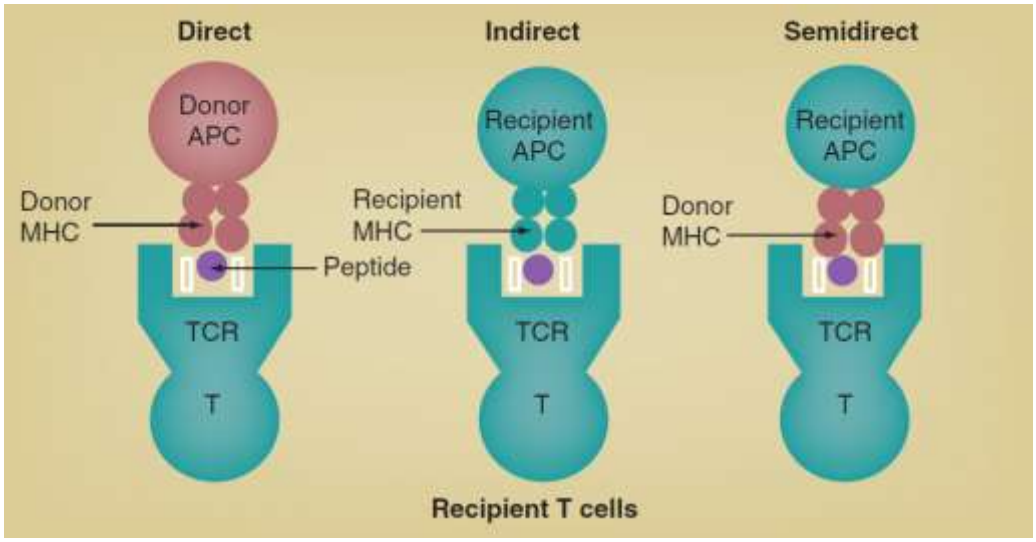

Figure 3: Pathways of T-cell allorecognition (explanation in text).

(Adapted from: Benichou et al., 2011)

Direct recognition is a process where recipient T cells recognize the allogenic MHC presented by the donor APC (APC migrate to recipient lymph nodes or the spleen from the graft) in secondary lymphoid organs. It comprises $CD4^+$ as well as $CD8^+$ T cells and recognizes the intact donor's MHC class I and class II molecules. This process of recognition induces a considerable amount of polyclonal T cell responses engaging up to 10% of the entire peripheral T cell repertoire, and is responsible for early acute rejection of the graft (Benichou et al., 2011). The direct recognition is limited over time (2 – 3 weeks) because the donor's APCs are eliminated by the recipient immune system. However, it can lead to the creation of memory cells which can later induce acute rejection after transplantation.

Indirect recognition is a process where recipient APC process a donor's MHC molecules. The donor HLA antigens are swallowed by the recipient APC and are then processed in the form of peptides. These are expressed in grooves of self-MHC II molecules which lead to the activation of $CD4^+$ T cells. In contrast to direct recognition, the indirect alloresponse is oligoclonal because it is induced by a limited set of T cell clones reacting to discrete TCR for antigens. The alloantigen can also be presented in the groove of self-MHC I by a phenomenon known as "cross-presentation." The MHC I molecules then activate $CD8^+$ lymphocytes which are important for the rejection process

(Benichou et al., 2011). Indirect recognition is a constant process which is active all the time during the presence of the graft in the recipient's body. This process of allorecognition is associated with chronic rejection.

Semidirect-recognition is the ability of the recipient's APC to obtain the intact MHC molecules of a donor. The transfer of donor HLA:peptide complexes can be mediated by endosomes or by cell-to-cell contact between donor and recipient cells (Geneugelijk et al., 2014). Consequently, a single recipient APC can present both allopeptides as self-MHC II to $CD4^+$ T cells (the indirect pathway) and directly stimulate cytotoxic $CD8^+$ T cells by an acquired donor MHC I molecule (Benichou et al., 2011).

$CD4^+$ helper T cells activate B cells which firstly produce low-affinity antibodies against donor HLA antigens of both class I and class II, and secondly function as APCs. Later B cells produce high-affinity antibodies. Naive B cells can be activated by $CD4^+$ T cells when they have been previously stimulated by the same antigen on APCs. $CD4^+$ helper T cells are activated via the direct recognition pathway to differentiate and expand to anti-donor $CD8^+$ cytotoxic lymphocytes.

The recipient immune system can also be activated by different antigens known as non-HLA molecules. These are antigens which are expressed on tissues (tissue-specific antigens) or they can be intracellular. Non-HLA antigens are for example MHC class I chain related (MIC) molecules – MICA/MICB (MHC class I polypeptide-related sequence A/B). Other known antigens are shown in Table 1.

	Cells / tissue distribution
Human Leukocyte Antigens (HLA)	Class I (all nuclear cells)
	Class II (antigen-presenting cells, activated T lymphocytes, etc.)
Non-HLA antigeny	AB0 antigens (erythrocytes, endothelial cells)
	MICA, MICB (endothelial cells, keratinocytes, fibroblastes, monocytes, etc.)
	Vimentin (cytoskeleton), Kα-1 tubulin (lungs)
	Angiotensin II Type 1 (AT1) receptor (blood vessels, brain, heart, kidney, etc.)
	Tissue-specific antigens (kidney, heart, liver, etc.)

Table 1: Summary of antigen types that play a role in transplantation.

(Adapted from: Slavčev, 2013)

The MHC class I chain related (MIC) molecules have a similar structure to MHC I molecules, although they are not associated with β_2-microglobulin and do not present peptides. They are stress molecules which are presented by endothelial and epithelial cells, monocytes, keranocytes etc. However, they are not presented on T cells. Higher levels of expression of MICA and higher production of anti-MICA antibodies may affect acute and chronic rejection (Slavčev, 2013).

1.3. Rejection

Rejection of the kidney graft is the most serious medical complication after transplantation because the kidney graft is damaged by components of both types of immunity - innate and adaptive. In the rejection process many types of cells are involved – monocytes, NK cells, macrophages, eosinophils and alloreactive T cells; however, antibodies are also involved. The most serious causes of the rejection are the alloantigens that can induce both cellular and humoral immune responses. HLA antigens are the main cause of rejection but the minor histocompatibility antigens can also

induce rejection even though they are weaker and slower (Abbas et al., 2012). About 1% - 2% of an individual's T lymphocytes are capable of recognitiying and responding to a single foreign HLA molecule. This high number of T cells, reactive with allogenic HLA molecules, is one of the reasons that allografts elicit strong immune response (Abbas et al., 2012).

The rejections are traditionally divided into hyperacute, acute and chronic rejection. In modern medicine another division is used on acute T cell-mediated rejection, acute antibody-mediated rejection and chronic antibody-mediated rejection. T cell-mediated and antibody-mediated rejection can occur simultaneously. The most frequent blood vessels of endothelia and graft parenchyma are targeted in the kidney graft by the patient's immune system. The immunology reaction against the graft results in progressive deterioration and demise in function of the graft.

1.3.1. Acute T cell-mediated rejection

Acute T cell-mediated rejection (TCMR) is characterized by the involvement of components of innate immunity (dendritic cells, macrophages, NK cells) and adaptive immunity ($CD4^+$ and also $CD8^+$ T lymohocytes). T lymphocytes are activated against a donor's HLA antigens in the lymphatic tissue of a recipient via direct or indirect allostimulation of dendritic cells.

Primarily, $CD4^+$ cells are activated by a donor's APC in the lymphatic organs or in the graft. $CD4^+$ T lymphocytes produce Th1-type cytokines, which are used for $CD8^+$ cytotoxic T lymphocyte activation. These cells can migrate to the graft where they kill nucleated cells in the graft. Only $CD8^+$ cytotoxic T cells, which are generated by a direct recognition pathway of HLA alloantigens, can kill these cells; whereas $CD8^+$ or $CD4^+$ T lymphocytes generated by either direct or indirect alloantigen recognition can cause cytokine-mediated damage to the graft. $CD8^+$ cytotoxic lymphocytes generated by direct allorecognition recognize graft alloantigens and therefore kill graft cells that express these same alloantigens (Abbas et al., 2012). The $CD8^+$ T cells use cytotoxic mechanisms

for killing cells – degranulation of the perforin/granzyme enzyme system, interaction of the Fas receptor and Fas ligand and the secretion of lymphotoxin to induce apoptosis in the cells of the graft. Molecules of perforin create a lytic pore in the membrane of the target cell. The granzyme A and B enzymes penetrate through the lytic pore to the target cell and activate caspases and fragmentation of DNA which leads to apoptosis. The binding of Fas to the Fas ligand works similarly by activation of caspases and induction of apoptosis.

In the case that the alloreactive T cells are stimulated via an indirect pathway of recognition, the rejection is not caused by cytotoxic activity but by inflammation. This is caused by cytokines that produce either $CD4^+$ or $CD8^+$ T cells and mechanisms of delayed-type hypersensitivity (DTH) are also involved. The macrophages that are stimulated by Th1 cells infiltrate the graft and produce toxic nitric oxide, reactive oxygen species and TNF-α.

The cellular infiltrates of mononuclear cells in the biopsy of the kidney graft are used as a marker of proceeding acute cellular rejection in the graft.

1.3.2. Antibody-mediated (humoral) rejection

1.3.2.1. Hyperacute rejection

Hyperacute rejection is mediated by preformed antibodies against mismatched (most commonly HLA) donor antigens. The preformed antibodies are formed as the result of blood transfusions, pregnancy or previous transplantations. This type of rejection develops within minutes to hours after transplantation. The preformed antibodies bind to the endothelia cells of the graft, following the activation of the complement cascade (thrombosis in microcirculation as a consequence) and graft necrosis.

A CDC test is used for the detection of preformed donor specific HLA antibodies in the patient's serum and for the prevention of the incidence of hyperacute rejection

before kidney transplantation. A positive result in the CDC test is considered contraindication of transplantation. Preformed antibodies against erythrocyte AB0 antigens can also exist; therefore a transplantation which is in accordance with the patient's and donor's blood group is the best solution for avoiding hyperacute rejection.

1.3.2.2. Acute antibody-mediated rejection

In contrast to hyperacute rejection, acute antibody-mediated rejection (AMR) is mediated by de novo created donor-specific antibodies forming against the donor's antigen in the graft. Alternatively, the titer of pretransplant DSA may be very low and thus may not be considered as a contraindication for transplantation. AMR develops quickly over a few days and occurs days to years after transplantation. The primary goal of AMR is the endothelium of the graft. The most common HLA class I antibodies participate in AMR because they are expressed constitutively, whereas HLA class II antibodies are not expressed on endothelium at normal conditions. However, antibodies against HLA class II antigens (especially DQ) are quite common in patients waiting for retransplantation. MICA antibodies can also be involved (Viklický et al., 2008).

Naive B cells require $CD4^+$ T lymphocytes for their full activation; however, they simultaneously recognize mismatched HLA graft antigens which bind to their immunoglobulin receptors. Mismatched HLA antigens are swallowed, processed and presented by the HLA class II. Firstly, T cells have to be activated by DCs (direct or indirect recognition) in order to produce various cytokines. Subsequently, an interaction occurs between T and B cells, which is enhanced by the binding of the co-stimulating CD40 receptor and the CD40 ligand. T lymphocytes produce then Th2 cytokines. These cytokines and co-stimulatory factors of T lymphocytes assist forming B memory and plasma cells, antibody class switching and affinity maturation. Long-lived plasma cells produce donor-specific antibodies, and migrate to the bone marrow and continue to produce antibodies indefinitely, without requiring T lymphocytes (Colvin and Smith, 2005).

The essential mechanism, which asserts itself in AMR, is the binding to alloantigens on the surface of the graft. In this way, the classic pathway of the complement cascade can be activated which leads to the infiltration and activation of neutrophils and the forming of thrombus and lysis of target cells by the MAC (membrane attack complex). Binding DSA can also change the function of the endothelium through the induction of intracellular signals which increase surface expression of inflammatory and procoagulant molecules, and which in turn leads to further damage of the endothelium. However, DSA can harm the endothelial cells without the activation of the complement and might contribute to the pathogenesis of graft rejection. In this case, NK cells and macrophages damage the endothelial cells by the antibody-dependent cellular cytotoxicity (ADCC) mechanism. When a low-affinity Fc receptor on their surface binds on the DSA (binding on endothelial alloantigens), it then releases a mediator which causes apoptosis (Colvin and Smith, 2005).

1.3.2.3. Chronic antibody-mediated rejection

Chronic antibody-mediated rejection (CHR) is considered the main obstacle to long-term graft survival. The risk factor of chronic rejection is not only the mismatching of HLA antigens but also the previous control of acute rejection (Viklický et al., 2008). Innate and also adaptive components of immunity are involved. The CHR develops over months to years after transplantation. Immunological (cellular and antibodies-mediated) and non-immunological factors (influence of the toxic effect of immunosuppression medicaments, chronic viral infection) are also involved. Damage occurs to endothelial blood vessels and the proliferation of intimal arteritis is stimulated. The decrease of blood flow, perfusion of the graft and subsequent hypoxia leads to replacement of functional tissue by non-functional fibrotic tissue.

1.4. Borderline changes

It is necessary to detect early rejection changes in the graft in order to provide the best possible treatment. For this purpose the term borderline changes (BCs) is used. BCs include some changes which are characterized by T cell mediated rejection, polyomavirus infection or urinary tract infection (Yokoyama et at., 2014). However, there is no consensus on a standard therapeutic approach for BC. According to the Banff 07 classification, BCs are defined as no intimal arteritis but a foci of tubulitis with minor interstitial infiltration or intestinal infiltration with mild tubulitis (Solez et al., 2008).

1.5. Diagnostics of humoral rejection

The diagnostics of acute humoral rejection is dependnt on several factors: clinical evidence of acute graft dysfunction (a level increase of serum creatinine and tenderness and pain in the graft); histological evidence of acute tissue injury: neutrophils, macrophages or thrombi in capillaries, fibrinoid necrosis, or acute tubular injury; and immunopathological evidence for the action of antibodies: complement component 4d (C4d) deposited in peritubular capillaries, or antibodies or C3 in arteries. There is serological evidence for the presence of circulating HLA-specific antibodies or other donor-specific antibodies at the time of biopsy (Colvin and Smith, 2005).

The most frequent method for detection of HLA donor-specific antibodies is the use of xMAP technology (Luminex). It is used for the complement-dependent cytotoxic test (CDC) and also for the crossmatch test using flow cytometry (FCXM).

The C4d component of the complement is an inactive cleavage product which binds in tissue by a covalent bond. C4d cleavage fragments of the complement are stored in the peritubular capillaries of the graft and it carry over in the tissue after complement activation. For that reason, deposits of C4d are used as a marker, which is proved immunohistochemicaly or imunofluorescently (Feucht et al., 1991). See Figure 4 for the proposed stages of AMR progress.

The uniform Banff classification is used for evaluation of histological cuts from the kidney biopsy. Every two years the Banff meeting takes place in order to modify the criteria and specific recommendations for the detection of rejections. The last meeting was held in Brazil in 2013.

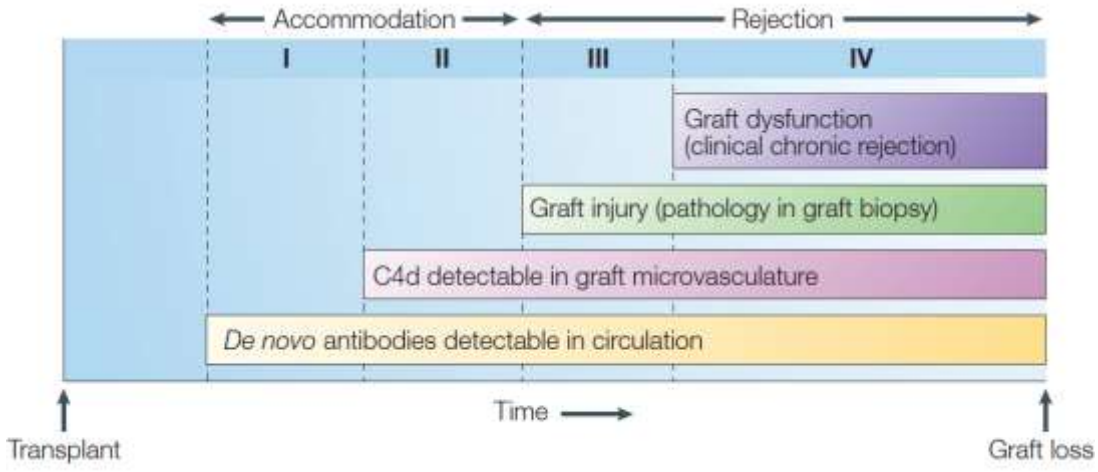

Figure 4: Proposed sequence of stages of antibody-mediated rejection: Donor specific antibodies (typically HLA molecules) might be produced at any time after transplantation. Pre-sensitized patients have circulating specific antibodies before transplantation. The time between the stages might range form days (acute) to months or years (chronic). The first two stages meet the criteria accommodation, because the graft is not pathologically injured despite circulating antibodies.

(Adapted from Colvin and Smith, 2005)

1.6. Immunosuppressive therapy of rejection

The success of organ transplantation depends (in addition to other clinical circumstances) on two important factors – the surgical procedure and the suppression of graft rejection by immunosuppressive therapy. Long-term immunosuppression is necessary in patients after transplantation and is often associated with side effects – infections, cardiovascular complications, nephrotoxicity and an incidence of tumors (lymphomas). Immunosuppressive therapy is divided into induction, maintenance and antirejection (Viklický et al., 2008).

1.6.1. Induction immunosuppressive therapy

Induction immunosuppressive therapy is used immediately after transplantation. It is based on the depletion or blocking of the function of T lymphocytes. M*etylprednisolone* is always used during operation and also a day after. Monoclonal (anti-CD52, anti-CD25) and polyclonal (rabbit anti-thymocyte globulin) antibodies can also be used against T cells. Monoclonal antibodies are sometimes used against B lymphocytes (*Rituximab* (anti-CD20)) as well, e.g. in the case of AB0 transplantation incompatibility (Viklický et al., 2008).

