New possibilities to improve the outcomes of renal transplantation

Ph.D. Thesis

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I. OBJECTIVES

Solid-organ transplantation for the treatment of patients with end-stage organ failure has been one of the most exciting medical advances in the 20th century. The number of patients awaiting transplantation has been increasing due to advances in immunosuppression while there is no good solution to expand the number of donors and to increase the number of transplantations. One possibility to ease the need for renal transplantation is to increase the life span of grafts. Better maintenance therapy as well as earlier diagnosis and effective treatment of rejection can save allografts by minimizing damage.

Evidence from several transplant centers has indicated that a substantial proportion of acute and chronic renal allograft rejection processes is caused by antibodies reactive to donor antigens. Antibody mediated acute rejection (AMR), a newly described entity, arises despite ongoing therapy with potent anti-T cell pharmacological agents. My thesis, which is based on the literature and our own experience, elucidates the mechanisms of AMR as well as novel diagnostic and therapeutic approaches to avoid, recognize and treat acute antibody mediated rejection. In our work, we have explored the factors influencing the occurrence of AMR and the useful methods and markers for diagnosing it. We have sought to compare the outcomes of a plasmapheresis (PP)-based versus a PP plus rituximab regimen to treat patients experiencing AMR and resistant to steroid plus anti-lymphocyte globulin treatments.

Post-transplant malignancy has become an important cause of mortality since newer, more potent immunosuppressive regimens have steadily reduced the incidence of acute rejections extending the life of allografts. My work also investigates possibilities to reduce the number and to manage post-transplant malignancies, particularly post-transplant lymphoproliferative disease (PTLD) a major complication of post-transplant
immunosuppression. In this thesis I have elucidated the efficacy of rituximab to encounter B-cell related processes such as AMR and PTLD.

However, while outcomes following transplantation have improved over the years, allograft loss is a problem ultimately confronted by many recipients. For such patients, repeat transplantation often provides the best chance for survival and good health. While re-transplantation offers hope, previous studies have demonstrated that outcomes following repeated transplantation are, in general, inferior to those observed with first transplants [1]. We have also investigated the influence of demographic and immunological factors on the patient and graft survivals as well as occurrence of acute and chronic rejections among primary and re-transplant patients.
II. ANTIBODY MEDIATED REJECTION, A NEWLY DESCRIBED ENTITY

II/1. Introduction

Sir Peter Medawar’s pioneer work brought together surgery and immunology, and gave ‘hope of progress’ to the clinical application of tissue and organ transplantation. At the beginning of World War II, treatment of burns patients had led him to the recognition of skin allograft rejection. The treatment of a badly burned patient was carried out using a combination of ‘pinch’ grafts taken from the patient herself (autografts), and those from another unrelated individual (allografts in current parlance). In addition, a second set of grafts was later transplanted from the same donor. The grafts were observed visually and, following appropriate biopsies, histologically. Surprisingly, this was the first systematic study of the process of rejection. The results showed that rejection of the first set of allografts was preceded by a latent period, with healing-in and vascularization indistinguishable from the autografts. However, rejection of the second set of allografts from the same donor took place much more rapidly, a hallmark of an immune response. These results established that the underlying basis of skin allograft rejection could be regarded as immunological rather than due to surgical problems or vague physiological incompatibilities [2]. He took this investigation further in experiments performed on outbred rabbits, confirming the specificity of second set rejection of grafts from the same donor as the first, and determining that graft rejection was preceded by an intense infiltration of lymphocytes [3].

The degree of immune response to a graft depends on the genetic disparity between the grafted organ and the host. Xenografts, which are placed between members of different species, show the greatest disparity and elicit the maximal immune response, undergoing rapid rejection. Autografts, which are grafts from one part of the body to another (eg, skin grafts), are not foreign tissues and, therefore, do not elicit rejection. Isografts, which are grafts
between genetically identical individuals (e.g., monozygotic twins), also undergo no rejection. Allografts are grafts between members of the same species that differ genetically. This is the most common form of transplantation. The degree to which allografts undergo rejection depends on the degree of histocompatibility between donor and recipient. The degree and type of response also vary with the type of the transplant. Some sites, such as the anterior chamber of the eye and the brain, are immunologically privileged (i.e., they have minimal or no lymphatics or vascularity, respectively); they tolerate the presence of mismatched grafts. In contrast to skin grafts which are not initially vascularized and so do not manifest rejection until the blood supply develops, the heart, kidneys, and liver are highly vascular organs producing vigorous immune responses in the host.

The antigens responsible for rejection of genetically disparate tissues are the products of histocompatibility genes which encode more than 40 loci. The loci responsible for the most vigorous allograft rejection reactions are located within the major histocompatibility complex (MHC). In man the system is denoted as human leukocyte antigen (HLA). In addition, minor histocompatibility antigens may also cause allograft rejection, as shown by the occurrence of rejection episodes among transplants exchanged between HLA-identical human donor and recipient pairs, although effective immunosuppression generally vitiates these responses.

The immune response to a transplanted organ consists of both cellular (lymphocyte mediated) and humoral (antibody mediated) mechanisms. The detection and treatment of allograft rejection has historically focused upon T-cell mediated process including natural killer cells, macrophages, antigen presenting cells (APC), antigen specific cytotoxic T-cells (CD 8 pos.), T helper (CD4 pos.) cells, cytokines. Cell mediated immunity has been extensively studied and long considered to be the main effector during acute allograft rejection. In fact, current induction and maintenance immunosuppression protocols seek to target T lymphocytes and to lesser degree, APCs [4]. Conversely, the role of alloantibodies in
acute rejection episodes has been far less clear, although the adverse effects of a positive cytotoxic antibody pre-transplant crossmatch - hyperacute rejections - have been recognized for 40 years. In my thesis I have dissected the mechanism, diagnosis and therapy of AMR.

II/2. Definitions

Multiple imprecise terms have been used to describe a rejection that may have a humoral component: vascular rejection, humoral rejection and accelerated acute rejection. The term antibody-mediated rejection (AMR) was eventually recommended [5] to describe all rejection reactions involving donor-specific antibody (DSA) or donor reactive antibodies, e.g. toward non-donor foreign anti-human leukocyte antigens (HLA), ABO isoagglutinins, and anti-endothelial antibodies. The spectrum of injury resulting from AMR includes hyperacute rejection occurring within two days after transplantation in patients with high levels of unrecognized DSA, as well as acute AMR generally occurring within the first weeks after transplantation sometimes later following an anamnestic increase in DSA activity in previously sensitized recipient. Chronic endothelial injury as a result of long term exposure to anti-HLA antibodies represents the other end of the spectrum - chronic AMR.

Hyperacute AMR is rare nowadays because of the sensitive crossmatch technique using recipient serum with donor lymphocytes. The modern crossmatching techniques, such as flow cytometry crossmatch, are capable of revealing even a low grade immunization.

Despite this sensitivity analyses with coated beads, save for avoiding HLA specificities to which antibody has been detected by routine serum screening, may be misleading due to insensitivity of the assay or altered antigen display on the particle producing a false negative result.

However, the subsequent development of acute antibody mediated rejection cannot be detected pre-transplant despite the improved understanding of cellular and humoral immune
mechanisms and the new immunosuppressive drugs. AMR rather than T cell-mediated variants has become the major hazard for long-term graft survival. Among the 8 to 10% of subjects who experience AMR, recognized due to a recent modification of the Banff classification [6], [7] 27 to 40% experience graft loss [6, 8]. Among all episodes of acute rejection, 20 to 30% show features of AMR [6]. This disorder is characterized by a sudden onset of rapidly progressive allograft dysfunction, which typically occurs within 3 weeks after transplantation [6],[9]. However, AMR may occur months or even years thereafter, particularly in the settings of non-adherence to the therapeutic regimen or altered absorption / metabolism of immunosuppressive drugs [6], [10].

II/3. Mechanisms of antibody mediated rejection

AMR results from the interaction of anti-donor antibodies, which are either preexistent at low titer or developed de novo after transplant, with allograft vascular endothelium [11, 12], the interface between the intravascular and extravascular compartments which is essential for proper renal function. Traffic of macromolecules, solutes, plasma proteins and cells between these compartments is tightly regulated, and depends on the integrity of the endothelial cell layer.

The intact endothelial surface is negatively charged, in part due to expression of electronegative molecules such as heparan sulfate. This electronegativity serves to repel negatively charged plasma proteins, including albumin and coagulation factors [13].

In humans, all endothelial cells constitutively express class I antigens. The capillary endothelium, but not the arterial endothelium, can express both class I and II antigens [14]. According to the humoral theory of organ transplantation, binding of antibodies to endothelial cell antigens can finally cause damage via four pathways (Figure1., Cai and Terasaki [12]. Briefly, it can be mediated directly by complement via forming membrane attack complex or inflammatory cells recruited by soluble complement fragments, or by phagocytes that
recognize complement fragments deposited on endothelial cells via complement receptors. The finding of complement fragment C4d in rejecting graft capillaries provided strong evidence to support this theory [15]. *Antibody-dependent cell toxicity* may also play a role in mediating endothelium damage without the involvement of complement [16]. Secondary pathological changes after endothelium damage include platelet activation and thrombosis, endothelial and smooth muscle cell proliferation, and humoral and/or cellular infiltrates mediated direct organ damage. These series processes contribute to thrombosis and vascular occlusion; therefore, the organ suffers irreversible ischemic injury.

The interactions of endothelial cells with DSA may activate complement causing cell lysis, loss of barrier integrity and subsequent ischemic injury [17]. The formation of intercellular ‘gaps’ and shedding of heparan sulfate molecules leads to loss of cell-surface electronegativity exposing the subendothelial matrix to plasma coagulation factors and platelets, resulting in thrombosis [18]. In addition to complement-mediated allograft injury, exposure to MHC class I antibodies induces vascular endothelial cell apoptosis, which is visible upon ultrastructural studies of human allografts experiencing acute AMR [19]. This phenomenon which occurs within days is complement independent. MHC class I antibodies may alter fibroblast growth factor receptor expression, thereby potentially inducing harmful proliferative changes [20]. Thus, variable levels of DSA activity may result in allograft injury through a variety of mechanisms, including both complement-dependent and independent pathways resulting in necrosis as well as endothelial cell apoptosis. This sequence of events provides logical targets for intervention in treatment of acute AMR. These interventions include lowering DSA activity through physical removal or inactivation as well as decreasing the production of DSA and complement inhibition.

In a prospective trial, it has already been found that by using antibody screening tests with flow cytometry or enzyme-linked immunosorbent assays, about 14%–23% of transplant
recipients with functioning grafts have detectable human leukocyte antigen (HLA) antibodies [21]. Within a 1-year follow-up period, 21 (8.6%) of 244 antibody-positive patients experienced graft rejection, which has significantly higher than the rate observed among the HLA antibody–negative patient group (43/1421 (3%); \( p = 0.00003 \)). These data suggest that some transplants may still function well in the presence of alloantibodies, which might be because of compensatory reactions of the transplanted organ to tissue injury. However, the graft may finally be rejected when the tissue repair system cannot fully compensate for the antibody-mediated injury. This damage-repair-damage process could take years to result in irreversible graft loss. This hypothesis has been supported by the study of Lee et al., who found that in some patients, it took many years for antibody-positive transplants to finally be rejected [22].

Due to proper patient selection and the highly effective immunosuppressive drug therapies unlike hyperacute rejection, acute and chronic graft function loss may not result mainly from thrombosis-related rapid vascular occlusion. Instead there is more likely to be a progressive damage-repair-damage pathological process, the speed of which depends on three factors: the level of alloantibodies; the capability of the transplanted organ for tissue repair; and the immunosuppressive and other supportive therapy.
II/4. Development of donor-specific antibodies

Following presentation of donor HLA antigens and in the presence of stimulatory cytokines corresponding clonotypic CD20+ B lymphocytes response internalize, process and subsequently express donor antigen-derived peptides complexed to cell surface MHC class II molecules (Figure 2, Gloor et al.[11]). The antigen - MHC II complex is then presented to helper T lymphocytes, which respond by releasing cytokines that stimulate proliferation and differentiation of B-cells into antibody-secreting plasma cells. This sequence of events requires the interaction of co-stimulatory molecules expressed on both B- and T-cells, including CD40 and CD40L (CD154) [23]. Additionally, B lymphocytes respond to T-cell help by migrating to lymphoid follicles, forming germinal centers and subsequently
undergoing mutations in cell surface immunoglobulin, resulting in the production of new clones of cells secreting antidonor antibodies (Figure 3, Gloor et al. [11]). Clones possessing increased specificity for their antigen are conserved, while less-specific clones are deleted. The costimulatory interactions between B-cell CD40 and T-cell CD40L are essential for isotype switching, whereby immature B cells expressing IgM and IgD differentiate into elements producing IgG, a more sustained immune reaction [11].

**Figure 2.** The interaction of T and B lymphocytes in development of donor-specific antibody-secreting plasma cells. (A). B lymphocytes expressing CD20 bind, internalize, process and then express the processed antigen-derived peptide complexed with MHC II molecule. (B). Antigen-derived peptide:MHC II complex. (C). T:B-cell interaction, under the influence of the co-stimulatory pathway (CD40-CD40L) results in production of cytokines which stimulate B cell proliferation and differentiation into DSA-secreting plasma cells. From Gloor et al.[11]
Figure 3. Production of donor-specific antibodies (DSA). Naïve B-cells (CD20+/27−) and memory B cells (CD20+/27+) located in secondary lymphoid tissue, when exposed to HLA antigen in the setting of appropriate T cell help either form germinal centers (GC) where they proliferate and differentiate into long-lived DSA-secreting plasma cells (CD138+/CD20−) which subsequently migrate to the bone marrow, or differentiate into short-lived DSA-secreting plasma cells without forming germinal centers. Helper T cells are required to permit differentiation of B cells into anti-HLA antibody-secreting cells. From Gloor et al.[11]

II/5. Humoral responses directed against non-human leukocyte antigens

Putative pathogenic antibodies that are not directed against the HLA system were observed among recipients who rejected HLA-identical kidneys more than three decades ago [24]. The relevance of non-HLA-related humoral immunity was recently confirmed among a large cohort of renal allograft recipients from the Collaborative Transplant Study [25]. However, characterization of non-HLA antibodies remains difficult. The antibodies rarely appear to recognize alloantigens, but often are directed against autoantigens. Similar to some autoimmune diseases, non-HLA antibodies may be diagnostically useful but not necessarily effector mechanisms. For this reason, it is important to identify their antigen specificity and potential pathogenicity of these moieties for rejection or other forms of allograft injury.
Aside from ABO blood group antigens and the HLA class I and II antigens, endothelial cells express minor histocompatibility antigens. However, the suspected existence of a common polymorphic non-HLA system in endothelial cells has not yet been confirmed by biochemical identification of the relevant antigens. Although several assays have been proposed to detect these determinants none has achieved acceptance possible due to the apparent heterogeneity of the endothelial antigens and their vascular bed-dependent distribution [26].

The evidence supporting a role for non-HLA antibodies against most common targets is based on observational trials performed in different aspects of solid-organ transplantation and experimental models. Among the other non-HLA antigens are the products of major histocompatibility complex class I chain-related gene A and gene B (MICA and MICB), which are highly polymorphic alleles that are closely linked to the HLA-B locus located on chromosome 6. In a recent study evaluating diagnostic posttransplant antibody tests in patients with renal allograft dysfunction, MICA did not correlate with C4d-positive rejection and the positivity was found in only one C4d-negative case of chronic allograft injury [27]. MICA/B apparently cannot fix complement, as C4d positivity is rare in MICA positive patients without anti-HLA antibodies. The antibodies may function as activating ligands that acts as potential target cells in the effector phase of natural killer (NK) cytotoxicity [28].

Antibodies against Angiotensin II type 1 receptor (AT1R-Abs) have been implicated as a trigger mechanism of non-HLA directed humoral rejection. AT1R is a seven transmembrane-spanning G-protein-coupled receptor including an extracellular, glycosylated region connected to the seven transmembrane-helices linked by three intracellular and three extracellular loops [29]. A recent study reported the presence of agonistic antibodies against the AT1R (AT1R-Abs) among 16 renal allograft recipients who underwent severe vascular rejection and malignant hypertension in the absence of anti-HLA antibodies [30]. AT1R-Abs
have also been associated with preeclampsia, malignant hypertension and occasionally seizures [31]. They experienced accelerated vascular rejection refractory to steroids and antilymphocyte antibody preparations despite transplantation of a “zero-mismatch” kidney. Rapid onset of malignant hypertension with seizures also accompanied the rejection process sometimes. Removal of AT1R-Abs by plasmapheresis combined with pharmacologic AT1R blockade improved renal function and graft survival in AT1R-Ab positive patients.

The identified AT1R-Abs belonged to complement-fixing IgG1 and IgG3 isotypes. Signal transduction studies employing IgG from transplant and preeclamptic patients implicate that genes regulated by AT1R-triggered transcription factors and not antibody-induced complement-directed cytotoxicity may account for a dominant effector pathway of the vascular injury [32].

In 40% to 50% of cases renal allograft biopsies showing severe vascular changes such as fibrinoid necrosis were C4d negative, implicating involvement of noncomplement-fixing antibodies or other mediators [33]. Non-HLA antibodies may mediate many biologic processes leading to obliteration of small and large vessels due to inflammation, thrombosis and facilitation of cell growth/migration. Nevertheless, there remains a great deal of investigation to achieve a more precise definition of the antigenic targets and suitable assays for non-HLA antibodies.

II/6. Clinical relevance of antibody-mediated processes

Patients with less than 10% panel-reactive antibodies show a significantly longer half-life than subjects with greater levels of sensitization [34], suggesting that pretransplant alloantibody levels are relevant to graft outcomes. While high levels of antibodies result in irreversible rejection, patients with lower levels of sensitization may experience rejection processes, although the graft may finally be rejected overcoming simultaneous tissue repair.
Hyperacute rejections continue to occur, according to The United Network for Organ Sharing (UNOS) kidney transplant registry particularly proportionate to increasing levels of panel reactive antibody (PRA) [35]. While it is unlikely that the crossmatch test was performed incorrectly, or that the test results were ignored or miscommunicated, there are other more likely reasons – antibodies directed against epitopes not arranged on lymphocytes or subclinical antibody levels. In contrast the presently applied FACS methodology may detect irrelevant antibodies that bind to lymphocytes but are unable to initiate graft destruction. The one-month failure rate 33% (n=52) among patients who were transplanted with a negative cytotoxicity crossmatch (that detects antibodies using complement-mediated target-cell killing) but a positive flow cytometry crossmatch was substantially greater than the 8% rate among 179 flow cytometry negative patients [35]. The UNOS registry revealed greater incidence of primary non-function correlated with increasing levels of preformed HLA antibodies [36], suggesting the involvement antibodies [35]. Many instances of primary kidney non-function, as evidenced by removal without ever functioning (8% of 7788 first, 14% of 1471 second and 20% in 224 third grafts in the UNOS registry) were caused by ‘hidden’ hyperacute rejection [35].

Among subjects with rejection episodes far fewer patients were without versus with HLA antibodies pretransplantation (n=98) or post-transplantation (n=98) [37]. Both acute humoral and acute cellular elements of rejection were observed among patients who displayed antibodies.

II/7. Diagnosis of AMR

In addition to its fulminant clinical course, AMR is characterized by evidence of antibody deposition in the kidney as evidenced histologically by accumulation of neutrophils in glomerular and peritubular capillaries, by vasculitis or by fibrinoid necrosis of arteries and arterioles. Accumulation of polymorphonuclear neutrophils and monocytes/macrophages in
dilated cortical peritubular capillaries (PTC) is suggestive of acute humoral rejection [38-44]. Other pathological features include as glomerulitis with neutrophils and/or monocyte infiltration, glomerular and arteriolar fibrin microthrombi and severe vasculitis with fibrinoid necrosis (Figure 4). However, these morphological features alone do not provide a specific and sensitive diagnosis.

![Figure 4](image)

**Figure 4.** Acute humoral rejection. (A) Peritubular capillaries contain numerous polymorphonuclear leukocytes and mononuclear cells. Glomerular capillaries contain some polymorphonuclear leukocytes and mononuclear cells and a fibrin thrombus. (B) Insert of A, left-middle part. Numerous polymorphonuclear leukocytes are observed in a peritubular capillary. Interstitial oedema is noted. Periodic acid-Schiff (PAS)-staining; original magnification ×200(A) and ×500 (B). (C) Immunofluorescence microscopy using monoclonal antibody to C4d. Intense linear circumferential staining of the peritubular capillaries (Fresh frozen tissue sample. Original magnification ×250). (D) Immunohistochemistry using polyclonal antibody to C4d. Intense staining of the peritubular capillaries (Paraffin-embedded tissue. Original magnification ×480). From: Moll and Pascual Humoral rejection of organ allografts Am J Transplant 2005;5(11):2611-2618.[14]
A more specific diagnosis of AMR is deposition of complement-dependent moieties (Figure 4) that leave a footprint of diffuse \textit{C4d deposition} along peritubular capillaries (PTC), [6, 9, 45, 46] C4d deposition in peritubular capillaries has been demonstrated to be a useful, sensitive marker for diagnosing AMR. This observation correlates with poor allograft survival [46, 47]. The prognosis of renal allografts demonstrating early post-transplant capillary C4d positivity was found to be significantly worse, with a 1-year graft survival of only 57% and 63% (diffuse and focal C4d deposition respectively), as compared to a 90% 1-year graft survival among kidneys without C4d deposits [46]. Although this study performed in the ‘cyclosporine, steroids, azathioprine’ era was confirmed using materials from the current era of newer immunosuppressive agents [6, 43, 48]. Moreover, the adverse predictive value of capillary C4d deposition has been shown to be independent of numerous other morphological and clinical factors [49].

Experimental animal studies have also suggested the role of complement in AMR. Complement fixation is shown to promote acute or hyperacute rejection [50]. Complement fixing isotypes of monoclonal anti-H-2 class I antibodies passively transferred the AMR toward mouse cardiac allografts [51], a reaction that was associated with C4d deposition in the microvasculature. These findings were similar to those observed among renal and heart grafts in humans [52, 53]. C4d is relatively durable due to the covalent bond to tissue element (C4d contains an internal thioester bond). In mice the C4d depositiob disappeared two weeks after passive transfer [52] and five days after the rat heart was re-transplanted back into an isogeneic recipient [53]. The acute effects of complement are well described and include chemoattraction of neutrophils and macrophages via C3a and C5a, vasospasm through the release of prostaglandin E2 from macrophages, and edema through the release of histamine from mast cells. C3a and C5a increase endothelial adhesion molecules, E-selectin, vascular
cell adhesion molecule-1 and ICAM-1, and production of cytokines and chemokines such as IL-6, IL-1α, CXCL8, and CCL5. The membrane attack complex, C5b-9, causes lysis of ECs. The local production of complement components, such as C6 by recipient macrophages, augments acute rejection. Protection from antibody-mediated rejection can be achieved in animal hosts by inhibition of the complement system, as shown by transgenic expression of the complement regulatory proteins CD46 (membrane cofactor), CD55 (decay-accelerating factor), and CD59 in pigs [54]. High expression of transgenic human CD46 prevented hyperacute rejection and thrombotic manifestations.

