# Donor specific HLA antibodies and renal transplant outcomes

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Thesis submitted for MD

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**2012**

**ABSTRACT**

Despite the use of potent modern immunosuppressive agents chronic rejection remains the leading cause of allograft failure after renal transplant. Historically T cells were considered to be the main pivot of rejection mechanisms; however, over the past decade the key role of B cells in allograft rejection has been much more fully described. Antibody mediated rejection [AMR] was first classified as a distinct entity in the Banff histological classification of allograft pathology as late as 2001. This was a consequence of the demonstration of the complement split product C4d in renal allografts giving indirect evidence of humoral injury, along with the development of newer assays to detect circulating HLA donor specific antibodies [DSAs]; both of which were subsequently shown to be associated with reduced allograft survival. The development of solid phase technologies, in particular single antigen beads has introduced a readily available assay which can detect HLA DSA with high sensitivity and specificity. The benefit of routine application of such assays in renal transplantation is not known and is the principal question of this thesis; that is to establish if the detection of HLA DSAs will help in predicting rejection and allograft loss.

Patients transplanted at Imperial College Renal and Transplant Centre were recruited into the studies. The underlying hypothesis is that donor specific antibodies identified by the more recently developed techniques are associated with inferior allograft survival, addressed in several related studies as follows:

1. Is C4d staining with no morphological features of rejection associated with risk of subsequent AMR and allograft loss?
2. Are preformed DSA detected by single antigen beads in the setting of a negative crossmatch associated with inferior allograft survival?
3. What is the relevance of de novo DSAs after renal transplantation?
4. What is the clinical significance of DQ DSA and should consideration be given to DQ mismatching in deceased organ allocation?
5. Do outcomes in patients with ACR with a humoral component differ when compared to those with pure ACR when treated in a uniform manner?

A summary of the results is as follows:

1. C4d in the absence of histological features is not associated with AMR. However, patients with acute tubular injury who have DSA are at risk of subsequent AMR.

2. Patients with preformed DSA are at significant risk of AMR and allograft loss and as such antibodies detected by single antigen beads alone pre-transplant should be considered a relative contraindication to transplantation.

3. Patients who develop de novo DSA are at risk of AMR, transplant glomerulopathy [TG] and allograft failure. Routine testing for the detection of DSA may allow the potential to augment immunosuppression in order to improve outcomes.

4. DQ DSA are the most common DSA detected after transplant and are associated with inferior allograft outcomes. Patients mismatched at both DR and DQ alleles are at risk of developing DQ DSA. Algorithms incorporating the level of DQ mismatch might reduce DQ DSA formation and therefore improve long term allograft survival.

5. Patients with ACR with a humoral element are at risk of subsequent AMR, TG and graft failure. Such patients might benefit from more aggressive therapies than those targeted at ACR alone.

**ACKNOWLEDGEMENTS**

I would like to thank all those who helped made the work in thesis possible. A special thank you to the following:

Paul Brookes and the staff of the Histocompatibility and Immunogenetics laboratory at Imperial College NHS Trust, who process the sera of the transplant recipients and have made this work possible.

Candice Roufosse and Terry Cook who report all the histology from the transplant biopsies.

David Taube for his guidance and input into the work carried out.

Janet Lee for help with the understanding and development of the affinity work.

I would finally want to thank my supervisors Tom Cairns and Charles Pusey.

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**ABBREVIATIONS**

**ABOi** ABO incompatible

**ACR** Acute cellular rejection

**AMR** Antibody mediated rejection

**ATN** Acute tubular necrosis

**CI** Class I HLA

**CII** Class II HLA

**CDC** Complement dependent cytotoxicity

**DSA** Donor specific antibodies

**FCXM** Flow cytometry crossmatch

**FK** Tacrolimus

**H&I** Histocompatibility and immunogenetics

**HLA** Human leukocyte antigen

**HLAi** HLA incompatible

**HLA MM** HLA mismatch

**Iv Ig** intravenous immunoglobulin

**MFI** Mean fluorescence index

**MICA** MHC class I related antigen A

**MHC** Major histocompatibility complex

**PCR** Polymerase chain reaction

**PTC** Peritubular capillaries

**SAg** Single antigen beads

**TCMR** T cell mediated rejection

**TG** Transplant glomerulopathy

**List of first author publications and oral presentations resulting from work in this thesis**

**Publications**

De novo DQ donor specific antibodies are associated with a significant risk of antibody mediated rejection and transplant glomerulopathy.
**Willicombe M**, Brookes P, Santos-Nunez E, Galliford J, Mclean A, Cook HT, Cairns T, Roufosse C and Taube D. Transplantation. 2012 Jul; 94: 172-177.

Antibody-mediated rejection after alemtuzumab induction: incidence, risk factors, and predictors of poor outcome.
**Willicombe M**, Roufosse C, Brookes P, Galliford JW, McLean AG, Dorling A, Warrens AN, Cook TH, Cairns TD, Taube D. Transplantation. 2011 Jul 27;92(2):176-82.

Outcome of patients with preformed donor-specific antibodies following alemtuzumab induction and tacrolimus monotherapy.
**Willicombe M**, Brookes P, Santos-Nunez E, Galliford J, Ballow A, Mclean A, Roufosse C, Cook HT, Dorling A, Warrens AN, Cairns T, Taube D. Am J Transplant. 2011 Mar;11(3):470-7.

**National and International Oral Presentations**

*Renal Association 2012, Gateshead:*
Surveillance biopsies and DSA screening predict rejection in patients receiving a minimalist immunosuppressive regimen.

*American Transplant Congress 2012, Boston:*
Surveillance biopsies and DSA screening predict rejection in patients receiving a minimalist immunosuppressive regimen.

*Royal Society of Medicine: Antibody mediated rejection symposium June 2011:*
Incidence, treatment and outcomes of acute antibody mediated rejection. London, June 2011

*American Transplant Congress 2011, Philadelphia:*

1. Association between transplant renal artery stenosis and donor specific antibodies.
2. De novo HLA-DQ donor specific antibodies are associated with a higher risk of rejection and transplant glomerulopathy.

*British Transplant Society 2011, Bournemouth:*

1. Detection of donor specific antibodies predicts antibody mediated rejection and transplant glomerulopathy.
2. De novo HLA-DQ donor specific antibodies are associated with a higher risk of rejection and transplant glomerulopathy.

*American Society of Nephrology 2010, Denver:*

De novo donor specific antibodies are associated with antibody mediated rejection.

*British Society of Histocompatibility and Immunogenetics' antibody symposium:*

Relevance id de novo DSAb post transplantation. Manchester, October 2010.

*Renal Association 2010, Liverpool:*

De novo donor specific antibodies are associated with antibody mediated rejection in patients receiving Campath induction.

*American Transplant Congress 2010, Boston:*

Preformed donor specific antibodies are associated with a high incidence of antibody mediated rejection in patients receiving Alemtuzumab induction.

*British Transplant Society 2010, London:*

C4d positive ATN does not predict acute rejection in patients with anti-HLA donor specific antibodies.

*American Society of Nephrology 2009, San Diego:*

Preformed donor specific antibodies are associated with a high incidence of antibody mediated rejection in patients receiving Alemtuzumab induction and tacrolimus monotherapy

## Introduction

Renal transplantation is the treatment of choice for patients with end stage renal failure with regards to both patient survival and quality of life [Thiruchelvam et al, 2011]. Unfortunately there remains a shortage of organs to meet demand; there are currently 6475 patients on the waiting list for a kidney transplant in the UK and they can expect to wait on average 1110 days [NHSBT, 2012] before receiving a transplant.

The use of current induction therapies and potent immunosuppression regimens, along with careful immunological selection of transplant recipients and successful treatment of acute rejection has meant improvement in short term allograft survival over the past decades. However, long term allograft survival has not improved [Meier-Kriescheet al, 2004; El-Zoghby et al, 2009], with the average lifespan of a kidney transplant being 8-15 years depending on the type of donor [NHSBT, 2012]. Chronic antibody mediated rejection remains the leading cause of allograft failure [Sellares et al, 2012; El-Zoghby et al, 2009].

Understanding the pathogenesis of antibody mediated rejection is important in order to identify the risk factors for its development. Identification of patients at risk can potentially offer an opportunity for prevention and intervention in order to reduce its occurrence.

In this thesis the role of donor specific antibodies in the development of antibody mediated rejection and allograft failure is examined. Risk factors associated with their development and associated risk of inferior allograft outcomes are considered, in the hope that this will allow the opportunity to try and prevent/minimise the risk of the development of donor specific antibodies in the future.

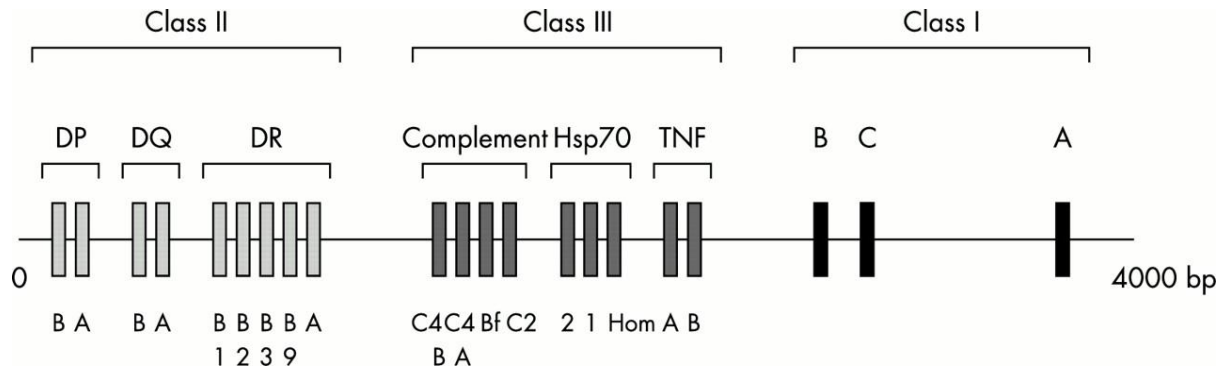
**CHAPTER 1 Transplant Immunology**

## 1.1.1 The major histocompatibility complex and its gene products

The discovery of a genetic impediment to organ transplantation developed from work carried out on tumour immunobiology in mice. When tumours were transplanted from one mouse to another in order to enable longer study than a life span of an affected mouse, it was found that the tumour did not grow but was rejected [Gorer 1937]. This phenomenon was also seen when healthy tissue was transplanted, except when the tissue was transplanted into highly inbred mice [Gorer 1955]. The genetic influence of tumour and tissue survival was found to be confined to a single genetic locus which was termed the 'histocompatibility locus' and named H-2 from the original mouse studies [Snell 1951]. This major histocompatibility complex was also identified in humans and its products were called human leucocyte antigens [HLA]. HLA antigens are fundamental in providing protection against pathogens but are also pivotal in the success of transplantation.

The MHC region is located on the short arm of chromosome 6 and spans 3.6 mega base pairs. Within the complex, three separate regions, class I, II and III are distinguishable as represented in figure 1.1. The MHC is highly polymorphic, with HLA-B being the most polymorphic gene in the human genome [Mungall 2003].

Figure 1.1. A simplified map of the major histocompatibility complex on chromosome 6p21, showing the genes arranged in three classes. [Taken from Kelly M A et al. Mol Path 2003;56:1-10]



The class III region does not contain any of the HLA genes, however it does encode for numerous proteins and cytokines associated with the immune response. These include complement components, tumor necrosis factor and heat shock proteins.

### 1.1.2 HLA class I antigens

Each HLA class I antigen is composed of an α heavy chain (≈45kDa) and a β light chain (≈12kDa). The class I region of the MHC encodes the α heavy chains of the class I antigens, mainly HLA –A, -B and –C but also –E, -F and –G. Whilst the β light chain or β2-microglobulin is encoded by a gene on chromosome 15 and is monomorphic [Marsh, 2000]. A diagramatic representation of the class I antigen is seen in figure 1.2. Class I antigens are expressed on all nucleated cells in the body. Class I HLA present endogenous peptides, including viral proteins, which have been processed by the endoplasmic reticulum to CD8+ T cells.