1.6.2. Maintenance immunosuppressive therapy

Maintenance immunosuppressive therapy is less intensive than induction therapy. The main aim is to prevent the incidence of acute rejection and ensure long-term survival of the graft. In clinical practice a combination of immunosuppressive medicaments (triple immunosuppression – inhibitor of calcineurin, anti-proliferative agents and corticosteroid) are commonly used. The combination of medicaments is necessary for affecting different levels of immune response. The advantage of triple immunosuppression is that only a small dose of medicaments can be used (Viklický et al., 2008).

Antirejection therapy is used only in case there is pre-existing knowledge of histological evidence due to side effects.

1.7. Immunological memory in transplantation

Immunological memory is the ability of the immune system to efficiently recognize and quickly respond to a secondary encounter with a pathogen. However, during the immunology reaction against the graft, long-lived memory T and B cells are also formed. The memory cells are essential for defending against invading pathogens, but on the

other hand can threaten the graft's survival. Many of the T cells that respond to an allogenic HLA molecule (even on first exposure) are memory T cells. It is likely that the memory cells are generated earlier during previous exposure to other foreign (e.g., microbial) antigens and cross-react with allogenic HLA molecules. These memory cells are more rapid and powerful than naive lymphocytes, and thus contribute to the strength of the alloreactive T cell response. The memory cells seem to be more resistant to immunosuppression than naive lymphocytes and the presence of a huge number of memory cells may lead to unfavorable transplantation outcomes (Abbas et al., 2012). Moreover, memory T cells demonstrate a much lower activation threshold than naive T cells. They are also less dependent on the CD28 co-stimulation signal. Furthermore, they are not confined to lymphoid tissue. Memory T cells inhabit both lymphoid and non-lymphoid tissue (e.g., the liver, the lung and the gut) which means that they are easily accessible to antigens. Additionally, memory T cells do not require secondary lymphoid organs in contrast to naive T cells. They are also less highly resistant to tolerance induction (Li et al., 2013).

So far, it is not known, whether the presence of antibodies, which are specific for graft antigens, is maintained as a consequence of the longevity of plasma cells or as a consequence of the continuous generation of new memory B cells. It is also possible that long-term grafts might become *neo-lymphoid organs* with organized lymphoid tissue. Stimulation through the indirect pathway of recognition can occur in such grafts through the infiltration of recipient DCs and the activation of the endothelium in response to present antigens (Colvin and Smith, 2005).

1.8. HLA-specific antibodies

HLA-specific antibodies can be developed before transplantation by blood transfusion, pregnancy or previous transplantations. Patients who are sensitized to HLA usually have a prolonged waiting time for a transplant and have an increased death rate due to the risks of long-term dialysis. In transplantation, HLA antibodies are created against HLA-mismatched antigens of the organ donor (DSA). Patients who produce even

low level of DSA are predisposed to increase incidenced of acute humoral rejection as well as chronic rejection, leading to graft failure (Süsal et al., 2009). In order to cerrecly determine DSA before transplantation, the CDC test is performed. A positive result of this test is considered as a contraindication for transplantation. In patients, who are inscribed into the transplantation kidney waiting list, panel of reactive antibodies (PRA)[1] test is regularly performed (four times per year). This test detects the level of HLA antibodies present in a patient's serum. Patients with high levels of HLA antibodies are considered hypersensitized patients. This special group of patients must undergo precise desensitization treatment before transplantation for the removal of antibodies. Desensitization treatments commonly used are plasmapheresis, immunoadsorption and the use of intravenous immunoglobulins. The presence of DSA HLA antibodies can also be determined by FCXM, or by using enzyme-linked immunoabsorbent assay (ELISA) and Luminex methods, which are more sensitive than the CDC test. As already mentioned, the specific HLA antibodies are associated with an incidence of AMR after transplantation, which can lead to graft damage. Therefore, the timely production and diagnosis of these antibodies is essential and useful for the adequate treatment of transplanted patients.

1.9. MICA antibodies

MICA antibodies are of the either IgG or IgM isotype. The clinical relevance of MICA-specific antibodies is still unclear. However, it has been reported that MICA antibodies pose a risk for rejection and are more frequent in patients who have rejected their graft than in those who have not (Zou et al., 2006). MICA antibodies are able to harm the endothelium of a graft by the mechanisms of complement cascade activation or ADCC (the antibody is bound on the endothelial cells and recognized by the Fc immunoglobulin fragment of NK cells which can liquidate these cells by lysis).

[1] PRA is the screening of antibodies against HLA antigens in sera of potential organ recipients. The test is usually performed using the CDC test. It is one of the essential examinations performed before inscription of patients onto kidney waiting list. After that it is repeated four times per year for every patient during the waiting time for transplantation.

1.10. Kidney graft allocation in the Czech Republic

In the Czech Republic, patients can receive kidneys from living or deceased donors. Kidney graft allocation applied for deceased donors is organized by the Coordinating Center of Transplantation (Ministry of Health). The main worldwide problem is that there are more patients waiting for organs than donors. In the Czech Republic, therefore, a few rules have been established for equitable allocation of kidneys.

The main aim of kidney allocation is to offer the kidney to the patient who has either the expectancy for the longest function of the organ or has a limited time to wait for transplantation due to medical reasons. There are two criteria taken into consideration – medical and nonmedical. Under medical criteria the following are considered: HLA match, panel reactive antibodies (PRA), blood group and clinical status (urgency, children). A match in blood group is ideal but the patient can also aquire an organ from a donor with a different blood group (Table 2) – ABO incompatibility donors (Maggiore et al., 2014). The nonmedical criteria involve the waiting time and balance between retrieved and transplanted kidneys of transplantation centers.

Donor	Recipient
A	A, AB
B	B, AB
AB	AB
0	0, B

Table 2: Possible blood groups in recipients.

The recipient selection process operates as follows: priority is given to urgent patients who are in an imminent life-threatening state due to impossibility of dialysis. Secondary priority is given to patients with a full HLA match (only HLA-A, -B,- DR), in donor and child patients up to 18 years old. After that come patients with special medical indications, i.e. indicated for combined transplantation – pancreas, liver, heart, etc. Alternatively, there are cases when the success of another operation (urological, angio-) is dependent on successful transplantation. The PRA are also taken into consideration – hypersensitized patients who have an actual PRA of 80-100 % are preferred. Finally, long-waiting patients who have been actively waiting longer than 5 years are selected. The period when the patient is, for various reasons, excluded from the waiting list is not taken into consideration. The balance between centers is also applied – the first kidney goes to the first patient from the allocation list and the second kidney to the first patient from the transplant center where the kidney was retrieved.

There is also the possibility of acquiring a kidney from a living donor - either from a relative donor (parents, siblings) or nonrelated donor, and alternatively within a kidney paired exchange program. Kidney transplantation from living donors has several advantages, of witch, the main advantage is longer graft survival (Viklický, 2010). Informed consent is required from all potential donors.

Before kidney transplantation from living donors, more time is allowed for extensive testing of both patients and potential donor(s), for planning the date of the transplantation and choosing the best donor. A living donation enables the possibility for better preparation of the patient (immunosuppression, immunoadsorption) and reduced of the risk of rejection. Another advantage is to perform transplantation before the initiation of dialysis therapy. Moreover, during the transplantation itself, there is only a brief perioda of kidney ischemia.

However, a living donation is also an advantage for donors because after nephrectomy (surgical removal of kidney) the donor (and also the patient) is monitored for a long period for potential medical complications which can be revealed earlier. Certainly, it is necessary to take into consideration the risk of kidney donation for donors in case the donor should be harmed by nephrectomy. This policy must be followed

strictly even if it means refusing a last chance to designate a living donor to a patient (Allen et al., 2014).

1.11. Kidney retransplantation

Kidney retransplantation is necessary in patients who have undergone kidney transplantation with subsequent graft failure and who have been enrolled in a new dialysis program. However, the mortality rate of patients who undergo dialysis after graft failure is equal to the mortality rate of patients who are not transplanted, so dialysis does not pose more risk for a retransplanted patient. The kidney grafts may fail due to various reasons: chronic rejection, acute rejection, surgical complications, vein/arterial thrombosis and recurrence of the original diseases – e.g. glomerulonephritis, diabetes mellitus, etc (Arce et al., 2010). Retransplantation reduces the mortality of patients by 23-45% (Fadli et al., 2014).

Before retransplantation, a nephectomy of the previous graft may be carried out; however, this depends on the clinic's decision and is not obligatory. Graft survival after repeated transplantation is not associated with patient age, time of functioning of the first graft, time between first and second transplantation, duration of dialysis before second transplantation or cold ischemia time. However, graft survival may decrease the post-transplantation presence of HLA class I and class II antibodies (Fadli et al., 2014).

The survival of the first graft is an important factor for the subsequent retransplant outcome. Patients who have had an acute rejection episode during their first transplantation are significantly more likely to develop acute rejection after retransplantation (Heaphy et al., 2014). Moreover, it has been reported that the patients who lose their first allograft within 36 months after transplantation, are at increased risk of a second allograft loss compared to patients with an initial allograft lasting more than 36 months. In patients whose first graft survival is longer than 3 years, the relative risk of repeated graft failure declines in a linear fashion (Arndorfe et al., 2001).

Patients who are indicated for retransplantation belong to a high-risk category because highly allosensitized patients pose an increased risk of rejection. The allosensitization of patients is higher in patients who are waiting for retransplantation, so it correlates with the number of received transplants, even in cases when the graft has failed due to non-immunological reasons. Almost every patient (about 90%) awaiting retransplantation produces alloantibodies (Moszkowska et al., 2014) and have higher PRA levels compared to primary recipients (Heaphy et al., 2014). Pretransplantation characterization of HLA-specific antibody profiles can be useful for finding the most suitable donor, for proper desensitization therapy and subsequently for better treatment (Moszkowska et al., 2014). It has also been confirmed that production of antibodies against both HLA class I and class II antigens is associated with impaired HLA mismatched graft outcome (Süsal and Opelz, 2002).

2. Aims of the diploma thesis

- Evaluation of the risk of development and the incidence of antibody-mediated rejection in patients after kidney retransplantation with respect to non-acceptable (forbidden) HLA antigens.
- Comparison of data from retransplanted patients with patients who awaiting a first kidney transplantation
- Comparison of the kidney graft outcomes (up to one year) in a retransplanted patient with forbidden HLA antigens with patients who were retransplanted without taking forbidden HLA antigens into consideration (transplanted before 2011).
- Assessment of the occurrence of HLA-C, HLA-DP and HLA-DQ antibodies in a retransplanted patient and their influence on the waiting time for kidney transplantation.

3. Materials and methods

3.1. Methodological approach

234 patients (162 males and 72 females) were included in the study for forbidden (non-acceptable) antigens. These patients were tested during the years 2011-2013. The patients not only came from IKEM, but also from other transplant centers in the Czech Republic. All patients were inscribed onto the waiting list for kidney retransplantation (they had been transplanted at least once).

All patients and their previous donors were HLA typed by polymerase chain reaction (PCR). The donors were typed by PCR with sequence-specific primers (PCR-SSP) and the patients were typed by PCR with sequence-specific oligonucleotide probes. DNA was isolated from non-coagulated blood (with EDTA). Testing for HLA-specific antibodies was performed in the following way: all patients inscribed into the waiting list were regularly (four times a year) tested for panel reactive antibodies by the complement – dependent cytotoxic test. For detection of donor-specific antibodies before and after transplantation, the CDC crossmatch and flow cytometry crossmatch tests were used. The FCXM test was also used for the diagnosis and monitoring of treatment of the humoral (antibody-mediated) rejection. Lymphocytes for CDC and FCXM tests were isolated from peripheral blood (living donors) or from lymph nodes or spleens (deceased donors).

All retransplanted patients had a negative result for the CDC crossmatch test and had been monitored for acute rejection and graft survival for a period of over one year. All patients were analyzed for the incidence of acute cellular rejection (ACR), acute humoral rejection (AHR), chronic humoral rejection (CHR) or borderline changes according to the updated Banff classification (Haas et al., 2014).

3.2. Forbidden (unacceptable) antigens

In our center, forbidden (non-acceptable) antigens for subsequent kidney transplantation are defined as mismatched HLA antigens of the previous kidney donor(s), against which patients produce HLA-specific antibodies. Forbidden antigens are determined using the Luminex method. Antibodies are evaluated against HLA-A, HLA-B and HLA-DR antigens because these antigens are taken into consideration in the organ allocation in the Czech Republic.

Example of forbidden antigens:

	HLA - A	HLA -B	HLA -DR
Patient	1	7, 18	8, 11
Donor 1.Tx	1	8, 18	11, 15
Donor 2.Tx	2	18, 35	4, 11
Mismatched Antigens	2	8, 35	4, 15
Forbidden Antigens	2	8	-

Table 3: Determination of forbidden antigens.

(Description in the text.)

Table 3 shows the principle of forbidding HLA antigens in patients with two previous transplantations. The HLA phenotype of a patient, who has been transplanted twice, is in the first row and now he is waiting for a third transplantation. The typing of

the previous two donors is indicated below. Antigens A2 and B8 (red in the table) are the forbidden antigens against which the patient produces antibodies, i.e. the patient has HLA-antibodies specific to these two antigens. The mismatched antigens: B35, DR4 and DR 15 (blue in the table), are allowed for the next transplantation because the patient does not produce antibodies against these antigens.

3.3. xMAP method (Luminex)

At present, xMAP (Luminex) technology is the most sensitive method for the detection of HLA specific antibodies. Fluorescently labeled polystyrene beads that are coated on their surface by HLA class I or class II antigens are used for antibody detection. These polystyrene beads are manufactured by recombinant technology and are also defined at the allele level (subtypes of antigens). The beads contain two fluorescent dyes mixed in different concentrations; therefore, the various subgroups of beads with HLA antigens on the surface are identified easily. If the antibody from patient serum binds to an HLA antigen on the bead's surface, it is detected by a secondary anti human IgG antibody, labeled with Phycoerythrin (PE). See Figure 5.

The intensity of fluorescence is measured using a double-laser cytometer (Luminex 200 IS 2.3.). The red laser excites the fluorescence inside the bead (it detects the concrete bead), while the green laser emits the fluorescence of PE on the secondary (anti-human IgG) antibody. A negative serum is also included in each test, thus the background data is set for every bead accordingly.

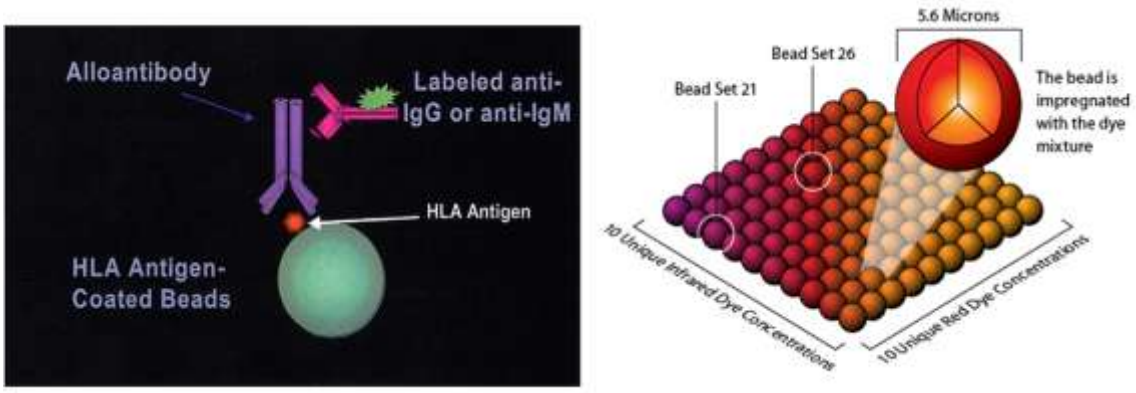

Figure 5: Principle of the xMAP method. Explanation in the text.

(Adapted from OneLambda Inc.).

The Luminex LABScreen Mixed test provides a basic serum screening for the presence or absence of HLA-specific antibodies. It determines whether the antibodies are directed against HLA class I, class II antigens or MICA antigens.

The LABScreen Single Antigen HLA Class I and Class II Antibody tests provide precise information on the specificity of anti-HLA antibodies. Every fluorescent bead has only one HLA antigen on its surface and a specific intensity of fluorescence, so it is identified easily.

Performance of the test:

Before starting the test, it is necessary to vortex the LABScreen beads thoroughly. 20 µl of the patient's sera are incubated in a 96-well plate with 5 µl of LABScreen beads for 30 minutes in the dark at 20°C on a rotor (slightly shaking). The negative control for each test is also tested– 20 µl of negative serum (commercially provided) and 5 µl of LABScreen beads. The assumption is that if there are antibodies against HLA-antigens in the serum, they bind to HLA antigens on the surface of the beads during the incubation. Negative control microbeads do not bind to HLA antigens on their surface.

After the first incubation the plate is washed three times. The first washing is performed with 150 µl wash buffer solution, while the second and third with 200 µl.

During the washing steps, the plate is centrifuged for 5 minutes at 1300 g and the supernatant is removed by flicking and draining on filter paper.

The Phycoerythrin (PE) labeled anti-IgG antibody is diluted 100 x with wash buffer solution. Each sample is incubated with 100 µl anti-human IgG for 30 minutes in the dark at 20°C on the rotor (slightly shaking).

The plate is centrifuged after the incubation and then washed twice with wash buffer solution.

The last step is the addition of 80 µl PBS to each sample. The samples are then ready for measurement and can be stored at 2°-8°C in the dark for 24 hours. The plate is measured by the double-laser Luminex 200 IS 2.3.

Evaluation of the samples is performed using HLA-FUSION software. The cutoff is defined based on the analysis of negative control serum and the ratio of fluorescence intensity of negative and positive control beads. The fluorescent signal of PE is interpreted as MFI (mean fluorescence intensity) and represents the intensity of fluorescence of the secondary (PE labeled) antibody. The MFI of the negative control (NC) should be lower than 500 MFI, the value of positive control (PC) should be higher than 500 MFI and the ratio of the positive/negative control should be higher than 2.5. The number of beads analyzed should be 100, but can be analysed at a minimally level of 50 beads.

An advantage of the Luminex technique is that no living donor cells are required (in comparison with the FCXM test, see below).