Mauiyyedi at al. performed a prospective study to evaluate the value of C4d staining for the diagnosis of AMR. 67/232 kidney transplanted patients developed acute rejection within the first 3 months among all acute rejection biopsies. Widespread C4d staining in PTC was present in 30% (20/67). A second metric of AMR is DSA which were present in 90% of the C4d+ cases (18/20) compared with 2% (1/47) of the C4d- acute rejection cases ($P = 0.001$). The sensitivity for the diagnosis of AMR of C4d staining was reported to be 95% versus 90% for donor-specific antibody tests [6].

Another index of humoral activity is the presence of putative donor-specific antibodies directed toward Class I or Class II human leukocyte specificities [6, 39, 55], as detected with HLA antigen-coated beads by flow cytometry. However, HLA class II-reactive antibodies are not always detected in conventional microtoxicity assays using panel cells, and may require more refined techniques (i.e. flow cytometric crossmatching and/or HLA antigen-coated fluorescent microparticles) [56-58]. The diagnosis of AMR has been aided considerably by technologic advances in histocompatibility testing and immunohistology. The increased sensitivity of assays and the ability to distinguish between antibody specific for the donor versus antibody to third-party HLA antigens or non-HLA antigens have been tremendous advancements [59, 60]. DSAs have been reported to be present in the majority of patients
whose renal biopsies are C4d positive. Despite the absence of vascular abnormalities, grafts with typical histological features of cell-mediated rejection may display a humoral component as evidenced by the C4d deposition [6, 9].

A firm diagnosis of AMR can be established when there is the presence of allograft dysfunction, characteristic histology features, presence of C4d and DSA. However, all of these data are not always available, and there may be a dangerous time to acquire this information.

Figure 5 shows an algorithm for the diagnosis of acute AMR caused by anti-HLA antibodies. The initial branch point in the algorithm is defined by the presence or absence of graft dysfunction. If graft dysfunction is present, the next step assesses the level of risk associated with the clinical setting of transplantation.

**Figure 5.** Algorithm proposed by the Antibody Working Group for the diagnosis of antibody-mediated rejection (AMR). From: Montgomery, Transplantation, 2004;78(2):181-185 [5]

*High-risk criteria* include husband to wife or child to mother donor-recipient pairs, history of a sensitizing event (pregnancy, transfusion, or previous transplant), and known
production of HLA-specific antibody, current or historical. If the transplant has occurred in a low-risk setting, there is greater a burden of proof for the diagnosis of AMR requiring both biopsy findings as well as the presence of DSA. In high-risk patients, the presence of characteristic features of AMR on light microscopy with (+) C4d staining represents sufficient diagnostic criteria. Even without DSA or characteristic biopsy features, one must maintain a high index of suspicion for AMR among patients with significant risk factors who show graft dysfunction [5].

In the absence of clinical graft dysfunction, routine biopsy or detection of DSA may suggest positive findings. The significance of these events remains incompletely elucidated. Light microscopic features consistent with AMR may appear on protocol biopsies obtained from patients with stable renal function, suggesting a subclinical or a preclinical antibody-mediated process that may or may not evolve into frank AMR. If DSA is detectable and there is evidence of injury on biopsy in the acute setting, graft dysfunction will likely occur. These patients may benefit from early or preemptive treatment seeking to eradicate the DSA or resolve the injury. In cases of ABO incompatible grafts, it is not unusual to see C4d staining without histologic evidence of injury [61]. This may represent accommodation, and it is unclear whether this findings warrants treatment, especially when isoagglutinin titers are stable. Anti-HLA DSA may be detected despite of a normal biopsy and unchanged graft function. This could also represent accommodation or a latent antibody response. In the early posttransplant period this may be an appropriate trigger for therapy. In most cases anti-HLA DSA can be durably suppressed except for ABO isoagglutinins in which antibody may return with adverse consequences.
II/8. Treatment of AMR

Traditional anti-rejection treatments, such as administration of steroid boluses or anti-lymphocyte antibodies are usually ineffective to treat AMR, since these modalities are primarily directed toward cellular immune mechanisms. Among the biopsies obtained to diagnose acute rejection episodes, 12-37% has been reported to occur in patients resistant to standard therapies; they experience a poorer prognosis [62]. Approaches to treat AMR seek to remove deleterious antibodies using plasmapheresis (PP) or immunoadsorption, and to modify B cell activation and antibody generation by administration of intravenous immunoglobulin (IVIg; 1 to 2 g/kg) or low-dose CMV hyperimmune globulin (100 mg/kg) [5, 6, 63-71] and to eliminate B-cells with rituximab (375 mg/m2;[72]) .

II/8.1. Primary immunosuppressant

While almost all currently used immunosuppressive drugs have direct or indirect effects to inhibit/deplete B cells. The most commonly used primary agents of maintenance immunosuppression, such as cyclosporine A, tacrolimus, and sirolimus, are powerful immunosuppressants that interfere with T-cell signaling. The successful prolongation of graft survival by using these agents has misled many clinicians and some immunologists into thinking that T cell is the only player that causes graft rejection. However, because many alloantigens eliciting antibody responses are proteins (e.g.,HLA, MIC) and antibody responses to protein antigens require antigen-specific T-cell help, T-cell targeting agents not only prevent T-cell but also antibody (B cell)-mediated immune responses. This mechanism explains why these primary agents may be used in combination with other therapies to treat antibody-mediated humoral rejection [12, 39].
II/8.2. Adjunct immunosuppressants

Unlike primary immunosuppressive agents, which block T-cell signaling and indirectly inhibit proliferation of B cell secondary to reduced cytokine production by T cell, adjunctive immunosuppressants interfere with nucleosid synthesis retarding cell division [73, 74]: namely azathioprine, cyclophosphamide, mycophenolate mofetil (MMF). These agents have direct inhibitory effects on B cell, an active dividing tissue cell. It is notable that because of its role in targeting the de novo purine biosynthesis pathway, mycophenolates can inhibit human lymphocytes (B and T cells) more specifically and efficiently than other cell types [73]. Clinical observations have suggested that immunosuppressive protocols with MMF inhibited antibody production and reduced allograft rejection episodes [75, 76].

II/8.3. Anti lymphocyte antibodies

Antibodies against lymphocyte surface molecules act in concert with complement to remove specific lymphocyte subsets or to inhibit cell function [67, 77-79]. The anti-CD3 reagent OKT3 blocks T-cell activation thereby inhibiting proliferation of naïve B-cells. Campath-1H which is directly reactive with CD52 surface marker on thymocytes, T and B as well as mast cells, directly depletes B-cells. Anti-thymocyte globulin (ATG) and anti-lymphocyte globulin (ALG) interfere with T-cell help, and due to cross-reactivity reduce and inhibit B-cells [12]. However, the anti T-cell effect of these reagents is so profound but they are unable to produce sufficient B-cell suppression to overcome a robust AMR.

II/8.4. Rituximab

II/8.4.1. Introduction

Rituximab (MabThera, F. Hoffmann-La Roche Ltd., Pharmaceuticals Division, Basel, Switzerland; Rituxan, IDEC Pharmaceuticals, San Diego, CA, and Genentech, Inc, San Francisco, CA) is a genetically engineered, monoclonal, chimeric (mouse/human) antibody
directed against the B-cell surface marker CD20 [80]. It was approved in the United States in 1997 and in the EU in 1998 only for the treatment of refractory or relapsed B-cell lymphomas [81]. After demonstration of its efficacy it was approved for the treatment of rheumatoid arthritis (RA) in 2006 by the FDA as well as the EU [82].

Rituximab has been used “off label” to treat transplant patients in several settings primarily based upon non-controlled clinical experiences.

1. post-transplant lymphoproliferative disorder;
2. rejection prophylaxis;
3. rejection reversal;
4. conditioning for ABO incompatible transplantation;
5. and desensitization in HLA sensitized patients.

II/8.4.2. Pharmacodynamics and pharmacokinetics

Rituximab comprises human IgG1Fck constant regions and Fab’2 small variable light and heavy chain regions of the murine anti-human CD20 antibody (IDEC-2B8). The construct was cloned into Chinese hamster ovarian (CHO) cells for the production of immunoglobulin [81]. Rituximab (molecular weight of 145 kDa) consists of two heavy chains (451 amino acids) and two light chains (213 amino acids). Its binding affinity for the CD20 antigen is approximately 8.0 nM [81]. Binding specificity for the CD20 antigen resides in the complementarily determining sequences with the variable murine regions, while the human portion triggers complement- and cell-mediated lysis mechanisms in vivo.

The majority of pharmacokinetic and pharmacodynamic studies have been performed in patients with B-cell lymphoma [80, 83]. Nine patients presented who received four doses of 375 mg/m2 as an IV infusion displayed a mean serum half-life of 59.8 hour (range=11.1 - 104.6 hour) after the first infusion and 174 h (range 26 to 442 h) after the fourth infusion. The serum concentration correlated directly with the response and inversely with tumor burden.
The wide range of half-lives was attributed to the variable tumor burden, especially the changes normal versus malignant B-cell populations upon repeated administrations. In a single-dose study in subjects with renal failure, a substantially longer half-life was observed ranging from 10 to 14 days [72]. In a study using rituximab for the treatment of rheumatoid arthritis, the half-life after the second dose was 20 days, which was similar to native IgG [84].

**II/8.4.3. Administration and adverse reactions**

The appropriate amount and the number of doses of rituximab are dependent on the clinical setting. For lymphoma, the approved dose is 375 mg/m2 as an IV infusion for four weekly doses. However a small single dose (50 mg/m2) study suggested similar efficacy with terms of degree and duration of peripheral B-cell suppression, and effect on antibody response [72]. In adult rheumatoid arthritis (and most autoimmune diseases) the suggested administration is two doses of 1 gm every other week [85], which is similar to the four-dose regimen in a 1.73 m2 adult. In this study body surface area was shown to contribute 19.7% to the variability in antibody clearance, an insignificant contribution to the variability in drug exposure as measured by AUC. Therefore, for adult rheumatoid arthritis, dosing by body surface area is not necessary.

**II/8.4.4. Clinical outcomes**

Garrett and colleagues reported a case of humoral rejection that was successfully treated with rituximab [77], followed by a series of 8 successfully treated heart transplant patients [86]. A Wisconsin group successfully treated 27 patients who were diagnosed with biopsy-confirmed rejection manifested with thrombotic microangiopathy and/or endothelialitis between February 1999 and February 2002. Twenty-four subjects received additional steroids, and 22/27 patients were also treated with plasmapheresis (PPH) and antithymocyte globulin. Only three patients experienced graft loss not associated with patient death during the follow-up period (605+/- 335.3 days). Among the 24 successful patients, the mean serum creatinine
decreased from 5.6±1.0 mg/dl at therapy initiation to 0.95±0.7 mg/dl at discharge. Authors predicted that the addition of rituximab may improve outcomes in severe, steroid-resistant or antibody-mediated rejection episodes after kidney transplantation [67]. A case report suggested that rituximab can be used successfully even in desperate, steroid and polyclonal antibody treatment resistant, C4d negative rejection episodes [87]. Among 8 renal transplant patients with C4d+ and DSA+ AMR treated with four infusions of rituximab (375 mg/m2 per week for four weeks) in addition to plasma exchange (2-17 sessions), steroids, mycophenolate mofetil and tacrolimus, Faguer et al. reported at a follow-up of 10 months (range 7-23), 100% and 75% patient and graft survivals, respectively. Renal function improved in 6 cases, graft loss occurred in two and four patients experienced infectious complications. DSA disappeared or decreased in four cases [8].

Rituximab and intravenous immune globulin has also been used for pre-transplant desensitization. Ashley et al. achieved among 20 highly sensitized patients (PRA=77±19% or with DSA) 100% and 94% patient and graft survival rates, respectively. Time to transplant after desensitization was 5±6 months. After the second infusion of intravenous immune globulin the mean PRA level was 44±8-30%. The authors concluded that a combination of intravenous immune globulin and rituximab may be an effective desensitization regimen for patients awaiting transplantation from a living or a deceased donor [88].

The optimal off label use or dosage of rituximab for AMR has not yet been established. The low AMR rates observed using current immunosuppression make such trials hard to recruit, enforcing the need for cooperative multicenter trials with long term follow up. As rituximab has no effect on plasma cells and little effect on circulating antibody, it is likely to be most effective for the treatment of rejection in combination with other strategies that include plasmapheresis and/or intravenous immunoglobulin (IVIg) therapy.
The ultimate resolution of the dosing regimen is important if for no other reason than cost, since rituximab is expensive. As currently available the 10 mg/mL concentrate containing either 10 mL (100 mg, average wholesale price of $568) or 50 mL (500 mg, average wholesale price of $2840). If using body surface area dosing, for an average 1.73 m² person, the required dose is 650 mg, which would translate to $3976 per infusion or $15 904 for four-treatments. If using the 1000 mg dose, it would be $5680, or $11 360 for the two-dose course.

As opposed to many chemotherapeutic agents, the dose is adjusted for lung, kidney, liver, or heart dysfunction. It is recommended to premedicate patients with acetaminophen and antihistamine before each infusion to prevent reactions. Adverse reactions related to rituximab usually occur during the first administration.

In the transplant setting corticosteroids are given in conjunction (e.g. treatment of rejection or at the time of transplant), the rate and severity of side effects is decreased. In rheumatoid arthritis patients, a single 200 mg dose of methylprednisolone with the first dose of rituximab did reduce the frequency of side effects [89].

Upon subsequent infusions, reactions are milder in intensity or do not arise at all [90]. Mild reactions can be treated symptomatically during the infusion: flu-like symptoms, fever, chills, rigors, nausea, headache, and rash. Agarwal et al. demonstrated increased cytokine levels particularly TNFα, immediately after the first dose of rituximab which can be associated with a febrile response [91]. Rare but more serious reactions include angioedema, hypotension, bronchospasm, and hypoxia can occur, usually within 30–120 min of beginning the first infusion. In these cases, rituximab should be stopped to initiate supportive care. After resolution of all symptoms, the infusion can be restarted at slower rate.

As of 2004, over 540,000 patients had received rituximab world-wide [92]. In a few lymphoma patients, rituximab therapy resulted in major complications, including tumor lysis
syndrome, infusion-related death, mucocutaneous reactions, delayed neutropenia, and lung injury, but their incidence was 0.5% upon post-marketing safety data analysis [92]. As rituximab affects both malignant and non-malignant CD20-positive B cells, there are concerns about infectious complications from the therapy. However, patients receiving maintenance rituximab for non-Hodgkin lymphoma have not been reported to show an increased infection rate after 2 years of continuous B-cell suppression [93]. Protective titers against immunized pathogens appear to be preserved in most instances.

Despite B-cell depletion for approximately 6 months, immunoglobulin levels in lymphoma patients remain stable after one cycle of rituximab treatment [94]. Extended use of the drug may result in decreased IgM levels [95]; therefore, measurement of serum IgG levels should be considered along with appropriate treatment with intravenous immunoglobulin if levels fall below 300 mg/dL [96].

In patients treated with rituximab rare case reports have described hepatitis B virus (HBV) reactivation resulting in liver failure [97], fatal varicella-zoster infection [98], pure red cell aplasia because of parvovirus B19 [99] and reactivation of latent JC virus. Based on these reports and several phase II trials, patients with PTLD appear to tolerate rituximab as well as de novo lymphoma patients. Of note, reactivation of cytomegalovirus infections resulted in the death of one PTLD patient after rituximab treatment [100]. Those PTLD patients who have an increased risk of HBV infection should be considered for HBV screening test prior rituximab.

Rituximab may be detected in the serum for many months after dosing [72]. The persistence of rituximab in the serum has implications for crossmatch and tissue typing analyses. These antibodies binding to B-cells could be detected using a second antibody suggesting the presence of alloreactivity. Since rituximab is cytotoxic in the presence of complement, sera that contain rituximab would produce a positive B-cell cytotoxic-positive
crossmatch. The human portion of the IgG1 can provide a target for the anti-human Ig fluorochromes in flow cytometric crossmatches again resulting in a false positive B-cell crossmatch. Flow and cytotoxic crossmatches and PRA determinations, however, can be attempted by either pronase treatment to reduce the cell surface CD20 or by removal of the circulating rituximab with immunomagnetic bead absorption [101].

II/8.4.5. Mechanisms of action

CD20 is a B-cell surface antigenic phosphoprotein that is restricted in its expression to pre-B and matures B-cells. It is neither present on stem cells nor on plasma cells [8, 67], although plasma blasts and stimulated plasma cells may express CD20 [102]. It does not rapidly modulate upon binding with anti-CD20 antibodies [103]. Studies in patients with non-Hodgkin’s lymphoma (NHL) have shown that rituximab treatment transiently and selectively depletes (CD20+) B-cells for up to six months. Thereafter, B-cell levels return to normal within 9–12 months [90]. Even when the total B-cell count returns to normal, there appears to be a change in the phenotype, with the B-cells present being relatively deficient in expression of CD27, a surface marker of memory B-cells [104], suggesting that the repopulating B-cells are primarily naive, at least as 2 years after a single dose.

CD20 does not shed, modulate or internalize after antibody binding; its expression is stable [105]. Anti-CD20 antibodies therefore remain bound to CD20 on the cell surface, where they initiate cell lysis. Free CD20 antigen is not found in the circulation [106].

There are no known natural ligands for CD20. The function of CD20 seems to be involved in the regulation of B lymphocyte growth and differentiation, possibly playing a role in Ca$^{2+}$ influx across plasma membranes to maintain intracellular Ca$^{2+}$ concentration necessary for B-cell activation [107]. The following effects of rituximab may contribute to its B cell depletion properties [103]:

\[ \text{B cell depletion properties} \]
1. Antibody dependent cell mediated cytotoxicity (ADCC, Figure 6), in which natural killer cells, macrophages, and monocytes are recruited through their Fc receptors bound to surface CD20. This induces CD20+ B cell lysis [108, 109].

![Rituximab-mediated ADCC and strategies to enhance the ADCC effect.](image)

**Figure 6.** Rituximab-mediated ADCC and strategies to enhance the ADCC effect.


Complement dependent cytotoxicity (CDCC) normally has little efficacy in rituximab therapy. This kind of cellular cytotoxicity can occur after binding of either the rituximab Fc region (in ADCC) or C1q, C3b, C4b, and iC3b (in CDCC) to their respective receptors, resulting in either phagocytosis or cell-mediated lysis of B-lymphocytes, depending on the effector cell type. The Fc region of cell-bound rituximab is recognized principally by either activating receptors Fc-RI/ Fc-RIII or the inhibitory receptor Fc-RIIB, whereas the byproducts of complement activation are recognized by C1qR, CR1, or CR3 on effector cell surfaces. The strategies to overcome resistance to ADCC or CDCC effects and thereby enhance rituximab effects
include: (1) anaphylatoxins (C5a, C3a); (2) delivering of cytokines (IL-2, IL-12, M-CSF) that activate effector cells; and (3) complement receptor 3 (CR3) - specific polysaccharides such as - glucan, which primes CR3 and therefore triggers cellular cytotoxicity [110].

This theory is also supported by data from an animal model with the significance of Fc receptor-deficient mice, in which rituximab shows significantly diminished activity [109]. Further, it has been demonstrated that Fcγ receptor polymorphisms affect cellular affinity for the Fc portion of rituximab, thereby impacting the time to progression among rituximab treated follicular lymphoma patients. It has been long recognized that the response to rituximab and its impact on time to progression is variable among various lymphoma types and different patients of each type due to Fcγ polymorphisms [111, 112]. In the future determination of Fcγ R polymorphisms might be used to predict the a priori response to rituximab which has shown variable efficac [113, 114]. The ability to predict response would not only allow for a rational patient selection and cost-effective therapy but also would enable us to choose alternative therapies for those patients with high probability of treatment failure. New anti-CD20 antibodies or conjugates are being developed with re-engineered Fc portions to enhance the efficacy of treatment by increasing binding affinity to Fcγ receptors [115].

2. **Complement dependent cytotoxicity** (Figure 7) is induced by complexed rituximab bound to surface CD20 and cousing C1q binding. Activation of the complement cascade and generation of the membrane attack complex, causing CD20+ B cell lysis [81]. C1q interacts with the rituximab Fc region exposed after binding to CD20 on the B-cell surface, thus activating the classical complement cascade and a membrane-attack complex (MAC) is inserted into the cell membrane, with multiple MACs (12- to
16-mers) leading to cytolysis. Strategies to overcome resistance to CDC effects include: (1.) inhibitors of the membrane complement regulatory protein (mCRPs), decay-accelerating factor (DAF), especially CD59, which enhances complement activation and augments MAC formation on the cell surface; (2) heteroconjugates of rituximab to cobra venom factor (CVF) or C3b, and other antigen-antibody complexes targeting tumor cells, which enhance complement activation; and (3) drugs that upregulates CD20 expression, namely the histone deacetylase inhibitor trichostatin A or the protein kinase C activator bryostatin-1 [110].

hCD20 transgenic mice have been generated through the integration of bacterial artificial chromosomes encoding the hCD20 locus in FVB mice. In this murine model, hCD20 expression mimics that of humans yet the expression of the transgene occurs at a 50% level of that of circulating human B cells.

Figure 7. Rituximab-mediated complement dependent toxicity (CDC) effect and strategies for enhancing the CDC effect. From: Zhou et al. The role of complement in the mechanism of

As this model preserved the CD20 epitopes recognized by rituximab, it is an invaluable tool to study the in vivo mechanisms of action of this drug [116]. Using this model, Gong et al. [116] have identified ADCC and CDCC as the most relevant in vivo depletional mechanisms of rituximab, and have shown that the susceptibility to depletion varies among the various lymphoid compartments. While CD20+ B cells in peripheral blood are rapidly depleted by rituximab [116], CD20+ B cells homing in lymphoid compartments are somewhat resistant to rituximab depletion, requiring longer, multiple-dose treatments to achieve effective killing. Of particular interest is the resistance to rituximab exhibited by B cells in the marginal zone (MZ). These cells are believed to be involved in natural antibody responses and maybe the sources of anti-ABO antibodies [116]) and germinal centers (GC) of the spleen [116, 117]. Depleting these cells is vital for the success of desensitization and antibody-mediated rejection protocols. MZ and GC B-cells are pivotal in the development of long-lived plasma cells and humoral responses against T-cell-dependent antigens [118]. Thus peripheral blood CD19 and CD20 absolute B-cell counts may not accurately reflect rituximab action. Although the assessment of lymphoid-bound B cells may be more accurate, this does not represent a clinically practical alternative.