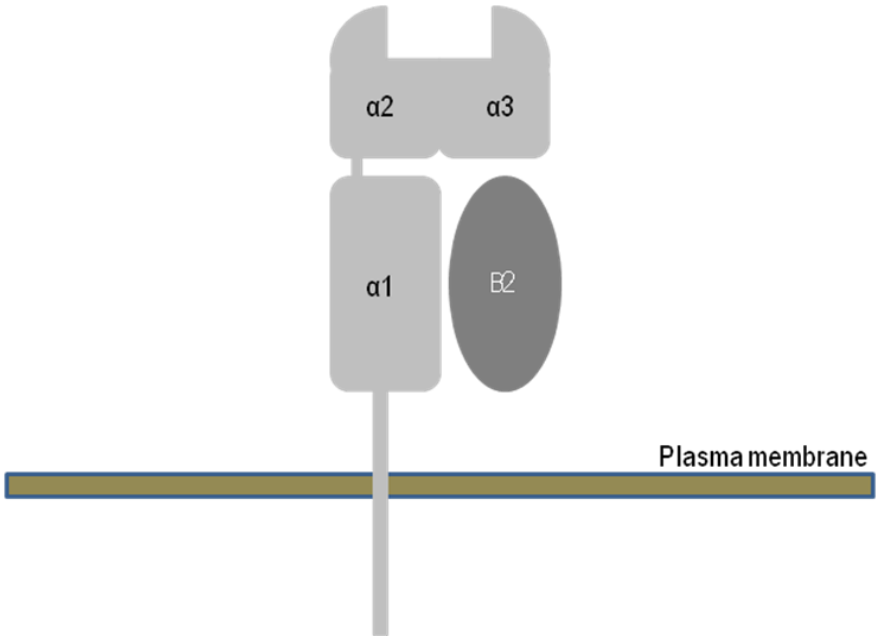



**Figure 1.2. Schematic representation of the HLA class I antigen**

### 1.1.3 HLA class II antigens

Class II antigens are also composed of a heavy α and a light β chain, both of which are encoded by genes in the MHC complex. The α chains are ≈33-35kDa and the β chains are ≈26-28kDa. The main class II antigens are HLA –DR, -DQ and –DP along with the less polymorphic HLA –DM and –DO. Unlike the class I antigens, the polymorphism of class II antigens is derived from both chains in DQ and DP antigens. However DR antigens have an invariant α chain with a polymorphic β chain, which is complicated further by the fact that there are several functional β chain genes which can be expressed simultaneously, with a DRB1 gene encoding one β chain whilst another being encoded by a separate DRB locus [Marsh, 2000]. A diagramatic representation of a class II antigen is seen in figure 1.3. A further major difference between class I and II antigens is their expression: class II antigens are only expressed on antigen presenting cells, namely dendritic cells, macropahges and B cells. However their expression may be induced on other cells as a result of an inflammatory response. Class II HLA present exogenous peptides by the lysosomal route [pathogens within phagocytic cells] to CD4+ T cells.

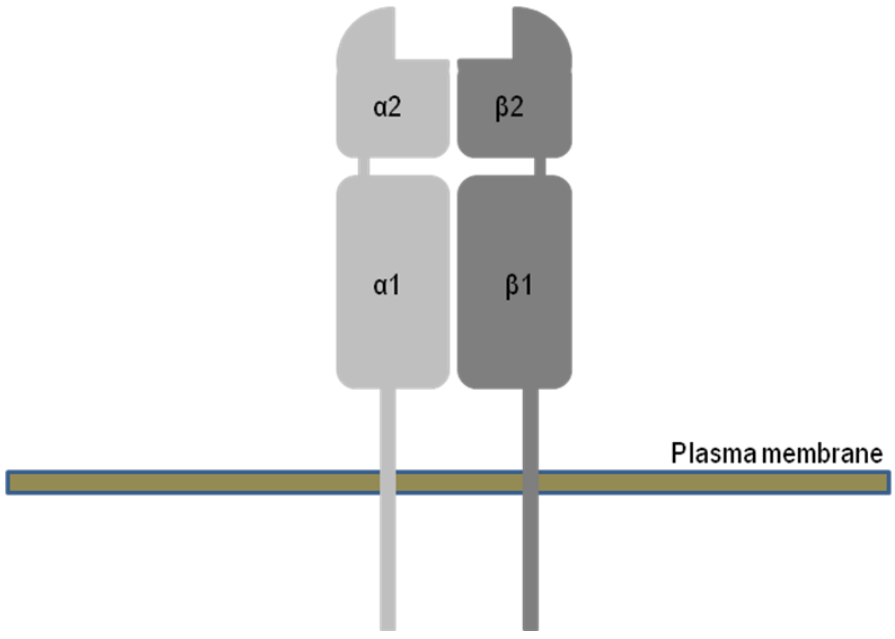



**Figure 1.3. Schematic representation of the HLA class II antigen**

### 1.1.4 Minor histocompatibility antigens

Endogenous proteins on recipient cells that are genetically polymorphic and can give rise to an immunological response to the donor are termed minor histocompatibility antigens. Although important in bone marrow transplantation [Gloumy et al, 1996], their role in solid organ transplantation and particularly renal transplantation is not well described [Dierselhuis et al, 2009]. Rejection can occur in recipients receiving a 'HLA identical' kidney, which suggests that minor histocompatibility antigens might have a role to play [Cecka, 2004]. Minor histocompatiblility antigens may be encoded autosomally or on the Y chromosome. The impact of the latter has been better studied. To answer the question of whether female recipients of male donor kidneys are at increased risk of rejection, Gratwohl and colleagues respectively analysed a large cohort and found that there was an increased risk of allograft failure in male into female kidneys than any other gender combination [Gratwohl et al, 2008]. Regarding autosomal minor histocompatibility antigens, one large study into their effect in transplants matched at the major histocompatibility antigens, found no risk of rejection or increased allograft failure in the 'minor' mismatched group [Heinold et al, 2008]. However work into minor antigens is by no means conclusive and should be the focus of further work. Non-HLA antigens will be discussed later in the chapter.

### 1.1.5 Methods of HLA typing

Methods of HLA typing have evolved over time and consequently more HLA alleles are continuously being identified. Currently over 7000 HLA alleles are recognised [IMGT/HLA database]. Typing initially was undertaken using serological methods but has since been superseded by molecular methods, which are undergoing constant refinement. Serological testing involved using sera from multiparous women and incubating the antibodies with lymphocytes of certain HLA types [Marsh]. Serological methods are still useful in determining whether a HLA allele detected by molecular methods is expressed [Elsner et al 2004].

The development of the polymerase chain reaction (PCR) has enabled rapid HLA typing with high resolution [Middleton, 1999]. Although all based on PCR amplification of HLA genes, there are a

number of different techniques used [Howell et al, 2010; Dunn, 2011]. These include: PCR with sequence specific primers (PCR-SSP), PCR with sequence specific oligonucleotide probes (PCR-SSOP) and PCR with sequencing based typing (PCR-SBT). The principle behind PCR-SSP is that amplification of DNA with a particular primer depends upon the presence of a particular allele. With PCR-SSOP, following amplification, a labelled sequence specific oligonucleotide probe is added and binds only if the sequence matches. PCR-SBT gives the highest resolution of all the techniques and involves amplification of specific coding regions with subsequent amplicon sequencing.

Currently for renal transplantation, typing at the HLA –A, -B and –DR loci only are required for allocation purposes in the UK and usually to the split specificity level. HLA splits are 2 closely structured HLA antigens which have been defined by more sensitive typing methods, and together they compose a parent antigen or 'broad' specificity level. With the advent of single antigen beads, and the association of class II DSA and chronic rejection [Pollinger et al, 2007], especially DQ donor specific antibodies, typing might need to be broadened prior to transplantation in order to determine risk.

## 1.2 Allorecognition and effector mechanisms

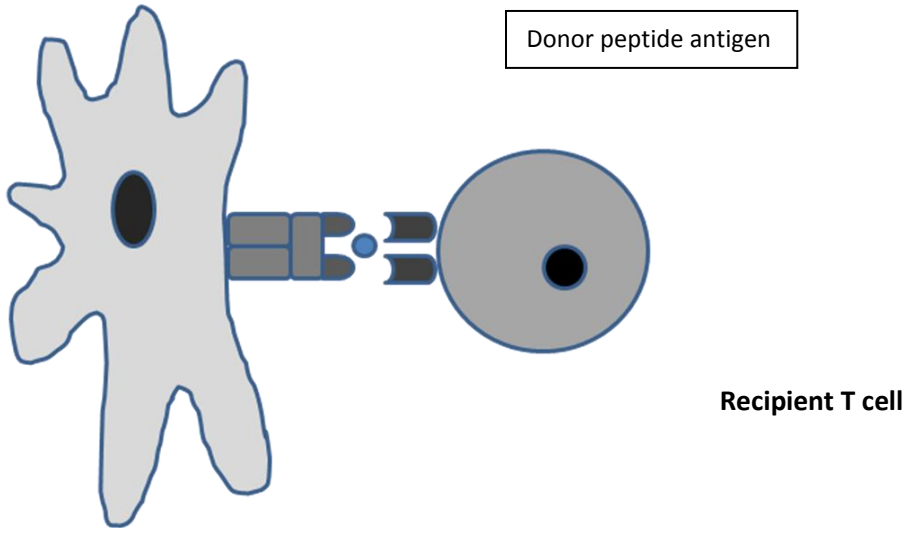
Allograft rejection remains the leading cause of allograft failure following renal transplantation. Historically rejection has been considered in terms of its timing (hyperacute, acute or chronic) or the putative primary immunological mechanisms (cellular or humoral). It is likely that multiple non-mutually exclusive pathways play a role in the rejection process which includes the innate along with the adaptive immune response, and incorporates both cellular and antibody effector processes. The following is a description of allorecognition along with the subsequent effector mechanisms.

### 1.2.1 Allorecognition

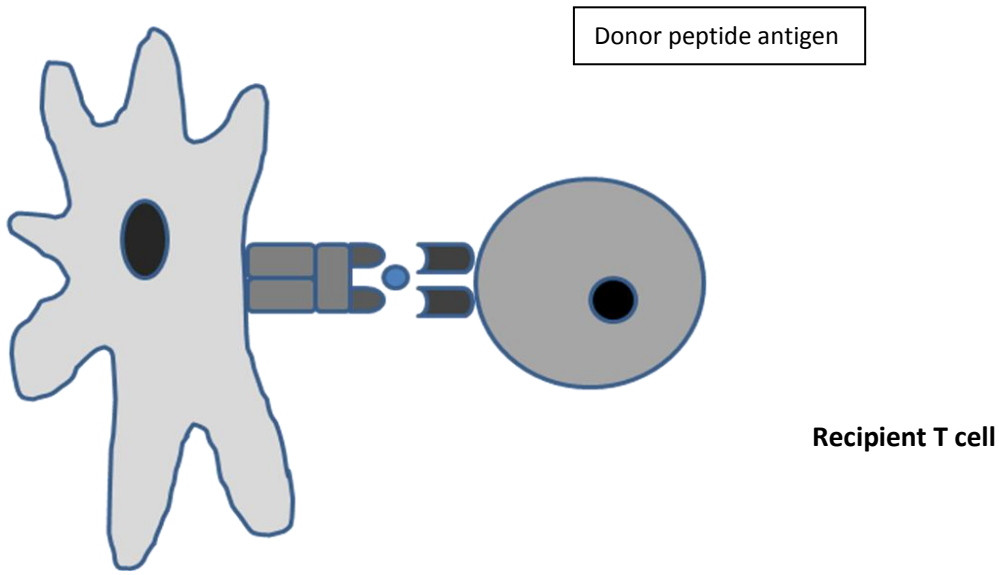
The recipient's T lymphocytes can recognise donor alloantigens by two major pathways as shown in figure 1.4. Direct allorecognition is the predominant pathway initially, when donor APC's are still present and can present alloantigen directly to recipient T cells [Janeway et al, 2001]. The indirect pathway resembles the normal immune response to antigens. Donor antigens from the graft and are taken up by recipient APCs and are presented to recipient T cells [Janeway et al, 2001]. More recently a third 'semi-direct' pathway has been described, the basis of which is that recipient APC can present donor peptide using both donor and recipient MHC [Afzali et al, 2008]. Recipient MHC can obtain donor intact MHC by cell to cell contact or following its uptake after its release in small vesicles by the donor APC cell. The recipient T cells than may be stimulated either directly by the donor MHC on recipient APC or indirectly by recipient MHC on recipient APC. Evidence for this phenomenon in vivo has just been published [Brown et al, 2011].