3.4. Isolation of lymphocytes from spleens, lymph nodes and whole blood (deceased and living donors)

The procedure for the isolation of donor lymphocyte suspension (necessary for the CDC and FCXM tests) is described below

3.4.1. Isolation of lymphocytes from spleens and lymph nodes

An injection syringe with needle and PBS is used for washing up cells from spleens or lymph nodes of deceased donors. The cell suspension from spleens is necessary to filter for removing of cell clots. The cell suspension is then layered on a Ficoll Paque gradient solution and centrifuged for 20 minutes at 700 g. After that, rings of lymphocytes are collected using a Pasteur pipette and washed twice with IMDM with 20% FBS (centrifuged for 10 minutes at 400g). The lymphocyte number is determined in a Bürker chamber using a phase contrast microscope. The lymphocytes are diluted to 1 x 10^6/ml for the FCXM test and 2-3 x 10^6/ml for the CDC test. The cell suspensions from spleens must be purified by magnetic separation using the EasySep kit for negative selection of T and B lymphocytes.

3.4.1.1. EasySep separation of T and B lymphocytes

A 50 µl cocktail of monoclonal antibodies is added to the cell suspension, vortexed and incubated for 10 minutes. 100 µl magnetic beads are then added to the suspension and incubated for 10 minutes. 1.5 ml 2% PBS with FBS are then added, inserted into the magnet and incubated for 5 minutes. The magnet is then inclined and the cell suspension is poured out carefully. The number of lymphocytes (T or B) is determined in the Bürker chamber. Then the lymphocytes are diluted to 1 x 10^6/ml or 2-3 x 10^6/ml for the FCXM and CDC tests respectively.

3.4.2. Isolation from whole blood

The heparin whole blood is diluted into a 1:1 ratio by PBS pH 7.2-7.4. The suspension is then layered on a Ficoll Paque gradient solution and centrifugated for 20 minutes at 700g. After centrifugation, the rings of the lymphocytes are collected using a Pasteur pipette and washed twice with 20% IMDM with FBS (centrifugated for 10 minutes at 400g). The lymphocytes are diluted to 1 x 10^6/ml for FCXM and 2-3 x 10^6/ml for the CDC test.

3.5. Complement-dependent cytotoxic crossmatch test (CDC crossmatch)

The CDC crossmatch test uses the principle of the complement-dependent microlymphocytotoxic test, which is one of the oldest and still commonly used methods in HLA laboratories (Terasaki a McClelland, 1964). The CDC test (also applied for serological HLA typing for many years) is now mostly used as a crossmatch test for the detection of performed cytotoxic antibodies against mismatched HLA antigens of the donor. The test is performed before kidney transplantation in order to exclude hyperacute rejection caused by preformed donor-specific antibodies and a positive result is usually a contraindication for transplantation.

Donor cells are incubated (1 µl/well) with the serum of the recipient (1 µl/well) on Terasaki microtiter plates for 30 minutes at room temperature. Positive and negative control sera (PC and NC) are also included. After incubation, a rabbit complement is added to each well (5 µl) and incubated for 60 minutes at room temperature. Eosin dye is added (1 µl/well) and after a 5-minute incubation period the reaction is stopped by 30% formaldehyde (1 µl/well).

If the antibodies in the patient serum bind to the cell surface antigens the complement cascade is activated which leads to lysis and cell death.

Donor cells are lymphocytes isolated from peripheral blood (living donors), lymph nodes or spleens (deceased donors). Rabbit serum, which is tested for the absence of anti-HLA antibodies, is used as a source for the complement. After dyeing the living/dead cells with eosin (or trypan blue), the reaction is evaluated under a microscope with a phase contrast.

The results are assigned according to percentage of dead cells as strongly positive (80-100% (8)), positive (40-80% (6)), weakly positive (20-40% (4)) and negative (0 – 10% (1)). It is necessary to assess the reaction with regard to both control reactions (PC and NC). The positive control is a serum which contains anti HLA antibodies against most HLA specificities (PRA 95% and higher). The negative control is a serum without HLA antibodies (usually male AB Rh- serum).

3.6. Flow cytometry crossmatch

Flow cytometry crossmatch (FCXM) is more sensitive (up to a hundred times, according to the procedure) than the CDC test. In contrast to the CDC test, it detects donor-specific antibodies that do not bind complement as well. FCXM is based on the principle of binding donor-specific antibodies to antigens of donor cells (mostly HLA antigens) and subsequently detecting them with fluorescently labeled (mostly Fluorescein Isothiocyanate - FITC) anti-human IgG antibody. T and B lymphocytes are incubated with fluorescently labeled monoclonal antibodies. This test is performed (in IKEM) in patients waiting for a living donor before transplantation and in patients with suspected antibody-mediated rejection.

Performance of the test:

The recipient's serum (50 µl) is incubated in tubes with donor lymphocytes (1 – 1.5 x 10^6/ml) for 30 minutes in the dark and at 20°C. A negative control is also tested (50 µl negative control serum and lymphocytes) with each donor sample. The positive

control (positive control serum and a pool of lymphocytes from 30 different donors) and the blank (PBS and a pool of lymphocytes) is tested at the same time. After incubation, the cells are washed three times with PBS (centrifugation for 5 minutes at 400 g).

After washing, 5 µl anti CD3 antibody labeled with Phycoerythrin (PE), 5 µl anti CD19 antibody labeled with Phycoerythrin-Cyanin 5,1 (PerCP) and 5 µl goat anti-human IgG antibody ($(Fab)_2$ fragment) labeled by FITC are added to each tube. Samples are again incubated for 30 minutes in the dark at room temperature. After the incubation, these samples are washed three times and 500 µl cellfix is added to each tube. The samples are then analyzed by a flow cytometer FC500 (Beckman Coulter).

It is necessary to assess the test with regard to both controls (positive and negative). Cutoff for positivity reaction is determined as an X-mean of fluorescence of the sample divided by the X-mean of the negative control (cutoff for T lymphocytes: x-mean > 2.0; cutoff for B-lymphocytes: x-mean > 2.5).

If present, IgG antibodies in recipient serum bind to donor cells during the first incubation. These antibodies are detected by the FITC-labeled goat polyclonal antibody $(Fab)_2$ against IgG heavy chains. Donor T cells are stained by the anti-CD3 monoclonal antibody labeled with PE. Positivity of T cells in FCXM indicates the presence of donor-specific antibodies against HLA class I antigens. B cells are detected by binding of anti-CD19 monoclonal antibody labeled by PC5. Since B cells express both HLA class I and class II antigens, positivity of B cells indicates the presence of donor-specific antibodies against HLA class I, class II antigens alone or both – class I and class II.

False positive reactions can be caused by patient treatment with humanized monoclonal antibodies (Rituximab (anti-CD20), Campath (anti-CD52), etc.).

3.7. Polymerase chain reaction (PCR)

The polymerase chain reaction is used for the purpose of typing HLA-A, HLA-B and HLA-DR loci of donors and recipients. For typing donors and recipients, different types of PCR reactions are used: PCR – sequence-specific primers for donors and PCR-sequence-

specific oligonucleotide probes for patients. In this paragraph, the general principles of PCR reaction are explained (Fig. 6). Different types of PCR are explained below.

PCR – performance of the test

The PCR reaction is a laboratory method which is, in principle, DNA replication performed in-vitro conditions. The PCR reaction mixture contains four deoxynucleotide triphosphates (dTTP, dGTP, dCTP and dATP), oligonucleotide primers, PCR buffer and Mg^{2+} ions. During the PCR reaction, only a defined DNA segment is amplified, not the whole molecule. The synthesis of the complementary strand is catalyzed by the thermostable DNA polymerase from the thermophilic bacterium, *Thermus aquaticus*. DNA denaturation is carried out by heating (90°C or higher, depending on the GC content of DNA). To define the concrete segment of DNA for amplification, two oligonucleotide primers are used – these are artificially synthesized single-strand DNA segments approximately 20 bp in length. The primers´ sequence is complementary to the 3' terminal sequence of both strands of the target DNA. By the binding of primers to denatured complementary segments (annealing), Taq DNA polymerase is activated and the amplification process is started. During the whole PCR reaction, precise changes of temperature in the reaction mixture are ensured by a PCR thermocycler.

The PCR reaction consists of the following steps: Denaturing of the double-strand DNA molecule is performed at temperatures ranging from 90°C to 95°C (15 seconds to 2 minutes). Annealing is executed at 40°C to 60°C (30 – 60 seconds). Synthesis of the second DNA strand is achieved at 72°C – 74°C (30 - 50 seconds). These three steps are repeated 20 – 40 times.

The PCR-SSP system consists of several independent PCR reactions; each reaction has a specific pair of primers to identify SNP (single nucleotide polymorphism). The conditions of the PCR reaction must be optimized (concentration of template DNA, concentration of Mg^{2+} ions, oligonucleotide primers and PCR buffer) to ensure the primer annealing to high specificity. The amplified fragments of DNA are then separated by agarose gel electrophoresis and visualized using UV-sensitive dye. The dye is

dispersed in an agarose gel, binds to the DNA and makes it visible under UV light. The result of electrophoresis is the presence or absence of a PCR product(s) of a certain size. The picture of a the gel is assessed using Score software.

Commercially manufactured kits were used for performing the test (according to manufacturer instructions). Briefly, genomic DNA was mixed with a PCR master mixture and Taq polymerase and aliqoted to the wells on the PCR plate with pre-pippeted lyophilized sequence specific primers.

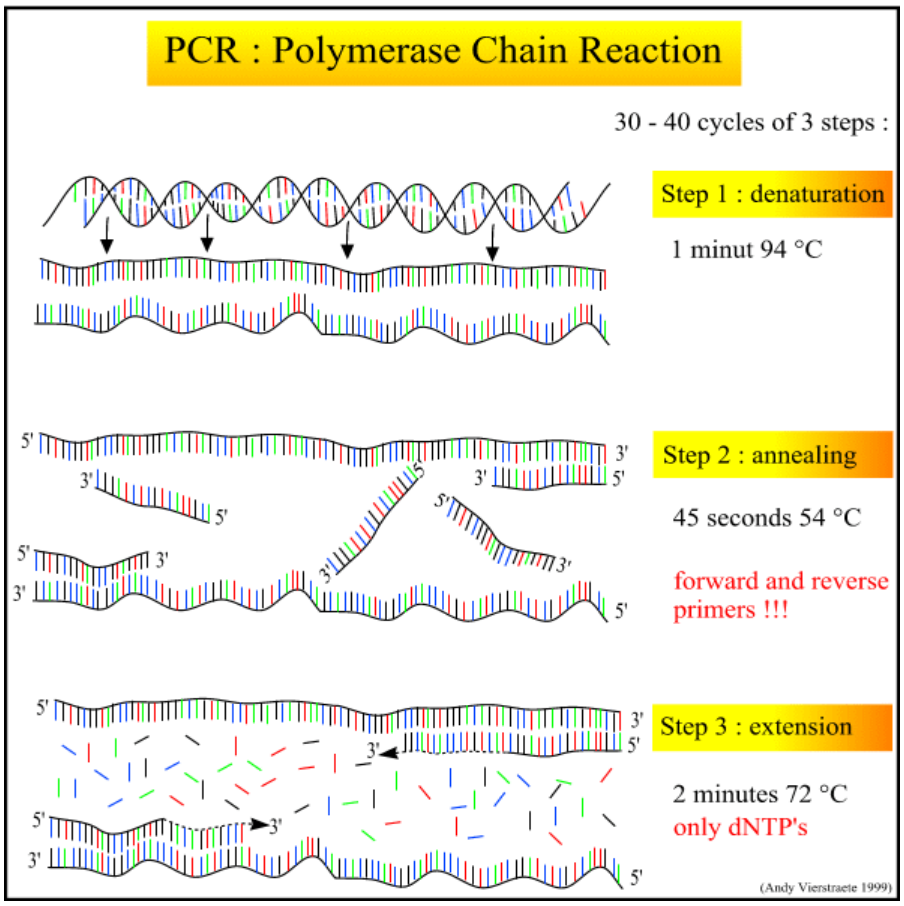

Figure 6: Principle of the PCR.

(Adapted from: http://users.ugent.be/~avierstr/principles/pcr.html)

3.7.1. PCR – SSP (Sequence specific primers)

The PCR – SSP method is used for typing A, B and DR HLA antigens of organ donors. The principle of this method is a PCR reaction which uses specific primers to detect SNPs. The definition of several SNPs allows an assignment of a group of alleles or an individual allele at a given locus. Testing at the level of allele groups is called low resolution, and high resolution typing at the allele level. Low resolution typing is used for recipient-donor searches. High resolution typing is used in uncommon cases, e.g. for the definition of allele-specific antibodies. Figure 7 is shows picture of electrophoretic gel in an SSP-PCR.

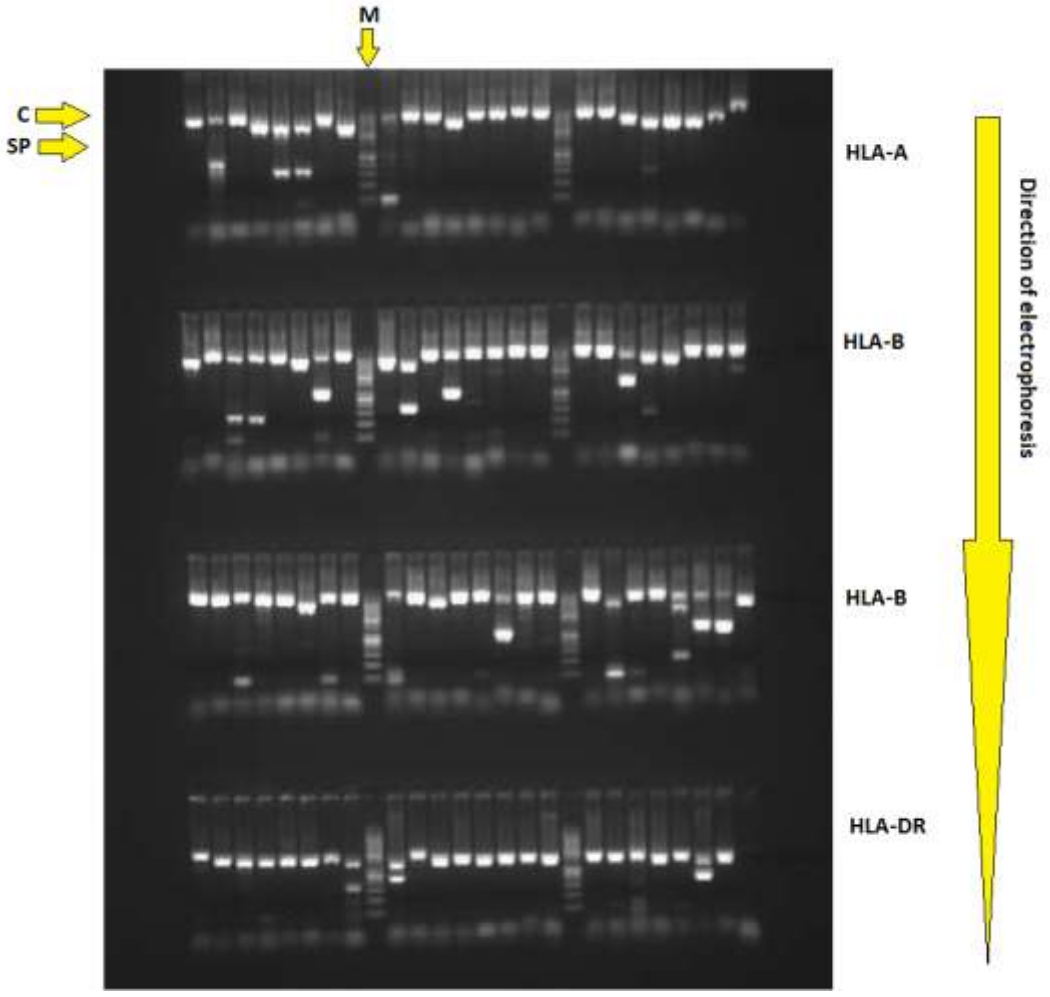

Figure 7: Picture of electrophoretic gel. First line from above belongs to the control bands (800 or 1000 bp) (C); specific products (SP) can be seen under the control bands (80 – 500 bp); M= DNA ladder.

3.7.2. PCR-Sequence-Specific Oligonucleotide Probes (SSOP)

The PCR-SSOP is another method of choice for A, B and DR HLA typing. The principle of this two-step method is the detection of SNPs using sequence-specific oligonucleotide probes which hybridize to PCR products. The target sequence of the whole gene (locus) is primarily amplified by the PCR reaction (described above) using specific primers in the first step. One of the primers is labeled with biotin for further detection in the hybridization step using R-phycoerythrin-conjugated streptavidin (SAPE). The PCR product is then denatured and hybridized with complementary DNA probes conjugated to fluorescently-labeled microbeads in the second step. These microbeads are measured using the flow analyzer Luminex 200 IS 2.3 flow analyzer. The set of two independent lasers identifies fluorescence intensity of both microbeads and phycoerythrin-labeled fragments on each microbead. The result of the Luminex measurement is evaluated using HLA-FUSION software and is based on each probe's comparison of reactivity of to published sequences of HLA genes.

Hybridization

The PCR product (5µl) is mixed with a denaturation buffer and incubated for 10 minutes at room temperature. The bead bixture is diluted with the hybridization buffer (beads have to be stored in dark). 5µl of the neutralization buffer is added to the suspension. Then 38µl of the hybridization mix is added to each sample and incubated for 15 minutes in a thermocycler. After incubation, 100µl of the wash buffer is added, vortexed and centrifugated for 5 minutes at 1454g. The supernatant is removed by flicking, this step is repeated twice. During the third washing step SAPE is prepared and stored in the dark. 50µl of SAPE is added to each sample, vortexed and incubated for 5 minutes in the thermocycler. After incubation and one wash, 70µl of the wash buffer are added to each sample and vortexed, after which the samples are ready for measuring on the Luminex.

3.8. Statistical analysis

The statistical software MedCalc (version 12.2.) was used for statistical evaluation. The Chi-square test, Fisher's exact test, the Chi-square test in a frequency table and the Chi-squared test for trend were used for comparing the groups of tested patients. We considered the value $p < 0.01$ as statistically significant.