3. Rituximab has also been shown to induce CD20+ B cell 

   \textbf{apoptosis} (Figure 8, [110, 119]).
Figure 8. Rituximab-induced apoptosis in therapy and strategies to enhance apoptosis.


Phosphoproteins associated with glycosphingolipid-enriched membrane microdomains (GEMs) normally recruit Csk to maintain the Src-family kinases Lyn, Fyn, and Lck in an inactivated state. CD20–rituximab crosstalking can redistribute lipid rafts transactivating these kinases and initiating downstream signaling pathways resulting in apoptosis. The redistribution of lipid rafts may also induce apoptosis by Fas molecule clustering, which leads to the formation of death-inducing signaling complexes (DISC) with subsequent recruitment of Fas-associated death domain proteins (FADD) and caspase-8 into DISC, thereby activating the downstream apoptosis pathway. Meanwhile, the redistribution of lipid rafts can also inhibit p38 mitogen- activated protein kinases (MAPK), extracellular signal–related kinases
(ERK-1/2), NFk-B, and Akt signaling pathways inhibiting both transcription and expression of many genes, particularly the anti-apoptotic genes Bel-2, Bel-xL, X-linked inhibitor of apoptosis protein (XIAP), and myeloid cell leukemia sequence 1 (Mcl-1), thereby making B-lymphocytes susceptible to apoptosis. In addition, inhibition of nuclear factor kappa B (NFk-B) pathway downregulates the transcription factor Yin Yang 1 (YY1), enhancing transcription of Fas and of death receptor 5 (DR5) as well as facilitating FasL- and TRAIL-induced apoptotic cascade.

Strategies to enhance apoptosis after rituximab treatment include: (1) scFvRit:sFasL (fusion protein); (2) mapatumumab, a humanized monoclonal antibody targeting tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptor 1; (3) a recombinant protein, Apo2L/TRAIL, all the above three drugs target DRs to trigger the apoptosis pathway; and (4) other means such as antisense oligonucleotides of related apoptotic molecules or bortezomib, a proteasome inhibitor that induces apoptosis.

4. Rituximab exerts regulatory effects on the cell cycle that induce direct growth arrest. Bezombes et al. [120] demonstrated direct inhibition of growth by rituximab in Daudi and RL B-lymphoma cells in vitro. After moderate accumulation of tumor cells in the G1 phase, there was a significant loss of clonogenic potential without apoptosis. Rituximab produced a rapid, transient increase in acid-sphingomyelinase activity and concomitant cellular ceramide generation in raft microdomains, suggesting a ceramide-triggered signaling pathway associated with the induction of cell cycle–dependent kinase inhibitors such as p27Kip1 through an MAPK-dependent mechanism. Therefore, rituximab can mediate direct growth arrest, which may contribute to its anticancer activity.
5. B cells are efficient antigen-presenting cells, particularly after they have been activated [121]. Such activation could occur at the time of rejection. Rituximab may mitigate this mechanism amplification. In addition loss of antigen presentation may reduce T-cell stimulation due to elimination of B-cell produced cytokines. Supporting the direct role of B cells in rejection is the report of Sarwal et al. showing that CD20 gene expression was associated with a worse prognosis [122]. More recently, Hippen et al. showed that subjects with CD20 positive rejection showed worse long-term renal graft survival [123]. Of 27 patients with biopsy-proven Banff 1-A or Banff 1-B rejection in the first year after transplantation, 6 had CD20-positive B-cell clusters in the interstitium and 21 did not. The CD20-positive group displayed reduced graft survival compared to the CD20-negative controls. Rituximab treatment of a patient with such CD20 cells in the biopsy was successful whereas the rejection had been resistant to steroids and anti-thymocyte globulin [124].

Possible mechanisms of rituximab action other than B-cell depletion:

1. Increases MHC II and adhesion molecule expression: lymphocyte function-associated antigens (LFA-1 and LFA-3) [125].

2. Downregulates B-cell receptors and CD16 and upregulates CD54 on NK cells leading to their exhaustion [125].

3. Depletes DSA or inhibit their production, preventing the maturation of new DSA-secreting plasma cells [126]. Since antibodies are primarily produced by plasma cells that have minimal expression of CD20 and therefore are not eliminated by rituximab, this mechanism has been questioned. However there is some debate whether plasma cells are long or short lived [127]. In the former case, a method to deplete plasma cells is needed, whereas in the latter, as CD27+ memory B-cells are
eliminated, and no source of alloantigen were present to re-stimulate newly appearing naïve B-cells, suggesting that alloantibody titer and PRA will fall at a rate controlled by immunoglobulin half-life and the half-life of plasma cells.

4. Down-regulates co-stimulatory B cell surface molecules expression, including CD40 and CD23 [128].

5. In some cases antitumor effects of rituximab persisted long after the antibody was cleared from circulation. These findings led some investigators to propose that rituximab-induced killing of malignant B-cells might result in priming of lymphoma antigen-specific T-cell responses in vivo. Generation of these T-cell responses or “vaccinal effects” of rituximab might in turn be responsible for an anti-lymphoma immunity that persists far beyond the initial cytotoxic effect of the antibody itself [129]. Supporting this mechanism, Selenko et al have shown that in vitro treatment of lymphomas with rituximab led to cell destruction and generation of apoptotic bodies processed by APCs with subsequent cross-presentation of tumor-derived antigens to T-cells [130]. It has been reported that rituximab administration may decrease the activated phenotype of peripheral and tissue-resident T-cells by abolishing antigen presentation by B cells, thereby enhancing the numbers and function of regulatory T cells leading to improvement in autoimmune disease [131].

6. Since rituximab has been used for various indications (with the exception of lymphoma where the target cells are actually eliminated), it has been claimed that the reagent acts as a nonspecific intravenous immunoglobulin (IvIg) [132]. While the clinically used doses seem too low to cause an effect, recent data have suggested that IvIg inhibits anaphylatoxin-C3a and -C5a-induced calcium responses in vitro and blocks cellular migration and lethal C5a-mediated circulatory effects in vivo in
mice and pigs [133] at 10 mg/mL concentrations, which are less than observed at the peak of a rituximab infusion [72].

II/8.4.6. Limitations

Current therapy may be limited by exhaustion of immune effector mechanisms rendering the patient susceptible to overwhelming. For example, high burdens of IgG-opsonized cells may deplete complement [134] and also exhaust cellular cytotoxicity mediated by NK cells and tissue macrophages [134, 135]. In contrast, ineffective rituximab therapy may lead to substantial reductions but not elimination of CD20 surface expression (shaving)[134]. Strategies to address these limitations include the development of novel anti-CD20 monoclonal antibodies that more potently recruit effector functions, the use of lower, more frequent antibody doses, and the combination of antibody therapy with immune modifying drugs (IL2 or IL2 activated cells) that mobilize or replenish effector functions[125]. Lessons learned from the strategies employed for CD20 immunotherapy may be useful for the optimization of other immunotherapeutic monoclonal antibodies such as the second-generation fully human, anti CD20 antibody namely ofatumumab [136].

II/8.5. Splenectomy

Splenectomy seeks to remove a major source of B-lymphocytes. Hume originally proposed this modality but Frye et al thought it to be more beneficial for re-transplant cases. The benefits of splenectomy to prolonge graft and patient survival remain controversial. Splenectomized patients displayed a reduced incidence and intensity of acute rejection episodes and better graft and patient survival rates in the azathioprine era [137]; however, this beneficial effect if anything was short-termed with various regimens. Recently, in combination with other treatment, splenectomy seems to play an important role to prevent humoral rejection and prolonging graft survival among ABO-incompatible transplantations.
although most current protocols have substituted rituximab treatment for the surgical procedure.

II/8.6. Removal or blockade of preexisting or newly developed antibodies

Methods to reduce or blocking their detrimental effects of existing antibodies include plasmapheresis and Immunoadsorption. The latter in vitro approach removes immunoglobulins from patient peripheral blood using blood group antigen A or B, protein A, or antihuman Ig-coated columns. Originally, it was primarily used as pre-emptive therapy for ABO-incompatible or presensitized patients [139-141]. But successful reversal of antibody-mediated rejection has also been reported [42, 142, 143]. However; an HLA antigen column, which specially removes HLA antibodies, is not yet commercially available.

Plasmapheresis (PP) removes antibodies and other plasma factors such as complements and cytokines. It is an effective treatment for humoral rejection. Burns et al. recently reported pre-transplant PP of 41 B- or T-cell crossmatch positive patients who displayed high DSA levels and 29 with DSA content. The incidences of AMR were 39% (16/41) in the High DSA group and 31% (9/29) in the Low DSA group. Overall the mean DSA levels decreased by day 4 post-transplant, remaining low in patients who did not develop AMR. By day 10, DSA levels increased in 92% (23/25) of patients developing AMR with positive B-cell flow cross-matches. The total DSA measured by single antigen beads correlated with responses suggesting that either could be used for monitoring. The authors concluded protocols that yielded lower level of DSA decreased the AMR incidence [144].

PP has also been used as a preemptive strategy to prevent potential rejection episodes [138, 145]. PP and/or IVIg have also been used to ‘desensitize’ highly sensitized patients in anticipation of living or deceased donor renal transplantation. Such strategies were successful in a number of individuals with positive crossmatches against their potential living donors or, who had been waiting for prolonged periods for a suitable deceased donor organ ([69, 146-
These modalities have also been used to reverse established antibody-mediated rejection, although they do not suppress antibody synthesis; which often rebounds after cessation of PP [67]. Abraham et al. reported 20/440 patients treated with PP alone with intensification of immunosuppressive regimen. Their approach to treat AMR was successful to reverse 78% of rejection episodes [149].

Augmentation of PP treatment with intravenous immunoglobulin has yielded more beneficial effect [66, 69, 149].

II/8.7. Intravenous immunoglobulin (IVIg)

Intravenous immunoglobulin preparations isolated from plasma pools of several thousand healthy blood donors contain an almost unlimited spectrum of antibody specificities. One mechanism by which IVIg may act is to suppress patient panel-reactive antibodies by anti-idiotypic antibodies in the IVIg [150]. However, a variety of other potential mechanisms have already been proposed that include inhibition of complement activation [151], blockade and downregulation of Fc receptors [152], and modulation of T- and B-cell activation and differentiation [153].

II/8.8. Avoidance or postponement of antibody-mediated primary and secondary tissue injury

Anticoagulation therapy. Since antibody-mediated responses are characterized by primary endothelial injuries transplant thrombosis is a major pathological characteristic of these forms of rejection [154]. Depending on the level of antidonor antibodies, antibody-mediated post-transplant thrombosis may result in vascular narrowing or occlusion. On the basis of the hypothesis that preventing clot formation may postpone graft rejection, anticoagulant therapy has been used to treat humoral rejections [155]. Recently, in combination with other antibody depletion or suppression treatment, anticoagulation therapy
reduced hyperacute/acute rejection episodes enabling ABO-incompatible transplantation with satisfying long-term graft survivals [138].

II/8.9. Glucocorticoids

Glucocorticoids are by far the most widely used agents to inhibit inflammatory responses associated with graft rejection. Although glucocorticoids may induce B-cell apoptosis [156] and alloantibody production, they primarily reduce infiltration of the parenchyma inflammatory cells by inhibiting the functions of elements of the nonspecific immune response including production of and responses to cytokins [157]. Because of its strong immunosuppressive and anti-inflammatory effects, glucosteroid is still in the first-line for transplant rejection. It is also a major component of maintenance immunosuppressive regimens. However, steroid therapy has many side effects, including fluid retention, weight gain, diabetes, and bone mineral loss, its benefits must be balanced against the serious, sometimes fatal, adverse reactions.

II/8.10. Proteosome inhibitors, a new therapeutic approach

Perry et al. has recently described a proteosome inhibitor. Two patients were treated with bortezomib for AMR after kidney transplantation. They demonstrated a transient decrease in bone marrow plasma cells suggesting persistent alterations in alloantibody specificities. They concluded that proteosome inhibition leading to plasma cell apoptosis and inhibition may represent a new technique for controlling antibody production in vivo [158].

II/9. Prevention of AMR

The principal methods of monitoring for rejection are periodic laboratory examinations and/or protocol biopsies. However, some evidence suggest that HLA antibodies may appear before there is an increase in the serum creatinine [21], suggesting the utility of routine testing for HLA antibodies. At any time after transplantation, approximately 20% of
patients can be expected to have HLA antibodies [21] and subjects are more likely to have grafts that fail as a result of humoral rejection. Ongoing humoral rejection is not apparent by laboratory indexes until the organ parenchyma is injured to some critical level, after which more difficult to reverse the process. The effectivity of PP and monoclonal antibodies such as rituxmab for treatment of chronic rejection remains to be seen. A drug treatment regimen that reduces antibodies would be the method of choice. An example to control DSA production is the use of FK 506 and MMF, as described by Theruvath et al. [159]. Drugs specifically aimed blockade of synthesis or reduction of antibodies require development. An important consequence of the humoral theory of chronic rejection is that if antibodies are not found, then immunosuppression might be reduced until production of antibodies begins. Many patients may be currently overimmunosuppressed, but we have no way of knowing which patients can be safely weaned from their current levels. If we can depend on circulating HLA antibodies to be a good test of responsiveness, decisions on drug levels can be based on it. It should be mentioned, however, that monitoring for HLA antibodies is likely not to be comprehensive because other antibodies, such as the MIC, Angiotensin II system, may also be involved. Assays for MIC antibodies are just now being developed and becoming widely available. Interestingly, the blood group A and B antigens seem to be different from HLAs because ABO-incompatible patients have slightly, but not significantly, lower long-term graft survival compared with those with ABO-compatible grafts [138].
II/10. Our experience, the impact of rituximab therapy for treatment of antibody mediated rejection

II/10.1. Introduction

The retrospective analysis presented in my thesis compared the outcomes of a plasmapheresis (PP)-based versus a PP plus rituximab regimen to treat patients experiencing AMR and resistant to steroid plus anti-lymphocyte globulin treatments.

II/10.2. Materials and methods

Clinical management:

This retrospective review describes the clinical courses of 54 (9.5%) kidney transplant patients who were treated for AMR at the University of Texas, Division of Immunology and Organ Transplantation between 2001 and 2006, a period during which 568 grafts were performed. This experience included 26 patients who received PP plus rituximab (Rituxan, Genentech Inc., South San Francisco, CA, USA; Group A) which was initiated after the inferior experiment results among an initial 28 patient cohort who underwent PP without rituximab (Group B). Group B patients more frequently had been supplemented with 0.5 gm/kg IVIg (Sandoglobulin, Novartis, Basel, Switzerland) or CytoGam (Genesis BioPharmaceuticals, Inc. Scarborough, Ontario) than those in group A, because their serum IgG levels drifted below 694 mg/dL or their IgM levels, below 60 mg/dL the lower limits of normal values in the clinical laboratory.

All renal transplants were performed following a negative cross-match by complement dependent cytotoxicity (CDC) enhanced with antihuman globulin (CDC-AHG) and flow cytometry using a fluorescein conjugated anti-human immunoglobulin reagent (One Lambda, Inc. Canoga Park, CA). Anti-Class I and Class II HLA PRA determinations were performed
before transplantation on all patients using CDC-AHG and flow cytometry techniques (One Lambda, Inc; FLOWPRA Screening Test; [160, 161]). DSA was also evaluated by microbead technology in flow cytometry with goat anti-human IgG-phycoerythrin (One Lambda, Inc FLOWPRA Specific Antibody Detection Test; combined single antigen kits (LS1AO1;AO2) [160, 161].

A similar distribution of induction therapies was observed: namely, Simulect (Novartis, Basel, Switzerland), a chimeric mouse-human anti-CD25 antibody (20 mg iv. on days 0 and 4; n=15) or for presensitized (PRA > 25%), African-American or re-transplant patients, Thymoglobulin (Genzyme, Boston, MA; 1.5 mg/kg, n=39). De novo baseline immunosuppression included: cyclosporine (CsA) + sirolimus (SRL) + prednisone (n=33); sirolimus + Mycophenolate mofetil (MMF) + prednisone (n=1); CsA + MMF + prednisone (n=9) or SRL + prednisone (n=11). CsA and SRL doses were adjusted based upon concentration monitoring [162], whereas the starting doses of MMF (2 grams/day) were reduced in the presence of adverse reactions. All antibody-treated patients were prescribed gancyclovir for 3 months. Every subject received chronic therapy with trimethoprim-sulfamethoxazole.

Diagnosis of AMR:

AMR was diagnosed when a sudden, rapidly progressive bout of renal dysfunction occurred particularly early after the transplantation in the absence of positive findings on ultrasound, renal scan or urinalysis/cultures which demonstrated another condition: Clinical criteria. All patients underwent a renal biopsy which was stained with hematoxylin-eosin or periodic acid-Schiff and Masson trichrome reagents. In most cases it showed a characteristic histologic appearance based upon the recent modification of the Banff 1997 criteria [7], including capillary-glomerulitis with margination and/or thromboses or transmural arterial
inflammation with fibrinoid changes. In addition we sought the presence of C4d deposits in peritubular capillaries, or serologic tests showing DSA to establish the diagnosis. Beginning in 2001, routine immunofluorescence staining was performed on another biopsy sample, using fresh-frozen tissue treated with a monoclonal anti C4d-antibody (clone 10–11; Biogenesis, Sandown, NH) [6, 9]. The same year testing for donor-specific antibody was implemented. However, treatment was initiated for AMR in cases wherein the diagnosis was strongly suspected based upon the clinical course, although the histology did not confirm it, albeit C4d and/or DSA testing results obtained shortly thereafter established this condition.

**Treatment of AMR:**

Except for 2 subjects in group A, every patient had previously received an initial course of anti-lymphocyte antibody therapy for either induction immunosuppression or treatment of an acute cellular rejection: Anti-thymocyte globulin (1.5mg/kg/day; n=38); or OKT3 (5 mg/day; n=18); or Campath (30 mg/every other day × 2; n=2).

When refractoriness to the ACR treatment was established, plasmapheresis was performed using a Cobe Spectra Continuous Flow Cell Separator (Gambro BCT, Lakewood, CO, USA). Prior to each exchange of one plasma volume (range = 2 to 4 L.), the patients were premedicated with diphenhydramine hydrochloride. Access and return were obtained using a functional arteriovenous fistula (46.4%) or a double lumen central venous dialysis catheter (53.6%). The replacement fluid for each procedure was 5% human albumin unless the patient was coagulopathic (INR >1.6), or the treatment was performed less than 24 hours post-renal biopsy, in which instances, fresh frozen plasma (FFP) was used alone or in combination with 5% human albumin. We prophylactically administered 10% calcium gluconate to each patient intermittently during the procedures.
The typical treatment protocol was a two week course: five daily plasma exchanges followed by a two day rest. Thereafter, thrice weekly procedures were performed for two to three weeks depending upon whether there had been a reduction of at least 30% in the serum creatinine. At the end of each PP cycle, the patients in Group A were administered rituximab (Rituxan, Genentech Inc., South San Francisco, CA, USA; 375 mg/m²). Resistance to two PP cycles was followed by a third 5-day course which was performed in 16 Group A patients and 6 Group B patients. During each procedure, the subject was monitored for adverse effects; cultures were routinely obtained to search for evidence of bacteremia.

**Data Analysis:**

Figure 9 shows a CONSORT description of the study, indicating the allocation of subjects and the two year follow-up. In addition to the clinical and demographic variables of gender, ethnicity, age at the time of rejection, donor type (living or deceased), diabetes mellitus, number of pre-transplant blood transfusions, cold ischemia time, donor age, donor gender, and etiology of ESRD, we compared: graft and patient survivals at two years post-diagnosis, Class I and II PRA (pre-transplant versus peri-rejection episode), DSA, C4d staining, HLA mismatches, time to rejection, Banff classification, serum creatinine and calculated GFR by the MDRD formula [26] at the nadir, at the time of diagnosis of rejection as well as at 3, 6, 12 and 24 months post-treatment among Group A versus Group B subjects. The minimum follow-up was 2 years.
Figure 9. CONSORT diagram of the study.

Statistical analyses were performed on a personal computer using SPSS 13.0 for Windows. For normally distributed results the data were summarized as mean values ± standard deviations (SD). Continuous variables were compared by the independent sample Student t- or ANOVA tests; categorical variables, using cross tabulation calculation or Fisher’s exact test. The regression coefficients of the slopes of MDRD GFR values over 2 years post-treatment were compared by a General Linear Model, Repeated Measures test. We estimated graft and patient survivals using the Kaplan-Meier method with significance evaluated by the Log-Rank and Breslow tests. A multivariate Cox-regression analysis was used to evaluate the impact of treatment components – administration of Rituximab or IVIg or the numbers of PP – on 2-year graft survival using all demographic or treatment covariates that showed $P \leq 0.1$ on univariate analysis.
II.10.3. Results

Pre-AHR courses:

There was no significant difference in the baseline demographic characteristics of the PP (Group B) versus the PP plus Rituximab (Group A) cohorts of the 54 renal transplant patients diagnosed with AMR (Table 1). Table 2 describes the early post-transplant clinical courses of the subjects. All patients had undergone induction therapy, but there was no difference in the proportions of Group A versus Group B subjects treated with anti-thymocyte polyclonal globulin or Basiliximab induction therapy. The baseline immunosuppressive regimens included significantly more cases treated with CsA de novo among Group B. The most striking feature prior to the onset of rejection was a decreased intensity of the immunosuppressive regimen by drug withdrawal or dose reduction, frequently associated with delayed graft function.