**Figure 1.4: Diagrams showing mechanisms of allorecognition**

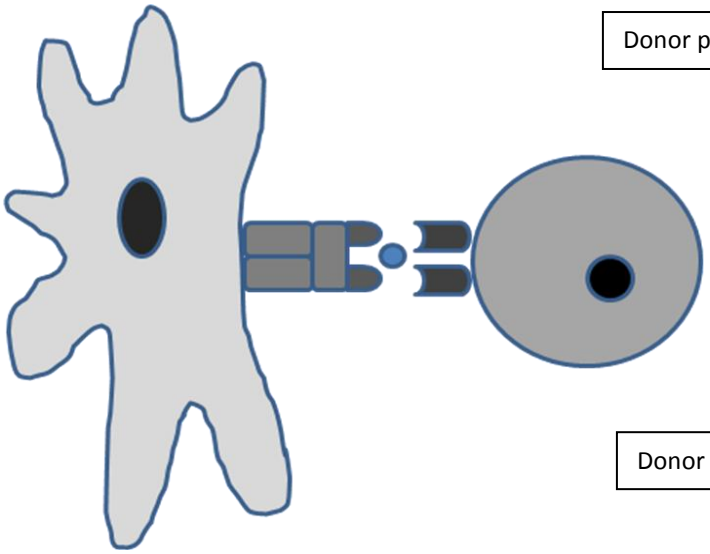
**a. Direct allorecognition**




**b. Indirect allorecognition**



c. **Semidirect allorecognition**




## 1.2.2 Role of T cells in rejection

Antigen is presented to T cells in the context of a MHC-antigen complex. In general class I HLA antigens present intracellular proteins to CD8+ cells, whilst class II HLA antigens present extracellular proteins to CD4+ cells. In order to become activated the T cell requires interaction between the MHC-antigen complex and the TCR and also a co-stimulatory signal. In the absence of a second signal T cells become anergic. Co-stimulation is achieved by the surface molecules on APCs. Two B7 molecules, CD80 and CD86, on the APCs bind to the CD28 molecule on the T cell. A further ligand on the T cell, CTLA-4, when bound either of the B7 molecules appears to down regulate the immune response [Walunas et al, 1998]. A further co-stimulatory molecule on APCs is CD40 and its

corresponding ligand CD40L (or CD154) [Li et al, 2009] with other, newer signal pathways having been decribed, for example the T-cell immunoglobulin mucin protein 1.

Once activated, T cells proliferate and differentiate into subgroups, each with their own cytokine profile and effector function. Type 1 helper T cells produce interleukin-2 (IL-2) and interferon gamma (IFN-γ) and mediate the cellular immune response. Type 2 helper T cells facilitate the humoral response with production of cytokines providing B cell help, namely IL-4, IL-5, IL-10 and IL-13. Th17 cells are thought to mediate rejection and produce IL-17, IL-21 and IL-22 [Heidt et al, 2010]. Mechanisms of allograft injury by T cells is via cell mediated cytotoxicity and effects of cytokines. In addition to the T cells which promote immune responses, a subgroup of T cells, regulate or control the response, T regulatory cells or Tregs.

### 1.2.3 Role of the innate immune system in rejection

Cells of the innate immune system have been shown to have an impact on allograft outcomes [Murphy et al, 2011]. This has also been shown clinically by the types of cells seen infiltrating rejecting allografts following alemtuzumab induction [Kirk et al, 2003]. The cells of the innate immune system most studied are NK cells, dendritic cells, macrophages and mast cells. The role of these cells is variable; they can act as APCs, produce cytokines and act as inflammatory cells [Liu et al, 2010]. An interesting study by Hirohashi et al, has shown that NK cells may play a pivotal role in chronic rejection. They found that in mice with donor specific antibodies, NK cells were necessary in order to produce the vasculopathy of chronic rejection independent of complement [Hirohashi et al, 2012].

## 1.3 B cells

### 1.3.1 B cell development

The majority of B cells are produced in the bone marrow, while a minority of B cells are developed during fetal life and are found in the peritoneal and pleural cavities. The former are often referred to as B2 cells and the latter B1 cells. B1 cells form part of the innate immune system and produce low affinity natural antibody independent of T cells. B1 cells are characterised by the CD5 molecule on the cell surface.

#### Central B cell development

B2 cells originate in the bone marrow from pluripotent haemopoietic stem cells, which under the influence of cytokines develop into pro-B cells. Pro-B cells express Pax-5 (a master transcription factor) along with RAG1 and RAG2 which catalyse D-JH rearrangements within the immunoglobulin heavy chain loci. Pro-B cells also acquire the surface expression of CD19 and HLA class II molecules. The next stage in B cell development is the pre-B cell stage in which a complex consisting of a Ig heavy chain and 'surrogate' light chains is expressed. Other cell markers which appear at this stage of development are: CD20, which regulates cell cycle progression [Tedder 1994], CD40 which regulates immunoglobulin class switching [Durie 1994] and CD22 which inhibits BCR signalling [Nitschke 2004]. From here the pre-B cells undergo light chain gene arrangement and proceed to express surface IgM to form an immature B cell. These B cells then undergo central selection based on the expressed BCR [B cell receptor]. B cells whose receptors bind with high affinity undergo apoptosis or undergo further light chain rearrangements [Louzoun 2002]. Under 10% of formed immature B cells in the bone marrow survive this process and leave to the periphery [Brahim 1970].

#### Peripheral B cell development

Once released from the bone marrow, mature B cells circulate lymphoid tissues until they encounter antigen. If B cells receive co-signals from antigen specific T cells they migrate to the germinal centres where, with T cell help, they undergo class switching and affinity maturation and differentiate into memory B cells and plasma cells. Such plasma cells are long lived and survive for months. Plasma

cells do not express typical B cell markers but are characterised by the presence of the CD38 molecule which is also present on thymocytes, immature myeloid cells and haematopoietic stem cells. Activated B cells which do not receive T cell help develop into short lived plasma cells (weeks), which secrete low affinity IgM antibodies as they do not undergo class switching. Memory cells are phenotypically like resting mature B cells, that is they express the surface molecules CD19, CD20, CD40, Fc gamma receptor II and MHC class II molecules. Memory B cells on re-exposure to antigen can generate plasma cells rapidly. Memory cells are extremely long lived and can survive for years.

### 1.3.2 B cell activation

B cell activation requires two signals. Different types of antigens may activate B cells and can be broadly divided into thymus dependent (TD) and thymus independent antigens (TI). The former account for the majority of protein and glycoprotein antigens and requires T cell help. TD antigens activate B cells by being processed by the B cell and presented on the MHC class II molecule to an activated T cell. A second signal is then provided by the interaction of the CD40 molecule on the B cell and the CD40 ligand on the T cell. TI antigens can be further subdivided into TI-1 and TI-2 antigens. TI-1 antigens, for example bacterial lipopolysaccharides, are also known as mitogens and stimulate B cells to undergo proliferation and differentiation. At high concentrations of antigen these B cells produce polyclonal antibodies and such activation does not produce memory B cells. TI-2 antigens, for example streptococcus pneumonia, activate B cells by cross linking of immunoglobulin receptors but also require non-cognate T cell help in order to produce antibody.

### 1.3.3 B cell homeostasis

The TNF-related cytokine BLyS [BAFF] is central to B cell homeostaisis and survival. There are 3 BLyS receptors: TACI (transmembrane activator I and calcium-signalling modulator and cyclophilin ligand-interactor), BCMA (B cell maturation antigen) and BR3 (BLyS receptor 3 or BAFF receptor). BLyS exists in both membrane and soluble forms and is produced by neutrophils, monocytes, macrophages and dendritic cells. Another cytokine, APRIL is also involved in B cell survival and is produced by dendritic cells and osteoclasts. BR3 only binds BLyS and not APRIL.

Animal models have shown that over expression of BLyS is associated with B cell autoimmune disease, conversely the use of a BLyS monoclonal antibody has demonstrated a blunting of

autoimmunity [Kalled 2005, Marino 2009]. This association has also been shown clinically in patients with SLE [Navarra 2011].

Treatment with rituximab is associated with an increased level of BLyS with B cell reconstitution, which has been seen with rituximab usage in both autoimmunity and also transplantation [Zarkhin 2009]. Enhanced levels of BLyS have also been seen in transplant recipients induced with alemtuzumab which depletes both T and B cells [Bloom 2009]. Furthermore, it has been shown that increased BAFF expression after transplant is associated with both anti-HLA antibodies and antibody mediated rejection [Xu 2009]. However, a more recent study has shown that BAFF is associated with the expansion of IL-10 producing B cells, which are shown to have regulatory properties [Yang 2010].

### 1.3.4 Role of B cells in rejection

The key role of B cells in allograft rejection has been increasingly recognised over the past decade, following decades where T cells alone were considered pivotal to the rejection mechanisms. It is now accepted that T and B cells are inextricably linked in the pathogenesis of allograft rejection. B cells are involved in both the initiation and effector mechanisms of rejection; their role is outlined below.

1. B cells are effective APCs and present antigen to CD4+ T cells. Also, B cells provide co-stimulatory signals via expression of a number of molecules (B7, CD40, and OX40L), which enable T cell activation. Whilst alloreactive T cells can be activated in the absence of B cells, differentiation into memory T cells is impaired [Ng 2010]. Conversely, CD4+ T cells are required for B cell antibody production and isotype switching from IgM to IgG [Steele 1996].

2. B cells produce pro-inflammatory cytokines IL-6, interferon γ and lymphotoxin α.

3. When B cells are activated they proliferate and differentiate into plasma cells which secrete antigen specific antibody. The period after initial B cell exposure to antigen until antibody is produced by plasma cells is 4-10 days and primarily results in the IgM antibody isotype. This contrasts with antigen re-exposure where there is rapid production of antibody, mainly IgG.

## 1.4 Antibody

### 1.4.2 Antibody structure

Each immunoglobulin molecule consists of 4 polypeptide chains, 2 heavy chains and 2 light chains linked together by disulphide bonds as shown in figure 1.5. Each chain can be divided into a variable and constant region. The variable region comprises the antigen binding site, whilst the constant region determines the immunoglobulin isotype.