4. Results

A cohort of 234 patients (162 males and 72 females) was included in the study for forbidden antigens, which were tested during the years 2011-2013. All patients were waiting for retransplantation, i.e. they had been transplanted at least once (mainly kidney transplantations – 283, 16 combined transplantations of the kidney and the pancreas, 2 transplantations of the liver, 2 transplantations of pancreatic islets, 1 combined transplantation of the kidney and pancreatic islets and 1 transplantation of the heart). Most patients (171 patients) were waiting for a second transplantation , 56 were waiting for a third transplantation, 6 patients for a fourth and 1 patient was waiting for a sixth transplantation (Fig. 8).

The age at the time of first retransplantation was similar in males and females (35 and 49 years respectively), as shown in Table 4. A mean of the number of previous donors is also shown.

Gender	Mean age at the time of 1st Tx	Mean age at the time of waiting for reTx	Mean number of previous Tx
Male	35,9	49,6	1,2
Female	34,4	48,4	1,5
Total	36,2	49,2	1,3

Table 4: Mean age at the time of 1st Tx and reTx in males and females, number of previsous Tx.

All patients were tested using the Luminex method to detect the level and specificity of HLA antibodies. The patients and all their previous donors were HLA typed at the A, B and DR loci (533 HLA typing - patients and donors together). According to the level and specificity of HLA antibodies and the HLA typing of the previous donor(s), we determined the donor-specific antibodies. These were subsequently banned for the next transplantation.

The data from our study group was compared with data from 267 patients (179 males and 88 females) waiting for a first transplantation. These patients were tested for the presence or absence of HLA antibodies using the Luminex method.

We also retrospectively evaluated the data from 63 patients (40 males and 23 females) who did not have determined forbidden antigens as the control group. They had undergone transplantation between the years 2009-2010.

The data from the study group of 50 (34 males and 16 females) patients retransplanted between the years 2011-2012 were compared with the control group. All retransplanted patients were followed up for incidence of acute rejection and graft survival over a period of one year.

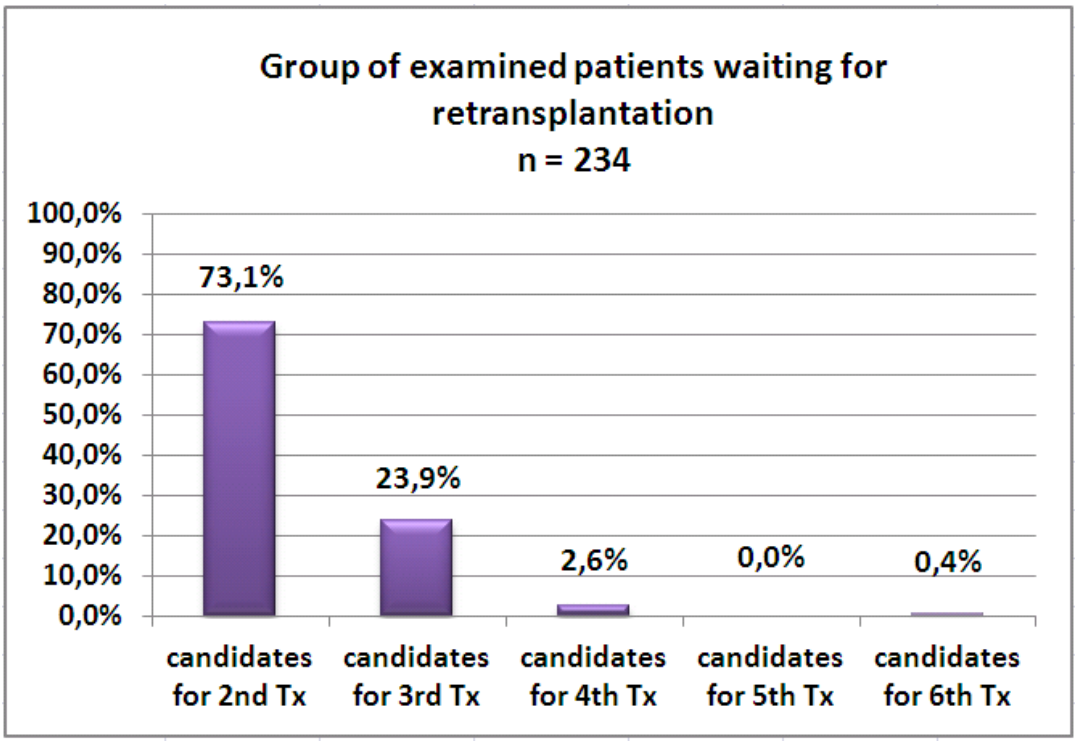

Figure 8: Distribution of patients (in %) waiting for retransplantation. 171 candidates for the 2nd transplantation, 56 for the 3rd, none for the 4th and 1 for the 5th.

4.1. Comparison of blood groups in the study group

The patients and donors blood groups were also evaluated to verify if there were any differences between the two cohorts. 501 deceased donors (tested in IKEM during in the years 2011-2013) were included in the group. Figures 9 and 10 show the distribution of blood groups for each of the tested groups. There were no significant differences between these two groups (p=0.13). Additionaly, the comparison with Caucasian populations shows a similar distribution between the blood groups, with the exception of blood group 0 (Fig. 11).

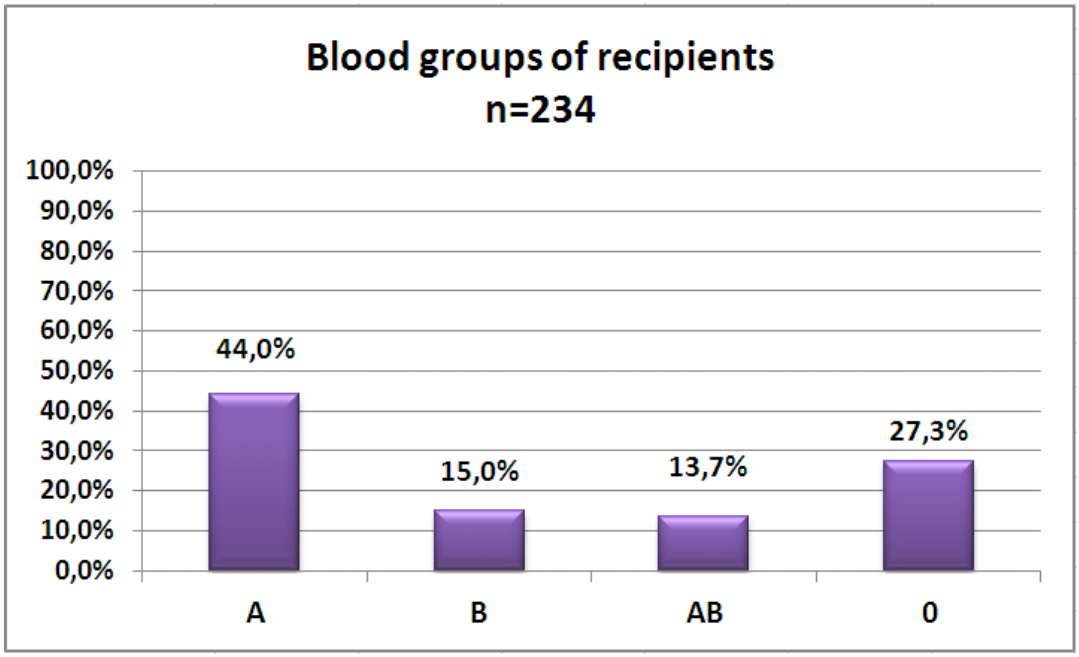

Figure 9: Distribution of blood groups in recipients (in %).

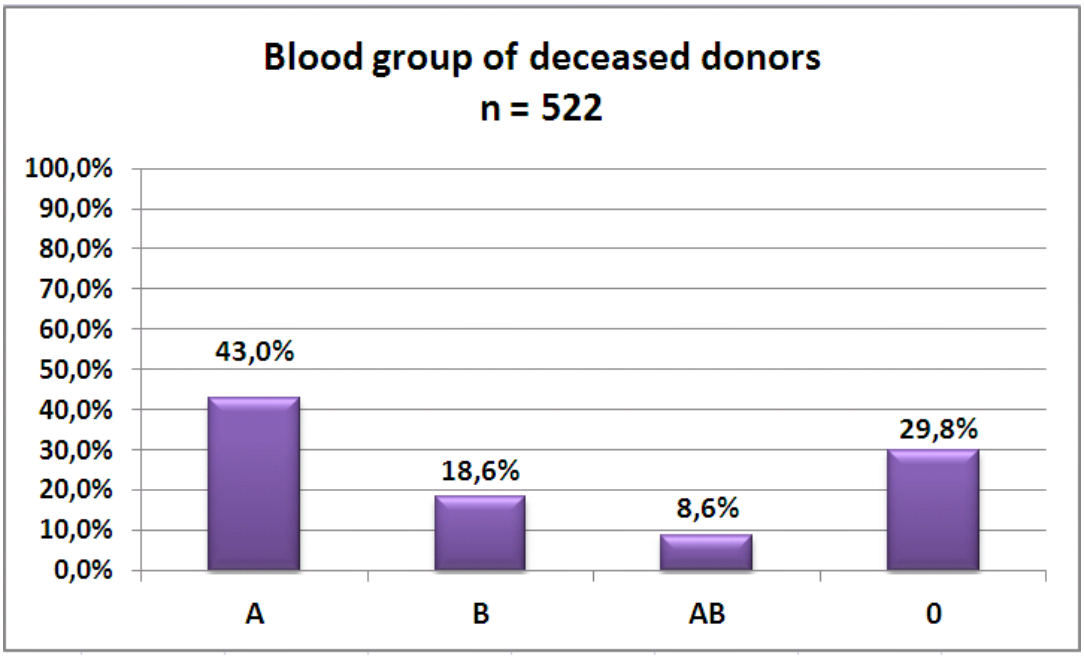

Figure 10: Distribution of blood groups in deceased donors (in %).

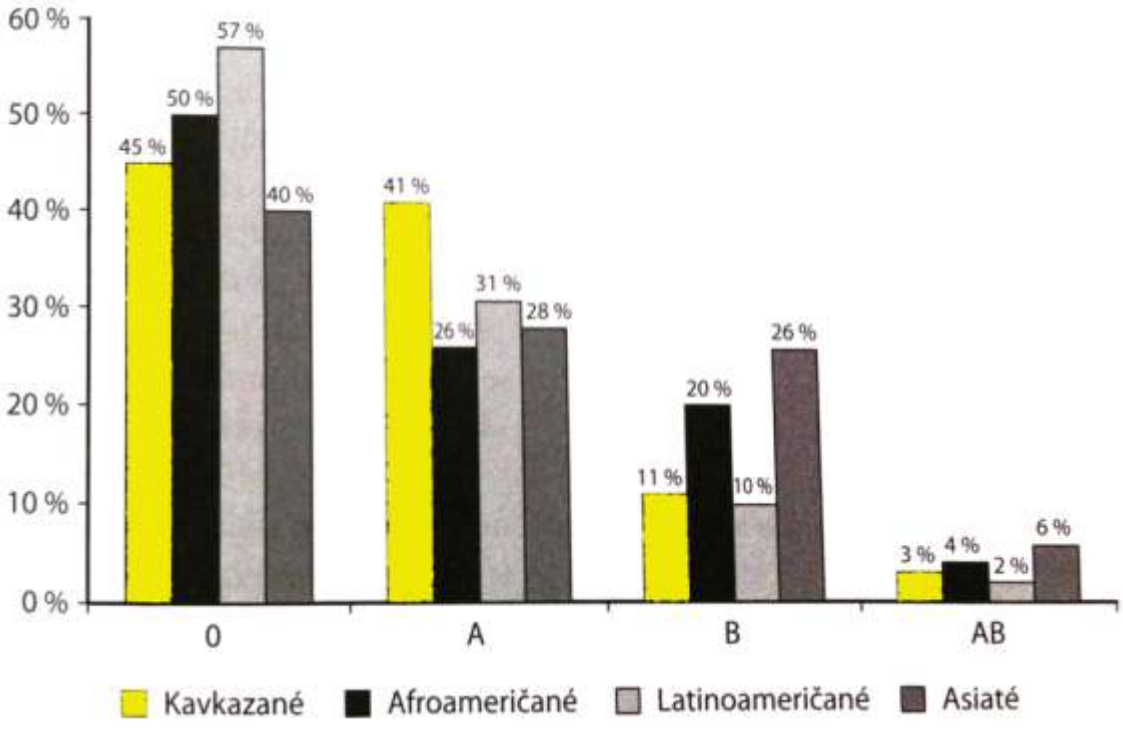

Figure 11: Blood groups representation according to race.

(Adapted from Penka and Tesařová, 2012)

4.2. Determination of antibodies and their specificity

Sera tested for HLA-specific antibodies were obtained during the waiting period for retransplantation. All samples were evaluated for the presence or absence of antibodies against HLA antigens. The majority of patients produced HLA-specific antibodies - 213 patients (91%); however, two patients produced only MICA antibodies. Moreover, females produced antibodies more often (Fig. 12), yet the result is non-significant (p-value p=0.34).

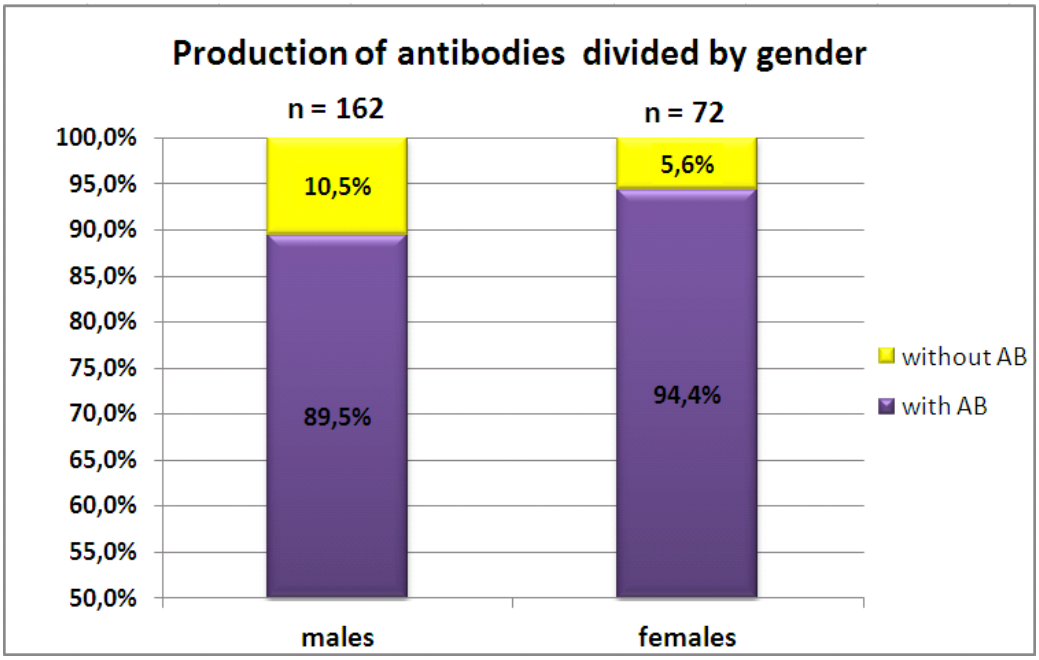

Figure 12: Comparison of antibodies production in males and females.

Patient sera were tested for the presence of antibodies against HLA class I, class II and MICA using the Luminex method. Primarily, sera were tested using the LABScreen Mixed (LS MIX) to detect the presence or absence of HLA and MICA specific antibodies. If the serum was positive for HLA class I antigens, it was then tested using the LABScreen Single Antigen Class I (LS SA1) to determine the specificity to HLA class I antigens. Specificities of antibodies were compared with the HLA antigens of previous donor/donors to determine whether antibodies were donor-specific. If the serum was

positive for HLA class II antigens in the LABScreen Mix examination, it was further tested using the LABScreen Single Antigens Class II (LS SA2) to determine the specificity to HLA class II antigens.

An example of the evaluation of antibody specificity against HLA antigens using software HLA-fusion software:

In the serum, antibodies against HLA class I and HLA class II were detected using the Luminex LABScreen Mixed test (Fig. 13). The cutoff for the LS MIX test was always 500 MFI. (Values of MFI were rounded to hundreds.) Every HLA antibody reaction measured above this threshold was considered positive.