Table 3 indicates similar methods were employed to diagnose AMR among the cohorts, except for the greater prevalence of ACR features observed on pathologic examination of biopsies among cohort B, which may have been due to more of these cases occurring before the delineation of AMR in the Banff criteria. No significant difference was observed comparing the two groups in terms of PRA (Class I and II) pre-transplant or at rejection. The median values of the time to rejection as well as the occurrences of AMR with or without ACR did not show significant differences. There were similar fractions of C4d and DSA positive cases, despite the fact that many group B patients were diagnosed before the availability of these serologic and histologic tools.
<table>
<thead>
<tr>
<th>Features</th>
<th>Overall</th>
<th>Group A</th>
<th>Group B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>54</td>
<td>26</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Age (years; mean ± SD)</td>
<td>41.5 ± 11.3</td>
<td>41.5 ± 13.1</td>
<td>41.5 ± 9.4</td>
<td>0.93&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male (n,%)</td>
<td>24 (44.4)</td>
<td>12 (46.2)</td>
<td>12 (42.9)</td>
<td>0.51&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Source of transplant (n,%)</td>
<td></td>
<td></td>
<td></td>
<td>0.21&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Living donor</td>
<td>15 (27.8)</td>
<td>9 (36.4)</td>
<td>6 (21.4)</td>
<td></td>
</tr>
<tr>
<td>Deceased donor</td>
<td>39 (72.2)</td>
<td>17 (65.4)</td>
<td>22 (78.6)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (n, %)</td>
<td></td>
<td></td>
<td></td>
<td>0.73&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>White</td>
<td>15 (27.8)</td>
<td>8 (30.7)</td>
<td>7 (25)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>17 (31.5)</td>
<td>6 (23)</td>
<td>11 (39.3)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>20 (37.1)</td>
<td>11 (42.3)</td>
<td>9 (32.1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>2 (3.6)</td>
<td>1 (4)</td>
<td>1 (3.6)</td>
<td></td>
</tr>
<tr>
<td>PRA, mean ± SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-TX Class I</td>
<td>22.8 ± 29.9</td>
<td>18.7 ± 25.9</td>
<td>26.6 ± 33.2</td>
<td>0.34&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pre-TX Class II</td>
<td>20.2 ± 30.7</td>
<td>17.5 ± 29.5</td>
<td>22.7 ± 32.3</td>
<td>0.54&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>HLA-MM (mean ± SD)</td>
<td>4.1 ± 0.2</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>0.90&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetes, (n, %)</td>
<td>7 (13)</td>
<td>3 (11.5)</td>
<td>4 (14.3)</td>
<td>0.54&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prior blood Transfusion (n, %)</td>
<td></td>
<td></td>
<td></td>
<td>0.40&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>24 (44.7)</td>
<td>9 (34.6)</td>
<td>15 (53.6)</td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>20 (37)</td>
<td>11 (42.3)</td>
<td>9 (32.1)</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>10 (18.5)</td>
<td>6 (23.1)</td>
<td>4 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Etiology of ESRD (n, %)</td>
<td></td>
<td></td>
<td></td>
<td>0.61&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertension</td>
<td>20 (37)</td>
<td>8 (30.8)</td>
<td>12 (42.9)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>1 (1.9)</td>
<td>0</td>
<td>1 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Hypertension and Diabetes</td>
<td>3 (5.6)</td>
<td>2 (7.7)</td>
<td>1 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>11 (20.4)</td>
<td>4 (15.4)</td>
<td>7 (25)</td>
<td></td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>4 (7.4)</td>
<td>2 (7.7)</td>
<td>2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Vesico-ureteral Reflux</td>
<td>2 (3.7)</td>
<td>2 (7.7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Systemic Lupus Erythematous</td>
<td>6 (11.1)</td>
<td>4 (15.4)</td>
<td>2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (5.6)</td>
<td>2 (7.7)</td>
<td>1 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Hepato-renal syndrome</td>
<td>1 (1.9)</td>
<td>1 (3.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>1 (1.9)</td>
<td>1 (3.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Alport’s syndrome</td>
<td>2 (3.7)</td>
<td>0</td>
<td>2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Donor age</td>
<td>36.2 ± 15.7</td>
<td>35.3 ± 14.2</td>
<td>37.0 ± 17.1</td>
<td>0.71&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male donor gender (n, %)</td>
<td>32 (59.2)</td>
<td>15 (57.7)</td>
<td>17 (60.7)</td>
<td>0.56&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cold ischemia time (mean±SD)</td>
<td>792.2±115.5</td>
<td>882.4±188.1</td>
<td>718.4±143.6</td>
<td>0.65&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> ANOVA test

<sup>2</sup> Cross tabulation, Fisher’s exact test

<sup>3</sup> Cross tabulation, Linear by linear association, Chi-Square test

**Table 1.** Demographic characteristics of renal transplant patients treated for AMR.
### Table 2. Clinical course of renal transplant patients prior to treatment for AMR.

<table>
<thead>
<tr>
<th>Features</th>
<th>Overall</th>
<th>Group A</th>
<th>Group B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>54</td>
<td>26</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Induction, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.23†</td>
</tr>
<tr>
<td>Thymoglobulin</td>
<td>39 (72.2)</td>
<td>19 (73.1)</td>
<td>20 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Basiliximab</td>
<td>15 (27.8)</td>
<td>7 (26.9)</td>
<td>8 (28.6)</td>
<td></td>
</tr>
<tr>
<td>Baseline IS, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sirolimus/everolimus</td>
<td>44 (81.5)</td>
<td>19 (73.1)</td>
<td>25 (89.3)</td>
<td>0.07²</td>
</tr>
<tr>
<td>cyclosporine</td>
<td>41 (75.9)</td>
<td>15 (57.7)</td>
<td>26 (92.9)</td>
<td>0.04²</td>
</tr>
<tr>
<td>MMF/MPA</td>
<td>10 (18.5)</td>
<td>7 (26.9)</td>
<td>3 (10.7)</td>
<td>0.17²</td>
</tr>
<tr>
<td>Prednisone</td>
<td>54 (100)</td>
<td>26 (100)</td>
<td>28 (100)</td>
<td>NA</td>
</tr>
<tr>
<td>Prograf</td>
<td>3 (5.6)</td>
<td>1 (3.8)</td>
<td>2 (7.1)</td>
<td>0.53²</td>
</tr>
<tr>
<td>Delayed graft function, n (%)</td>
<td>10 (18.5)</td>
<td>1 (3.8)</td>
<td>9 (32.1)</td>
<td>0.012²</td>
</tr>
<tr>
<td>Maintenance IS, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sirolimus/everolimus</td>
<td>46 (85.2)</td>
<td>25 (96.2)³</td>
<td>21 (75.0)</td>
<td>0.008⁴</td>
</tr>
<tr>
<td>cyclosporine</td>
<td>33 (61.1)</td>
<td>15 (57.7)</td>
<td>18 (64.3)</td>
<td>0.75⁴</td>
</tr>
<tr>
<td>MMF/MPA</td>
<td>15 (27.8)</td>
<td>8 (30.8)</td>
<td>7 (25.0)</td>
<td>0.57⁴</td>
</tr>
<tr>
<td>Prednisone</td>
<td>47 (87.0)</td>
<td>24 (92.3)</td>
<td>23 (82.1)</td>
<td>0.12⁴</td>
</tr>
<tr>
<td>Prograf</td>
<td>1 (1.9)</td>
<td>1 (3.8)</td>
<td>0 (0)</td>
<td>0.29⁴</td>
</tr>
</tbody>
</table>

† Cross tabulation, Linear by linear association, Chi-Square test
² Fisher’s exact test
³ Data missing on one patient
⁴ Kruskal-Wallis non-parametric test comparing distributions between Groups A and B

**Treatment regimens for AMR:**

Among cohorts A and B, 14 and 15 patients had experienced an ACR episode prior to the diagnosis of AMR, respectively. All patients received initial therapy with monoclonal or polyclonal anti-lymphocyte immunoglobulin antibodies at similar mean doses prior to recognition and during treatment of the AMR (Table 4).

Significantly more Group A patients received IVIg (IVIg/Cytogam; Fisher’s exact test, \( P=0.02 \)) as well as a greater number (\( t \)-test; \( P=0.003 \)) and length (\( t \)-test; \( P=0.009 \)) of plasmapheresis treatments to treat AMR. Despite these differences, the multivariate analysis documented that only the prescription of Rituximab was a major factor to improve outcomes (Table 5).
**Features** | **Group A**<br>(n=26) | **Group B**<br>(n=28) | **P**
---|---|---|---
PRA, mean% ± SD | | |
Class I | 28.1 ± 29.1 | 37.9 ± 37.1 | 0.26<sup>4</sup>
Class II | 34.9 ± 34.9 | 35.5 ± 38.6 | 0.95<sup>4</sup>
Time to first rejection (days) | Median (Range) | 23 (2-7767) | 27 (1-5051) | 0.82<sup>2</sup>
Onset<30 days, n (%) | 13 (50) | 16 (57) | 0.40<sup>3</sup>
Pathological dx of first rejection episode, n (%) | | | 0.02<sup>4</sup>
ACR | 2 (8) | 9 (32) | 0.04<sup>3</sup>
AMR + ACR | 12 (46) | 6 (21) | 0.08<sup>3</sup>
AMR | 12 (46) | 13 (49) | 0.21<sup>3</sup>
Histopathologic dx AMR | 24 (92) | 15 (54) | 0.05<sup>3</sup>
DSA positive (n/checked N; %) | 16/24 (62) | 3/5 (60) | 0.52<sup>3</sup>
C4d positive (n/checked N; %) | 22/26(85) | 13/20 (65) | 0.17<sup>3</sup>
C4d + DSA + histologic dx | 26 (100) | 28 (100) | 1.0<sup>3</sup>

*PRA = panel reactive antibody; ACR = acute cellular rejection; AMR = acute humoral rejection; dx = diagnosis; DSA = donor-specific anti-HLA antibody*

<sup>1</sup>ANOVA test  
<sup>2</sup>Mann-Whitney U-test  
<sup>3</sup>Fisher’s exact test  
<sup>4</sup>Cross-tabulation, Linear by linear association, Chi Square test

**Table 3.** Comparison of metrics for diagnosis of AMR.

To evaluate independent risk factors for 2-year renal allograft survival, a multivariate analysis was performed using a Cox regression model (Table 5). The relative risk of graft failure at 2 years was 5-fold greater for patients who were not treated with Rituximab; none of the other variables showed a significant impact.

Although Group A underwent significantly more PP treatments, this finding was not the major factor determining graft survival (regression analysis, ANOVA test P=0.076).

Despite the treatments, 6 subjects in Group A (2 AHR, 2 ACR, 2 ACR/AHR) and 7 in Group B (5 AHR, 1 ACR, 1 AHR/ACR) experienced a second episode. Indeed, two subjects in each cohort experienced even a third rejection episode.
Table 4. The therapy for AMR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall</th>
<th>Group A</th>
<th>Group B</th>
<th>P Doses</th>
<th>P Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td><strong>Overall</strong></td>
<td><strong>Group A</strong></td>
<td><strong>Group B</strong></td>
<td><strong>P</strong></td>
<td><strong>P Mean</strong></td>
</tr>
<tr>
<td><strong>Initial antibody</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymoglobulin, (n) mg mean ± SD</td>
<td>(38) 1229.5 ± 480</td>
<td>(19), 1249.1 ± 548</td>
<td>(19), 1209.9 ± 513</td>
<td>0.56^2</td>
<td>0.52^1</td>
</tr>
<tr>
<td>OKT3, (n) mg mean ± SD</td>
<td>(18) 56.4 ± 27</td>
<td>(5), 56.1 ± 27</td>
<td>(13), 60.6 ± 27</td>
<td>0.03^2</td>
<td>0.33^1</td>
</tr>
<tr>
<td>Campath, (n) mg mean ± SD</td>
<td>(2) 92.5 ± 24.7</td>
<td>(2), 92.5 ± 24.7</td>
<td>0</td>
<td>0.21^2</td>
<td>0.14^1</td>
</tr>
<tr>
<td>IVIg/Cytogam Total</td>
<td>22</td>
<td>17</td>
<td>5</td>
<td>0.02^2</td>
<td>NA</td>
</tr>
<tr>
<td>Immunoglobulin (Sandoglobulin), (n) mg mean ± SD</td>
<td>(11) 205.4 ± 151</td>
<td>(7), 217.7 ± 191</td>
<td>(4), 187 ± 47</td>
<td>0.32^2</td>
<td>0.74^1</td>
</tr>
<tr>
<td>Cytogam, (n) mg mean ± SD</td>
<td>(11) 38.5 ± 40</td>
<td>(10), 28.1 ± 21</td>
<td>(1) 142.5</td>
<td>0.02^2</td>
<td>0.02^1</td>
</tr>
<tr>
<td>Plasmapheresis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean PP, n ± SD^3</td>
<td>17.1 ± 8.3</td>
<td>21.5 ± 8.4</td>
<td>12.1 ± 6.4</td>
<td>0.003^1</td>
<td></td>
</tr>
<tr>
<td>Days of PP, n ± SD^4</td>
<td>37.4 ± 41.4</td>
<td>52.1 ± 50.8</td>
<td>21.4 ± 18</td>
<td>0.009^1</td>
<td></td>
</tr>
<tr>
<td>Rituximab, (n) mg mean ± SD</td>
<td>NA</td>
<td>(94; 3.61) 2385.8 ± 1141</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Bonferroni correction P=0.01 [163]

1 Independent sample Student’s t-test
2 Fisher’s exact test
3 Mean dose of group—actual total dose for an individual patient varied from 30 to 585 mg for IVIg
4 Mean PP is the number of PP treatments
5 Days of PP refers to the length of the overall course

Figure 9 shows the consort analysis of the study. Figure 10 shows the graft and patient survivals at 2-years. Among the 26 patients in Group A there were 2 therapeutic failures and one death due to fungal sepsis in a patient who experienced a third episode and was treated with Campath.

Among the 28 Group B subjects there were 3 deaths - ileus/sepsis, pneumonia/sepsis, hepatitis C liver failure - as well as 8 other graft losses due to 3 therapeutic failures, 1 abandoned graft due to post-transplant lymphoproliferative disease and 4 instances of chronic allograft nephropathy. Panel A indicates the patient survival rates among Group A versus Group B to be 100 versus 90% (P=NS), respectively.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Rituximab</td>
<td>0.009</td>
<td>0.042</td>
<td>5.0</td>
</tr>
<tr>
<td>Pathological dx (Banff)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACR</td>
<td>0.045(^1)</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>ACR/AHR</td>
<td>0.054(^1)</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>AHR</td>
<td>0.40(^1)</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>No evidence</td>
<td>0.21(^1)</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>IVIg/Cytogam (given vs. not given)</td>
<td>0.05(^1)</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Number of PP</td>
<td>0.003(^2)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Age (&lt;40 vs. &gt;40)</td>
<td>0.10(^1)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>C4d (positive vs. negative)</td>
<td>0.42(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRA Class I (&gt;30% vs. &lt;30%)</td>
<td>0.74(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRA Class II (&gt;30% vs. &lt;30%)</td>
<td>0.90(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A,B mismatch (0 vs. 1&lt;)</td>
<td>0.40(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA DR mismatch (0 vs. 1&lt;)</td>
<td>0.08(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymoglobulin (given vs. not given)</td>
<td>0.53(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OKT 3 (given vs. not given)</td>
<td>0.28(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early rejection (&lt;30 vs. &gt;30 days)</td>
<td>0.49(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethnicity (Blacks vs. Others)</td>
<td>0.50(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (yes vs. no)</td>
<td>0.77(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood transfusion (given vs. not given)</td>
<td>0.24(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induction therapy (Thymoglobulin vs. Simulect)</td>
<td>0.82(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re-rejection vs. None</td>
<td>0.40(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased Immunosuppression vs. not</td>
<td>0.78(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed graft function vs. not</td>
<td>0.88(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sirolimus/everolimus based therapy vs. Other</td>
<td>0.30(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclosporine based therapy vs. Other</td>
<td>0.46(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor sex (male vs. female)</td>
<td>0.70(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor age (&lt;40 vs. &gt;40 years)</td>
<td>0.60(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold ischemic time (&lt;10 vs. &gt;10 hours)</td>
<td>0.74(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESRD etiology(^3)</td>
<td>0.561(^1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Kaplan-Meier analysis

\(^2\) By Independent samples t-test (see Table 4)

\(^3\) Comparing the most frequent reasons for ESRD with Kaplan-Meier analysis:
1. Hypertension, Diabetes;
2. Glomerulonephritis;
3. Polycystic kidney disease;
4. Other, less frequent reasons

**Table 5.** Results of univariate and Cox regression multivariate analyses related to 2 year allograft survival.
The respective overall graft survival rates at 2 years were 90 versus 60% for Groups A versus B (P=0.005), respectively. Upon multivariate analysis the administration of IVIg or Cytogam alone was associated with improved graft survival compared with non-treated, PP only subjects (Panel B; 90% vs. 66%, P=0.05). However, administration of Rituximab yielded significantly better graft survivals (Panel C; 92% vs. 60%, P=0.009). Even greater outcomes were obtained with the combination of Rituximab and IVIg/Cytogam compared with non-treated, PP only subjects (Panel D; 94% vs. 53%, P=0.025).

Neither the slope values (regression coefficients: -0.347 ml/min/month (ANOVA test; P=0.29) versus -0.420 ml/min/month (ANOVA test; P=0.25), nor the actual MDRD estimates of GFR values (General Linear Model, Repeated Measures test, P=0.42) of group A versus group B showed a significant difference over time (3, 6, 12 or 24 months after completion of AMR treatment) or between groups. (Figure 11; Table 6).

Complications of Plasmapheresis:

Among the 54 patients who underwent 838 plasma exchange procedures, the 124 complications occurred in 29 patients, who were similarly distributed between groups A and B (Table 7). No deaths occurred as a result of the plasma exchange procedures, although one treatment was discontinued due to severe nausea and vomiting. The most common complications were tachycardia (21.8%), bleeding/bruising (16.9%), nausea/vomiting (16.1%), pruritus/urticaria (12.9%), hypotension (12.9%), bacteremia (11.3%), and hypocalcemia (8.1%). Other events occurred among less than 1% of subjects.

There was also no significant difference between the two groups comparing the total number of patients with complications (P=0.25 within 3 months and P=0.787 within 6 months; Fisher’s exact test) and the number with multiple complications (P=0.14 within 3 and P=0.17 within 6 months after the completion of treatment; Fisher’s exact test; Table 7).
Figure 10. Kaplan-Meier allograft survival at 2 years post-transplantation treatment for rejection. (A) Cumulative patient survival with Rituximab (Group A) and without Rituximab (Group B). $P=0.09$; Log-Rank test. (B) Treatment with IVIg/Cytogam (solid line) and without IVIg/Cytogam (dashed line). $P=0.05$; Log-Rank test. (C) Treatment with Rituximab (Group A) and without Rituximab (Group B). $P=0.009$; Log-Rank test. (D) Treatment with Rituximab + IVIg/Cytogam (solid line) and without Rituximab and IVIg/Cytogam (dashed line) $P=0.025$; Log-Rank test.
Figure 11. MDRD GFR over the course of time. Comparison of the evolution of MDRD GFR values at 3, 6, 12 and 24 months after rejection treatment. Panel A: Group A; Regression coefficient: -0.347 ml/min/months (ANOVA test $P=0.29$); Panel B: Group B; Regression coefficient: -0.42 ml/min/months (ANOVA test $P=0.25$).
Table 6. Renal function over time

<table>
<thead>
<tr>
<th>MDRD GFR (^1,2)</th>
<th>Overall</th>
<th>Group A.</th>
<th>Group B.</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (n), mean ± SD</td>
<td>38.4 ± 23.7</td>
<td>37.6 ± 22.9</td>
<td>39.1 ± 24.7</td>
<td>0.82</td>
</tr>
<tr>
<td>At rejection (n), mean ± SD</td>
<td>18.8 ± 16.3</td>
<td>18.0 ± 10.9</td>
<td>19.6 ± 20.3</td>
<td>0.73</td>
</tr>
<tr>
<td>At resolution (n), mean ± SD</td>
<td>44.03 ± 24.1</td>
<td>49.6 ± 23.05</td>
<td>38.7 ± 24.3</td>
<td>0.09</td>
</tr>
<tr>
<td>3 months (n), mean ± SD</td>
<td>42.8 ± 24.9</td>
<td>46.2 ± 23.6</td>
<td>39.8 ± 26.08</td>
<td>0.36</td>
</tr>
<tr>
<td>6 months (n), mean ± SD</td>
<td>36.7 ± 26.5</td>
<td>43.4 ± 24.5</td>
<td>30.7 ± 27.2</td>
<td>0.09</td>
</tr>
<tr>
<td>12 months (n), mean ± SD</td>
<td>36.2 ± 27.05</td>
<td>42.0 ± 22.3</td>
<td>31.3 ± 30.01</td>
<td>0.17</td>
</tr>
<tr>
<td>24 months (n), mean ± SD</td>
<td>32.6 ± 29.4</td>
<td>38.3 ± 22.8</td>
<td>28.3 ± 33.3</td>
<td>0.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum Creatinine, mg/dl(^3)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline mean ± SD</td>
<td>3.0 ± 2.4</td>
<td>3.1 ± 2.8</td>
<td>2.9 ± 2.0</td>
<td>0.70</td>
</tr>
<tr>
<td>At rejection mean ± SD</td>
<td>5.2 ± 3.2</td>
<td>5.0 ± 3.1</td>
<td>5.3 ± 3.3</td>
<td>0.71</td>
</tr>
<tr>
<td>At resolution mean ± SD</td>
<td>2.1 ± 1.5</td>
<td>2.0 ± 1.6</td>
<td>2.3 ± 1.5</td>
<td>0.45</td>
</tr>
<tr>
<td>3 months mean ± SD</td>
<td>1.9 ± 1.5</td>
<td>1.9 ± 1.3</td>
<td>1.9 ± 1.6</td>
<td>0.86</td>
</tr>
<tr>
<td>6 months mean ± SD</td>
<td>1.7 ± 1.5</td>
<td>1.7 ± 1.0</td>
<td>1.7 ± 1.8</td>
<td>0.89</td>
</tr>
<tr>
<td>12 months mean ± SD</td>
<td>1.8 ± 2.1</td>
<td>1.8 ± 1.2</td>
<td>1.8 ± 2.6</td>
<td>0.98</td>
</tr>
<tr>
<td>24 months mean ± SD</td>
<td>1.8 ± 1.9</td>
<td>1.8 ± 1.3</td>
<td>1.8 ± 2.3</td>
<td>0.91</td>
</tr>
</tbody>
</table>

\(^1\) MDRD GFR Formula: [26]

\[
\text{MDRD-GFR (ml/min/1.73m}^2\) = 170 \times [\text{PCr}]^{0.999} \times [\text{age}]^{-0.176} \times [0.762 \text{ if patient is female}] \times [1.180 \text{ if patient is black}] \times [\text{SUN}]^{-0.176} \times [\text{Alb}]^{-0.318}
\]

Where PCr=serum creatinine concentration (mg/dl); SUN=serum urea nitrogen concentration (mg/dl); Alb=serum albumin concentration (g/dl).

\(^2\) Lost grafts designated as MDRD GFR = 0.

\(^3\) SCr values among surviving grafts.

In 756 (90.2%) procedures, 5% albumin was used for replacement, while FFP was used alone or in combination with albumin in 121 (14.43%). Pruritus and urticaria as well as hypocalcemia were exclusively associated with the use of FFP. There was no other significant association between the use of 5% albumin or FFP and the various complications (data not shown).