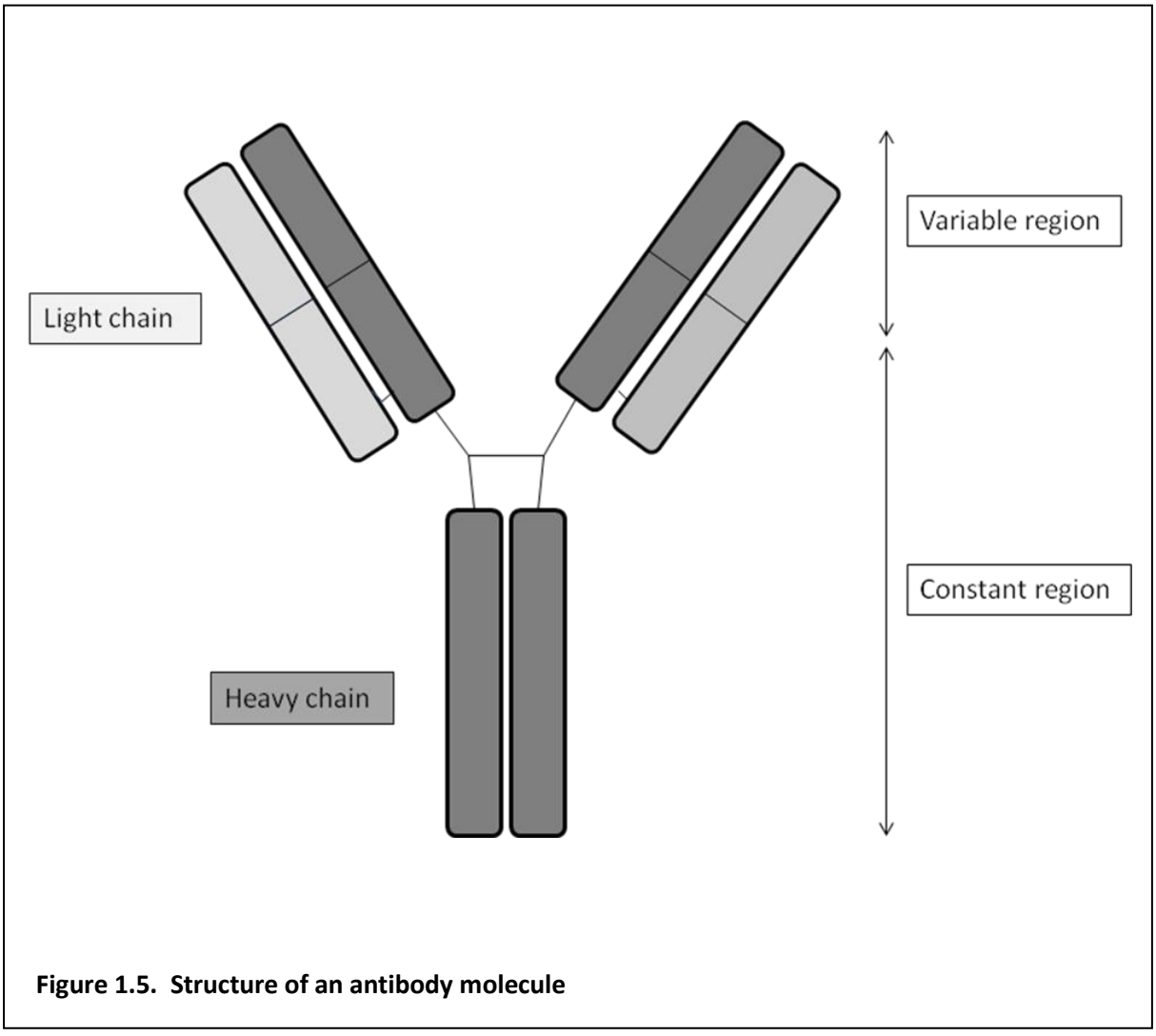



**Figure 1.5. Structure of an antibody molecule**

### 1.4.2 Antibody isotypes

The protease papin can cleave the antibody molecule into 3 fragments; 2 Fab fragments [Fragment antigen binding] and the Fc fragment [Fragment crystallisable]. The Fc fragment contains part of the constant region of the heavy chains which differs between the isotypes and is the region which interacts with effector cells and molecules. There are 5 main different heavy chains; gamma (γ), alpha (α), mu (μ), epsilon (ε) and delta (δ) which correspond to the major classes of iummunoglobulins IgG, IgA, IgM, IgE and IgD respectively. In humans IgG can be further subdivided into subclasses IgG1, IgG2, IgG3 and IgG4 and IgA into IgA1 and IgA2.

### 1.4.3 Antibody function

Antibodies protect by a number of different mechanisms. They can neutralise a pathogen by binding to its surface and prevent it from entering cells. They also cause opsonisation, where binding to the pathogen enables recognition of the Fc receptors on phagocytic cells. Also antigen-antibody complexes can activate complement. Table 1.1 is a summary of the functional differences between the classes and subclasses.

**Table 1.1. Function of the immunoglobulin isotypes** [Taken from Janeway, Travers, Walport, Shlomchik. Immunobiology 5. Churchill Livingston]

| Functional activity | IgM | IgD | IgG1 | IgG2 | IgG3 | IgG4 | IgA | IgE |
|---|---|---|---|---|---|---|---|---|
| Neutralisation | + | - | ++ | ++ | ++ | ++ | ++ | - |
| Oponisation | - | - | +++ | * | ++ | + | + | - |
| Sensitisation for killing by NK cells | - | - | ++ | - | ++ | - | - | - |
| Sensitisation for mast cells | - | - | + | - | + | - | - | +++ |
| Activates complement system | +++ | - | ++ | + | +++ | - | + | - |

*IgG2 can act as an opsonin in the presence of Fc receptors of a particular allotype, found in ~50% of white people.

### 1.4.4 Detection of HLA donor specific antibodies

The most widely used methods of detecting pathogenic donor specific antibodies remain the cell based assays [Gebel et al, 2003], although the advent of solid phase techniques and in particularly the single antigen beads have resulted in assays so sensitive their relevance is not yet known, which is one of the starting points of this thesis. The various techniques available are briefly described below.

The CDC (complement dependent cytotoxicity] crossmatch was the original test which revolutionised transplantation when presented by Patel and Terasaki in 1969 [Patel, 1969]. The basis of the cell based crossmatch is that any potential antibodies in the recipient's serum are detected by exposing the donor antigens expressed on lymphocytes in vitro pre-transplantation. In the case of the CDC test, complement is added to the serum and lymphocytes, and if donor specific antibodies of the appropriate subclass are present then the complement cascade will be activated and result in lysis of the lymphocytes. The basic CDC test was made more sensitive by the addition of anti-human globulin (AHG) in order to detect low titre antibody [Johnson et al, 1972]. AHG is added prior to complement and binds to any antibody-antigen complex on the lymphocytes, therefore amplifying the response to complement by increasing the number of Fc receptors available. The flow cytometry assay was developed in order to enhance further the sensitivity of the pre-transplant crossmatch, and was able to detect low titre as well as non-complement fixing antibodies [Garavoy et al, 1983]. With the flow cytometry assay, fluorescein labelled anti-human IgG is incubated with the patient's serum and donor lymphocytes. If there is any binding to donor specific antibodies, these will then be detected by flow cytometry.

With solid phase techniques HLA molecules are bound either to plates (ELISA) or beads. Single antigen Luminex beads are the most sensitive method for detecting HLA antibodies [El Awar et al, 2005]. The beads are bound with HLA molecules which may be of a single specificity or a mixture of HLA molecules which correspond to the single antigen and mixed screening beads respectively. The technique involves incubating test serum with the beads and if donor specific antibodies are present they bind to the bead, fluorescein labelled AHG is then added. Following this the beads are then passed through a dual beam laser, as each bead contains a unique fluorochrome, one laser picks up antibody binding and the other identifies the corresponding bead and therefore its HLA specificity. The advent of single antigen beads, which detect HLA antibodies with high sensitivity and specificity have enabled the use of the 'virtual crossmatch' [Amico et al, 2009].

Other technologies already established might become more widely used. This includes the HLA Matchmaker program, which involves a computer program which considers the structural basis of HLA molecules [Duquesnoy, 2011].

### 1.4.5 Non-HLA antibodies

Studies into HLA identical transplantation have shown that non-HLA immune responses play an important role in allograft rejection [Opelz et al, 2005]. However the extent to which non-HLA antibodies contribute to allograft failure and other forms of allograft injury is yet to be determined. Non-HLA targets are expressed on endothelial and epithelial cells and can be categorised into non-HLA antigens and tissue specific autoantigens [Zhang et al, 2010]. Below is summary of studies into non-HLA antibodies in renal transplantation and their effect on outcomes.

MICA and MICB

The MHC class I-related chain A (MICA) and B (MICB) are the product of an HLA related polymorphic gene, which is located in close proximity to the HLA –B locus on chromosome 6 [Sumitran-Holgersson, 2008]. MICA is expressed on monocytes, fibroblasts and endothelial cells but not on resting lymphocytes [Zwirner et al, 1999].

Zou et al demonstrated that the presence of MICA antibodies at the time of transplantation is associated with graft failure in patients well matched at the HLA loci [Zou et al, 2007]. In a prospective study as part of the international workshop, Terasaki et al showed that patients with anti-HLA and anti-MICA antibodies who were followed up had a significant increased risk of allograft failure at 1 and 4 years [Terasaki et al, 2007]. A more detailed study has investigated the impact of MICA mismatching by determining MICA alleles, which also allowed for the identification of donor specific MICA antibodies [Cox et al, 2011]. They found that mismatching at the MICA allele level was a risk factor for de novo MICA antibody formation, which was subsequently associated with rejection and inferior allograft function.

Anti-endothelial antibodies

Anti-endothelial cell antibodies [AECAs] are a heterogeneous group of antibodies directed against various antigenic targets on the endothelium, they comprise both IgM and IgG antibodies [Bordron et al, 2001]. A multi-centre trial investigating the usefulness of a pre-transplant endothelial cell

crossmatch, showed that those patients found to have donor reactive AECA's were at significant risk of rejection [Breimer et al, 2009]. The development of de novo AECA's after transplant has also been shown to be associated with rejection [Sun et al, 2011].

Tissue specific antibodies

Vimentin is a non-polymorphic protein expressed in the cytosol of endothelial and vascular smooth muscle cells [Zhang et al, 2010]. Anti-vimentin antibodies have been studied in more depth in the context of cardiac transplantation. However, anti-vimentin antibodies have also been shown to be associated with renal allograft loss [Carter et al, 2005]. An interesting observation is the association between the preferential detection of anti-vimentin antibodies in recipients of non-heart beating transplants when compared with heart beating donors [Mi et al, 2005].

Angiotensin II type I receptors are located in the glomeruli along with the proximal tubules and vasculature [Zhang et al, 2010]. The gene encoding the receptor is located on chromosome 3 and a few polymorphisms of the gene have been described [Dragun, 2008]. AT1R antibodies have been found in cases of acute AMR in patients with no detectable HLA or MICA antibodies [Reinsmoen et al, 2010].

## 1.5 Complement System

### 1.5.1 Complement components

The complement system incorporates a number of plasma proteins which form part of the innate and adaptive immune response. It is activated through 3 main pathways: classical (C1q binding pathogen surface/Ab-Ag complex), alternative (pathogen surfaces) and the mannan binding lectin pathway (binding of MBL to mannose containing carbohydrates on pathogens) along with other proposed mechanisms. Its main role regardless of the route of activation is opsonisation of pathogens, production of anaphylactoids, along with cell lysis from the formation of the membrane attack complex. A schematic diagram and description of the complement cascade is seen in figure 1.6.

### 1.5.2 Role of complement in renal allografts

The demonstration by Feucht and colleagues of the complement split products C4d and C3d in peritubular capillaries [PTC] as indirect evidence of immunological activation highlighted the role of antibody in renal allograft rejection [Feucht , 1991]. As a consequence C4d was included into the Banff criteria of allograft rejection in 2001.

There are 2 different methods used to detect C4d, which are indirect immunofluorescence [IF] using a monoclonal antibody on frozen tissue sections and immunoperoxidase using a polyclonal antibody on paraffin embedded tissue. C4d scoring is dependent upon the technique used, with IF being more sensitive, therefore focal C4d staining by immunoperoxidase may equate to diffuse scoring by IF [Seemayer, 2007; Solez, 2008]. C4d scoring is classified as negative, minimal with 0-10% staining of PTC, focal with 11-50% and diffuse with >50% of PTC staining.

There have been numerous studies showing the predictive value of C4d positive staining in renal allografts, with C4d positivity being associated with poor outcome. Feucht's group analysed the relevance of C4d deposition in 93 patients with early renal allograft dysfunction. There was a significant number of graft losses in the C4d+ group when compared with the C4d- group at 1 year, 18/51 [42.9%] and 4/42 [9.5%] respectively, p=0.0027 [Feucht, 1993]. In a follow up study of 218 patients, Lederer showed that C4d was strongly associated with alloantibodies. C4d positive allografts had an inferior survival to C4d negative grafts and they found that C4d positivity within 6 months of transplantation gave the worse prognosis [Lederer, 2001]. Collins et al stained for C4d in 16 biopsies from high risk patients with classical histological features of AMR along with DSAs and compared these with a control group comprising ACR, CNI toxicity and no abnormality. None of the ACR group was C4d positive or was found to have DSA, compared with all in the AMR group. It was concluded that C4d was a specific and sensitive marker of DSAs and antibody mediated injury [Collins, 1999]. Nickelait in their study looking at the impact on management of C4d positive patients, highlighted a small group with no histological features of rejection who did not require therapy. However the majority of C4d positive patients had morphological evidence of rejection. They also found that the C4d status of allografts could change within a matter of days to weeks [Nickelit, 2002]. Mauiyyedi's group was the first to show that C4d staining was associated with DSA and specific histological features [Mauiyyedi, 2002]. These initial studies contributed to antibody mediated rejection being introduced to the Banff classification for the first time in 2001. Later publications have unanimously demonstrated that in ABO compatible grafts C4d is predictive of poor

outcomes and associated with donor specific antibodies [Haririan, 2009; Kedainis, 2009; Kayler, 2008].