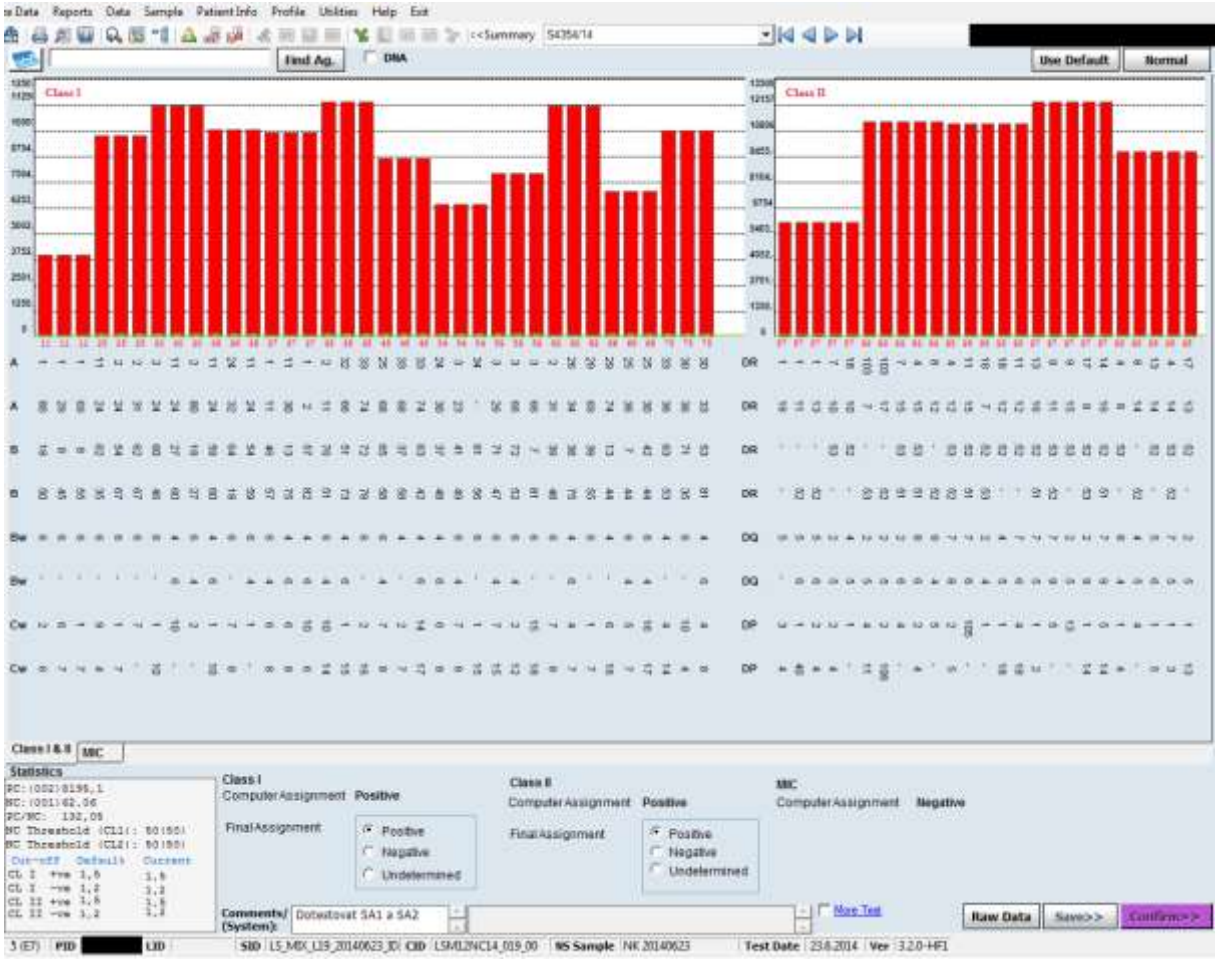

Figure 13: Luminex LABScreen Mixed (Class I and Class II).

PC = 8195 MFI

NC = 62

PC/NC = 132

The MFI value of specific antibodies against HLA class I measured in the serum reached 11250. The value of specific antibodies against HLA class II reached 12150 MFI. MICA antibodies were negative.

As the serum was positive for both HLA class I and class II, it was necessary to test it for a more specific level - LS SA1 and LS SA2. These tests are very similar, they differ only in the setting of cutoffs (LS SA1 – 1000 MFI and LS SA2 – 2000 MFI). For briefness, we are showing only the LS SA1 evaluation (Fig. 14).

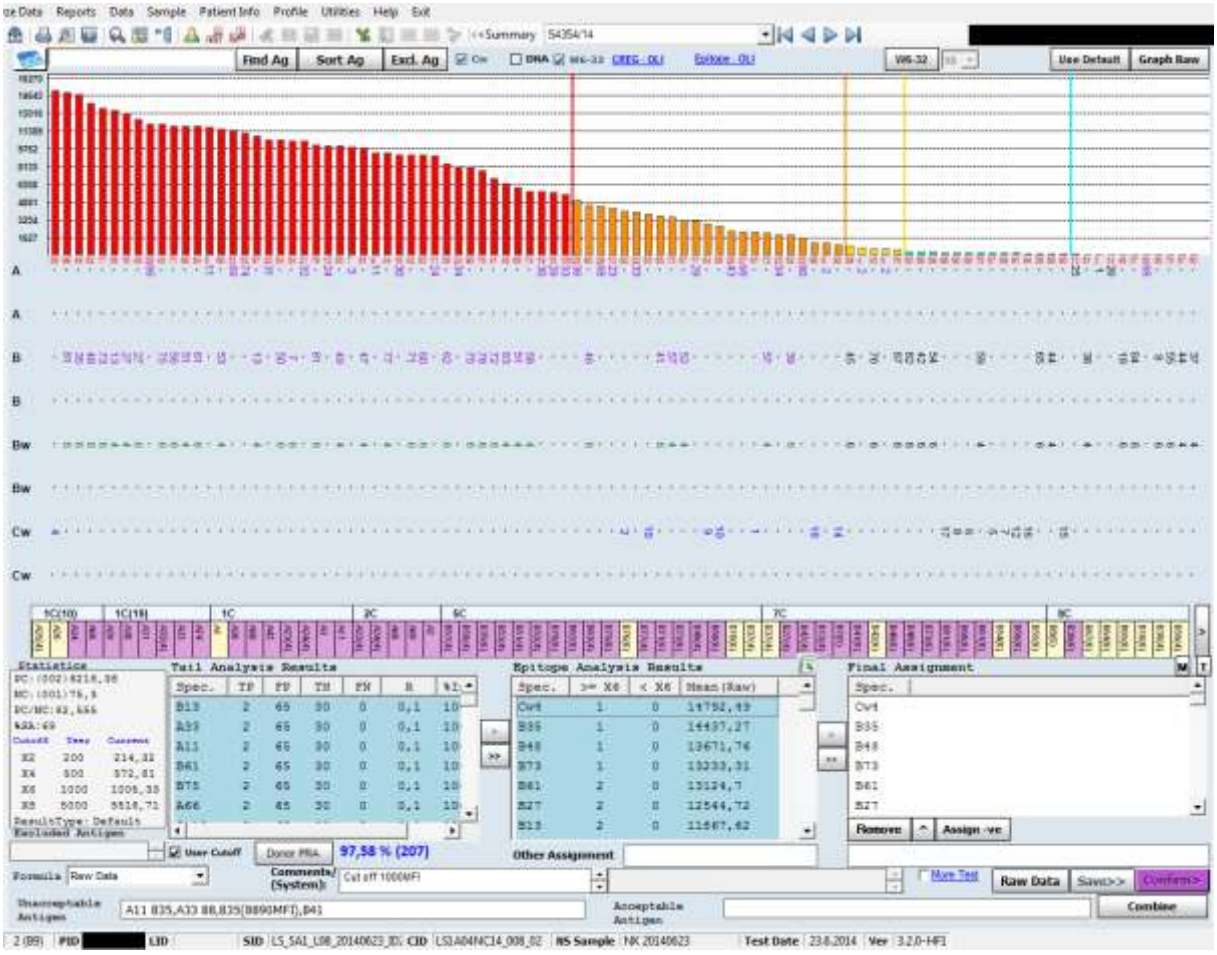

Figure 14: Luminex LABScreen Single Antigen Class I.

PC = 6216 MFI

NC = 75

PC/NC = 83

Many antibodies against HLA class I were detected because the patient serum was strongly positive, e.g. Cw4, B35 and B38.

Example of determination of DSA:

HLA typing of patient	*HLA typing of 1st donor*	*HLA typing of 2nd donor*
A1, A28	A2, A11	A1, A33
B14, B37	B35, B60	B8, B41
DR7, DR13	DR7, DR13	DR3, DR4

DSA 1 (against 1st donor) were determined as follows: A2 (1200 MFI), A11 (10400 MFI), B35 (14400 MFI) and B60 (9100 MFI).

DSA2 (against 2nd donor) were determined were detected: A33 (4700 MFI) and B41 (3600 MFI).

4.3. Relation between production of antibodies and forbidden HLA antigens

The majority of the study group produced antibodies (Fig. 15) – 211 patients (90.2%). HLA antibodies to both class I and class II antigens (Class I+II in the figure) were most often, 157 patients. However, production to single class – I or II antigens was also observed (class I 25 patients; class II 29 patients). 23 patients did not produce any HLA antibodies.

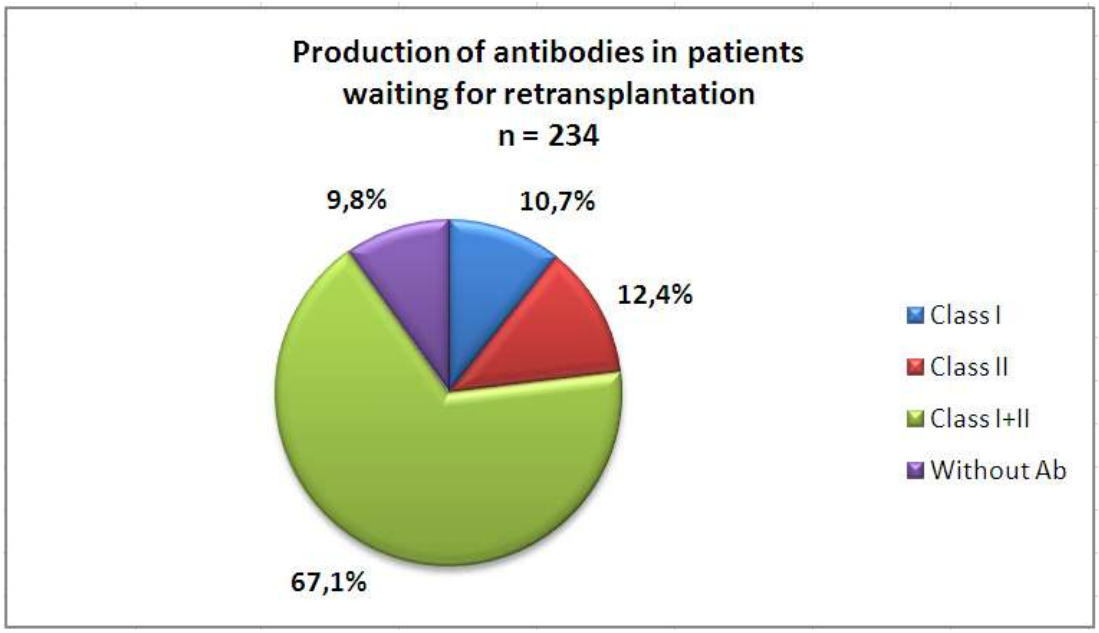

Figure 15: Production of antibodies in the examined group (in %).

Despite the fact that the production of HLA antibodies in the study group was high, the number of patients with forbidden antigens was significantly lower (Fig. 16) – 167 patients. This is due to fact that not all produced antibodies were donor-specific and therefore the respective HLA antigens were not considered as forbidden. In 67 patients no HLA antigens were forbidden. In 78 patients, both class I and class II antigens (Class I+II) were forbidden. Only HLA class I antigens were forbidden in 85 patients, and only single HLA class II antigens were forbidden in 4 patients.

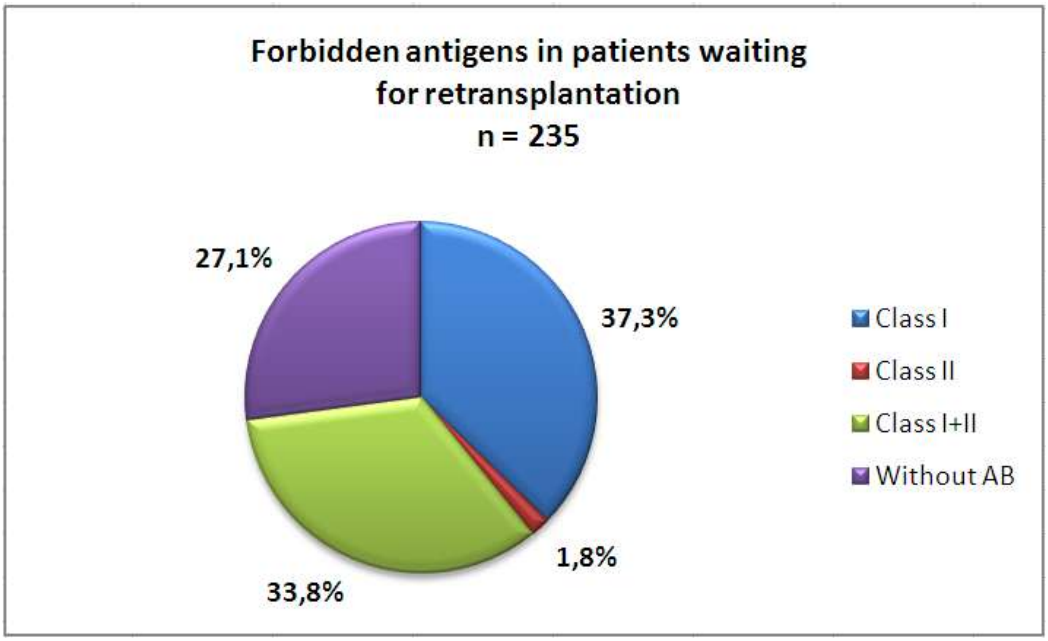

Figure 16: Number of forbidden antigen shown (in %).

If patients only produced HLA class antibodies, only HLA class I antigens were forbidden. Figure 17 shows that only 25 patients produced antibodies against HLA class I, and a specific HLA antigen(s) was forbidden in 19 patients. In Class II – out of 29 patients with the production of antibodies to HLA Class II, only 4 patients had forbidden HLA antigens. The rest of the patients were either without antibodies (23 patients) or with the production of HLA antibodies to both class I and class II – 157 patients and 78 of these patients were forbidden antigens.

Fisher's exact test was used for the statistical evaluation of the single production of HLA class I and class II antibodies and corresponding forbidden HLA antigens. The p-value was $p<0.0001$, so the comparison was statistically significant. It was confirmed that despite the fact that the production of antibodies in both HLA classes was similar, the number of forbidden HLA class II antigens was lower. This is related to the kidney allocation system in the Czech Republic, where HLA-DR antigens are taken into consideration as the principal.

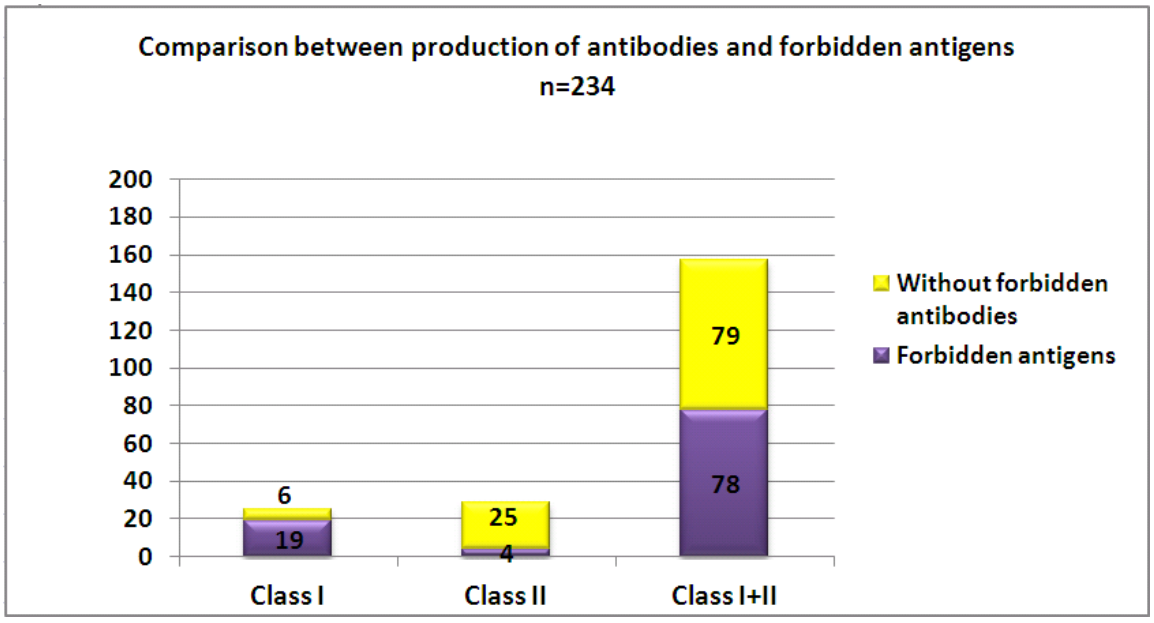

Figure 17: Comparison between production of HLA antibodies and forbidden antigen.

Comparison of only Class I and Class II was statistically evaluated.

Figure 18 shows the percentage of forbidden HLA class I antigens. The rate of forbidden HLA antigens in single HLA-A and HLA-B antigens is not different since - they are forbidden in about 20% of patients, while HLA-A and HLA-B antigens were simultaneously forbidden in about 59% of patients.

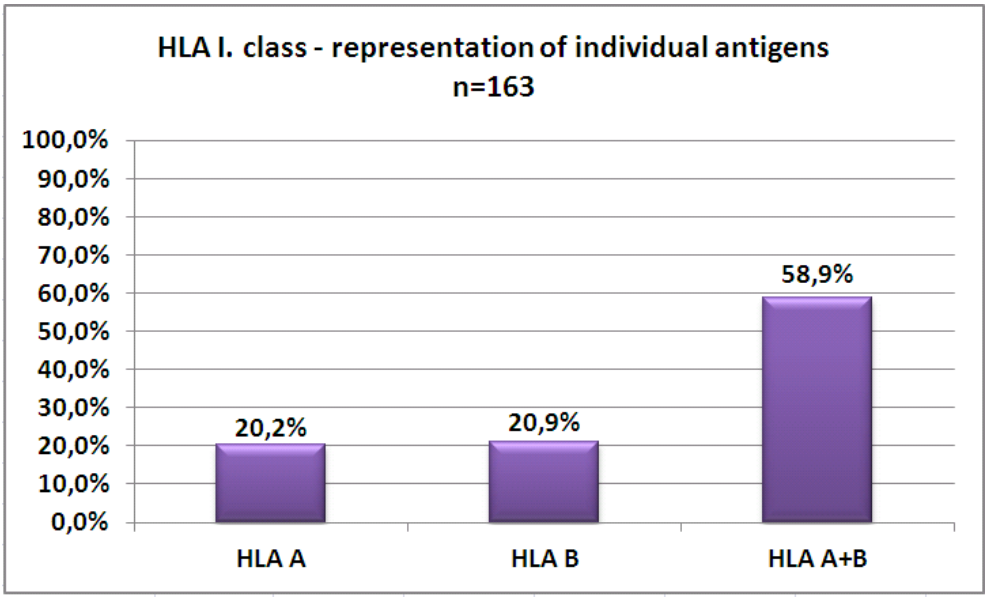

Figure 18: The rate of forbidden antigens in HLA-A and HLA-B does not differ.