Infectious complication(s) within 6 months showed no significant impact on graft survival at 2 years, using the Kaplan-Meier method (\(P=0.66\), Log-Rank test; data not shown). Furthermore, there was no significant difference regarding the rates/types of infectious complications between the two groups within 3 or 6 months after completion of treatment (\(P=0.24\) and \(P=0.78\), respectively; Cross tabulation calculation, Fisher’s exact test). Administration of IVIg showed a trend toward decreasing the appearance of infectious complications in Group B (\(P=0.058\)) but not in Group A (\(P=0.42\); Fisher’s exact test).
<table>
<thead>
<tr>
<th>Type</th>
<th>Overall (n=838)</th>
<th>Group A (n=560)</th>
<th>Group B (n=278)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Patients with ≥ 1 complication¹</td>
<td>64.8</td>
<td>69.2</td>
<td>57.1</td>
<td>0.72²</td>
</tr>
<tr>
<td>Complications, n</td>
<td>124</td>
<td>73</td>
<td>51</td>
<td>0.29²</td>
</tr>
<tr>
<td>Tachycardia, n</td>
<td>27</td>
<td>16</td>
<td>11</td>
<td>0.47²</td>
</tr>
<tr>
<td>Bleeding/bruising, n</td>
<td>21</td>
<td>14</td>
<td>7</td>
<td>0.43²</td>
</tr>
<tr>
<td>Nausea/vomiting, n</td>
<td>20</td>
<td>11</td>
<td>9</td>
<td>0.30²</td>
</tr>
<tr>
<td>Pruritus/urticaria, n</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>0.40²</td>
</tr>
<tr>
<td>Hypotension, n</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>0.57²</td>
</tr>
<tr>
<td>Bacteremia, n</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>0.66²</td>
</tr>
<tr>
<td>Hypocalcemia, n</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>0.14²</td>
</tr>
</tbody>
</table>

B. Infectious complications

<table>
<thead>
<tr>
<th>Type</th>
<th>Overall</th>
<th>Group A</th>
<th>Group B</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3 mo</td>
<td>17(31.5)</td>
<td>22(40.7)</td>
<td>6(23.07)</td>
<td>10(38.4)</td>
<td>11(39.2)</td>
</tr>
<tr>
<td>Multiple n (%), %</td>
<td>9 (16.7)</td>
<td>11 (20.4)</td>
<td>2 (7.7)</td>
<td>3 (11.5)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Upper respiratory, n</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Skin, n</td>
<td>5</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Wound, n</td>
<td>6</td>
<td>11</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Lower respiratory, n</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Urinary tract, n</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Diarrhea, n</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sepsis, n</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total number of infections</td>
<td>26</td>
<td>41</td>
<td>9</td>
<td>43</td>
<td>17</td>
</tr>
<tr>
<td>Viral infections, n</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Bacterial infections, n</td>
<td>21</td>
<td>32</td>
<td>7</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Fungal infections, n</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

¹ Number of complications / Total number of PP
² Fisher’s exact test

Table 7. Complications of plasmapheresis treatments.

II/10.4. Discussion

Kidney transplant rejection is a multifarious process. Although T cells are usually implicated in the acute rejection process, a more pernicious form, resulting in a greater rate of early loss or reduced long-term graft survival, [6, 8, 63-65] is mediated by humoral antibodies—acute antibody-mediated rejection. This syndrome includes sudden, progressive deterioration of renal function is associated with the presence of vascular lesions and/or C4d
deposition along peritubular capillaries in a graft biopsy, a positive post-transplant cross-match or DSA. The biopsy may not be consistent since the process may be patchy and the sampling is limited. Furthermore, the lack of detectable C4d does not exclude mediation by non-complement-dependent antibody vectors such as those dependent on cell-mediated cytotoxicity. Finally, the DSA may not necessarily be positive [87], because it is measured with beads coated with prototypic but not necessarily donor-type HLA A, B or DR but not HLA-DP or DQ antigens. In addition, even the cross-match may not be positive, since the AMR may be directed toward non-lymphoid, polymorphic tissue-specific antigens [30]. Future prospective studies must examine whether a protocol of serial DSA tests prognosticates the early and/or long-term outcomes of AMR treatment.

Patients undergoing AMR tend to be refractory to steroid and anti-lymphocyte antibody therapies which are useful for cell-mediated rejection episodes. Based upon favorable experiences in inflammatory and autoimmune disorders, plasmapheresis treatments have been used to remove humoral mediators from the circulation, although they do not suppress antibody synthesis; which often rebounds after cessation of PP [67]. Treatment of AMR with IVIg and/or T cell-depleting antibodies in conjunction with PP has yielded 1-year graft survivals of 75 to 88% [63-67, 71] and 1-year renal function similar to that observed after acute cellular rejection, namely, median creatinine levels of 1.6 to 1.8 mg/dL [8, 64, 66, 67]. The present series achieved an actual 2-year graft survival rate of 90% among Group A patients with serum creatinine values of 1.8 mg/dL. Although the graft survival rate was better with rituximab, the level of renal function was only slightly greater for the PP plus rituximab group versus the PP alone cohort. Both values were indicative of impairment, suggesting that there was incomplete reversal and ongoing immunologic responses; they trended downward over time.
Beneficial effects of IVIg on B cells have been suggested to result from Fc binding, from feedback inhibition of antibody synthesis, and from stimulation of immuno-regulatory mechanisms that dampen new IgG synthesis [63]. Jordan et al reported successful reversal of AMR in 5 renal transplant recipients who were treated with IVIg in addition to anti-lymphocyte therapy [68]. Using the combination of PP and IVIg to treat AHR, Lehrich et al reported 78% graft survival at 2 years, an outcome that was superior to a PP alone cohort [65]. Montgomery et al described an extensive experience with 3-year follow-up of 66 patients undergoing AMR treatment with plasmapheresis plus CMV-IgG [69]. Actuarial patient survival was 90.4% and graft survival, 78.3%. Herein, we also documented a beneficial contribution of either IVIg or CMV-Ig together with plasmapheresis to reverse AMR episodes, although the IVIg therapy was only prescribed for patients with low immunoglobulin values.

In this retrospective analysis of our experience, a beneficial effect was observed with plasmapheresis in addition to treatment with rituximab, which inhibits B cell proliferation, destroys circulating B-cells and induces cellular death through complement binding [161]. A number of recent studies have identified the presence of intra-renal CD20 positive clusters, without DSA or C4d positivity, to be associated with steroid and/or anti-lymphocyte globulin-resistant vascular rejection. These cell aggregates, which may serve to present antigen to T-cells and generate antibodies that directly injure or coat phagocytic macrophages, have been shown to resolve following rituximab therapy [164].

Early success with rituximab therapy was described in two case reports: a heart transplant [165] and a lung recipient [77]. Alausa et al. reversed refractory acute humoral rejection in one kidney transplant case [124]. The benefit of one rituximab infusion plus plasmapheresis was reported by Becker et al in 27 patients with biopsy-proven AMR, who were resistant to steroid and Thymoglobulin therapy. [17] The reported 2-year graft survival
rate was 85%. The diagnosis was based on histological evidence of rejection; DSA or C4d immunostaining was not performed. Faguer et al also reported 8 patients successfully treated with 4 doses of rituximab, PP, steroid, MMF and tacrolimus. Allograft survival was 81% at 20 months follow-up. [3] A recently published case report showed a successful outcome of Rituximab, PP and IVIg for treatment of AMR in an HIV and hepatitis C virus infected patient [166].

Clinical trials have shown that administration of this antibody for treatment of non-Hodgkin lymphoma leads to a significant, rapid decline in B cells (70 to 80% decrease) followed by full repopulation over the following 12 months [67, 80, 167, 168]. Despite this finding, serum immunoglobulin concentrations in this study did not change significantly from normal [168]. Interestingly we noted a greater need for IVIg supplementation among the rituximab cohort possibly related to the therapeutic reduction in the number of B cells and/or to the greater number of plasmapheresis treatments.

In conclusion, this retrospective study showed a 2-year graft survival of 92% for patients treated with rituximab plus plasmapheresis, which was significantly greater than that observed among the group who did not receive anti-CD20 therapy. The 100% patient survival at 2 years as well as the absence of a greater incidence of major complications among individuals treated with rituximab supports the effective, safe use of this monoclonal antibody for AMR. Although our data support the primacy of rituximab treatment, the contributions of PP and/or IVIg to this outcome must be established in randomized controlled trials.
III. OTHER USES OF RITUXIMAB POST-TRANSPLANTATION FOR LYMPHO-PROLIFERATIVE DISORDERS (PTLD)

III/1. Introduction

Development of lymphoma after renal transplantation was first described by Doak et al. [169] in 1968. The term post-transplant lymphoproliferative disorder or disease (PTLD) was introduced by Starzl et al. in 1984 [170]. PTLD has been broadly defined as a benign or malignant lymphoid proliferation that develops as a consequence of pharmacological immunosuppression following the solid organ or bone marrow transplantation [171]. Its occurrence among engrafted patients is 12- to 20-fold higher than that among the general population [172]. The histologic subtypes of PTLD range from early Epstein–Bar virus (EBV)-associated polymorphic lymphoid proliferations to EBV-positive or -negative monomorphous B-cell or less often T-cell lymphomas (Table 8).

III/2. Pathogenesis

The majority of PTLDs are EBV-associated, of B-cell origin, and expressing CD20 antigen [173]. The pathogenesis of EBV-associated PTLD is linked to T-cell dysfunction. The suppressed EBV-specific immune response results in uncontrolled EBV reactivation in adults or primary EBV infection in children [173]. Early-onset PTLDs are mainly regarded as EBV-driven lymphoproliferations that are frequently, though not always, polyclonal or oligoclonal, whereas most late-onset PTLDs are true monoclonal lymphoid malignancies that are not necessarily associated with EBV infection. The prognosis of the late onset PTLDs is worse [174].

A special category of patients at particular risk for PTLD development (10- to 50-fold increased risk) are EBV-seronegative patients who receive allografts from EBV-seropositive donors, consequently leading to primary EBV infection [175]. This is also the main reason for
the greater incidences of PTLD in the early post-transplant period among paediatric recipients, who more often are still EBV-seronegative at the time of transplantation.

Correlations of morphologic and molecular features of PTLDs have contributed to recognition of specific disease categories and have provided prognostic indicators for these disorders [176]. The outcomes of monoclonal B-cell PTLDs depend upon the interval from transplantation, the histologic features, and the site of lymphoma origin.

Table 8. World Health Organization classification of post-transplant lymphoproliferative disease (PTLD)[177].

1. Early lesions
   - Reactive plasmacytic hyperplasia
   - Infectious mononucleosis-like
2. Polymorphic PTLD
3. Monomorphic PTLD
   - B-cell neoplasms
   - Diffuse large B-cell lymphoma
   - Burkitt/Burkitt-like lymphoma
   - Plasma cell myeloma
   - Plasmacytoma-like lesions
   - T-cell neoplasms
   - Peripheral T-cell lymphoma
   - Other types
4. Hodgkin-like PTLD

Oncogenic viruses known to be involved in PTLD pathogenesis include EBV and HHV-8, which act through direct mechanisms. The virus directly infects the tumor clone exerting B-cell transformation. Viral infections exploit several strategies to ensure persistent infection: namely, prevention of death of infected cells, enhanced proliferation to maintain the
infected reservoir, and evasion of the immune system [178-180]. Several lines of evidence have suggested that EBV infection has a major pathogenetic role in PTLDs: (a) EBV infects 60%–80% of PTLD patients, including 100% of early-onset cases [181]. (b) In many cases of monomorphic PTLD, EBV infection is monoclonal, consistent with the hypothesis that the virus was present in tumor progenitor cells since the early phase of clonal expansions. (c) A decrease in EBV-specific cytotoxic T lymphocytes (CTLs) and an increase in the EBV viral load are strongly associated with PTLD development [182]. (d) Treatment of PTLDs with autologous EBV-specific CTLs controls viral load and reduces tumor size.

An interesting observation in this respect is the strong relationship between EBV-infected proximal tubular cells of the transplanted kidney (chronic EBV nephritis), even months before the onset of PTLD, and subsequent localization of the PTLD in or near the graft [183]. It has been suggested that chronic EBV infection of renal proximal tubular cells evokes a cellular immune response that not only damages the graft interstitium, but also leads to a local inflammatory environment thereby facilitating PTLD development [184]. It is unknown whether other viruses, such as the oncogenic BK virus (frequently observed in kidney transplant recipients [185]) might also contribute to the development of PTLD as a result of providing a local inflammatory environment. It would be of interest to study this relationship in the next future.

The greatest risk of developing PTLD occurs during the first year after transplantation [186, 187]. Among solid organ transplant recipients, the median time of onset of PTLD is about 6 months [187, 188]. In bone marrow transplant recipients, the median time to onset is about 2 months and these patients tend to have widespread, rapidly progressive disease [188]. The frequency of PTLD varies depending on the type of transplant and the level of immunosuppression. There seems to be a relationship between the occurrence of PTLD and treatment of rejection episodes with increased immunosuppression [189]. An analysis of 3796
solid organ transplant patients revealed that the highest frequency of PTLD was in lung (8.2%) and the lowest frequency (1.3%) in renal transplant patients [187] with 3% among liver and 5% among heart recipients [189]. Within a group of 2030 patients after renal transplantation, a greater level of immunosuppression was associated with an increased risk of developing PTLD [190]. About 30% of pediatric patients can develop PTLD after small intestinal transplantation [113].

The typical evolution of disease occurs in patient on calcineurin inhibitor therapy who suffers a rejection episode which either does not respond to steroid or rapidly relapses after tapering steroids, requiring treatment with one or two courses of antilymphocyte globulin or OKT3. Shortly thereafter the patient develops PTLD [189]. Interestingly, induction therapy with the more recently introduced interleukin (IL)2-receptor antibodies does not seem to lead to a higher incidence of PTLD [191]. The total amount of immunosuppression for induction or anti-rejection therapy rather than the use of a single maintenance agent has been correlated with an increased risk of PTLD [191, 192].

A genetic predisposition may also play a role in the development of PTLD. Patients with an inherently lower immune capacity might be at an increased risk for PTLD development [193]. For example, subjects with a low capacity to produce cytokines due to genetic polymorphism (interleukin-2 and interferon-c), have been reported to display an increased risk of PTLD development [194].

Another report suggested that increased levels of IL-10 might be predictive for PTLD risk [195]. Although the exact relationship between IL-10 and the development of PTLD has not been fully elucidated yet, IL-10 can act as a putative autocrine growth factor for EBV-transformed B-cells [196].
Furthermore, increased total numbers of HLA mismatches have also been observed to be associated with PTLD development [191] presumably due to excessive immunosuppression relative to the antigenic disparity.

### III/3. Clinical manifestations

Clinically, PTLD can present in a number of ways:

1. oropharyngeal hyperplasia or lymphadenopathy which resembles infectious mononucleosis,
2. a fulminant, rapidly progressive polyclonal lymphoid hyperplasia,
3. Or most commonly as a single or metastatic polyclonal or clonal tumors which most often is observed in an extranodal locations within the brain, gastrointestinal tract, or allograft [197].

The fulminant form of PTLD is characterized by peripheral lymphadenopathy, severe metabolic acidosis, organ failure or allograft dysfunction [198]. Approximately 90% of patients show an association of active EBV infection, either primary or by reactivation [189]. Early changes of PTLD represent, a spectrum of EBV driven lymphoid B-cell proliferations arising as polyclonal expansions of EBV infected cells, which progresses to a monoclonal, monomorphous B-cell lymphoma [189].

In some instances proliferating B-lymphocytes have been shown to be of donor origin apparently originating from “passenger” lymphocytes [199]. PTLD development within the allograft is higher (30%) in the first post-transplant year among kidney and lung transplant recipients [200]. These lymphomas may result from EBV-infected donor B-lymphocytes transplanted within the graft escaping host surveillance, due to immunosuppression [201]. Consistent with this hypothesis, PTLD of donor origin tends to arise early after transplantation and is more often localized in or near the allograft, and rarely shows dissemination as compared with recipient-derived PTLD [202].
The polyclonal type is commonly polymorphous but many have been shown to have a monoclonal component by genotypic analysis. In some cases different monoclonal and polyclonal populations are present in the affected organs or sites [203].

Overall, the mortality rate for PTLD has been estimated to be 40-70% after solid organ [175] and 80% after bone marrow transplantation [177]. A multivariate model for predicting survival using three adverse factors including poor performance status (response to therapy and tolerability of therapy), monomorphic disease, and graft organ involvement has been developed recently [204].

III/4. Diagnostic methods

The diagnosis is usually established from biopsy material obtained from patients suspected of having PTLD or a rejection episode. Fine needle aspiration biopsy also can be sensitive for the diagnosis when reviewed by experienced cytopathologist [205]. Grossly, PTLD usually presents either as a tumor mass or an infiltrating lesion indistinguishable from other lymphomas. PTLD is characterized by a dense inflammatory infiltrate with a spectrum histologically ranging from that found in infectious mononucleosis to that of lymphoma [197, 203].

1. Early reactive-like lesions mainly involve the oropharynx and lymph nodes with preservation of nodal architecture. They may be difficult to differentiate from simple reactive hyperplasia. The lesions contain evidence of multiple EBV infections without alterations of oncogenes or tumor suppressor genes [206].

2. Polymorphous B-cell hyperplasia and lymphoma produces effacement of lymph node architecture and dense lymphoproliferative infiltrates of many organs often with associated extranodal necrosis. The infiltrate shows a full range of B- and T-cell phenotypes ranging from immature to mature lymphocytes. There may be extensive necrosis associated with numerous neutrophils and histiocytes are seen. Most cases are monoclonal although rarely
polyclonal lesions are seen as well. The lesions usually contain a single form of EBV but lack oncogene (c-myc, ras) and tumor suppressor gene (p53) alterations [206].

3. Monomorphous, monoclonal lymphomatous infiltrates have the features of high-grade lymphomas. The monomorphous infiltrate consists of large atypical B-cells, frequently with immunoblastic features. They may also show extensive necrosis. The monomorphous infiltrate contains a single form of EBV but unlike the polymorphous PTLD it contains oncogene (c-myc, c-ras) and tumor suppressor gene (p53) alterations [207]. On rare occasions T-cell lymphomas have been observed in this category[208].

Analysis of EBV is especially useful for the diagnosis of early cases of PTLD. Early detection of EBV DNA in peripheral blood leukocytes or plasma uses quantitative polymerase chain reactions (PCR) to indirectly identify patients at risk for PTLD and to monitor responses to therapy. PCR assays include those EBV DNA, for in situ hybridization to EBV RNA (EBER-1) or for EBV latent membrane protein (LMP) have been used to identify patients at risk for PTLD, especially in the pediatric and allogeneic bone marrow transplant populations [209]. These two patient populations are particularly at high risk for PTLD, given the lack of EBV immunity in most pediatric patients and the high level of immunosuppression and the altered immune system seen in T-cell depleted or mismatched allogeneic bone marrow transplants.

Among pediatric liver and bone marrow transplant patients, EBV PCR has been shown to identify patients at risk for PTLD, allowing that early intervention such as reductions in immunosuppression or treatment with EBV cytotoxic T cells [210, 211].

A recently conducted retrospective analysis [209] has also reported the importance of peripheral blood EBV PCR for the diagnosis and monitoring of post-transplant lymphoproliferative disorder among adult solid organ transplant patients. Peripheral blood leukocytes obtained from 35 subjects were tested by EBV PCR at the time of initial
Eighteen of 35 (51%) patients were ultimately diagnosed with a post-transplant lymphoproliferative disorder by tissue biopsy. Fifteen of 18 (83%) patients showed EBER-1 positive tumors by *in situ* hybridization. EBV PCR was positive in 7 of 15 patients, suggesting a sensitivity of 39%. Among 17 patients without post-transplant lymphoproliferative disorder and three with EBER-1 negative posttransplant lymphoproliferative disorder, all displayed negative EBV PCR tests, suggesting a specificity of 100%. The authors also reported that declines in EBV DNA load were associated with response to therapeutic interventions, such as reduction in immunosuppression, rituximab therapy and/or chemotherapy.

An exact cutoff value of EBV DNA load critical for the development of PTLD in the individual patient cannot be defined due to the many variables that may influence the immune responses of the individual transplant recipient, such as level of immunosuppression, time after transplantation, concomitant infections, type of organ transplanted, but also genetic factors. Therefore, an increase in EBV-DNA content in an individual patient is more appropriate to identify the risk rather than a cutoff value [212].

Analysis of clonality cannot be used to reliably predict tumor behavior in an individual patient [189]. To create more confusion in diagnosis and treatment it has been demonstrated that lesions at various sites in an individual patient, may show different histologic appearances and/or clonality [213].

Conventional diagnostic methods are utilized to visualize PTLD include ultrasound, endoscopy, magnetic resonance imaging (particularly in case of CNS involvement), computed tomography (CT). Positron emission tomography (PET) scanning is increasingly being used to visualize malignant lymphomas, especially to detect extra nodal localizations and for post-treatment evaluation. It has reported to be superior to conventional diagnostic methods for differentiation between residual masses containing vital tumor cells versus scar tissue [214].
III/5. Treatment options

III/5.1. General considerations

Early identification of patients with PTLD is important for the treatment of this disease, as it allows early intervention. When detected in an early state, reduction in immunosuppression is an effective therapy, with response rates as high as 89% among low-risk patients [187]. There are no large, prospective, randomized trials to provide clear guidelines for PTLD treatment. Reduction in immunosuppression (RI) is considered the first line therapy [187]. It seeks to reconstitute the immune system. The technique is usually tailored to the clinical course of the individual patient and the transplant type. In general, RI involves discontinuation of azathioprine or mycophenolate mofetil and minimization of the calcineurin inhibitor and steroid. The magnitude of RI is patient-specific and may be limited in those with history of organ rejection or when the graft is indispensable for survival. In kidney transplant patients, where graft rejection is compatible with life, RI may be aggressive including complete cessation of immunosuppression. The time to seek a response to RI is not well defined. In one series, the median time to documentation of a response was 3.6 weeks [187]. Patients, however, may show signs of clinical improvement within 1–2 weeks. The strategy of RI alone can result in high response rates (RR) ranging from 0% to 89% depending upon the prognostic factors such as elevated lactate dehydrogenase (LDH), multi-organ involvement by PTLD, and organ failure at the time of diagnosis [187]. Other treatments might need to be used in conjunction with RI if the patient is not a candidate for RI alone.

Targeting EBV by antiviral agents such as ganciclovir or acyclovir has been attempted for prophylaxis and treatment of PTLD [211]. In order to prevent development of PTLD, 18 high-risk pediatric liver transplant patients received 100 days of i.v. ganciclovir at 6–10 mg/kg/ day [211]. None developed PTLD as opposed to the 10% rate of PTLD among
an historical cohort of control subjects. In 198 adult patients who received either ganciclovir or acyclovir during immunosuppressive therapy with antilymphocyte globulin, only 0.5% developed PTLD as opposed to 7% among historical controls [215]. It is difficult to make conclusions based on these non-randomized studies since the definition of ‘high-risk’ patients and the dosing regimen were inconsistent. It is unlikely that antiviral agents are effective monotherapy to treat PTLD [216]. Latent EBV-infected B-cells, which carry the viral genome and express a limited number of viral proteins are not eliminated by antiviral agents. However, arginine butyrate, which selectively activates the EBV thymidine kinase gene in latently EBV-infected human lymphoid cells and tumor cells, has been used in combination with ganciclovir in six PTLD patients, who were resistant to conventional radiation and/or chemotherapy [217]. The combination produced complete responses (CR) in four of six patients, with a partial response (PR) occurring in the fifth patient. Infusion of patient-derived EBV-specific T-cells has been reported for the management of patients with EBV-associated PTLD [218].