The relevance of focal C4d in comparison with diffuse staining remains controversial. Kayler and Haririan have both looked at the relevance of focal C4d using immunoperoxidase. Kayler found that focal C4d was associated with DSA, although found no difference in allograft loss between the diffuse, focal and negative groups [Kayler, 2008]. Haririan and colleagues showed that focal C4d staining had a comparable effective on graft survival than diffuse C4d. However they also showed that DSA status predicted allograft survival despite C4d staining [Haririan, 2009]. Using the more sensitive IF method, Kedainis showed that focal C4d was associated with circulating alloantibody and there was a trend for inferior graft survival in the focal group when compared to the negative group [Kedainis, 2009].

C4d is not always considered detrimental in renal allografts. Peritubular capillary C4d staining is often seen in ABO incompatible [ABOi] grafts in protocol and indication biopsies in the absence of histological features of rejection [Haas, 2006; Setoguchi, 2008]. Furthermore, in one study it was shown that C4d positivity was associated with less scarring than those ABOi grafts who were C4d negative [Haas, 2009]. The presence of C4d deposition with circulating anti-donor [HLA, blood group etc] with stable graft function and no other morphological features of rejection is coined accommodation [see section 1.7.3].

The relevance of C4d staining in the absence of rejection in ABO compatible graphs has not been as extensively studied, probably since C4d, when present, is often associated with features of rejection even in protocol biopsies [Mengel, 2005]. Only a few papers have highlighted patients with C4d with acute tubular necrosis ; Nickeleit in their analysis of 398 indication biopsies only found 3 patients with C4d+ ATN [Nickeleit, 2002], likewise Mauiyyedi found 2/20 patients with diffuse C4d showing ATN only [Mauiyyedi, 2002]. Dickenmann, in a retrospective study of ABO compatible transplants, looked at the outcomes of C4d positive indicative biopsies lacking features of rejection. Of 22 patients studied, only 1 had diffuse C4d with 21 having focal C4d. 5 of the patients received augmented immunotherapy. They found there was no difference in allograft survival but there was an improvement in allograft function in those patients who received treatment.

More recent advances in the role of complement in transplantation have come from the finding of its role in ischaemic reperfusion, intra-renal synthesis of C3 and enhanced complement gene expression in deceased donor kidneys and its subsequent effect on allograft outcomes.

**1.5.3 Complement cascade**

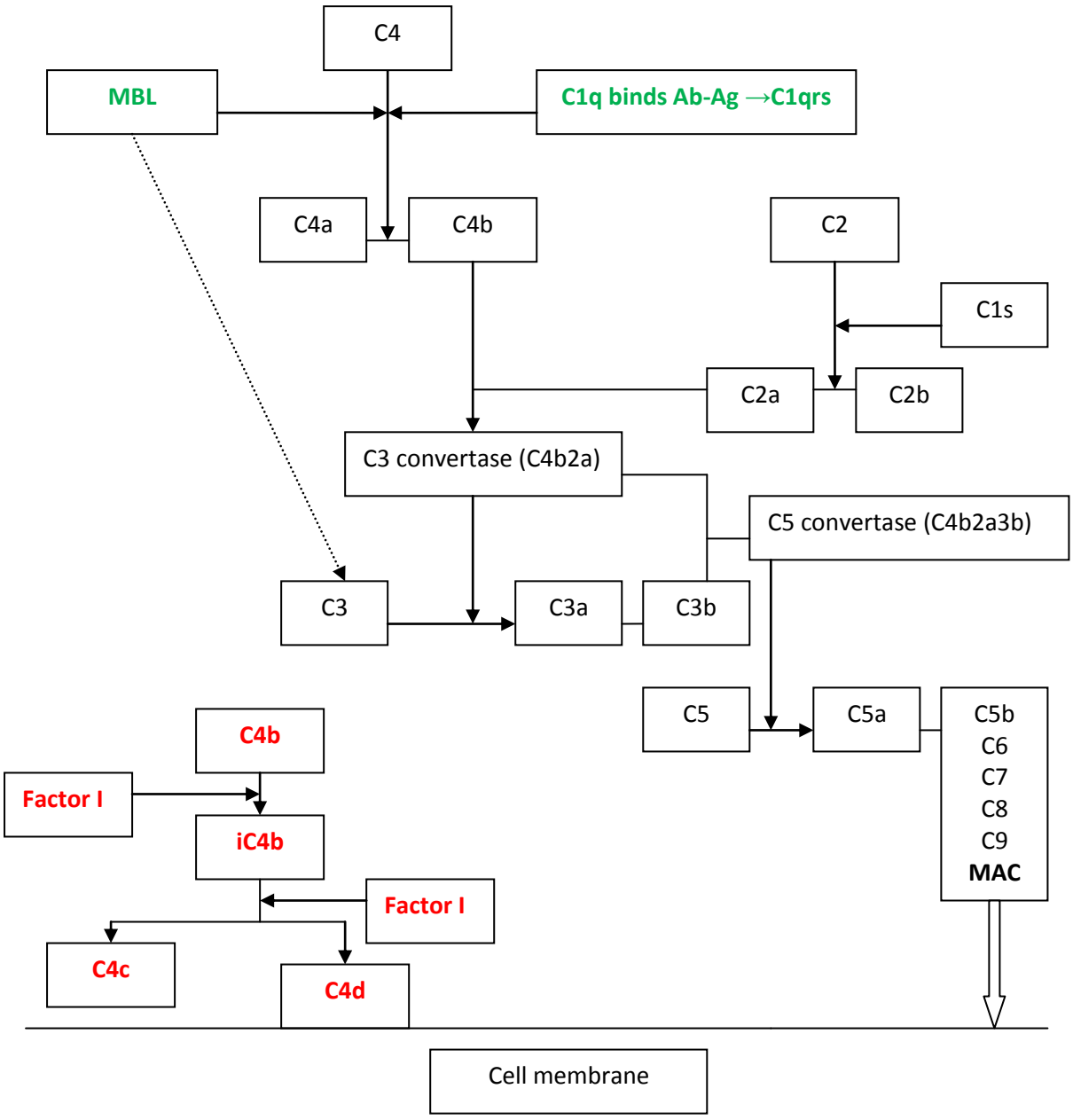



**Figure 1.6. The classical pathway is activated when C1q binds directly to the Fc portion of immunoglobulin or the pathogen which causes C1r to cleave and activate C1s. C1s in turn cleaves C4 into C4a and C4b, the latter which binds to the pathogen surface. C4b then binds C2 which is cleaved by C1s forming C3 convertase, which cleaves C3 forming the C5 convertase [C4b2a3b]. This cleaves C5 into C5a [anaphylactoid] and C5b which couples with C6,C7,C8 and C9 to form the membrane attack complex.**

### 1.5.4 Non-complement fixing antibodies

The role of non-complement fixing HLA antibodies in renal transplantation is yet to be fully appreciated. A potential drawback of detecting HLA antibodies by single antigen assays is that they do not differentiate between the different subclasses of IgG. It is known that the 4 subclasses of Ig G differ in their ability to fix complement, with IgG1 and IgG3 able to activate complement whilst IgG2 and IgG4 subclasses are considered non-complement fixing [Heinemann et al, 2007]. The detection of non-complement fixing antibodies in the setting of rejection may be a result of class switching, with activated lymphocytes first secreting IgM antibodies followed by IgG 1/3 and then IgG 2/4 [Malisan et al, 1996]. Further studies have shown that non-complement antibodies synergistically activate complement in combination with complement activating antibodies [Murata et al, 2007; Rahimi et al, 2004]. Also, HLA antibodies against class I HLA antigens on the endothelium may cause rejection by stimulating proinflammatory and proproliferation signals [Zhang et al, 2009]. Finally, antibodies have also been shown to activate NK cells, neutrophils and macrophages through their Fc receptors [Murata et al, 2007]. The mechanisms involved in chronic rejection may be different from those in acute rejection. Hirohashi et al, presented data from an animal study that class I HLA antibodies can cause chronic arterial lesions in cardiac transplants in the absence of complement [Hirohashi et al, 2009].

## 1.6 Allograft rejection

### 1.6.1 Definition of clinical rejection

Thus far I have described the mechanisms behind allorecognition and the subsequent effector responses. Rejection can manifest clinically in numerous ways; from a rapid deterioration in allograft function with or without oliguria, to a more insidious decline in function with or without proteinuria. In fact, in the era of protocol biopsies it has been shown that despite histological evidence of rejection, there may be no clinical evidence of rejection with no allograft dysfunction or proteinuria, and this is defined as subclinical rejection. The limited evidence that exists on protocol biopsies suggests that even in the absence of allograft dysfunction, histological rejection should be treated

[Henderson et al, 2011]. For the purposes of this thesis, rejection is defined as biopsy proven rejection in the setting of allograft dysfunction.

### 1.6.2 Banff classification of allograft pathology

Renal transplant biopsies are widely classified according to the Banff criteria. The first meeting took place in Banff in 1991 and the official categorisation of allograft pathology was established in 1997 [Racusen et al, 1999]. Since that time there have been continued updates of the classification as necessary, with working groups formed in order to address unmet needs, for example whether the use of molecular markers can or should be integrated into the classification [Sis et al, 2010].

Table 1.2 is taken from the current classification [Sis et al, 2010]. There are six, non-mutually exclusive categories; normal, antibody mediated rejection, borderline rejection, T cell mediated rejection, interstitial fibrosis and tubular atrophy and 'other'.

**Table 1.2:** Banff 97 diagnostic categories for renal allograft biopsies—Banff '09 update [Sis et al, 2010]

1. **Normal**
2. **Antibody-mediated changes** (may coincide with categories 3, 4 and 5 and 6)
Due to documentation of circulating antidonor antibody, C4d and allograft pathology

C4d deposition without morphologic evidence of active rejection
C4d+, presence of circulating antidonor antibodies, no signs of acute or chronic TCMR or ABMR (i.e. g0, cg0, ptc0, no ptc lamination (<5 layers by electron microscopy), no ATN-like minimal inflammation). Cases with simultaneous borderline changes are considered as indeterminate

Acute antibody-mediated rejection
C4d+, presence of circulating antidonor antibodies, morphologic evidence of acute tissue injury, such as (Type/Grade)
I. ATN-like minimal inflammation
II. Capillary and or glomerular inflammation (ptc/g >0) and/or thromboses
III. Arterial – v3

Chronic active antibody-mediated rejection
C4d+, presence of circulating antidonor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries

3. **Borderline changes:** 'Suspicious' for acute T-cell mediated rejection (may coincide with categories 2 and 5, and 6)
This category is used when no intimal arteritis is present, but there are foci of tubulitis (t1, t2 or t3) with minor interstitial infiltration (i0 or i1) or interstitial infiltration (i2, i3) with mild (t1) tubulitis

4. **T-cell mediated rejection** (TCMR, may coincide with categories 2 and 5 and 6)

Acute T-cell mediated rejection (Type/Grade:)
IA. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of moderate tubulitis (t2)
IB. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of severe tubulitis (t3)
IIA. Cases with mild to moderate intimal arteritis (v1)
IIB. Cases with severe intimal arteritis comprising >25% of the luminal area (v2)
III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)

Chronic active T-cell mediated rejection
'chronic allograft arteriopathy' (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)

5. **Interstitial fibrosis and tubular atrophy**, no evidence of any specific etiology
(may include nonspecific vascular and glomerular sclerosis, but severity graded by tubulointerstitial features)
Grade
I. Mild interstitial fibrosis and tubular atrophy (<25% of cortical area)
II. Moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area)
III. Severe interstitial fibrosis and tubular atrophy/ loss (>50% of cortical area)

6. **Other**: Changes not considered to be due to rejection- acute and/or chronic (For diagnoses see table 14 in (49); may include isolated
g, cg, or cv lesions and coincide with categories 2, 3, 4, and 5)

### 1.6.3 Accommodation

'Accommodation' in transplantation refers to the absence of effector immune mechanisms despite the presence of antibody against graft antigens. Most extensively studied in the field of xenotransplantation, it was first described clinically following ABO incompatible transplantation [Chopek et al, 1987]. It is proposed that accommodation may occur as consequence of either changes within the graft endothelium or the characteristics of the donor directed antibody. One mechanism by which the former may occur is by the upregulated expression of protective genes; A20, Bcl-2, Bcl-xL and heme oxygenase 1 [Bach et al, 1997; Salama et al, 2001].