4.4. Production of MICA antibodies in patients waiting for retransplantation

From all patients producing antibodies, 61 also produced antibodies against MICA antigens. There is an association between simultaneous production of antibodies against HLA antigens and MICA, because only 2 patients produced single MICA antibodies, see Figure 19. Most patients (46) produced MICA antibodies together with HLA class I and class II antibodies. Production of MICA and HLA class I antibodies was observed in 4 patients, and MICA and HLA class II antibodies was observed in 9 patients.

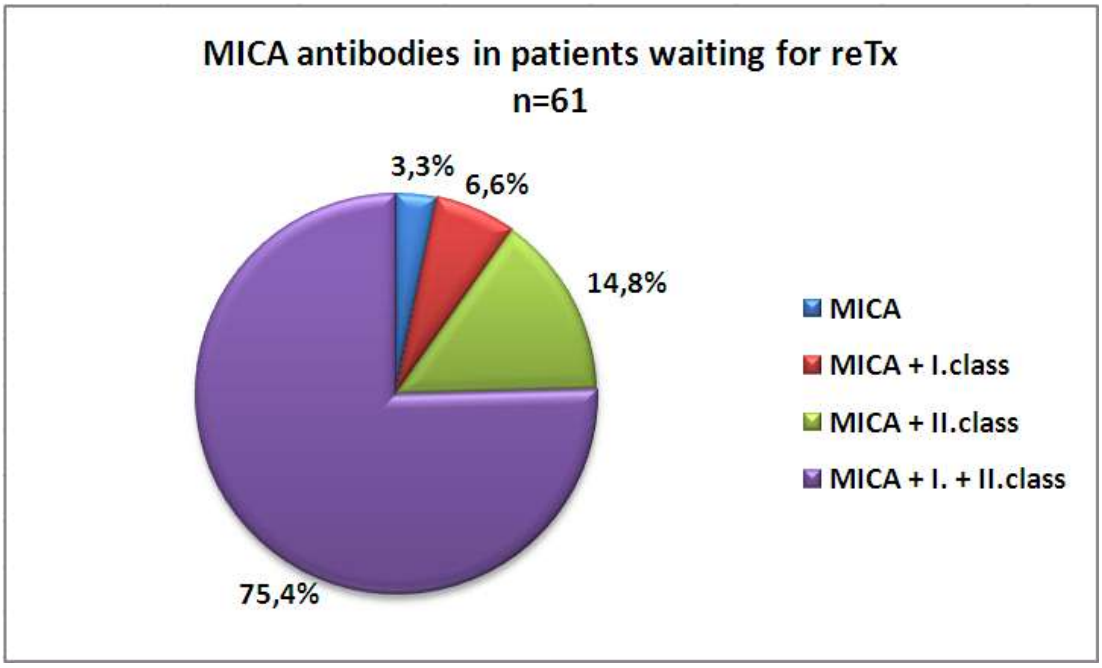

Figure 19: Production of MICA antibodies with HLA antibodies shown (in %).

4.5. Production of HLA-C, HLA-DQ and HLA-DP antibodies

213 patients from the tested group also produced antibodies against HLA-C, HLA-DQ and HLA-DP antigens, see Figure 20. Most patients produced antibodies against HLA-DQ antigens - 164. Overall, 95 out of 213 patients produced antibodies to HLA-C antigens.

One third of 213 patients produced HLA-DP antibodies (75 patients). These antigens were not forbidden for the next transplantation because of the allocation policy in the Czech Republic.

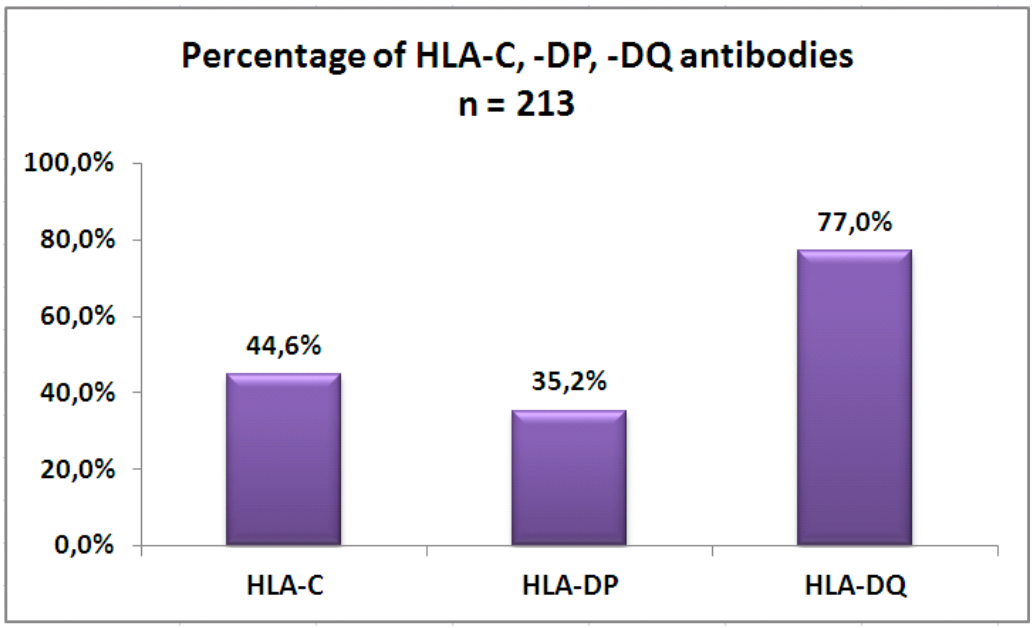

Figure 20: Production of HLA-C, DP and DQ antibodies (in %).

4.6. Association between DR and DQ antigens

Our study also confirmed the strong association between DR and DQ loci (as expected). Altogether, 173 patients produced antibodies against HLA-DR and HLA-DQ alone, or HLA- DR and HLA-DQ simultaneously (Fig. 21). Antibodies only to HLA-DR produced 13 patients and 39 patients created antibodies against single HLA-DQ. However, the majority (121 patients) produced HLA-DR and DQ antibodies simultaneously. This fact was also confirmed statistically by the Chi-square test ($p<0.001$).

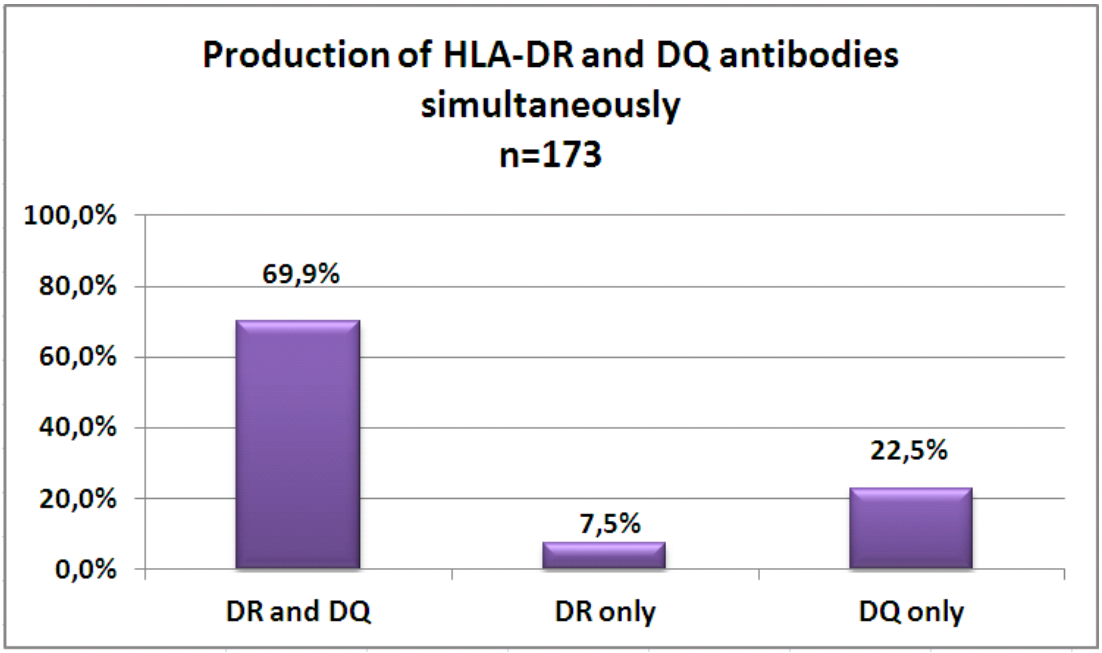

Figure 21: Production of HLA-DR/DQ antibodies (in %).

4.7. Number of transplanted patients with forbidden antigens

The study of forbidden antigens lasted for three years (2011-2013). During this period, 234 patients were tested but only a minimum of patients were retransplanted (Fig. 22). The share of retransplanted patients displayed a decreasing trend –29% in the year 2011, 18.6% in the year 2012 and 12.5% in the year 2013. So there was a significant decrease p=0.001, confirmed by the Chi-square test.

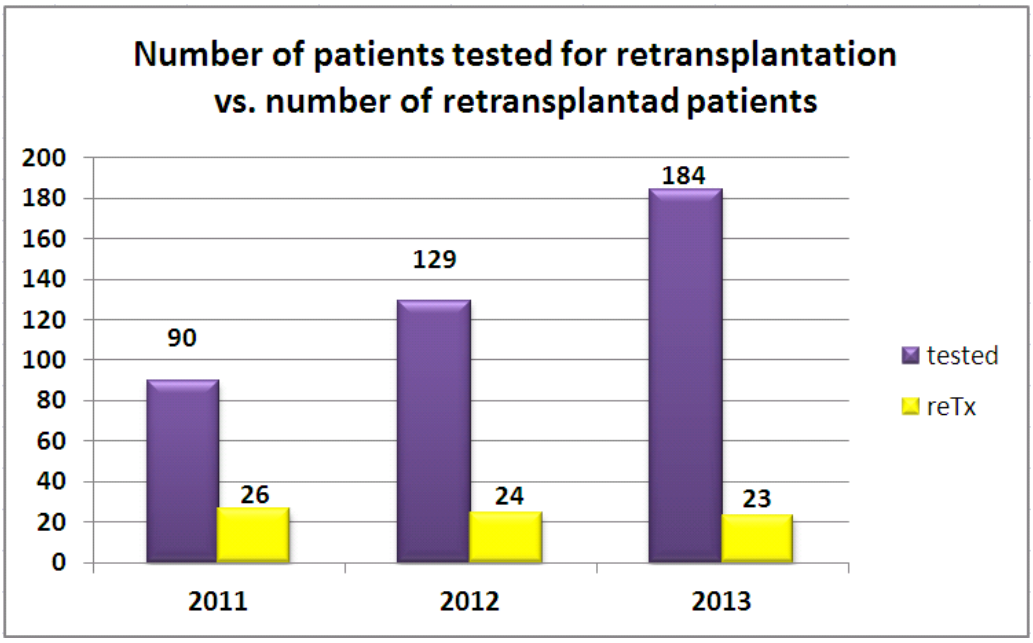

Figure 22: Numbers of tested and retransplanted (reTx) patients. The number of retransplanted patients did not change over time. However in the context of the increasing number of patients waiting for reTx, the percentage of retransplanted patient displayed a decreasing trend.

4.8. Incidence of antibodies over time

Figure 23 shows that patients were still producing donor-specific antibodies and non-donor-specific antibodies for a long time after transplantation. Surprisingly, there were patients in the tested group who were transplanted about 23 years ago and still produced HLA antibodies. This data include patients who were waiting for a second transplantation and who were also producing antibodies – 171 patients. However, in 6 patients the date of transplantation was not known, so they were excluded from these data.

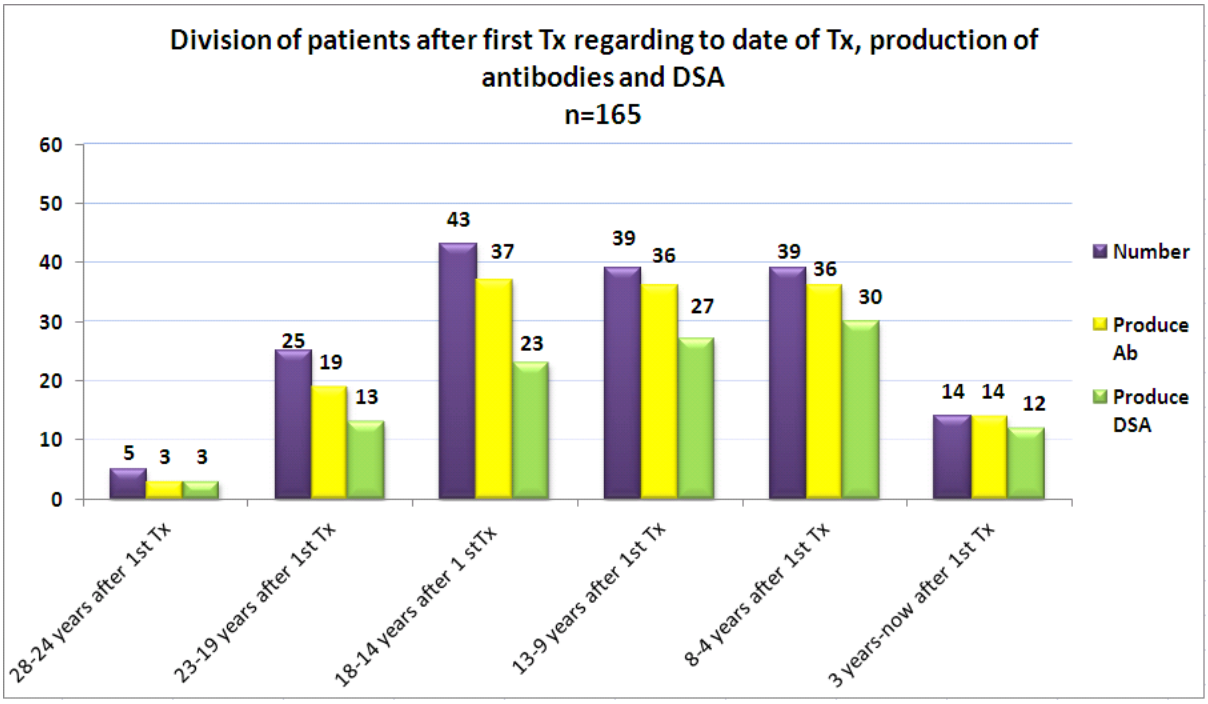

Figure 23: Production of antibodies over time.

4.9. Repeated mismatches

The study group included patients who had repeated mismatches with their previous donors. This means that the patients had been transplanted for a second time at least and there was a common mismatched HLA antigen with the next donor. 63 patients in the group had been retransplanted with a second or more transplants. From these patients, only 16 patients (25.4%) had repeated mismatches and only 7 patients produced antibodies against the repeated mismatches (Fig. 24).

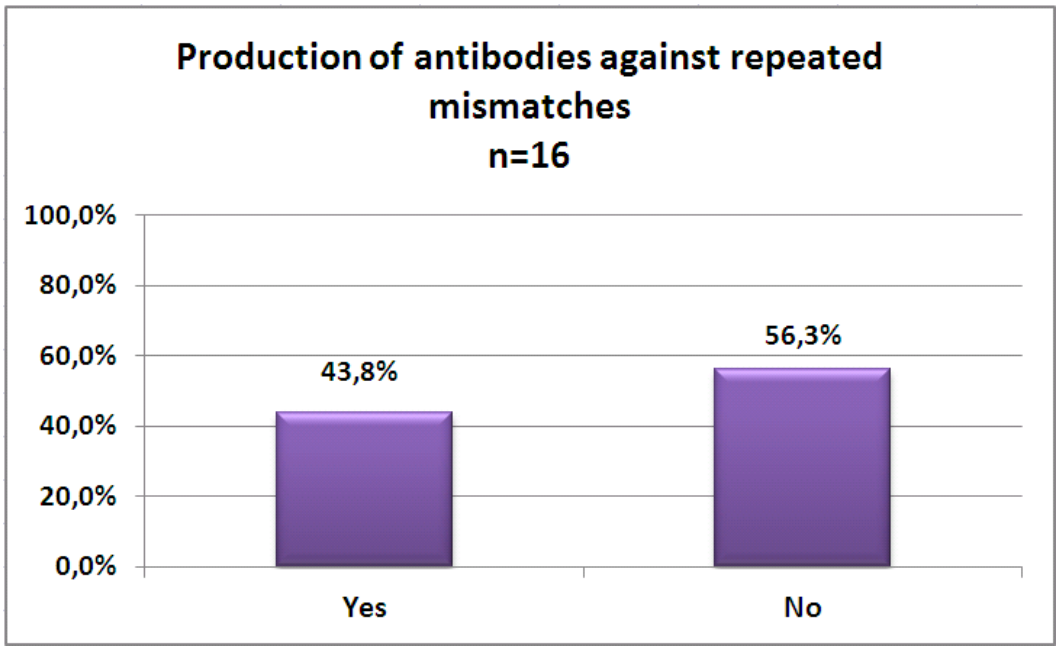

Figure 24: Antibodies against repeated mismatches (in %).

4.10. Comparison between the cohorts with and without forbidden antigens

From the study group of 234 patients, only 50 underwent retransplantation between the years 2011 - 2012 (21.4%). For comparison, a control group comprising 63 patients who were retransplanted without forbidden antigens between the years 2009 – 2010 was used. Both groups were similar to each other (Table 5). However, both groups differ insignificantly in donor and living/deceased donor categories (Table 6). The groups are similar in the number of transplantations; almost all patients underwent a second transplantation (Figure 25 and 26.)

Gender	With forbidden antigens	Without forbidden antigens
Male	34	40
Female	16	23
Total	50	63

Table 5: Distribution of males and females in groups with/without forbidden antigens.

Type of donor	With forbidden antigens	Without forbidden antigens
Deceased	40	62
Living	10	1
Total	50	63

Table 6: Type of donors in groups with/without forbidden antigens.