Non-specific immune stimulants such as interferon-alpha can enhance immune system in PTLD patients [171, 219]. Since interferon use has the unfortunate side effect of inducing allograft rejection, it has not been widely employed. Other treatment modalities such as external beam radiation and surgery have been used in settings of localized PTLD [171, 187].

Chemotherapy is the standard salvage therapy after failure of reduction in immunosuppression, but carries significant mortality and morbidity rates in the organ transplant population [220]. Conventional cytotoxic chemotherapy which has been shown to be curative for many lymphomas in non-PTLD setting can be administered to PTLD patients who fail or are not amenable to RI. In certain aggressive PTLD subtypes (i.e. Burkitt lymphoma-like disease), conventional cytotoxic chemotherapy is used as the first line treatment as less aggressive approaches appear to be ineffective [221]. Various multi-drug
regimens such as CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) have been used in PTLD patients. In spite of the high response rate (up to 70%), the associated toxicity is significant including treatment-related deaths in about 25% of patients [220, 222]. The high mortality of standard chemotherapy regimens in the PTLD population may occur because of various factors including baseline pharmacologic immunosuppression, graft dysfunction, and colonization with resistant or hospital acquired infectious organisms.

### III/5.2. Monoclonal antibodies for treatment of PTLD

In the late 1980s and 1990s the initial use of anti-B-cell monoclonal therapy (anti-CD21 and anti-CD24) demonstrated high response rates with 50% long-term survivals [223]. However, these antibodies were not commercially available. In the late 1990s, the anti-CD20 monoclonal antibody, rituximab, became available world-wide. It has primarily been used to treat PTLD [224].

Its mechanisms of action have been reviewed in detail earlier are summarized in Figure 12.

![Rituximab: three potential mechanisms of action include apoptosis, complement activation and antibody-dependent cell-mediated cytotoxicity (from Svoboda et al.)](image-url)

**Figure 12.** Rituximab: three potential mechanisms of action include apoptosis, complement activation and antibody-dependent cell-mediated cytotoxicity (from Svoboda et al.).

Rituximab was first approved for the treatment of relapsed low-grade CD20-positive non-Hodgkin lymphomas with reported overall response rates of up to 50% and complete remission rates of 5% [90]. However, the duration of the response in patients with low-grade
lymphoma was limited; the median time to progression was 13 months [90]. Since the initial approval, it has been widely used as a single agent or in combination with chemotherapy for the treatment of various CD20-positive hematological malignancies [93, 225, 226].

Rituximab also has an expanding role in management of various non-malignant diseases, especially autoimmune condition in which B-cells play important role, including rheumatoid arthritis [227], Sjogren’s syndrome [228], systemic lupus erythematosus [229], myasthenia gravis [230], autoimmune hemolytic anemia [231], idiopathic thrombocytopenic purpura [232] and dermatomyositis, polymyositis [233, 234]. The beneficial effect of rituximab in antibody mediated rejection episodes has been reviewed earlier.

In 1998, Fay et al. reported the use of rituximab in a pediatric patient with Fanconi disease who developed PTLD 6 months after matched unrelated donor kidney transplantation [235]. The patient developed a tonsillar mass and cervical lymphadenopathy. The biopsy was consistent with a polymorphic CD20-positive B-cell PTLD. Nearly all B-cell nuclei contained EBV RNA and high levels of EBV DNA were detected in peripheral blood. The patient received rituximab at the standard dose (375 mg/m2 for 4 weekly doses), experiencing tumor regression at only 3 days after the first infusion. He achieved complete response without relapse at 6 months [235].

Over the recent years many case reports and series described the use of rituximab in PTLD [236-243]. Most patients also underwent concurrent RI, some concurrent antiviral therapy [241]. Many subjects experienced clinical improvements within a few days of the first infusion [240, 242], but in some instances the benefit was not observed for a few months [243]. Most patients in the case reports were treated with the standard dose of rituximab (375 mg/m2) once a week for four consecutive weeks. The majority of the case reports described the use of rituximab for early onset PTLD, but suggested that it might also be effective for patients with late onset PTLD. Dotti et al. presented five patients who had late onset CD20-
positive PTLD beyond 2 years after solid organ transplantation. After treatment with rituximab [238], 2 patients with advanced disease had only PR, but 3 patients who underwent either successful prior surgical debulking or radiotherapy displayed excellent clinical outcomes [238].

While large retrospective analyses suggested a benefit of rituximab [244-249], Phase II trials prospectively confirmed its clinical benefit in PTLD [114, 250, 251]. The RR of single agent rituximab treatment in PTLD patients ranged from 44% to 75% with CR rate ranging from 28% to 75%. The duration of CR varied depending on the trial, but clearly some patients displayed prolonged disease free survivals after single agent treatment. The major differences in the results of the phase II trials are likely secondary to the heterogeneity of patients enrolled, small sample sizes, and short time of follow-up.

A large prospective trial using rituximab in PTLD was recently published by Choquet et al. [114]. This multicenter, open label, European phase II trial enrolled 46 pediatric and adult patients with PTLD after solid organ transplantation who did not improve after RI. They were treated with 375 mg/m2 weekly for 4 weeks. Most PTLD cases were of relatively late onset; only 14 (35%) patients had a PTLD diagnosis within 1 year after transplantation. At day 80, the RR was 44% including 12 (28%) patients with CR. A normal LDH was a significant predictor of a response. At day 360, the responses were maintained in 68% of patients. The overall survival rate at 1 year was 67%. Rituximab was well tolerated; thereby only two grade three to four adverse events related to the treatment [114]. In the USA, a smaller phase II trial involving 11 patients revealed a 64% response rate [252].

Another prospective, multicenter, phase II trial has been published recently [248]. Patients were treated with reduction of immunosuppression and four weekly infusions of rituximab. Those patients who did not achieve complete remission (CR) received a second course of four rituximab infusions. The primary end-point of the study was the CR rate.
Among 38 evaluable patients the only severe adverse event was one episode of grade 4 neutropenia. After the first course of rituximab, 13 (34.2%) patients achieved CR, 8 did not respond, and 17 had partial remissions. Among these last 17, 12 were treated with a second course of rituximab with 10 (83.3%) achieving CR, yielding an intention-to-treat CR rate of 60.5%. Eight patients excluded from the trial because of absence of CR were treated with rituximab combined with chemotherapy; six (75%) achieved CR. Event-free survival was 42% and overall survival was 47% at 27.5 months. Fourteen patients died including ten with progressive post-transplant lymphoproliferative disorders. The authors concluded that extended rituximab treatment obtained a high rate of CR among post-transplant lymphoproliferative disorders without increased toxicity, recommending it as initial therapy.

A French PTLD Registry prospectively enrolled 230 affected adult kidney recipients between January 1, 1998, and December 31, 2003 to analyze the incidence, risk and prognostic factors for the disorders. The cumulative incidence of PTLD was 1.18% at 5 years. Older age (per year, hazard ratio (HR)=2.19, CI=1.22–3.94) and recipient Epstein-Barr virus seronegativity (HR=3.01, CI=1.57–5.08) were associated with an increased risk of PTLD. Patients with PTLD showed a reduced survival rate: 61% at 5 years. Graft PTLD displayed the best prognosis, namely 81% survival rate after 5 years. Infections with hepatitis C or B virus (HCV or HBV), late-onset PTLD, multiple sites of involvement and high Ann Arbor staging were risk factors for patient death. Use of azathioprine was associated with a poorer survival rate. This incidence and the risk factors among French recipients were consistent with international or American PTLD series.

The single agent rituximab may be effective to prevent fulminant PTLD. Among 49 allogeneic bone marrow transplant patients, 17 experienced EBV reactivation as detected by positive quantitative PCR, namely, more than or equal to 1000 genome equivalents per milliliter. As virus reactivation is believed to correlate with an increased risk of PTLD, these
patients underwent ‘pre-emptive’ rituximab treatment [253]. When compared with an historical cohort with the same risk profile, this strategy achieved a significant reduction in PTLD incidence and eliminated PTLD-associated mortality [253]. A study of 56 allogeneic stem cell transplant patients, that included PCR, monitoring of EBV reactivation and of CD8 positive T-cell immune responses provided indices to initiate rituximab before the immune response was overwhelmed by the viral burden [254]. Patients with EBV-specific T cells at the onset of reactivation controlled viral reactivation without rituximab.

Apart from its use as a single agent, rituximab has been reported to have chemosensitizing effect on several lymphoma cell lines, possibly by augmenting apoptosis [255]. Clinically, patients with CD20-positive non-Hodgkin lymphomas who received combination treatment of rituximab with cytotoxic chemotherapy showed superior outcomes to those treated with cytotoxic chemotherapy alone [256]. A pilot trial added rituximab to cyclophosphamide and prednisone therapy in six solid organ transplant patients [257]. Every three weeks they received two to six courses of cyclophosphamide (600 mg/m2, on day 1 of each course) and prednisone (1 mg/kg, every 12 h for 10 doses). The first two courses were given in combination with 4–6 weekly doses of rituximab (375 mg/m2). At a median follow-up of 12.5 months (range=4–29 months), all patients responded, including five with CR [18]. The one patient who did not achieve CR had a PR, but eventually progressed succumbing to fulminant disease. There were no infectious complications; all allografts in surviving patients were functional [257]. Preliminary data were presented recently from an ongoing phase II trial of sequential rituximab followed by CHOP chemotherapy combined with granulocyte colony-stimulating growth factor (G-CSF) [258]. Among the 25 evaluable PTLD patients, nine (36%) experienced severe infections and three (12%) died of treatment-related causes [258].

III/5.3. Rituximab versus chemotherapy
Until recently, patients with PTLD who failed RI were treated with cytotoxic chemotherapy [259]. There are no prospective, randomized trials that compare chemotherapy to rituximab for patients who do not respond to RI. However, a recent retrospective study analyzed data on 35 PTLD patients who underwent treatment with rituximab, chemotherapy, or both [222]. The findings confirmed that either single agent rituximab or chemotherapy was effective treatment for patients who failed RI. Both types of therapies resulted in prolonged disease-free survivals and cures in a number of PTLD patients. The 22 patients who received rituximab displayed an RR of 68% with 13 (59%) patients in CR. Their median OS was 31 months. The 23 patients who received cytotoxic chemotherapy had RR 72% with 13 (57%) patients in CR at a median OS of 42 months. While rituximab was well tolerated, 26% of patients who received chemotherapy died from treatment-related toxicities [222]. An important observation in this study was that patients who failed treatment with rituximab were subsequently able to receive salvage chemotherapy later [222]. These results suggested that, rituximab should be considered a first line treatment for EBV-related, CD-20-positive cases of PTLD.

III/5.4. Case report: successful treatment of post-transplant lymphoproliferative disorder and quiescence of dermatomyositis with rituximab and sirolimus

III/5.4.1. Introduction

I have presented herein the course of a patient with a chronically disturbed immune system owing to dermatomyositis who underwent en bloc pediatric donor renal transplantation and subsequently developed PTLD. Our patient was at increased risk for PTLD, not only due to antithymocytic globulin (ATG) induction and cyclosporine (CsA)-based immunosuppression, but also due to the autoimmune disease, which although it primarily affects muscle, skin, and lungs, is associated with a greater incidence of malignancy.
In addition to its efficacy to treat PTLD, rituximab has been reported to be useful for reversal of relapses of dermatomyositis, presumably because of its effects to deplete B-cells.

### III/5.4.2 Case history

A 65-year-old Caucasian woman underwent primary en bloc kidney transplantation from a deceased 2-year-old donor on October 23, 2006. The recipient had a history of a ruptured arteriovenous malformation in her brain, medication-controlled hypertension, atopic dermatitis, dermatomyositis, and asthma. In addition, her history included degenerative disc disease, a bout of trigeminal neuralgia, osteoarthritis, and hypothyroidism. She had previously undergone a right oophorectomy, appendectomy, knee arthroscopy, arteriovenous fistula creation, and removal of a basal cell carcinoma in the left nasolabial crease followed by facial and septal reconstruction. The diagnosis of dermatomyositis was obtained after her presentation with fatigue, weakness, and myalgia in October 2003. Not only was the creatine phosphokinase (CPK) level 4400 U/L, but an electromyogram revealed myopathy of the proximal muscle groups. The dermatomyositis had been well controlled for 2 years before transplantation using prednisone (5 mg/d) and azathioprine (75 mg/d); CPK levels remained in the range of 30 to 60 U/L. Her end-stage renal disease was secondary to autosomal-dominant polycystic kidney disease. After transplantation, immunosuppressive induction was achieved with ATG (100 mg X 3 and 50 mg X 5), followed by inception of sirolimus (SRL; 2 mg/d), CsA (75 mg AM and 50 mg PM), and prednisone (30 mg/d). She was discharged with good renal function, namely a serum creatinine (SCr) of (1.4 mg/dL) 123.7 µmol/l. Shortly after transplantation the maintenance regimen was changed from SRL to mycophenylate mofetil (MMF; 1 g bid), owing to lower extremity swelling and for putatively better control of the dermatomyositis. She continued to experience satisfactory renal function with a SCr of (1 to 1.5 mg/dL) 88 to 132 µmol/l until July 2007, when her level increased to (2.0 mg/dL) 176 µmol/l and she offered a history of fever that had persisted for several weeks. The MMF dose
was increased from 1 to 1.5 g bid by her rheumatologist who felt that the fever was secondary to the polymyositis. She was initially admitted to the transplant center in August 2007 with plans for a renal biopsy owing to the elevated SCr. However, the intake history revealed that she was injured by a rose bush thorn in February with the onset of cough and then fever soon thereafter. Indeed, computed tomography (CT) of the chest performed in July at an outside facility was discovered to show pulmonary infiltrates, suggesting an interstitial process or pneumonia. Although bacterial cultures were negative, bronchoscopic lavage revealed *Candida albicans*, which was treated with caspofungin (50 mg IV daily for 10 days). Despite the relevant antifungal therapy, the fever persisted. Abdominal CT revealed a 6-cm mass in proximity to or within the inferior transplanted pediatric kidney. Repeat chest CT as well as magnetic resonance imaging of the brain was negative. Histologic and immunohistochemical examinations of an ultrasound-guided biopsy showed a polyclonal-PTLD, which displayed EBV and CD20 markers as well as both κ and λ immunoglobulin light chains. The treatment strategy for this polyclonal localized neoplasm included replacing MMF and CsA with SRL therapy, as well as prescription of oral acyclovir and four weekly intravenous rituximab doses (375 mg each). She did not experience an adverse reaction to the treatments; her serum creatinine remained in the (1.5 to 2 mg/dL) 132 to 176 µmol/l range and she became afebrile. The CPK level was consistently within normal limits (35 to 53 U/L), suggesting that the dermatomyositis was not reactivated despite the withdrawal of MMF. In retrospect, examination of deceased donor records revealed reactive EBV and cytomegalovirus (CMV) immunoglobulin G levels. Unfortunately, there was no information on the recipient’s EBV status before transplantation, but the CMV serologies were negative. She had received valganciclovir HCl (450 mg daily for 6 weeks) after transplantation because of ATG induction therapy and CMV disparity. Two follow-up CT scans at weekly intervals upon completion of the rituximab course revealed the size of the mass to be unchanged, prompting
a decision for surgical exploration to obtain a biopsy to determine the status of the lesion. At operation, a biopsy showed over 90% necrosis of the infiltrating lymphocytes, documenting the efficacy of the therapeutic regimen to control the PTLD, although there had been emergence of a moderate acute cellular rejection.

III/5.5. Summary of PTLD and its treatment with rituximab

The above case report has reported successful treatment of PTLD associated with an en bloc renal transplant performed in a patient with a history of recurrent bouts of dermatomyositis. What made it unique is the multiple disturbed immune system which eventually has led to development of PTLD. Additional risk factors for malignancy were the intense MMF regimen to control the autoimmune disease, the advanced age of the recipient, CMV mismatch, and an EBV-positive donor. Rituximab and SRL were chosen due to the antibody’s favorable actions on non-Hodgkin lymphomas and dermatomyositis [234, 239, 260]. This strategy permitted discontinuation of MMF without a relapse of dermatomyositis. The effects of the anti-CD20 monoclonal antibody were enhanced by the antineoplastic and immunosuppressive actions of the SRL. However, although the combination overcame the PTLD and kept the dermatomyositis in remission, it did not prevent the emergence of a moderate allograft cellular rejection reaction. In conclusion, this case demonstrated the efficacy of rituximab combined with SRL to treat PTLD — over 90% of neoplastic cells were necrotic — while suppressing activation of the immunologic comorbidities associated with dermatomyositis.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Publication</th>
<th>Transplant type</th>
<th>N</th>
<th>Response</th>
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<tr>
<td>Elstrom et al. [222]</td>
<td>Article/retrospective analysis</td>
<td>Solid organ and bone marrow (adults)</td>
<td>22</td>
<td>RR was 68% and 13 (59%) patients had CR; when compared with patients who received chemotherapy, the RR was similar with less toxicity</td>
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<td>Webber et al. [250]</td>
<td>Abstract/retrospective Analysis</td>
<td>Solid organ (pediatrics)</td>
<td>26</td>
<td>Response rate was 75% and 18 (69%) patients had CR; four non-responders included two Epstein–Bar virus negative one fulminant disease, and one Burkitts-like disease</td>
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<tr>
<td>Ferry et al. [261]</td>
<td>Abstract/retrospective analysis</td>
<td>Bone marrow (pediatric and adult)</td>
<td>26</td>
<td>Overall survival (OS) at 180 days of 26 patients who received rituximab was 46% (vs. 0% for seven patients who did not received rituximab); patients with less advanced disease and low viral load had better response rate</td>
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<tr>
<td>Milpied et al. [249]</td>
<td>Article/retrospective analysis</td>
<td>Solid organ and bone marrow (adults)</td>
<td>32</td>
<td>Response rate was 69% and 20 (63%) patients had complete responses CR; projected OS was 73% at 1 year; four patients relapsed and three died while in remission</td>
</tr>
<tr>
<td>Gonzalez-Barca et al. [248]</td>
<td>Abstract/retrospective analysis</td>
<td>Solid organ (adult)</td>
<td>36</td>
<td>The 36 patients who received rituximab had improved OS when compared with the total 108 PTLD patients (76% vs. overall 21% with median follow-up 15 months)</td>
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<td>Morrison et al. [262]</td>
<td>Abstract/phase II trial</td>
<td>Solid organ (adults)</td>
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<td>Response rate was 75% with three CRs, three PRs, one progressive disease, and one death</td>
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<td>Horwitz et al. [263]</td>
<td>Abstract/phase II trial</td>
<td>Solid organ (pediatrics and adults)</td>
<td>14</td>
<td>Response rate was 62% (three with CRs, five with PRs); one patient had stable disease at 1 month; four patients progressed on therapy and went on to receive chemotherapy, resulting in two septic death</td>
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<tr>
<td>Webber et al. [250, 252]</td>
<td>Abstract/phase II trial</td>
<td>Solid organ (pediatric)</td>
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<td>Nine (75%) patients had CR, OS was 83% with median follow-up 18 months</td>
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<td>Blaes at al. [252]</td>
<td>Article/phase II trial</td>
<td>Solid organ (adult)</td>
<td>11</td>
<td>10 months follow-up; RR 64%, 6 CR, 1 PR, 2 progressive disease, 2 death; median survival 14 months; median duration of CR 10 months</td>
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<tr>
<td>Oertel et al. [251]</td>
<td>Article/phase II trial</td>
<td>Solid organ (adult)</td>
<td>17</td>
<td>Nine (53%) patients achieved CR, one with PR, one with progressive disease; mean OS 37 months, no severe adverse events</td>
</tr>
<tr>
<td>Choquet et al. [114]</td>
<td>Article/phase II trial</td>
<td>Solid organ (pediatric and adult)</td>
<td>43</td>
<td>At day 80, RR was 44% with 12 patients in CR; at day 360, responses were maintained in 68%; the treatment was well tolerated</td>
</tr>
</tbody>
</table>

**Table 8.** Efficacy of rituximab in the treatment of PTLD in various settings.
Many case reports, retrospective analyses, and several prospective trials have demonstrated that rituximab is effective for CD20-positive PTLD in various settings (Table 8). PTLD patients can achieve long-term CR and potential cure after single agent rituximab treatment. When compared with cytotoxic chemotherapy, rituximab has comparable RR, but significantly reduced toxicity and treatment-associated mortality. Limited data suggest that patients with fulminant, advanced disease, EBV-negative or late-onset tumors are less likely to respond to single agent rituximab [114, 204, 222, 238, 249]. For these patients, cytotoxic chemotherapy might be necessary early in the treatment course.

For the majority of EBV-associated CD20-positive PTLD patients, rituximab as the second line of treatment right after RI is favorable. Given the significant toxicity, chemotherapy is best reserved for use in patients who are ineligible or fail rituximab. In the future, trials with combination therapies involving rituximab and other immune-based treatments as well as early detection and possible prevention will hopefully improve the clinical outcome of patients with PTLD.
IV. THE ROLE AND EFFICACY OF SIROLIMUS IN RE-TRANSPLANTATION

IV/1. Introduction

Re-transplantation offers a better survival benefit compared with continuous dialysis after kidney transplant failure [264]. Patients who lose their first grafts have three options: hemodialysis, which results in a poor quality of life and is the least cost effective [265]; peritoneal dialysis which is often complicated by recurrent peritonitis and other intra-abdominal complications but is cost-effective and allows a more active lifestyle [266]; or repeat renal transplantation, which has obvious quality of life benefits, but inherent risks relative to graft survival and is expensive at least in the first year [267]. Using the Canadian Organ Procurement Registry, Rao, et al [264] observed that, except during the first year thereafter, retransplantation conferred a covariate-adjusted reduction in the hazard ratio (HR) for mortality compared with dialysis (HR=0.50, \( P<0.0001 \)). Despite this benefit, the number of patients electing retransplantation is relatively low [268]. In 2003, the United Network for Organ Sharing (UNOS) reported that 15.3% of patients awaiting a kidney and 13.5% of those receiving a graft had undergone a previous transplantation [1]. One obstacle for these candidates to receive a re-transplantation is their higher content of panel reactive antibody (PRA) following rejection of the first graft, namely 31.5% of subjects displayed a PRA value of at least 20% (Figure 13).