Whilst the beneficial effect of accommodation in ABOi transplantation is indirectly observed by the good outcomes, HLA incompatible transplantation is not associated with the same success [Montgomery, 2010]. There is however a vital difference between blood group antibodies when compared to HLA antibodies, the former being carbohydrates (as are xenoantigens) whilst the latter are proteins [Rose et al, 2012]. They differ in their immune response, carbohydrates elicit T-cell independent immune responses whilst proteins induce T-cell dependent mechanisms [Rose et al, 2012]. Only one study has demonstrated potential accommodation in response to HLA antibodies in sensitised patients who underwent antibody removal pre-transplant. In a small study of 7 patients, 4 patients had DSA returning post-transplant, with 3 of these patients demonstrating upregulation of Bcl-xL in the absence of acute rejection [Salama et al, 2001]. Further studies are required into the mechanisms of accommodation in HLA incompatible grafts.

# Chapter 2 Materials and methods

## 2.1 Patient selection

Patients studied in the thesis were all transplanted at Imperial College Renal and Transplant Centre between 2005 and 2011. All patients receiving a kidney alone transplant against a negative crossmatch were included. All patients receiving an antibody incompatible graft, which we define as a kidney transplanted against an incompatible blood group or a positive flow or CDC crossmatch were excluded from the analyses. Inclusion criteria for each of the individual studies are defined later in the thesis. The number of patients included in each of the studies differs slightly because of the different timepoints at which the analyses were carried out.

## 2.2 Histocompatibility and Immunogenetics methods

### Crossmatch Techniques

All patients studied were transplanted with a negative T and B cell CDC crossmatch and a negative T cell FCXM performed in the Histocompatibility and Immunogenetics [H&I] laboratory at Imperial NHS Trust. B cell FCXMs are not routinely performed in our laboratory as we have found they lack specificity, similar to the experience of other groups [Delgado et al, 2008; Riethmüller et al, 2010].

### Detection of HLA donor specific antibodies

The presence of anti-HLA antibodies were detected using LABScreen® mixed beads (One lambda, Inc) in the H&I laboratory. Patients with a positive screen had the specificity of their anti-HLA antibody identified using LABScreen® single antigen beads. Each anti-HLA antibody detected by the Luminex fluroanalyzer had its signal intensity recorded [by mean fluorescence index (MFI)]. A MFI of more than 300 was considered positive. In patients who had more than one DSA detected, the individual MFIs of the separate DSAs present were summed for Class I and Class II separately in order to calculate the overall intensity level. Taylor et al. found that summing the MFIs of multiple antibodies improved the predictive value of Luminex [Taylor et al, 2009]. Our laboratory protocol is to type for HLA -A,-B,-Cw,-DRB1, DRB3/B4/B5 and -DQ antigens. Patients are typed for DQA and DP antigens when indicated.

## 2.3 Immunosuppression regimens

All study patients received monoclonal antibody induction with either alemtuzumab [Campath-1H®, Cambridge, MA] 30mg perioperatively or Daclizumab [Zenax®, Roche Inc, NJ] 2mg/kg on day 0 and day 14. Maintenance immunosuppression in the alemtuzumab group was tacrolimus [Prograf®; Astellas Inc, Japan] monotherapy with an aim to achieve a mean trough level of 5-8ng/ml. Daclizumab treated patients received tacrolimus with a mean trough level of 8-12ng/ml along with mycophenolate mofetil [Cellcept®, Roche Inc, NJ] with a mean trough level of 1.2-2.4mg/l by HPLC-MS [High performance liquid chromatography with tandem mass spectrometry].

All patients received a week of steroids starting at the time of transplantation. Our steroid sparing protocol consists of methylprednisolone (500 mg) iv preoperatively, followed by prednisolone 1mg/kg/day (maximum 60 mg/day), reduced to 0.5mg/kg/day (maximum 30mg/kg/day) on day 4, and then discontinued after day 7.

Alemtuzumab [Campath-1H®] is a monoclonal anti-CD52 antibody which is increasingly used as an induction agent in renal transplantation [Sampaio et al, 2009; Cai et al, 2011]. The CD52 antigen is expressed on B and T lymphocytes, natural killer cells, macrophages, monocytes and neutrophils [Hale et al, 1990; Ambrose et al, 2009]. Following administration there is depletion of all affected mononuclear cells, with discordant recovery of the different cell types, T lymphocytes failing to fully replete even after long term follow up [Bloom et al, 2006]. Although initial hypotheses that such radical depletion would enable tolerance were unfounded [Kirk et al, 2003], it has allowed successful transplantation in the setting of minimal immunosuppressive regimens [Calne et al, 1998 and 1999].

It has been reported that alemtuzumab is associated with an excess of antibody mediated rejection [AMR]. However these reports were limited to patients receiving calcineurin inhibitor [CNI] free immunosuppressive regimens [Knechtle et al, 2003; Flechner et al, 2005]. Randomised control trials assessing the efficacy of alemtuzumab and CNI monotherapy have shown results in allograft and rejection free survival that are equivalent to other standard regimens [Margreiter et al, 2008; Vathsala et al 2005; Thomas et al, 2007].

## 2.4 Histopathological methods

All biopsy specimens were examined by light microscopy (haematoxylin and eosin, periodic acid-Schiff, Jones methenamine silver and Sirius Red-elastin van Gieson), and classified by the histopathology department at Imperial NHS Trust using the Banff 07 Classification of Renal Allograft Pathology [Solez et al, 2008], as seen in table 1.2.

Immunohistochemistry for C4d was performed by the histopathology laboratory on paraffin sections from all biopsies, using polyclonal rabbit anti-C4d antibody at 1/40 (Oxford Biosystems, BI-RC4D). The slides were subjected to 20 mins microwave antigen retrieval, then placed on the Biogenex i6000 autostainer. The Biogenex Non-Biotin detection kit was employed. Slides were counterstained with haematoxylin and cover slipped. C4d staining was quantified and classified using the scoring proposed in the Banff 2007 Classification of Renal Allograft Pathology [Solez et al, 2008]. Namely, C4d was classified as negative/minimal [C4d0 and C4d1 with peritubular capillary (PTC) staining of 0% and <10% respectively], focal [C4d2 with PTC staining of 11-50%] or diffuse [C4d3, PTC staining >50%].

Antibody mediated rejection (AMR) was diagnosed according to the Banff criteria (for Type II or Type III AMR – as discussed further in Chapter 5) when either there was focal or diffuse C4d staining or DSA positivity with any of the following histological features; capillary and/or glomerular inflammation or thromboses, in the case of type II AMR and arterial involvement in the case of type III AMR.

## 2.5 Treatment of allograft rejection

Biopsy proven acute cellular rejection was treated with 500mg iv methylprednisolone on 3 consecutive days along with the introduction of mycophenolate mofetil [Cellcept®; Roche, Nutley, NJ] to achieve 12 hour predose mycophenoleic acid levels of 1.2-2.4mg/l measured by HPLC-MS. After intravenous steroids, oral prednisolone was started (30mg/day) and weaned to 10mg by 3 months and continued thereafter. Patients had their tacrolimus dose adjusted if necessary in order to achieve a 12 hour trough level of 8-12 ng/ml. Antibody mediated rejection was treated as above with the addition of 5 to 10 plasma exchanges with an exchange volume of 50mls/kg (maximum of 4.5l) and a total of 2g/kg iv immunoglobulin (Vigam®; Bio Products Laboratory, Hertfordshire, UK) in divided doses for every 5 plasma exchanges.

## 2.6 Statistical Analysis

All statistical analyses in this thesis were performed using Medcalc version 10.4.3. Comparisons of means and frequencies of normally distributed variables were calculated using t-tests and chi-squared/Fisher's exact tests. Survival analysis was determined using Kaplan-Meier plots [Kaplan et al, 1958]. If more than two survival curves are compared the logrank test for trend in survival scores across the groups is performed. Cox proportional regression plots were used for multivariant analyses. A p value of $<0.05$ was deemed statistically significant.

# Chapter 3 Relevance of C4d with minimal histological changes

## 3.1 Introduction

Acute antibody mediated rejection [AMR] occurs as a result of antibodies directed against antigens expressed on endothelial cells, with or without complement activation [Teraskai, 2003; Solez et al, 2008]. The classic histological features of acute AMR include neutrophils or mononuclear cells in the peritubular capillaries, glomerulitis, vascular thrombosis and fibrinoid necrosis [Racusen et al, 2003]. Peritubular capillary C4d deposition is a marker of antibody mediated injury [Feucht et al, 1991; Feucht et al, 1993]. The Banff criteria for acute AMR were first established in 2001 and included a category comprising the presence of C4d deposition, evidence of circulating donor specific antibodies [DSAs] with morphological evidence of acute tissue injury [Racusen et al, 2003]. The category of "acute tissue injury" includes acute tubular necrosis (ATN) with no other features (acute or chronic) of antibody mediated damage. The prognosis of C4d positive/DSA positive ATN without other features of rejection in antibody compatible allografts is not well established. Certainly it is accepted that C4d positivity with no histological findings of rejection is not associated with adverse outcomes in ABO incompatible grafts and furthermore it has been shown in that setting to be a protective finding at least in the short term [Haas eta l, 2006; Hass et al, 2009; Haas, 2010].

There are no published studies which have looked specifically at the prognostic significance of indicative biopsies in antibody compatible renal allografts showing C4d positivity and ATN only, incorporated with DSA data. The purpose of this study was to determine the relevance of C4d+ATN in terms of subsequent likelihood of developing morphological features of antibody mediated injury, allograft dysfunction or allograft failure.

## 3.2 Methods

### Patients

All indicative renal allograft biopsies of patients transplanted at Imperial College Renal and Transplant Centre between October 2005 and January 2011 were retrospectively studied. All biopsies with a histological diagnosis of ATN without prior rejection and subdivided patients on the basis of C4d positivity were identified. Patients with more than one biopsy with the described histology had their index biopsy defined in the C4d+ group as the biopsy with the strongest C4d staining and in the C4d- group as the first biopsy after transplant. The medical records of patients were then reviewed in order to clarify that there was no enhancement of immunosuppression following the biopsy until the end point was reached. As a result 7 patients were excluded as they subsequently received iv Ig for pyelonephritis or BK nephropathy on further biopsy and 1 patient went on to receive plasma exchange for recurrent FSGS. 48 patients were identified who were C4d+ with a histological diagnosis of ATN, the control group was the 123 patients who had ATN but were C4d negative. The baseline patient demographics are shown in table 3.1.

Histological and immunological screening methods are as described in chapter 2. Table 3.2 shows the distribution by focal or diffuse C4d staining of the patients studied.

**Table 3.1 Table showing patient demographics**

| | | C4d+ [N=48] | C4d- [N=123] | p value |
|---|---|---|---|---|
| **Gender** | Male<br>Female | 36<br>12 | 97<br>26 | 0.73 |
| **Age at transplant** | Years | 50.28 ± 13.40 | 47.94 ± 12.30 | 0.46 |
| **Ethnicity** | Indoasian<br>Afrocarribean<br>Caucasian<br>Other | 15<br>5<br>26<br>2 | 44<br>19<br>55<br>5 | 0.69 |
| **Cause of ESRF** | Diabetes<br>APKD<br>GN<br>Urological<br>Other<br>Unknown | 8<br>7<br>15<br>2<br>2<br>14 | 28<br>11<br>31<br>7<br>5<br>41 | 0.71 |
| **Type of graft** | DD<br>LD<br>SPK | 30<br>17<br>1 | 68<br>45<br>10 | 0.97 |
| **Graft number** | 1<br>≥2 | 44<br>4 | 108<br>15 | 0.59 |
| **Sensitisation** | Non-sensitised<br>Sensitised<br>Preformed DSA | 36<br>11<br>1 | 92<br>19<br>12 | 0.15 |
| **HLA mismatch** | Mean | 2.96 ± 1.73 | 3.64 ± 1.57 | 0.22 |
| **Induction** | Alemtuzumab<br>Daclizumab | 39<br>9 | 102<br>21 | 0.97 |

**Table 3.2 Distribution of C4d scoring in C4d positive patients**

| C4d scoring | % PTC staining | Number of patients [n=48 (%)] |
|---|---|---|
| **C4d2** | 11-50% | 39 (81.3) |
| **C4d3** | >50% | 9 (18.8) |