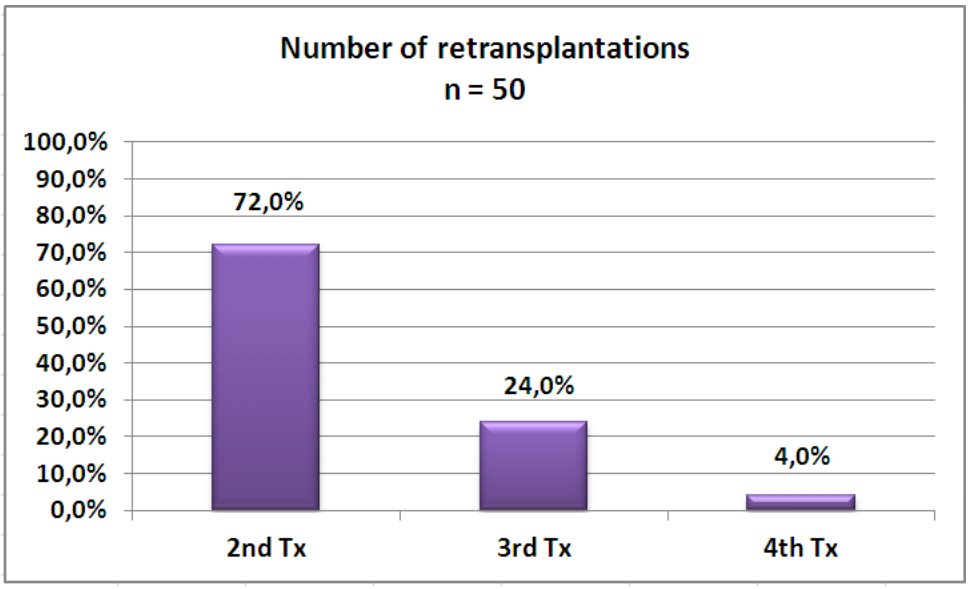

Figure 25: Number of transplantations – retransplanted group of patients. 36 patients underwent a 2nd retransplantation, 12 patients underwent a 3rd and 2 patients underwent a 4th.

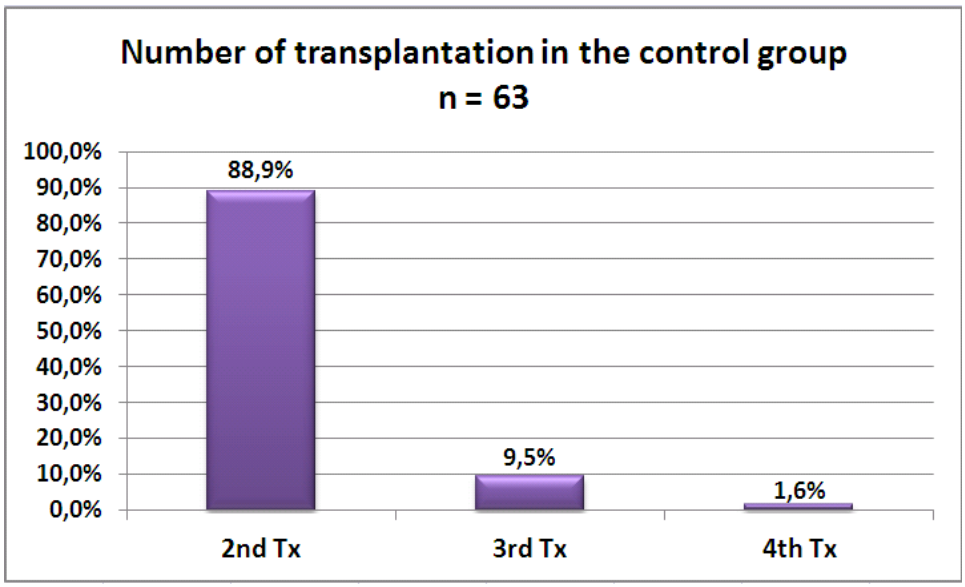

Figure 26: Number of transplantation in control group. 56 patients underwent a 2nd retransplantation, 6 patients underwent the 3rd and 1patinet underwent 4th.

Figures 27 and 28 show that the occurrence and type of rejection was similar in both groups. In the retransplanted group of patients with forbidden antigens ACR developed in 6 patients and in the control group without forbidden antigens it developed in 7 patients. AMR occurred in 11 patients with forbidden antigens and in 15 in the control group. 2 patients displayed an incidence of CHR in the group with forbidden antigens and 4 patients in the control group. Borderline changes were also observed - 5 patients from the group with forbidden antigens and 8 patients in the control group. 26 patients in the group with forbidden antigens did not experience rejection along with 29 patients from the control group.

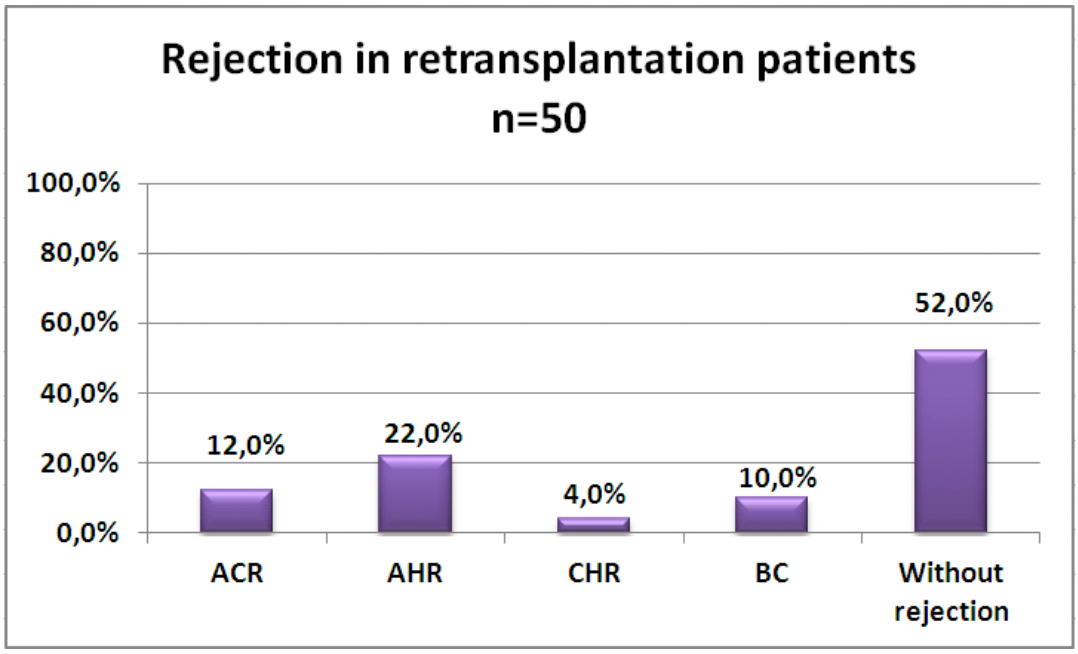

Figure 27: Type of rejection in the retransplanted group of patients (in %).

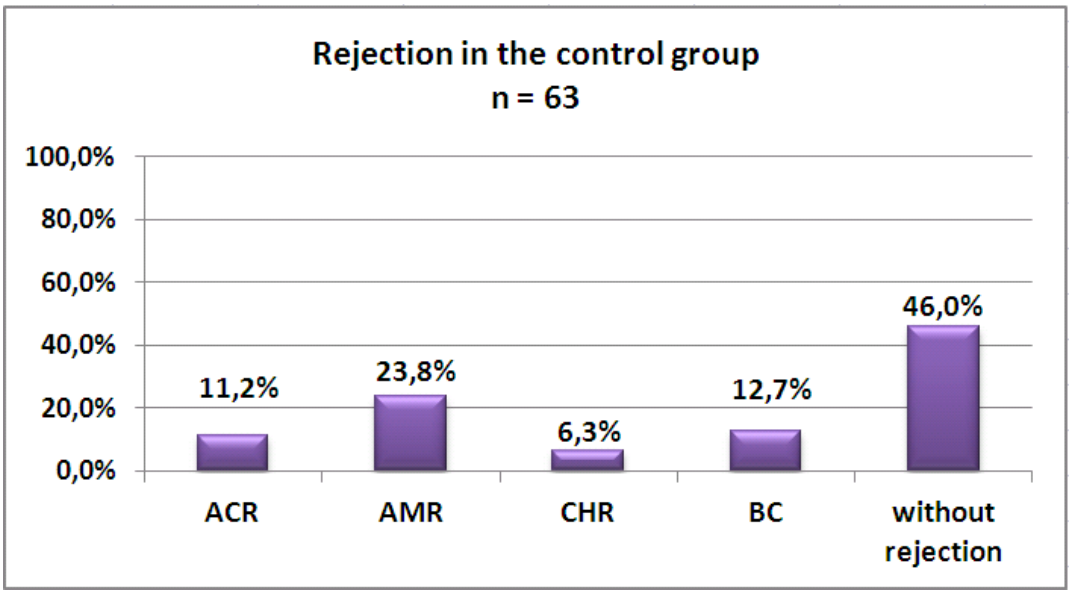

Figure 28: Type of rejection in the control group of patients (in %).

4.11. Production of antibodies in retransplanted patients

Overall, 32 (64%) out of 50 retransplanted patients were tested using the Luminex method afterwards. From these patients, only 10 (31.3%) produced DSA against their new donor. DSA and AMR occurred in 8 patients (Table 7). A statistically significant correlation between the occurrence of AMR and DSA was proved (p-value < 0.001) using the Fisher's exact test. However, only two patients produced the antibodies de novo.

	DSA +	DSA -
AHR +	8	4
AHR -	2	36

Table 7: The number of patients with DSA+/- and AHR+/-.

23 patients (46%) had previous grafts left in situ from previous transplantations at the time of retransplantation. Approximately half of these patients produced DSA before

retransplantation – 10 patients (43.5%). However, only 2 patients produced DSA after retransplantation. One produced antibodies against both donors but not de novo (without rejection), and the other only against the second donor and also not de novo (AMR).

4.12. Comparison of patients waiting for retransplantation with patients waiting for a first transplantation

The comparison of data from the retransplanted patients group with patients who were awaiting a first kidney transplantation was also made. These data were obtained from the kidney waiting list (second quarter of 2014). The whole waiting list (342 patients) was tested using the Luminex method in order to determine the level of HLA antibodies in each patient. However, for our purposes, only data from patients waiting for a first transplantation were used.

The group of patients, who were waiting for a first transplantation consisted of 267 people (179 males and 88 females). Only about a third of the patients (29.6%) produced HLA antibodies. Production was also different in males and females (Fig. 29). It was statistically confirmed that females produce antibodies more frequently than males (p-value: $p < 0.001$).

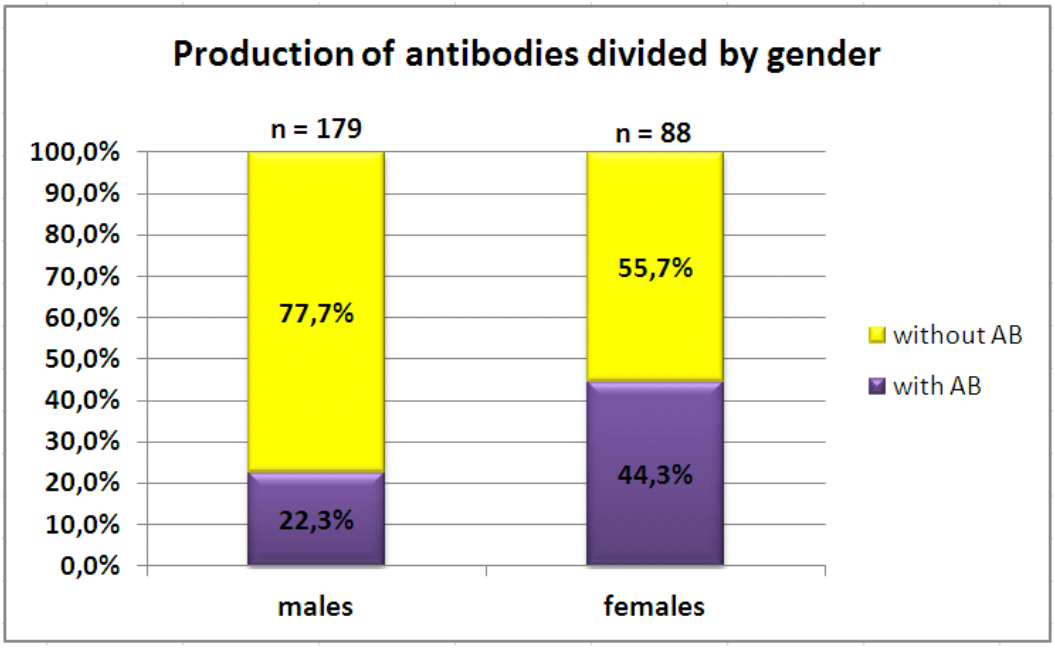

Figure 29: Comparison of antibodies production in males and females (patients waiting for a first transplantation).

In this group of patients, there was also a difference in production of HLA antibodies in contrast to the study group. In our study group of patients waiting for retransplantation the majority produced HLA antibodies, while in patients waiting for first transplantation almost 67% did not produce HLA antibodies (Fig. 30) and the production of HLA class I antibodies was higher than in the study group of patients ($p < 0,0001$).

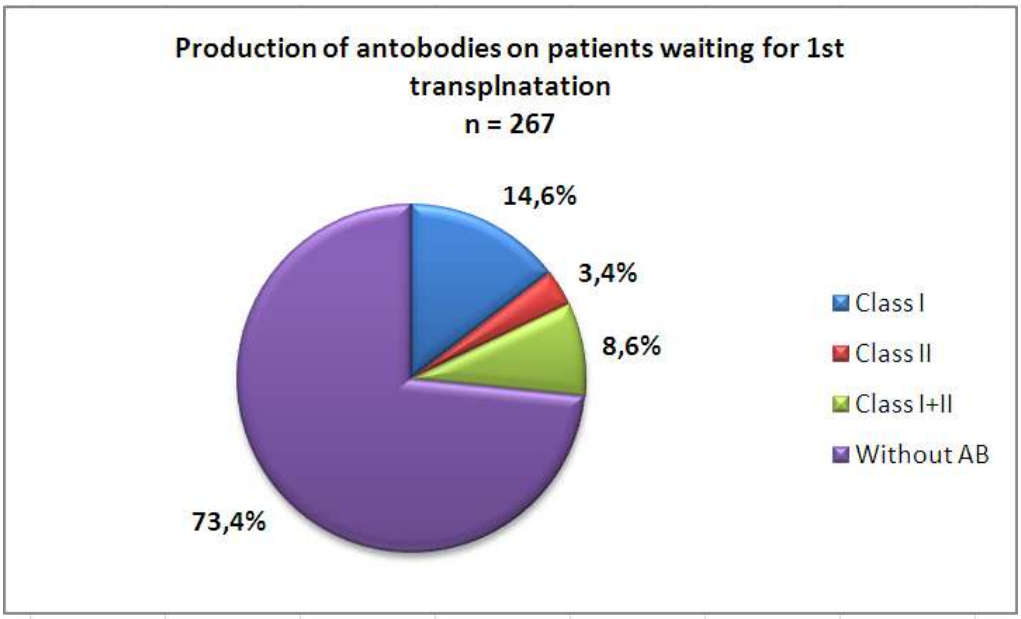

Figure 30: Production of antibodies in patients waiting for a 1st Tx.

There was also a difference in the production of MICA antibodies between these two groups. In the group of patients waiting for retransplantation only 2 patients produced MICA antibodies (see above) independently, whereas in this group of patients 8 patients produced only MICA antibodies (Fig. 31). Overall, 12 patients (4.5%) waiting for first transplantation produced MICA antibodies.

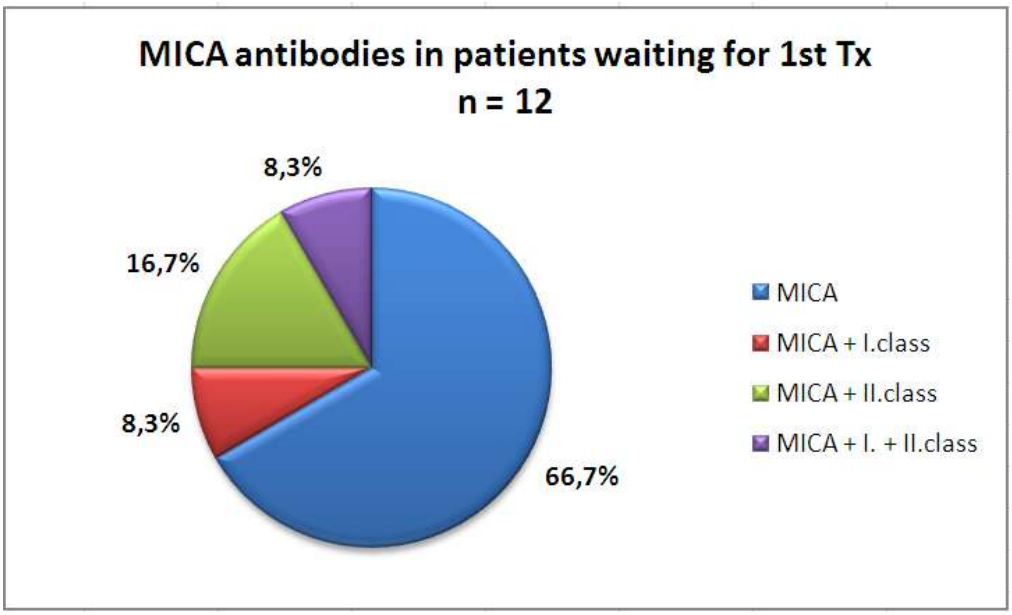

Figure 31: Production of MICA antibodies with HLA antibodies (in %).

5. Discussion

The Forbidden HLA Antigens project was originally aimed at decreasing the incidence of AMR in patients waiting for retransplantation. Patients waiting for retransplantation are at risk because they more often produce higher levels of anti-HLA antibodies. The reason for this is because their immune systems have met foreign antigens in the form of mismatched donor antigens. To the best of our knowledge, there are very few literal sources which independently study the influence of forbidded/non-forbidding HLA antigens before repeated transplantation. Therefore, this thesis provides a unique insight into this issue.