Figure 13. Deceased donor kidney transplants in 2005, by peak PRA at listing. OPTN Analysis, November 2006.
The outcomes of re-transplantation tend to be inferior to primary grafts (Figure 14). In the cyclosporine-prednisone era in 1985, primary versus second graft survivals at 1, 2 and 5 years were reported to be 79, 72 and 72% versus 69, 58 and 58%, respectively [269]. In a 12 year series from Guy’s Hospital (London), the 1, 2, 3 and 4 year survivals of 100 matched first renal transplants were superior to 163 re-transplantations, namely 92, 84, 74 and 60% versus 82.3, 67.3, 55.97, and 42.14%, respectively [270]. Using more intense recent immunosuppressive regimens, the 1, 3 and 5 year survivals reported to UNOS in 2005 were 95, 89 and 81% versus 93, 83 and 76%, respectively ($P<0.01$; $P<0.0001$ and $P=0.01$) [1]. Whereas graft failures among both groups in the first year were usually attributed to acute rejection and graft thrombosis, thereafter chronic rejection and allograft nephropathy appeared to be more frequent among the re-transplant cohort.

Sirolimus, a potent immunosuppressive drug, inhibits the multifunctional kinase mammalian target of rapamycin (mTOR). In combination with a calcineurin antagonist, sirolimus has been shown to reduce the incidence of acute allograft rejection among primary renal graft recipients [271-273].

![Figure 14. Unadjusted living donor (LD) and deceased donor (DD) graft survival for first and second kidney transplants, 2000–2005. SRTR Analysis, May 2006.](image-url)
Additionally, in vivo animal and in vitro experimental models have demonstrated that the drug mitigates intimal hyperplasia and mesangial cell proliferation, suggesting that it may prevent the development of chronic allograft nephropathy [274, 275]. Unlike calcineurin inhibitors which are known to be nephrotoxic due to vasomotor and tubular toxic effects, sirolimus seems to produce only minimal renal injury which has been attributed to inhibited mitosis during acute tubular nephropathy. Other features of the drug are its potent anti-proliferative properties for most rapidly-dividing cells and its blockade of the activities of transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) which together contribute to a reduced incidence of malignancies compared with other immunosuppressive regimens [276].

Until recently, the only high-risk population that had been established to show the efficacy of sirolimus was African Americans/Blacks [277], an ethnic group that displays inherent adverse genetic, pharmacokinetic, pharmacodynamic, cultural and socio-economic factors. In the present study, we sought to examine the outcomes of de novo sirolimus-based immunosuppression between high risk retransplant versus primary renal allograft recipients.

IV/2. Materials and methods

Patients

Between May 1994 and November 2005, a cohort of 162 (15%) subjects underwent renal re-transplantation within the overall population of 1,062 grafts at the University of Texas, Division of Immunology and Organ Transplantation. Within this cohort 98 (64%) received de novo sirolimus-based immunosuppression. None of the re-transplant patients had been previously treated with sirolimus and only 5 had been primarily engrafted at another center. The 900 patients who underwent primary transplantations included 576 (64%) who were enrolled in de novo protocols of sirolimus immunosuppression. From these 576 subjects
we selected a control population of 200 patients who were matched to the 98 repeat graft recipients based upon month of grafting and demographic features (Table 9).

**Immunosuppressive protocol**

The sirolimus regimen (Rapamune, Wyeth, Philadelphia, PA), generally began with a pre-transplant loading dose of 15 mg followed on day 1 with 10 mg once daily. After initial adjustment to achieve target trough levels from 10 to 15 ng/mL, the schedule evolved by 6 months to 10 ± 2 ng/mL for maintenance therapy. Among patients whose grafts displayed immediate function, Cyclosporine (CsA; Neoral, Novartis, Basle, SZ) was initiated on day 1 at reduced doses, \(^{16}\) employing 50 mg every 12 hours. The exposure was subsequently tailored to achieve trough concentrations of 150 ± 25 during the first 6 months, 100 ± 25 from 6-12 months post-transplant and 50 ± 25 ng/mL thereafter. Cyclosporine was routinely initiated by day 5 even when the patient remained on dialysis.

The induction and maintenance steroid regimens during the first 90 days for primary transplants were tailored according to the perceived risk of a biopsy-proven acute rejection episode (BPAR) based on the organ donor source and the recipient’s immunologic status. While low-risk recipients underwent steroid withdrawal at 30 days, high-risk patients were delayed until after 90 days. Steroids were continued indefinitely in all patients who had experienced a BPAR or were retransplant recipients. The addition of mycophenolate mofetil (MMF) was necessary only in rare cases, namely, subjects unable to tolerate sirolimus and/or CsA. MMF tended to be avoided due to its apparent potentiation of sirolimus-induced myelosuppression and its effects to foster cytomegalovirus (CMV) and BK virus infections as well as post-transplant lymphoproliferative disease, conditions that occurred only rarely under sirolimus-based therapy [273, 278].
Five daily doses of Thymoglobulin (Genzyme, Boston, MA. 1.5mg/kg/dose) were administered to high-risk recipients from day 0, unless they displayed leukopenia or thrombocytopenia in which case we prescribed a 50 to 75% dose reduction or cessation of therapy. High-risk patients were deemed to be African-Americans younger than 65 years, retransplant subjects, or recipients whose grafts experienced a cold ischemia time greater than 24 hours. Low-risk patients comprised primary graft recipients who displayed a peak PRA less than 20% and were Caucasian, Hispanic, Asian or African-Americans over 65 years of age. Induction therapy in low-risk patients utilized basiliximab (Simulect, Novartis, Basle, SZ; 20 mg administered at surgery and on post-operative day 4). All retransplant patients received thymoglobulin induction and chronic steroid therapy.

Adjuvant therapy

All patients were prescribed a first generation cephalosporin (or vancomycin for penicillin allergic patients) as a peri-operative antibiotic. *Pneumocystis carinii* prophylaxis included trimethoprim-sulfamethoxazole (Bactrim SS; orally every other day). Oral antifungal prophylaxis was prescribed for 3 months as was a course of valgancyclovir for prophylaxis for all CMV-mismatched or thymoglobulin-induced patients.

Clinical follow-up, diagnosis and treatment of rejection

After hospital discharge patients were seen in the outpatient transplant clinic twice a week for the first month, every week for the second month, monthly for a year and every 4-6 months thereafter. A complete blood count, basic metabolic panel, and whole blood levels of sirolimus and cyclosporine were monitored during each visit using high-performance liquid chromatography and a fluorescence polarization immunoassay, respectively.

When the serum creatinine had increased by more than 30% above the baseline, the patient underwent a percutaneous biopsy. All acute episodes were biopsy-confirmed and
graded for severity according to the Banff 1997 criteria with subsequent modifications. Mild bouts were treated with pulse steroid therapy alone; moderate or severe ones or those refractory to steroids were generally addressed with thymoglobulin (1.5 mg/kg for 10 to 14 days) or if it was not-tolerated, with ATGAM (Pharmacia, Kalamazoo, MI), or OKT3 (Orthoclone, Johnson and Johnson, Raritan, NJ). Antibody-mediated acute rejections were treated by a 14-day course of thymoglobulin together with at least two 5-day cycles of plasmapheresis accompanied by injections of anti-CD20 monoclonal antibody (Rituxan, Roche, Basle, SZ). The diagnoses of chronic rejection (CR)/chronic allograft nephropathy (CAN) were established by biopsies performed to establish a cause for renal dysfunction.

Statistical Analyses

The database included patient demographic features, post-operative clinical conditions, serial serum creatinine concentrations, acute and chronic rejection incidences, as well as graft and patient survivals. Median values and ranges or mean values and standard deviations of continuous variables were analyzed by independent sample Student’s-t tests. Percentage incidences of categorical features were subjected to Fisher exact chi-square tests. Distribution analyses were performed with the cross tab method with Fisher exact tests. Univariate analyses were followed by multivariate Cox regression techniques with forward and backward, stepwise elimination methods, including all variables that showed $P<0.20$ on univariate analysis. Kaplan-Meier time to event analyses were evaluated by log-rank statistics. Results were regarded as significant when $P<0.05$.

IV/3. Results

Demographic features

Table 9 summarizes the demographic features of both populations. The majority of recipients in both groups were males, and the major source of kidneys was deceased donors,
all of whom had experienced brain death. The significant demographic differences between repeat versus primary graft recipients included greater average PRA value (21.1 ± 27 versus 7.3 ± 17%; \( P = 0.001 \)), lower mean age (40.3 ± 1.3 versus 47.4 ± 13.3 years; respectively, \( P = 0.001 \)), greater proportion of <3 HLA mismatches (20.2 versus 10%, \( P = 0.042 \)) and more pre-emptive transplantations (17.3 versus 9%, \( P = 0.036 \)). The mean times on dialysis prior to primary or re-transplantation were about 3 years. There were no significant differences in donor/recipient gender or ethnicity or their mismatches; in number of combinations with an age disparity greater than 10 years; in mean or distribution of cold ischemia times, as well as among the incidences of diabetes mellitus, hypertension or anti-CMV antibody pre-transplantation. Repeat versus primary recipients had mean post-transplant follow-ups of 65.5 ± 29 versus 64.8 ± 31 months (\( P = 0.85 \)).

**Primary end-points**

Figure 13 reveals that the patient survival rates for primary versus re-transplant cases at 1 year (Fisher-exact test; 96 versus 94%; \( P = 0.49 \)) and at 5 years (Fisher-exact test; 88 versus 86%; \( P = 0.68 \)) were not significantly different (Log-Rank, \( P = 0.68 \), Panel A). The graft survival rates at 1 year (Fisher-exact test; 90 versus 90%; \( P = 0.96 \)) and 5 years (Fisher-exact test; 78 versus 77%; \( P = 0.92 \)) were also comparable (Kaplan-Meier analysis; Log-Rank, \( P = 0.98 \); Panel B).

Among the re-transplantations the causes of 9 graft losses in addition to the 14 deaths with a functioning graft were one case of primary non-function, and eight of CR/CAN. Among the primary transplantations, the etiologies in addition to 28 deaths were CR/CAN (n=10), primary nonfunction (n=3), surgical complications (n=2), and other medical problems (n=3).
<table>
<thead>
<tr>
<th>Feature</th>
<th>Re-transplant (n=98)</th>
<th>Primary Tx (n=200)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age, years, (mean±SD)</td>
<td>40.3±11.3</td>
<td>47.4±13.3</td>
<td>0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt;60, n (%)</td>
<td>6 (6.1)</td>
<td>42 (21.0)</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>40-60, n (%)</td>
<td>39 (38.8)</td>
<td>99 (49.5)</td>
<td>0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18-39, n (%)</td>
<td>53 (53.6)</td>
<td>59 (29.5)</td>
<td>0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Panel Reactive Antibody, %</td>
<td>21.1±27.1</td>
<td>7.3±17.5</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;10, n (%)</td>
<td>53 (54.1)</td>
<td>163 (81.5)</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10-20, n (%)</td>
<td>7 (7.1)</td>
<td>12 (6.0)</td>
<td>0.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>21-50, n (%)</td>
<td>20 (20.4)</td>
<td>15 (7.5)</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt;51, n (%)</td>
<td>17 (17.3)</td>
<td>10 (5.0)</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HLA mismatch, (mean±SD)</td>
<td>4.1±1.8</td>
<td>4.4±1.6</td>
<td>0.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;3, n (%)</td>
<td>18 (20.2)</td>
<td>20 (10.0)</td>
<td>0.042&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3, n (%)</td>
<td>18 (18.4)</td>
<td>32 (16)</td>
<td>0.177&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt;3, n (%)</td>
<td>70 (71.2)</td>
<td>148 (74)</td>
<td>0.64&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>No Pre-transplant Dialysis</td>
<td>17 (17.3)</td>
<td>18 (9)</td>
<td>0.036&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recipient gender, male, n (%)</td>
<td>54 (55)</td>
<td>123 (62)</td>
<td>0.29&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recipient ethnicity, n (%)</td>
<td></td>
<td></td>
<td>0.24&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caucasian</td>
<td>43 (44)</td>
<td>75 (38)</td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>18 (18)</td>
<td>57 (28)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>32 (33)</td>
<td>55 (28)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>5 (5)</td>
<td>13 (6)</td>
<td></td>
</tr>
<tr>
<td>Deceased donor source, n (%)</td>
<td>67 (68)</td>
<td>140 (70)</td>
<td>0.43&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Donor age, years (mean±SD)</td>
<td>33.7±15</td>
<td>35.6±15.7</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt;60, n</td>
<td>3</td>
<td>13</td>
<td>0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>40-60, n</td>
<td>31</td>
<td>69</td>
<td>0.62&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18-39, n</td>
<td>64</td>
<td>118</td>
<td>0.30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Donor gender, male, n (%)</td>
<td>58 (59.2)</td>
<td>106 (53)</td>
<td>0.28&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Donor ethnicity, n (%)</td>
<td></td>
<td></td>
<td>0.41&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caucasian</td>
<td>63</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>15</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>17</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Age mismatch &gt;10 years, n</td>
<td>57</td>
<td>127</td>
<td>0.38&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender mismatch, n</td>
<td>43</td>
<td>101</td>
<td>0.26&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethnicity mismatch, n</td>
<td>45</td>
<td>92</td>
<td>1.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cold ischemia time, min. (mean±SD)</td>
<td>729.4±609.1</td>
<td>787.2±613.0</td>
<td>0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;1440, n (%)</td>
<td>87 (88.2)</td>
<td>168 (84.0)</td>
<td></td>
</tr>
<tr>
<td>1441-2160, n (%)</td>
<td>9 (9.4)</td>
<td>30 (15.0)</td>
<td></td>
</tr>
<tr>
<td>&gt;2160, n (%)</td>
<td>2 (2.4)</td>
<td>2 (1.0)</td>
<td></td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>21 (21)</td>
<td>56 (28)</td>
<td>0.22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pre-Tx HTN, n (%)</td>
<td>95 (97)</td>
<td>186 (93)</td>
<td>0.16&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Pre-Tx CMV, n (%)</td>
<td>74 (76)</td>
<td>150 (75)</td>
<td>0.77&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Pre-transplant Dialysis Time (mo±SD)</td>
<td>36.0±39.8</td>
<td>31.0±42.2</td>
<td>0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a. Independent sample Student’s t-test  
b. Cross tab/Fisher exact test

Table 9. Demographic features of patients undergoing re-transplants versus primary grafts.
Figure 13. Patient and graft survivals among first (●) versus repeat (▲) renal transplants

(A) Percent patient survival (B) Percent graft survival.

Figure 14. Incidence of patients free of a biopsy-confirmed acute rejection episode comparing first (●) versus repeat (▲) renal transplants (A) Percent free from acute rejection (B) Percent free of chronic allograft nephropathy.
The causes of death among the re-grafted subjects were: malignancy (n=3), diabetic complications (n=2), sepsis (n=4), pneumonia (n=2), liver failure (n=1), and cardiac complications (n=2). The causes of death among primary recipients were cardiovascular (n=5), malignancy (n=1), pneumonia (n=2), ruptured aortic aneurysm (n=1), stroke (n=1), sepsis (n=4), adult respiratory distress syndrome (n=1), liver failure (n=1), acute intracerebral bleed (n=1), and unknown (n=7).

Figure 14 shows that the incidences of BPAR (Panel A) and CR/CAN (panel B) were similar between the cohorts (Log-Rank, $P=0.12$ and $P=0.99$, respectively). While the curves depicting CR/CAN overlapped, the freedom from acute rejection was slightly, albeit not significantly, greater for the group of primary grafts. Among the repeat transplants there were 5 (5%) humoral-vascular rejection episodes compared with 8 (4%) among the primary grafts. Four primary and 4 re-transplant patients underwent plasmapheresis—anti-CD20 monoclonal antibody treatment (data not shown).

Multivariate analysis for graft and patient survival of all transplant recipients included the significant variables in Table 9 as well as those with $P$ values $\leq 0.2$, namely, donor gender ($P=0.059$), age $>60$ years ($P=0.066$), and pre-transplant diabetes ($P=0.17$). A beneficial effect on patient survival was observed for age less than 60 years at 2 ($P=0.033; HR=0.18$) with decreased impact at 3 and 4 ($P=0.052; HR=0.22$ and $P=0.052; HR=0.25$, respectively); and no significance at 5 years. The 5 year graft survival was better among subjects without diabetes mellitus ($P=0.034; HR=0.307$); whereas, an HLA-mismatch $<3$ showed a trend toward a protective effect at 5 years ($P=0.076; HR=0.164$).

Factors predisposing to graft or patient loss as well as BPAR: re-transplants

Table 10 shows the impact of various clinical features on the outcome of re-transplantations. Upon univariate analysis the risk factors for graft loss included prior
transplant loss within 6 months ($P=0.0001$), older mean recipient age ($P=0.01$), occurrence of an BPAR ($P=0.049$) and donor ethnicity ($P=0.05$). Upon multivariate analysis, graft loss at 5 years was significantly increased among recipients who experienced BPAR ($P=0.034$, HR 2.42). Patient survival at 2, 3 and 4 years showed the benefit of recipient age <60 years ($P=0.033$, HR 0.185; $P=0.05$, HR 0.22; $P=0.05$, HR 0.22), and at 5 years, the absence of diabetes mellitus ($P=0.034$, HR 0.037). Freedom from an acute rejection episode at 5 years tended to be associated with an HLA mismatch <3 ($P=0.07$, HR 0.164). None of the other factors was significant.

**Factors predisposing to graft or patient loss as well as BPAR: first grafts**

Table 11 shows a univariate analysis of factors affecting the survivals of first renal grafts, revealing the beneficial effects of Caucasian ethnicity ($P=0.016$); shorter cold ischemia time ($P=0.03$); pre-emptive transplantation ($P=0.035$); younger mean recipient age ($P=0.05$); and freedom from BPAR ($P=0.02$). Multivariate analysis for graft loss showed hazardous effects of cold ischemia time >24 hours at 1, 2, 4 and 5 years ($P=0.034$, HR=3.14; $P=0.02$, HR=3.7; $P=0.042$, HR=2.08; $P=0.023$, HR=2.20). Only at 5 years was the occurrence of BPAR a significant risk factor for graft loss ($P=0.049$, HR=1.89). Among patient survival data for primary transplantations, age >60 years was a risk factor at 1, 4 and 5 years ($P=0.02$, HR 6.08; $P=0.002$, HR 3.46; $P=0.003$, HR=3.42, respectively) and occurrence of an acute rejection episode at 1 and 3 years ($P=0.023$, HR 5.6; $P=0.046$, HR 2.63, respectively). None of the identified factors were significantly associated with the occurrence of an acute rejection episode.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Graft Outcome</th>
<th></th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loss (n=25)</td>
<td>Survival (n=73)</td>
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</tr>
<tr>
<td>Recipient age</td>
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<tr>
<td>18-40, n</td>
<td>11</td>
<td>42</td>
<td>0.68a</td>
</tr>
<tr>
<td>41-60, n</td>
<td>12</td>
<td>28</td>
<td>0.42a</td>
</tr>
<tr>
<td>&gt;60, n</td>
<td>2</td>
<td>3</td>
<td>0.066a</td>
</tr>
<tr>
<td></td>
<td>Mean±SD (years)</td>
<td>48.3±13.1</td>
<td>43.9±12.9</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Primary graft loss within 6 months, n</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Occurrence of BPAR, n</td>
<td>11</td>
<td>17</td>
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<td>Donor ethnicity, n</td>
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<td>Recipient Gender, n</td>
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<td>Cold ischemia time(min)</td>
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<td>4.2±1.7</td>
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<td>PRA (%), n</td>
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<td>14</td>
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<td>Blood transfusion, n</td>
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<td>66</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Pre transplant dialysis, n</td>
<td>20</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Pre transplant CMV (+), n</td>
<td>17</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

a. Cross tab the chi<sup>2</sup> exact Fisher for difference in distribution
b. Independent sample Student’s t-test

**Table 10.** Factors impacting graft loss among second transplantations (N=98).
<table>
<thead>
<tr>
<th>Factor</th>
<th>Graft Outcome</th>
<th></th>
<th></th>
<th></th>
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</thead>
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<td></td>
<td>Loss (n=53)</td>
<td>Survival (N=147)</td>
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<td></td>
</tr>
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<tr>
<td>Recipient age</td>
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<td></td>
<td></td>
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<tr>
<td>18-40, n</td>
<td>16</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-60, n</td>
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<td>78</td>
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<td></td>
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<tr>
<td>&gt;60, n</td>
<td>15</td>
<td>25</td>
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<td></td>
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<tr>
<td>Mean (±SD)</td>
<td>50.44±13.5</td>
<td>46.3±13.13</td>
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<td></td>
<td>.016$^a$</td>
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<td>Hispanic</td>
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<td></td>
<td></td>
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<tr>
<td>Other</td>
<td>3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cold ischemia time, min</td>
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<td></td>
<td></td>
<td>.04$^a$</td>
</tr>
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<td>&lt;1440</td>
<td>41</td>
<td>131</td>
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<td></td>
</tr>
<tr>
<td>&gt;1440</td>
<td>12</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>965.54±677.85</td>
<td>730.44±582.86</td>
<td></td>
<td>.03$^b$</td>
</tr>
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<td>Occurrence of ARE, n</td>
<td>17</td>
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<td></td>
<td>.02$^a$</td>
</tr>
<tr>
<td>Pre-emptive transplant, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>1</td>
<td>17</td>
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<td>.035$^a$</td>
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<tr>
<td>No</td>
<td>52</td>
<td>130</td>
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<td>Recipient Gender, n</td>
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<td>.43$^a$</td>
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<td>Female</td>
<td>18</td>
<td>59</td>
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<td>Donor Source, n</td>
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<td></td>
<td>.75$^a$</td>
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<tr>
<td>Deceased</td>
<td>38</td>
<td>102</td>
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<td>Living</td>
<td>15</td>
<td>45</td>
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<td></td>
<td></td>
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<td>58</td>
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<td>Hispanic</td>
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<tr>
<td>Other</td>
<td>3</td>
<td>10</td>
<td></td>
<td></td>
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<td>18</td>
<td>38</td>
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<td>.26$^a$</td>
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<td>136</td>
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<td>.65$^a$</td>
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<td>Pre Transplant CMV+, n</td>
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<td>112</td>
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<td>.5$^a$</td>
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<td>.48$^a$</td>
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<td>23</td>
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<td>.82$^a$</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>23</td>
<td></td>
<td>.82$^a$</td>
</tr>
<tr>
<td>&gt;3</td>
<td>40</td>
<td>108</td>
<td></td>
<td>.77$^a$</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>4.5±1.44</td>
<td>4.32±1.56</td>
<td></td>
<td>.50$^b$</td>
</tr>
<tr>
<td>PRA (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10, n</td>
<td>41</td>
<td>122</td>
<td></td>
<td>.37$^a$</td>
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<tr>
<td>11-20, n</td>
<td>3</td>
<td>9</td>
<td></td>
<td>.90$^a$</td>
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<tr>
<td>21-50, n</td>
<td>5</td>
<td>10</td>
<td></td>
<td>.53$^a$</td>
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<td>&gt;50, n</td>
<td>4</td>
<td>6</td>
<td></td>
<td>.32$^a$</td>
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<tr>
<td>Mean (±SD)</td>
<td>8.9±18.45</td>
<td>6.75±17.17</td>
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<td>.45$^a$</td>
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<td>Blood transfusions, n</td>
<td>24</td>
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<td>.89$^a$</td>
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<td>Donor gender, n</td>
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<td></td>
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<td>.07$^a$</td>
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</table>
Table 11. Factors impacting graft loss among primary transplantations (N=200).