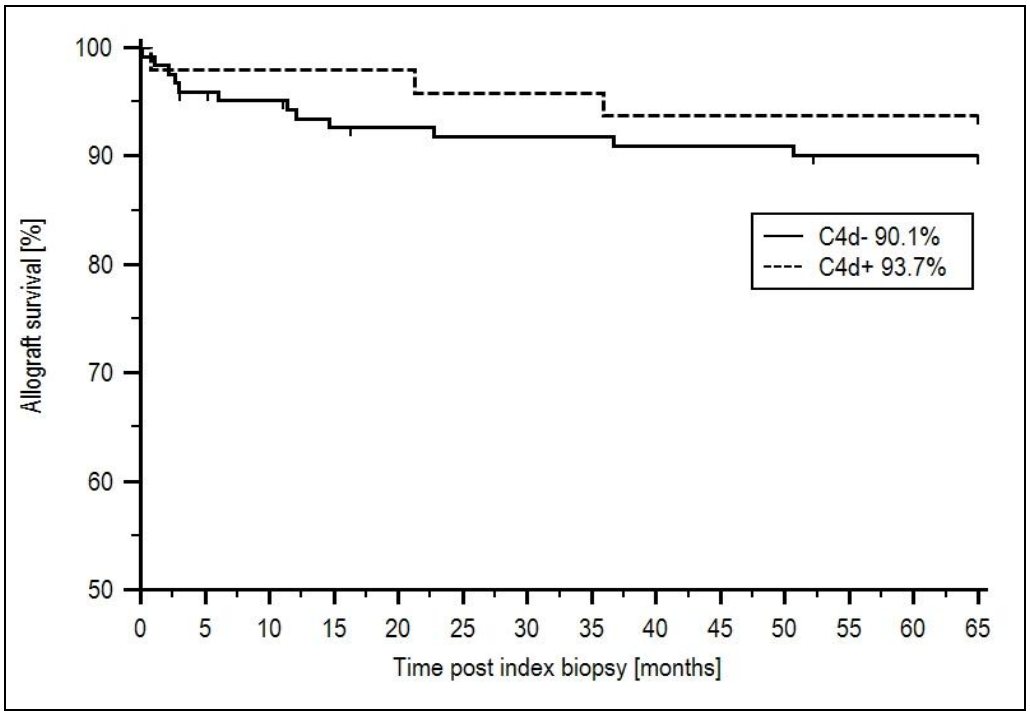
## 3.3 Results

171/800 [21.37%] patients were found to have at least one indicative biopsy with a histological diagnosis of ATN. Of these, 48 [26.97%] were found to have at least one biopsy with C4d positive (focal or diffuse) ATN, whilst 123 [71.93%] patients had C4d negative ATN only. The mean time to index biopsy after transplantation was 4.51 ± 8.36 months, with no difference in timing between the C4d positive and negative groups, p=0.75. The median follow up after the index biopsy was 30.38 ± 15.62 months.

### Patient and allograft survival

There was no difference in patient survival between the two groups. There were 5 patient deaths in the C4d- group and 1 death in the C4d+ group, p=0.52. Figure 3.1 shows a Kaplan Meier curve demonstrating no difference in death censored allograft survival between the C4d+ and C4d- groups, which was 93.7% and 90.1% respectively, p=0.45. At the end of follow up 3 grafts were lost in the C4d+ group compared with 12 in the C4d- group. The causes of allograft failure in the C4d+ group were non-compliance, rejection and progressive scarring in a marginal donor organ. In the C4d- group, 4 of the grafts were lost as a consequence of rejection, one complicated by macrovascular compromise. The other causes of failure were BK nephropathy, renal vein thrombosis, ureteric leak, immunosuppressive withdrawal, haemorrhage after renal biopsy and progressive scarring of unknown aetiology.

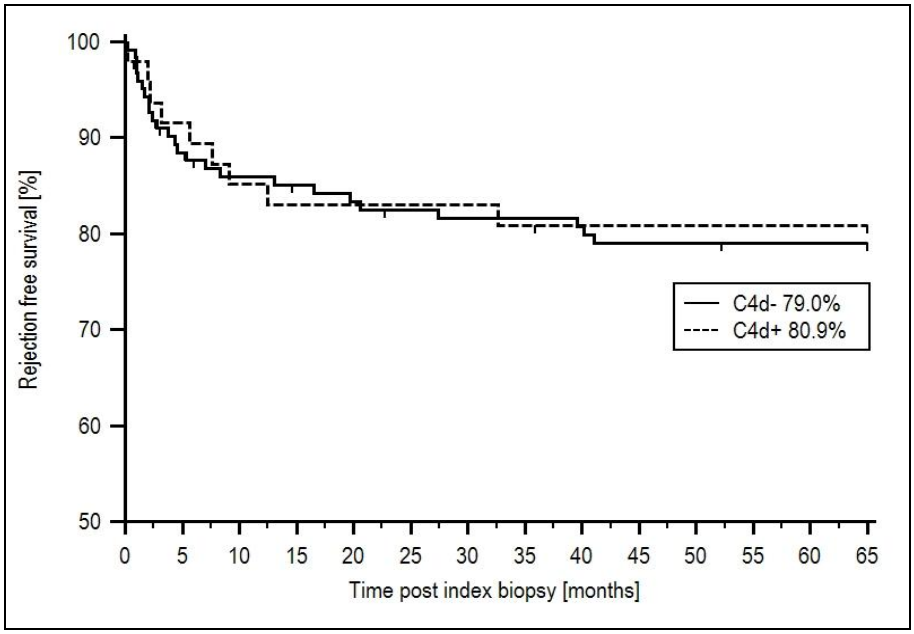
**<u>Figure 3.1</u> Allograft survival by C4d staining.** There was no difference in allograft loss between the C4d+ and C4d- groups, 93.7% and 90.1% respectively, p=0.45.




**Rejection free survival**

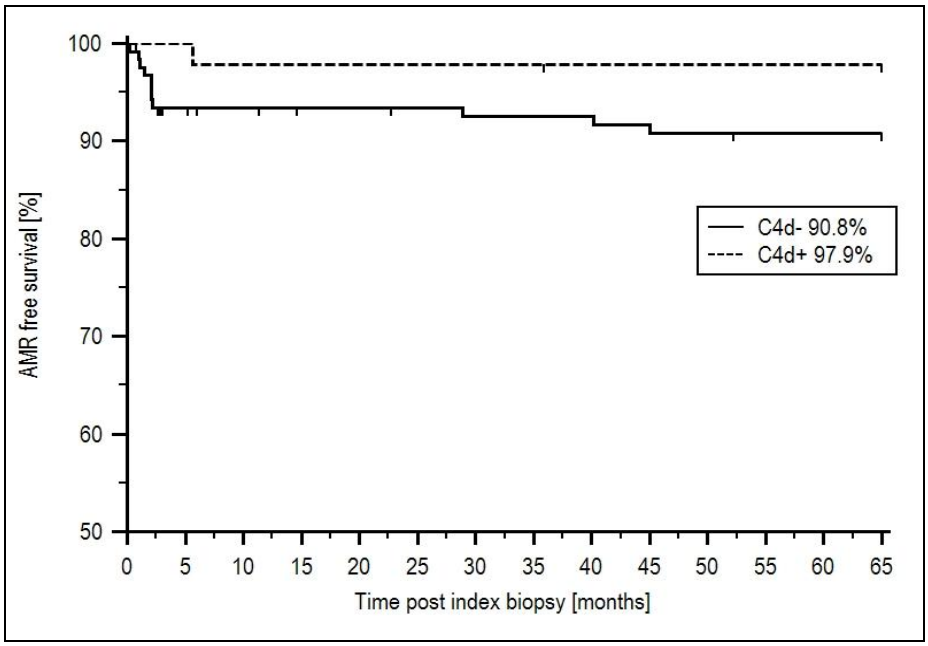
Analysing all rejection types, 9 patients in the C4d+ group experienced acute rejection after the index biopsy compared with 25 in the C4d- group, p=0.80 as shown in figure 3.2. 1/9 [11.1%] of diffuse C4d+ patients had rejection which was comparable with 8/39 [20.5%] of the focal C4d+ group, p=0.86. When considering episodes of rejection by cellular and antibody components, again there was no significant difference. ACR free survival was 82.8% and 83.0% in the C4d- and C4d+ groups respectively, p=0.97. Whilst considering antibody mediated rejection, 11 of the C4d- group subsequently developed biopsy proven AMR compared with only 1 in the C4d+ group, p=0.11, as shown in figure 3.3. There was no difference in the development of transplant glomerulopathy between the two groups at the end of follow up, p=0.13, as shown in figure 3.4.

**<u>Figure 3.2</u> Rejection free survival by C4d status at time of index biopsy demonstrating ATN only**. There was no difference in all rejection episodes between the C4d+ and C4d- groups, 80.9% and 79.0% respectively, p=0.80.



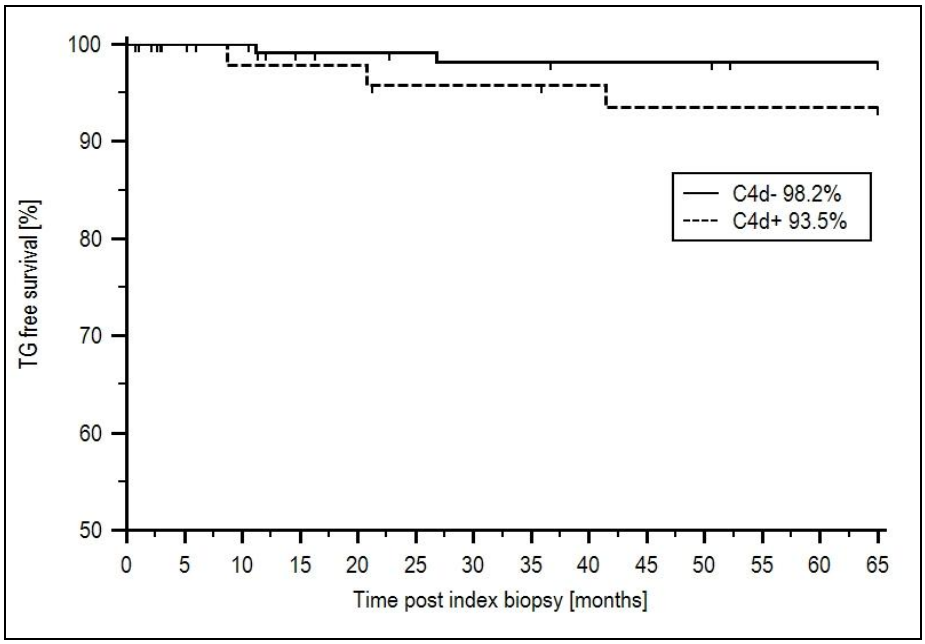

**<u>Figure 3.3</u> AMR free survival post index biopsy of C4d positive ATN.**

There was no difference in subsequent AMR between the C4d+ and C4d- groups, 97.9% and 90.8% respectively, p=0.11.



**<u>Figure 3.4</u> Transplant glomerulopathy free survival post index biopsy showing C4d positive ATN.**

There was no difference in subsequent TG between the C4d+ and C4d- groups, 93.5% and 98.2% respectively, p=0.13.




**Allograft function**

At the time of index biopsy allograft function as determined by serum creatinine in the C4d- and C4d+ groups was 257.0 ± 172.8 µmol/l and 245.7 ± 179.5 µmol/l respectively, p=0.70. Function was compared at 1, 3, 6, 9 and 12months after the index biopsy as shown in figure 3.5. At no time point during the 12 months after the index biopsy was there a significant difference between the groups.

**Focal versus Diffuse C4d**

Analysis was then repeated in order to determine if there was an effect of focal when compared with diffuse C4d staining. There was no significant difference in terms of allograft survival, rejection, AMR or TG between the focal and diffuse groups as seen in figure 3.6.