The main aim of this diploma thesis is to determine the forbidden (non-acceptable) HLA antigens in patients waiting for retransplantation and evaluate their effect on the incidence of rejection in the context of the next transplantation. 234 patients were included in this study and the majority produced anti-HLA antibodies (211 patients i.e. 90.2%). However, there was no significant difference in antibody production between males and females, in contradiction to the literature data (Triulzi et al., 2009). However, data from our patients waiting for a first transplantation shows that females produced antibodies more often (p-value: $p < 0.001$). The preformed antibodies may be created as a result of blood transfusion, previous transplantation(s) and pregnancy. In our group of patients waiting for retransplantation, all patients had already undergone one transplantation at least; therefore, the different level of production of HLA antibodies between males and females was not so noticeable.

In our study group, the majority of patients produced HLA antibodies against both class I and class II antigens (67.1%). The production of antibodies against HLA antigens class I or class II alone was 10.7 % and 12.4 %, respectively. Our results are in concordance with a similar study of the Polish National Waiting List, where, out of 112 patients (waiting for retransplantation) who were negative in PRA-CDC but positive in the LS assay, 70 of them (63%) produced antibodies against HLA class I and class II simultaneously (Moszkowska et al., 2014). Such high production of antibodies against

both HLA classes may be a result of the direct and indirect pathways of recognition of alloantigens. Normally, HLA class I antigens are expressed on all nucleated cells, whereas HLA class II antigens are expressed on antigen-presenting cells – APCs. The HLA class II antigens are expressed in higher frequency only in special conditions when the adaptive immune response is induced. The expression of HLA class II antigens may be also stimulated on non-APCs in the presence of IFN-γ (Neefjes et al., 2011). However, during the immunological reaction against the graft T lymphocytes, mismatched HLA antigens recognized by the direct pathway are in high frequencies (these are cross-reacting, viral-specific T lymphocytes). Although the immune response caused by the direct pathway of recognition disappears over time, the production of HLA antibodies is still maintained by the indirect pathway of recognition and the immunological reaction does not disappear.

The HLA antibody production in patients waiting for retransplantation was also compared with the production of HLA antibodies in patients waiting for a first transplantation. 267 patients were tested for the presence of HLA antibodies before a first transplantation using the Luminex and only 26.6% of them produced antibodies. The remaining patients (73.4%) did not produce HLA antibodies. It was also statistically confirmed that patients waiting for retransplantation produced HLA antibodies more frequently ($p < 0.0001$). Patients before a first transplantation produce HLA antibodies only on the basis of blood transfusion or pregnancy, so they do not provide a strong immunogenetic stimulus for organ transplantation.

Despite the fact that the production of HLA antibodies in the group of patients waiting for retransplantation was high, the number of patients with forbidden HLA antigens was lower – 167 patients (71.4%). HLA class I were forbidden in the majority of patients – 85 (36.3%). On the contrary, – in 78 patients (33.4%) HLA class I and class II antigens were forbidden. However, HLA class II only were forbidden in 4 patients (1.7%). This finding is related to the allocation system of kidney grafts in the Czech Republic because there the HLA-DR antigens are taken into consideration in the first place (HLA-DR > HLA-B > HLA-A). To prove the fact that HLA class II antibodies were minimally

forbidden, a statistical comparison of production of HLA class I alone (and the number of corresponding forbidden antigens in this group) with HLA class II alone (and the number of corresponding forbidden antigens) was made. The p-value was $p < 0.0001$, so the comparison was statistically significant. Moreover, it has been reported that preformed HLA class I antibodies appeared to be harmful, contrast to HLA class II antibodies (Süsal et al., 2009). 28.6% of patients did not have any forbidden HLA antigens, so not all produced antibodies are DSA.

We also evaluated whether the distribution of forbidden HLA-A and HLA-B differs. The rate was practically the same; HLA-A antigens were forbidden in 20.2% of cases, while HLA-B were forbidden in 20.9% of cases.

50 patients (21.4%) from our study group with forbidden HLA antigens underwent the retransplantation. These data were compared with data from 63 retransplanted patients without forbidden HLA antigens. The occurrence of rejection was very similar. ACR developed in 6 patients in the group with forbidden HLA antigens and in 7 patients in the control group (12% and 11.2%, respectively). AMR developed in 11 patients with forbidden HLA antigens and 15 patients in the control group (22% and 23.8% respectively). The incidence of CHR was determined in 2 patients in the group of forbidden HLA antigens and in 4 patients in the other group (4%, 6.3%, respectively). Borderline changes were diagnosed in 5 patients in the group of forbidden HLA antigens and in 8 patients in the control group (10% and 12.7%, respectively). Cases without rejections were recorded in 26 patients in the group of forbidden HLA antigens and in 29 patients in the other group (52% and 46%, respectively). Our data indicate that forbidden HLA antigens had no effect on the development of rejection after retransplantation. The higher percentage of patients without rejection in patients with forbidden HLA antigens may be due to better and timely immunosuppressive treatment and may “mask” the influence of forbidden antigens. It will be necessary to determine a new approach for this highly sensitized group of patients waiting for retransplantation because the number of these patients will increase to with better diagnostic methods and treatment.

Our data also confirms that the production of donor-specific HLA antibodies correlates with the incidence of AMR. Out of 32 retransplanted patients tested using the Luminex method after transplantation, 10 patients (31.3%) produced DSA and 8 of them developed AMR (p-value < 0.001). However, only two patients produced DSA de novo. This result indicates that not only de novo produced antibodies may induce AMR. It has been reported that patients with DSA have increased rates of acute AMR, chronic graft dysfunction and graft loss. Moreover, donor-specific antibodies against HLA class II or both class I and class II antigens have a strong association with graft loss (Fidler et al., 2013).

Although the study was carried out in good faith, the effect was not favorable for patients, as our data indicate. The number of rejection episodes was not reduced and waiting list times were even prolonged. The study lasted three years and during this period the low number from 234 patients was retransplanted (73 patients; 31.2%). Moreover, the number of retransplanted patients displayed a decreasing trend – in the year 2011, 26 patients (29%) were retransplanted; in the year 2012, 24 patients (18.6%); and in the year 2013. 23 patients (12.5%). This was statistically significant (p = 0.001). Therefore, many patients were not retransplanted and new patients were added to the study group over the duration of the study. At the end of the project (year 2013), 184 tested patients were still waiting for retransplantation and only 23 of them were actually retransplanted.

HLA mismatches were repeated in 16 of the 63 patients (25.4%) in our study group and 7 of these patients produced HLA antibodies against repeated mismatches. This result suggests that a policy which forbids all antigens of all previous donors for the next transplantation used in some transplant centers might not be appropriate (Moszkowska et al., 2014). This policy is still applied in transplant centers in Germany, the UK and France. There are still not sufficient data to support or overturn this finding. However, our data suggests that according to the immunosuppressive protocols in our center, the

risk of AMR incidence is not increased and forbidding HLA antigens extends the time patients spend on the waiting list. Our practical suggestion is that patients should be tested for levels of donor-specific antibodies (which can often cause AMR) using the Luminex method; however, the respective HLA antigens should be forbidden. This approach, in combination with a negative pretransplant CDC test, can increase the chance of obtaining a compatible kidney donor.

The production of antibodies is a long-term process, as our data suggests. Out of 171 patients waiting for retransplantation, 165 (96.5%; of these patients date of transplantation was known) produced HLA antibodies in the long term, even though they underwent the transplantation more than 20 years before. Therefore, the production of donor-specific antibodies is a long-term process, but occurs in approximately 75% of patients. Obviously, the production of antibodies correlates with the immunological memory of T and B lymphocytes. According to the literary data, memory lymphocytes are long-living cells which demonstrate a lower activation threshold then naive cells and are more resistant to immunosuppression than naive T and B cells. Moreover, these cells may be found in non-lymphoid tissue; so they are easily accessible to antigens (Abbas et al., 2012; Li et al., 2013).

In our study group, the production of HLA antibodies against HLA-DQ, -C and –DP was higher than we expected, especially against HLA-DQ antigens (77%; 44.6%; 35.2% respectively). Similar results were reported in the study of the Polish National Waiting List – HLA-DQ 65%, HLA-C 50% and HLA-DP 39% (Moszkowska et al., 2014). These loci have not been forbidden for the next transplantation because they are not taken into consideration in the allocation system in the Czech Republic. Furthermore, the expression of these antigens is not considered as strong immunogenetic factor because their expression on lymphocytes is weak and their clinical relevance remains unclear (Moszkowska et al., 2014; Duquesnoy et al., 2008). On the other hand, it has been reported that donor-specific HLA-DQ antibodies may contribute and are responsible for AMR of the graft (DeVos et al., 2012; Tagliamacco et al., 2014). It may be beneficial for

patients waiting for retransplantation to also take also HLA-DQ locus into consideration. However, on the other hand this would probably extend the time on the waiting list because of polymorphism of the HLA locus. Alternatively, pretransplant antibodies to DQ antigens have to be evaluated with caution. It has also been mentioned that high levels of donor-specific anti-HLA-C antibodies before transplantation may also lead to AMR during the first year after transplantation (Aubert et al., 2014). Furthermore, donor-specific HLA-DP antibodies have also been reported to cause AMR (Mierzejewska B., 2014). Consequently, antibodies to HLA-DQ, -C and –DP may play a specific role in AMR, in particular if they are donor-specific and are produced before (re)transplantation.

The strong linkage disequilibrium between HLA-DR and HLA-DQ antigens was (as expected) confirmed by our data ($p < 0.001$). This fact is also reported in the literature (Carlquist et al., 1991). Linkage disequilibrium occurs between neighboring HLA loci and causes non-random association of alleles at two or more loci (the occurrence of some combination of alleles is more or less frequent in the population than would be expected from a random formation of haplotypes from alleles) (Wassmuth, 2010).

Our results indicate that the production of MICA antibodies is associated with the production of antibodies against HLA class I and II antigens in patients waiting for retransplantation. In our study group, 61 patients produced MICA antibodies and the majority (75.4%) of them had antibodies to both HLA class I and class II antigens. MICA antibodies were produced more frequently in patients waiting for retransplantation than in patients without previous transplantation (26.1%, 4.5% respectively). However, the production of MICA antibodies independent of HLA antibodies was different between these groups. In patients waiting for retransplantation, only 2 patients (from 61) produced MICA antibodies without HLA antibodies. However, in patients waiting for first transplantation it was 8 patients (out of 12). Unfortunately, this data could not be statistically compared for two reasons. Firstly, the group of patients without previous transplantation contained a low number of patients for comparison with the group of 61 patients. Secondly, during the year 2014 the cutoff for evaluations had changed from

500 MFI to 1000 MFI. In spite of this, our results indicate that the production of MICA antibodies can be related to graft failure as has been previously reported (Terasaki et al., 2007; Panigrahi et al., 2007).

The same results – 26% (out of 235 patients) production of MICA antibodies in patients waiting for retransplantation has been reported in a similar study testing the Polish National Waiting List (Moszkowska et al., 2014).

We also compared the distribution of blood groups in our patient's cohort with data from 501 deceased donors (tested in IKEM between in the years 2011-2013). As expected, we did not find statistically a significant difference between the two groups. These findings were also compared with several Caucasian populations (Penka and Tesařová, 2012; Řeháček and Masopust, 2013). With the exception of blood group 0 our data corresponded to the distribution of European Caucasian populations.

The problem with an algorithm for testing and forbidding HLA antigens in patients awaiting retransplantation is quite relevant, because the number of patients with failed grafts is increasing worldwide. Furthermore, because of the lack of literature data, there is no consensus between countries and even between transplant centers in the same country (for example in the UK) concerning the policy for forbidding HLA mismatches form previous transplants. Therefore, during the 26th EFI Annual Meeting in Liverpool in 2012 it was agreed that six different laboratories publish their non-acceptable HLA antigen determination algorithms. São Paulo, Basel and Berlin have used Luminex to determine forbidden HLA antigens (but the cutoff can be different); whereas Leiden, Vienna and Barcelona use the CDC screening against a panel of about 50 HLA-typed blood donors in combination with the Luminex method (Süsal et al., 2013). An approach to determine of acceptable antigens also exists. That means that transplantation is HLA-antigens, against which the patient does not produce any antibodies, are allowed for the subsequent. Alternatively, they are determined as low level antibodies under the cutoff. However, there is no consensus concerning the cut off for positivity, so every laboratory sets the cut off at different empirically-defined levels of MFI. Oneadvantage of the

acceptable HLA antigen approach is also the fact that patients transplanted according to this policy have excellent graft survival rates (Class et al., 2004). The policy (The Acceptable Mismatch Program) is routinely applied within Eurotransplant (an organization responsible for allocation of donor organs in Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia). The Acceptable Mismatch Program is an allocation system based on a HLA typing and matching strategy that allows about 60% of highly sensitized patients (CDC-PRA ≥ 85%) to be transplanted within 2 years. However, the recipient should actively wait at least 2 years on the waiting list (defined by the date of the first dialysis) before inclusion into the program. Within Eurotransplant the acceptable HLA mismatches are defined as HLA antigens that are mismatched at the broad HLA antigen level, but which have compatible epitopes between donor and recipient at the structural level (Maggiore et al., 2014). They are defined by extensive serum screening (more than 100 panel cells) and they find the "holes" in the immune repertoire of patients, i.e. HLA antigens against which hypersensitized patients do not have antibodies.

6. Conclusion

Our study focused on evaluating whether forbidden HLA antigens before kidney retransplantation would have a positive influence on the incidence of cellular and antibody-mediated rejection after transplantation. The forbidden antigens were determined using the Luminex method.

1. Even though the study was designed with the good intention of reducing the incidence of rejection in retransplanted patients, the determination and forbidding of “non-acceptable” HLA antigens did not prove to be justified. The rate of cellular and antibody-mediated rejection was not reduced and time spent on the waiting list was prolonged.

2. Patients waiting for retransplantation produced HLA antibodies more frequently than patients waiting for a first transplantation However, not all produced HLA antibodies were donor-specific. The most common antibody production was simultaneously against both HLA class I and class II antigens. Production antibodies against HLA class II antigens alone was lower than production antibodies against HLA class I antigens because the allocation system in the Czech Republic ensures preferential matching according to HLA-DR. Consequently, the forbidden HLA antigens were determined less frequently in HLA class II antigens and more often against HLA class I antigens.

3. The production of HLA-DQ, -C and –DP-specific antibodies was observed more frequently in patients waiting for retransplantation than was expected.

4. MICA antibodies awere more often produced in combination with HLA antibodies (both HLA class I and class II).

5. Production of de novo donor-specific antibodies was rarely observed in retransplanted patients.

The determination of forbidden HLA antigens in patients awaiting retransplantation did not have an influence on the incidence of cellular and antibody-mediated rejection. Determination of forbidden HLA antigens may decrease the chance of successfully finding a kidney. Therefore, a new approach is needed in order to get a better graft outcome for these highly sensitized patients.

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Appendix 1: List of Figures and Tables

Appendix 2: Used materials, equipment and chemicals

1. Materials

Sera for testing for presence/absence of antibodies are obtained by centrifugation of full coagulated patient blood (1600g 10min)

Lymph nodes or pieces of spleens of deceased donors

DNA isolated by MPC Nucleic Acid Isolation Kit I. (Roche) – automatic isolating machine MagNA Pure Compact from full blood on the principle of magnetic bead isolation

Heparin whole blood

2. Chemicals

Kit producer - One Lambda Inc, Los Angeles, CA, USA

LABScreenMixed, cat. No. OL-LSM12

LACScreen Single Antigen Class I Antibody detection, cat. No. OL-LS1A04

LABScreen Single Antigen Class II Antibody detection, cat. No. OL-LS2A01

LABScreen PE-Conj. Goat anti Human, cat. No. OL-LS-ABS2PE

LABScreen Neg. Control serum, cat. No. OL-LS-NC

LABType SSO A locus, cat. No. RSSO1A

LABType SSO B locus, cat. No. RSSO1B

LABType SSO DRB1 locus, cat. No. RSSO2B1

Sheath fluid

PBS pH 7.2-7.4

Lymphocyte Separation medium LSM 1077, cat. No. J15-004, Bio Tech

IMDM cat. No. E15-819, BioTech

Fetal bovine serum, cat. No. A15-101, BioTech

EasySep, Negative selection Human T cell enrichment Kit, StemCell Technologies (Canada, Vancouver)

EasySep, Negative selection Human B cell enrichment Kit, Stem Cell Technologies (Canada, Vancouver)

Trypan Blue 0.3% solution

Aqua pro injectione, cat. No., 3500080, B. Braun Medical (Prague)

anti-CD3-PE, cat. No. 347647, Becton-Dickinson (USA, San Jose)

anti-CD19-PC5, cat. No. A07771, Beckman Coulter (France, Marseille)

Goat anti human IgG-FITC F(ab')2, cat. No. 109-096-098, Jackson Immunoresearch Lab. (Jakcsonville, USA)

EBSS (Earle's Balanced Salts), cat. No. E6263, Sigma Aldrich

CellFix, cat. No. 340181, Becton-Dickinson

Olerup SSP Typing Kit – Genovision (Qiagen) contains test tubes with lyophilized primers and PCR Master Mix without Taq polymerase

Taq polymerase at concentration 5 U/µl, TOP BIO

DNA-ladder GeneRulerTM 50bp DNA Ladder, Biogen Praha s.r.o.

TBE buffer

Gel Red nucleic acid, cat. No. Biot41003, Lab Mark

Electroforetic set, BioRad

3. Equipment

Uniplate 96-well V bottom, white polystyrene, Whatman

Films on the PCR plates

Luminex 200 IS 2.3. (Luminex corp., Netherlands)

Centrifuge CL 30 with rotor for PCR plates, Thermo electron

Centrifuge Minispin+, Eppendorf

Centrifuge Thermo CL30, Trigon plus

Shaker ZX3, Velp Scientifica

Analysis software HLA-Fusion (OneLambda Inc.)

Analysis software Score (Olerup, Sweden)

Pipettes 1 – 10 µl, 10 – 100 µl and 20 – 200 µl

Electronic pipette 10-100µl

Pipette tips

Centrifuge Thermo CL30, Trigon plus

Centrifuge Minispin+, Eppendorf

Bürker chamber

Microscope Olympus BX 41 with phase contrast

Sterile test tubes

Pasteur's pipettes

Laminar flow cabinet biohazard HS 18, Haereus

Thermocycler GeneAmp PCR system 9700, Applied Biosystems

Full spectral spectrophotometer ASP 3700 Avans Biotechnology for measuring concentration and purity of isolated DNA