Renal function

The post-transplant serum creatinine levels at 1, 3, 12, 24, 48 and 60 months among repeat versus primary recipients were not significantly different (Table 12A). Kidney function at these times were also similar for both populations, as computed by the abbreviated MDRD formula, considering lost grafts as GFR=0 ml/min (Table 12B).

A. Serial serum creatinine values

<table>
<thead>
<tr>
<th>Time Post-TX</th>
<th>Re-transplant (n=98) (mg/dl) µmol/l</th>
<th>Primary (n=200) (mg/dl) µmol/l</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>(2.5 ± 1.8) 221±159</td>
<td>(2.6 ± 2.1) 229±185</td>
<td>0.95</td>
</tr>
<tr>
<td>3 months</td>
<td>(1.9 ± 1.1) 168±97</td>
<td>(2.1 ± 1.3) 185±114</td>
<td>0.13</td>
</tr>
<tr>
<td>6 months</td>
<td>(2.0 ± 1.0) 177±88</td>
<td>(2.1 ± 1.3) 185±114</td>
<td>0.45</td>
</tr>
<tr>
<td>1 year</td>
<td>(1.8 ± 0.8) 159±70</td>
<td>(1.7 ± 0.9) 150±79</td>
<td>0.40</td>
</tr>
<tr>
<td>2 years</td>
<td>(2.1 ± 0.9) 185±79</td>
<td>(1.7 ± 1.2) 150±106</td>
<td>0.52</td>
</tr>
<tr>
<td>4 years</td>
<td>(1.9 ± 0.7) 168±63</td>
<td>(1.7 ± 1.0) 150±88</td>
<td>0.68</td>
</tr>
<tr>
<td>5 years</td>
<td>(1.9 ± 0.7) 168±62</td>
<td>(2.0 ± 1.3) 176±114</td>
<td>0.90</td>
</tr>
</tbody>
</table>

B. Creatinine clearance calculated by abbreviated MDRD

<table>
<thead>
<tr>
<th>Time Post-Tx</th>
<th>Re-transplants (n=98) ml/min/1.73m²</th>
<th>Primary Tx (n=200) ml/min/1.73m²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>44.7 ± 28.4</td>
<td>43.8 ± 26.4</td>
<td>0.65</td>
</tr>
<tr>
<td>3 months</td>
<td>52.0 ± 24.2</td>
<td>49.9 ± 26.6</td>
<td>0.37</td>
</tr>
<tr>
<td>1 year</td>
<td>52.0 ± 24.3</td>
<td>52.0 ± 22.1</td>
<td>0.99</td>
</tr>
<tr>
<td>5 years</td>
<td>42.6 ± 16.7</td>
<td>47.0 ± 22.6</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 12. Post-operative renal function among re-transplant versus primary graft recipients.
IV/4. Discussion

Allograft survival rates for renal re-transplantations have historically been lower than those of primary allograft recipients, particularly beyond 2 years [264, 266, 269, 279-284]. The 77% survival rate for re-transplantations at 5 years described herein was similar to the OPTN report of 76%. These results demonstrated the efficacy of sirolimus in a second high-risk population in addition to our prior report among Black recipients [277]. The observation that a matched cohort of primary graft recipients showed a 78% graft survival at 5 years reported herein, which was lower than the 81% in the recent UNOS report [1], was possibly due to the present cohort including our initial experience with sirolimus dating to 1994, when we prescribed exposures greater to calcineurin antagonist resulting in potentiation of nephrotoxicity. Due to improved understanding of the pharmacokinetic and pharmacodynamic interactions between cyclosporine and sirolimus, it now appears that greater than 80% reduction in calcineurin inhibitor concentrations de novo yields superior results over full [273] or even halved exposure [285]. These effects may have attenuated graft survival at five years.

The present data revealed a low incidence of chronic nephropathy as shown by biopsies performed for increases in serum creatinine. The overall level of renal function over 5 years, albeit stable, was impaired in both groups, namely calculated glomerular filtration rates of 40 to 50 ml/min. We attributed this observation to excessive cyclosporine exposure. In an ongoing study of 636 patients employing minimization of CsA dose by 83%, the mean 4-year GFR calculated by the MDRD formula was $65 \pm 24$ ml/min/1.78m$^2$ among patients treated de novo with <2.5 mg/kg CsA. (Kahan et al, unpublished results), demonstrating that optimization of exposures to cyclosporine and sirolimus can result in excellent longer term graft function.
Improved outcomes of re-transplantation [268] over the past decade may be due to several factors. The benefit of uniform administration of thymoglobulin has been shown by Ott, et al [286]. However, their acute rejection rate of 44.4% among re-transplant patients treated with thymoglobulin induction, tacrolimus, mycophenolate mofetil and corticosteroids, was significantly greater than the 28% in the present study. Unfortunately the UNOS database does not include the incidence, type or treatment of acute rejection episodes, so that it is impossible to judge their occurrence in the general experience versus the 28% among repeat versus 22% BPAR among primary recipients in the present study. Another significant factor promoting the success of re-transplantations is the improved methods of cross-matching. Additional considerations are the more precise histologic criteria for and tailored therapy of BPAR.

The risk factors detected upon univariate analysis included several that have been previously noted to be significant among re-transplant cases: greater degrees of HLA mismatch, graft loss within the first 6 months [266], freedom from an acute rejection episode, pre-emptive re-transplantation and black donor ethnicity [286, 287]. In contrast, lack of sensitization as evidenced by a PRA <10% at the time of re-transplantation was a protective factor. Younger recipient age and absence of diabetes mellitus were significant prognostic features for patient survival. However, the use of living donors did not appear to yield better results [288].

Upon multivariate analysis the risk factors for an adverse outcome among re-transplantation patients were BPAR, age greater than 60 years, diabetes mellitus and HLA mismatches \( \geq 3 \).

In our experience there was no greater incidence of surgical complications among second procedures (data not shown), although this had been previously reported [270]. Furthermore, we neither confirmed attenuated survivals of repeat kidney grafts among women.
undergoing second versus first transplantations [279], nor of re-exposure to foreign HLA antigens present on the prior graft [284, 289]. Expansion of our experience over the coming decade is likely to reveal which of the factors identified on univariate analysis, but not confirmed by multivariate techniques, are robust. Furthermore, precisely tailored exposures to immunosuppressive drugs with calcineurin inhibitor withdrawal may yield superior long term outcomes.
V. CONCLUSIONS AND NOVELTIES

The number of patients awaiting transplantation has been persistently increasing. We have sought to improve the outcomes of different aspects of transplantation.

Substantial proportion of acute and chronic renal allograft rejection processes is caused by antibodies reactive to donor antigens. Antibody mediated acute rejection, a newly described entity, arises despite ongoing therapy with potent anti-T cell pharmacological agents. The detection and treatment of allograft rejection has historically focused upon T-cell mediated process and partly because of this humoral mechanisms are less clear. Our goal was to examine the influencing factors of AMR and to compare the impact of different treatment modalities on the outcomes.

New findings:

1. This retrospective study showed a 2-year graft survival of 92% for patients treated with rituximab plus plasmapheresis, which was significantly greater (Log-Rank test; \( p=0.025 \)) than that observed among the group who did not receive anti-CD20 therapy.

2. The 100% patient survival at 2 years as well as the absence of a greater incidence of major complications among individuals treated with rituximab supports the effective, safe use of this monoclonal antibody for AMR.

3. There was no significant difference regarding the rates/types of infectious complications between the two groups within 3 or 6 months after completion of treatment (\( P=0.24 \) and \( P=0.78 \), respectively). Administration of IVIg showed a trend toward decreasing the appearance of infectious complications in Group B (\( P=0.058 \)) suggesting the beneficial effect of it.
4. C4d staining and DSA detection and monitoring seem to be very helpful markers for the diagnosis of acute antibody mediated rejection.

In conclusion, our work at The University of Texas, Division of Immunology and Organ Transplantation has revealed the primacy of *rituximab* in the treatment of AMR besides the previously established therapeutic effect of plasmapheresis and IVIg.

Post-transplant malignancy, particularly *post-transplant lymphoproliferative disease*, has become an important cause of mortality since newer, more potent immunosuppressive regimens have steadily reduced the incidence of acute rejections and have extended the life expectancy of allograft recipients. In this thesis I have reviewed the literature for pathogenesis, diagnosis and possible treatment modalities of PTLD. I also have elucidated utilization and the efficacy of *rituximab* in the treatment of PTLD. Majority of EBV-associated, CD20-positive PTLD patients benefit from rituximab as the second line of treatment right after or besides the reduction of immunosuppression. Our successfully treated patient also provided evidence for the favorable effect of this treatment modality. Given the significant toxicity, chemotherapy is best reserved for use in patients who are ineligible or fail rituximab.

*Re-transplantation* offers hope for transplant recipients who have had a graft fail. Unfortunately such failures, in addition to the suffering they place on the recipient, contribute to the overall demand for organs. Given the shortage of donor organs, re-transplantation can create tension, especially when outcomes following re-transplantation are below those observed for primary recipients.

New findings:

1. Upon univariate analysis the risk factors for graft loss included prior transplant loss within 6 months (*P*=0.0001), older mean recipient age (*P*=0.01), occurrence of an BPAR (*P*=0.049) and donor ethnicity (*P*=0.05). The use of living donors did not appear to yield better results.
Upon multivariate analysis, graft loss at 5 years was significantly increased among recipients who experienced BPAR ($P=0.034$, HR 2.42). Patient survival at 2, 3 and 4 years showed the benefit of recipient age <60 years ($P=0.033$, HR 0.185; $P=0.05$, HR 0.22; $P=0.05$, HR 0.22), and at 5 years, the absence of diabetes mellitus ($P=0.034$, HR 0.037). Freedom from an acute rejection episode at 5 years tended to be associated with an HLA mismatch <3 ($P=0.07$, HR 0.164).

2. In our experience there was no greater incidence of surgical complications among second procedures. Furthermore, we neither confirmed attenuated survivals of repeat kidney grafts among women undergoing second versus first transplantations, nor of re-exposure to foreign HLA antigens present on the prior graft.

3. Patient survival rates for primary versus re-transplant cases at 1 year (96 versus 94%; $P=0.49$) and at 5 years (88 versus 86%; $P=0.68$) were not significantly different (Log-Rank, $P=0.68$). The graft survival rates at 1 year (90 versus 90%; $P=0.96$) and 5 years (78 versus 77%; $P=0.92$) were also comparable (Log-Rank, $P=0.98$).

4. Incidences of biopsy proven acute rejection and chronic rejection/chronic allograft nephropathy were similar between the cohorts (Log-Rank, $P=0.12$ and $P=0.99$, respectively). Among the repeat transplants there were 5 (5%) humoral-vascular rejection episodes compared with 8 (4%) among the primary grafts.

5. The post-transplant serum creatinine levels at 1, 3, 12, 24, 48 and 60 months among repeat versus primary recipients were not significantly different. Kidney function at these times was also similar for both populations, as computed by the abbreviated MDRD formula.

In conclusion, the novelty of our findings was that a sirolimus-based regimen yielded similar efficacy and outcomes among re-transplanted patients compared with first renal transplantations with a mean 5-year follow-up.
VI. ABBREVIATIONS

Abs - antibodies
ACR - acute cellular rejection
ADCC - antibody-dependent cell-mediated cytotoxicity
ALG - ant-lymphocyte globulin
AMR - antibody mediated rejection
AT1R - Angiotensin II type 1 receptor
ATG - anti-thymocyte globulin
BPAR - biopsy proven acute rejection
CDCC - complement-dependent cell-mediated cytotoxicity
CMV - Cytomegalovirus
CNS - central nervous system
CR - complete response
CTL - cytotoxic T-lymphocytes
CVF - cobra venom factor
DAF - decay accelerating factor
DSA - donor specific antibody
EBV - Epstein-Barr virus
FACS - Fluorescence-activated cell sorting
FDA - Food and Drug Administration (USA)
FDG-PET - Fluorodeoxyglucose - positron emission tomography
FK506 - Prograf, tacrolimus
GC - germinal center
G-CSF - granulocyte colony-stimulating factor
HHV-8 – Human Herpesvirus-8
HLA - human leukocyte antigen
LDH - lactate dehydrogenase
MAC - membrane attack complex
M-CSF – macrophage colony-stimulating factor
MHC - major histocompatibility complex
MIC A and B - Major histocompatibility complex class I chain-related gene A and B
MMF - mycophenolate mofetil
MZ - marginal zone
OS - overall survival
PAS - Periodic acid-Schiff stain
PCR - polymerase chain reaction
PR - partial response
PRA - panel reactive antibody
PTC - peritubular capillaries
PTLD - post-transplant lymphoproliferative disorder
RA - rheumatoid arthritis
RI - reduction of immunosuppression
RR - response rate
SCr - serum creatinine
SRL - sirolimus, rapamycin, Rapamune
VII. REFERENCES


leukocyte antigen staining and microsatellite analysis. Transplantation, 2005. 79: p. 79.


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VIII. PUBLICATIONS AND PRESENTATIONS

Publications

1. Zs. Káposztás, L. Cseke, K.Kalmár, P. Ö. Horváth
   Neoadjuvant chemotherapy for gastric cancer – a case of complete response

2. Zs. Káposztás, Ö.P. Horváth
   An unusual case of oesophageal metastasis from gastric adenocarcinoma

3. Zs. Káposztás, L. Cseke, Ö. P. Horváth
   Oesophageal metastasis from gastric adenocarcinoma

   Neoadjuvant Chemotherapy for locally advanced gastric cancer
   Magyar Sebészet 56, 177-184; 2003.

5. Zs. Káposztás, T. Tornóczky, Horváth Örs Péter
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8. D. Kelemen, Z. Kaposztas, O.P. Horvath

A modified technique of pancreatojejunostomy

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Comparing aboral versus oral pouch with preserved duodenal passage after total gastrectomy: does the position of the gastric substitute reservoir count?


**Publications in relation to thesis**

10. Z. Kaposztas, W. B. Etheridge, B. D. Kahan

Case report: successful treatment of post-transplant lymphoproliferative disorder and quiescence of dermatomyositis with Rituximab and sirolimus


Impact of Rituximab Therapy for Treatment of Acute Humoral Rejection

Clinical Transplantation, 2009 Jan;23(1):63-73. **IF:1.923**

12. Sirolimus Provides Similar Immunosuppressive Efficacy for Repeat Versus Primary Renal Allograft Recipients

V. Petero, Z. Kaposztas, B. Kahan

Clinical Transplantation, The paper just has been accepted for publication. **IF: 1.923**
13. Primary Synovial Sarcoma of the Native Kidney with Renal Vein and Inferior Vena Caval Extension in a Renal Transplant Recipient


14. Sirolimus therapy predisposes to new onset diabetes mellitus after renal transplantation: A ten year analysis

E. Gyurus, Z. Kaposztás, B.D. Kahan


Citable abstracts:

1. Z. Káposztás, K. Kalmár, L. Cseke, D. Kelemen, Ö.P. Horváth

Randomised study to compare aboral and oral pouch construction after total gastrectomy (abstract)


2. Z. Káposztás, K. Kalmár, L. Cseke, D. Kelemen, Ö. P. Horváth

Aboral versus oral pouch construction with duodenal passage preservation after total gastrectomy: randomised clinical trial, preliminary results (abstract No.:491)

Disease of the Esophagus 2004, Jun Vol. 17, Supplement 1 \textbf{IF:0.797}


Effect of aboral pouch construction with or without duodenal passage preservation after total gastrectomy: prospective, randomised trial (abstract No.: 224)

Disease of the Esophagus 2004, Jun Vol. 17, Supplement 1 \textbf{IF:0.797}
Citable abstracts in relation to thesis:

M. Reyes, O. Illoh, Z. Kaposztas, B. Kahan

Impact of plasma exchange and rituximab therapy for treatment of acute humoral rejection (S136-040E)

Transfusion 2008, Sept., Vol 48, Special Issue, p: 51 IF:3.374

Oral Presentations

1999, February, University of Medicine, Pécs, Hungary

Scientific Students’ Association Conference

The characterisation of low molecular weight (10-50kDa) human serum proteins in kidney transplanted patients

Z. Káposztás

1999, April, Budapest, Hungary:

Frigyes Korányi Scientific Conference

The characterisation of low molecular weight (10-50kDa) human serum proteins in kidney transplanted patients

Z. Káposztás

1999, July, Zadar, Croatia:

Summer University On Enzymes

The characterisation of low molecular weight (10-50kDa) human serum proteins in kidney transplanted patients

Zs. Káposztás, A. Ludány, T. Kőszegi

1999, September, Szeged, Hungary

Hungarian Surgical Research Congress

The effect of early enteral alimentation on early postoperative complications
following esophageal resection
A. Tavakoli, L. Cseke. Zs. Káposztás, Ö. P. Horváth

2000, March, University of Medicine, Pécs, Hungary
Scientific Students’ Association Conference
The effect of early enteral alimentation on early postoperative complications following esophageal resection
Zs. Káposztás

2000, June, Győr, Hungary
55th Annual Congress of the Hungarian Surgeons Society
The effect of early enteral alimentation on early postoperative complications following esophageal resection
Zs. Káposztás, L. Cseke, Ö. P. Horváth

2001, April, Debrecen, Hungary
Casuistical Meeting of Young Surgeons in English
Neoadjuvant chemotherapy for gastric cancer – a case of complete response
Zs. Káposztás, K. Kalmár

2001, November, Szeged, Hungary
Scientific Meeting of Young Surgeons
The use of peritontectomy and intraperitoneal chemotherapy in carcinosis peritonei
Zs. Káposztás

2001, December, Pécs, Hungary
Regional Meeting of Young Surgeons
The use of peritoneectomy and intraperitoneal chemotherapy in carcinosis peritonei
Zs. Káposztás

2002, June, Budapest, Hungary
56th Annual Congress of the Hungarian Surgeons Society

Aboral pouch construction with duodenal passage preservation – searching for the optimal type of reconstruction after total gastrectomy

L. Cseke, K. Kalmár, Z. Káposztás, Ö. P. Horváth

2004, March, Budapest, Hungary

12th Congress of the European Society of Surgical Oncology

“Effect of pouch construction and preservation of the duodenal passage on the nutritional and motility parameters and quality of life after total gastrectomy”

K. Kalmár, Zs. Káposztás, L. Cseke, J. Németh, Ö.P. Horváth

2004, May, Madrid, Spain

IX World Congress of the ISDE

Aboral versus oral pouch construction with duodenal passage preservation after total gastrectomy: randomised clinical trial, preliminary results

Zs. Káposztás, K. Kalmár, L. Cseke, D. Kelemen, Ö. P. Horváth

2004, May, Madrid, Spain

IX World Congress of the ISDE

Effect of aboral pouch construction with or without duodenal passage preservation after total gastrectomy: prospective, randomised trial

K. Kalmár, Zs. Káposztás, L. Cseke, J. Németh, Ö. P. Horváth

2004, June, Pécs, Hungary

57th Annual Congress of the Hungarian Surgeons Society


2004, June, Balatonaliga, Hungary

46th Annual Meeting of the Hungarian Gastroenterologic Society

Aboral versus oral pouch construction with duodenal passage preservation after total gastrectomy: randomised clinical trial, preliminary results
Zs. Káposztás, K. Kalmár, L. Cseke, D. Kelemen, Ö. P. Horváth

2004, June, Balatonaliga, Hungary

46th Annual Meeting of the Hungarian Gastroenterologic Society

Neoadjuvant chemotherapy in locally advanced gastric cancer to make it resecable
K. Kalmár, L. Cseke, A Papp, G Varga, Zs Káposztás, G Horváth, Ö.P. Horváth

2005, April, Pécs, Hungary

II nd International Congress of the Society of the Hungarian Molecular and Predictive Epidemiology

Epidemiology of gastric cancer

2006, September, Budapest, Hungary

58th Annual Congress of the Hungarian Surgeons Society

Prognostic factors in the treatment of gastric cancer – 13 years experience
Zs. Kaposztas, K. Kalmar, L. cseke, L. illenyi, D. Kelemen, O.P. Horvath

2006, September, Budapest, Hungary

58th Annual Congress of the Hungarian Surgeons Society

The effect of laparoscopy on the operability of carcinoma of the gallbladder
K. Kalmar Nagy, A. Papp, Zs. Kaposztas, P. Heigl, M. Imre, O.P. Horvath

Oral presentations in relation to thesis

2007, April 24-27, Cambridge, MA, USA

Genzyme Annual Fellows Conference 2008, Advances in Organ Transplantation

Sirolimus Decreases Serum Prostate-Specific Antigen (PSA) Level Post-Transplant

2008, October 4-7, Montreal, Canada

American Association of Blood Bank (AABB) – Annual Meeting

Impact of plasma exchange and rituximab therapy for treatment of acute humoral rejection. O. Illoh, M. Reyes, Z. Kaposztas, B.D. Kahan
Poster presentations

1998, March, University of Pécs, Hungary

Scientific Students’ Association Conference

Electrophoretic characterisation of the Low Molecular Weight (10-40 kDa) human serum proteins

Zs. Káposztás

1998, September, Kecskemét, Hungary

48th Annual Meeting of the Hungarian Association of Laboratory Diagnostic

Electrophoretic characterisation of the Low Molecular Weight (10-40 kDa) human serum proteins

A. Ludány, T. Kőszegi, Zs. Káposztás, M. Kellermayer

1998, September, Karlovy Vary, Czech Republic

5th International Congress of Clinical Chemistry and Laboratory Medicine

ALPS-ADRIA ’98

Electrophoretic characterisation of the Low Molecular Weight (10-40 kDa) human serum proteins

A. Ludány, T. Kőszegi, Zs. Káposztás, M. Kellermayer

2004, April, Budapest, Hungary

12th Congress of the European Society of Surgical Oncology

Randomised study to compare aboral and oral pouch construction after total gastrectomy

Zs. Kaposztas, K. Kalmar, L. Cseke, D. Kelemen, Ö.P. Horváth

2006, September, Budapest, Hungary

58th Annual Congress of the Hungarian Surgeons Society

Modification of pancreatico-jejunostomy
Poster presentations in relation to thesis

2008, August, Sydney, Australia

XXII International Congress of The Transplantation Society

Accepted poster

Sirolimus Decreases Serum Prostate-Specific Antigen (PSA) Level Post-Transplant


2009, May, Boston, USA

American Transplant Congress

Accepted poster

Adverse impact of sirolimus on development of new onset diabetes after transplantation

Z. Kaposztas, E. Gyurus, B.D. Kahan
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