**Figure 3.5. Comparison of allograft function by serum creatinine [µmol/l]**

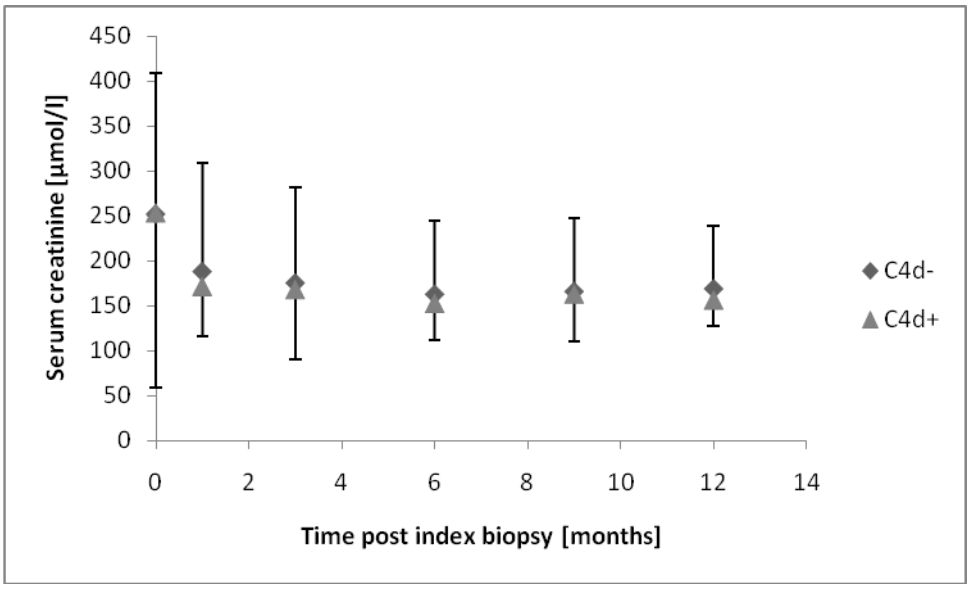
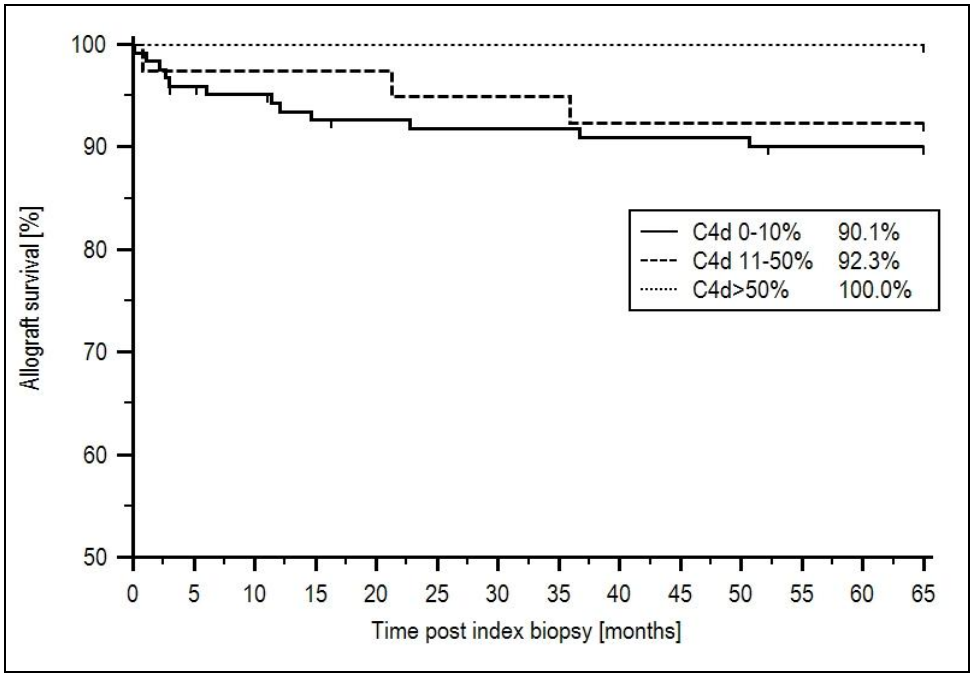
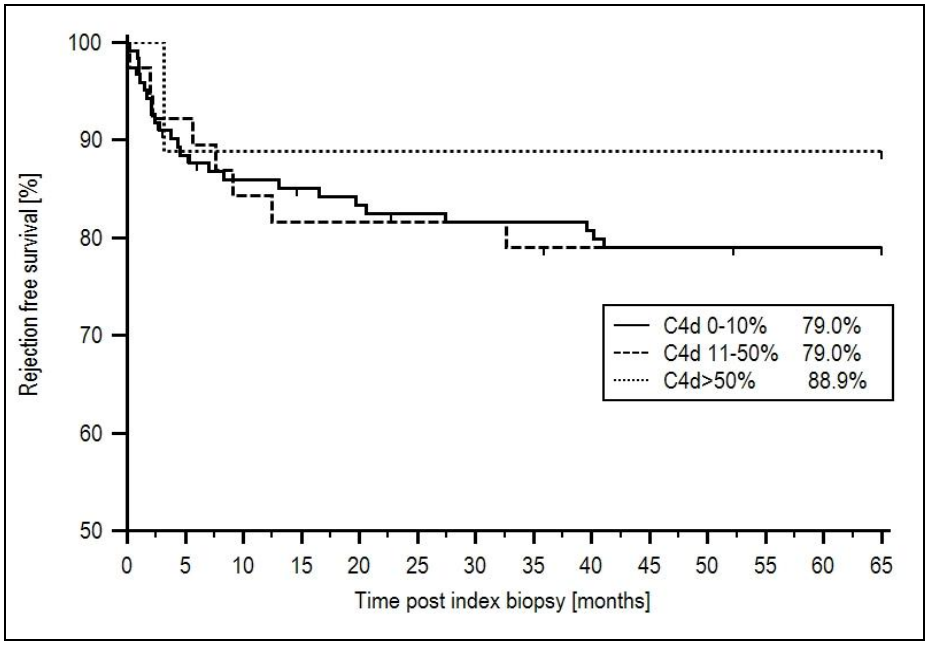



**Figure 3.6 Allograft outcomes by C4d score**

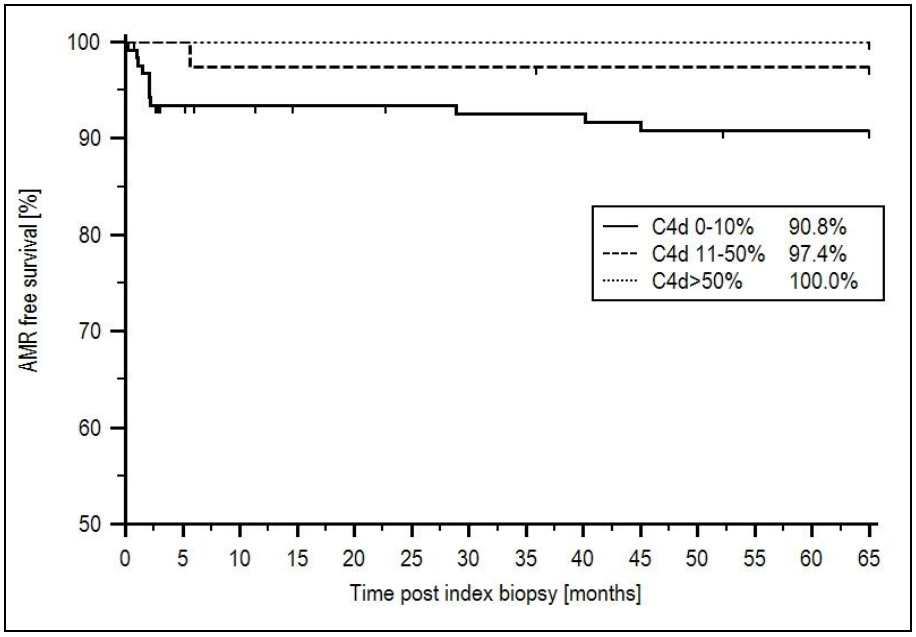
**3.6.1 Allograft survival by C4d score.** There was no difference in allograft loss between the diffuse C4d+, focal C4d+ and C4d- groups at 100.0%, 92.3% and 90.1% respectively, p=0.58.



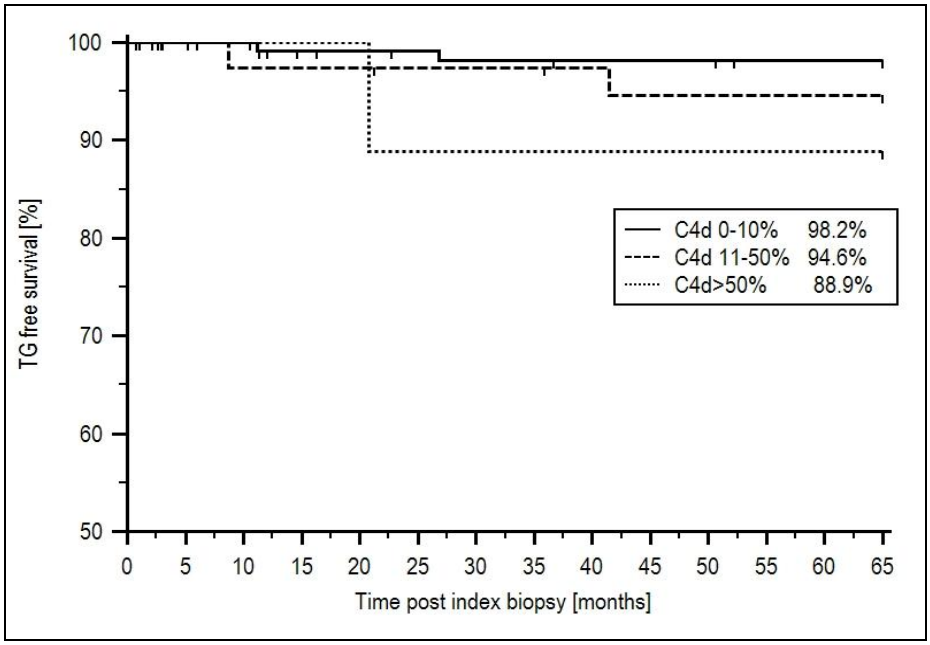
**3.6.2 Rejection free survival post index biopsy by C4d score.** There was no difference in all rejection episodes between the diffuse C4d+, focal C4d+ and C4d- groups at 88.9%, 79.0% and 79.0% respectively, p=0.79.




**3.6.3 AMR free survival post index biopsy by C4d score.** There was no difference in subsequent AMR between the diffuse C4d+, focal C4d+ and C4d- groups at 100.0%, 97.4% and 90.8% respectively, p=0.28.



**3.6.4 Transplant glomerulopathy free survival post index biopsy.** There was no difference in subsequent TG between the diffuse C4d+, focal C4d+ and C4d- groups, 88.9%, 94.6% and 98.2% respectively, p=0.21.




**Influence of donor specific antibodies on outcomes**

**Association of C4d and DSA**

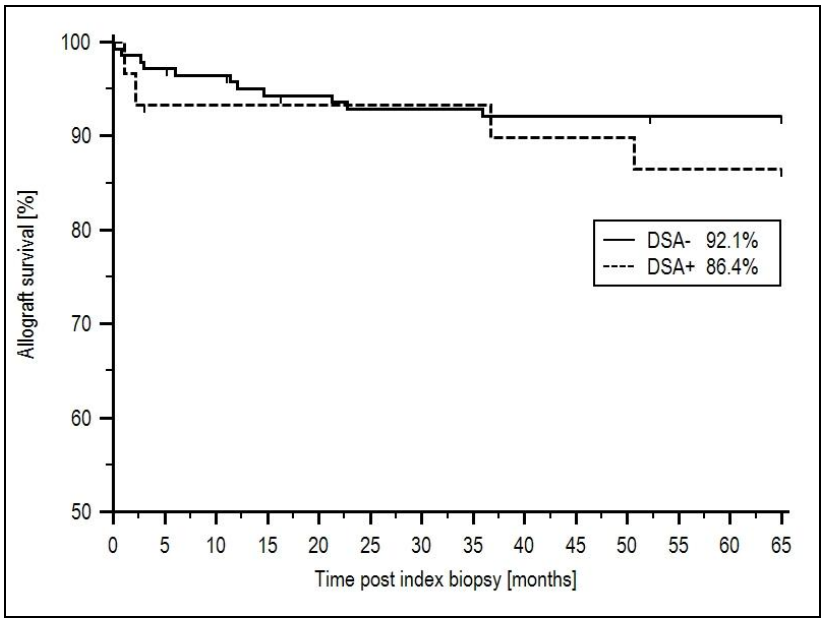
22/123 [17.9%] of the C4d- patients compared with 8/48 [16.6%] of C4d+ patients had DSA at the time of index biopsy, p=0.97. Looking at the association between C4d scoring and DSA, 4/9 [44.4%] of diffusely C4d+ patients had DSA compared with 4/39 [10.3%] of focally positive patients. Therefore, there was a significant association between ATN with diffuse C4d and detectable DSA, p=0.03.

**Outcomes by DSA positivity alone**

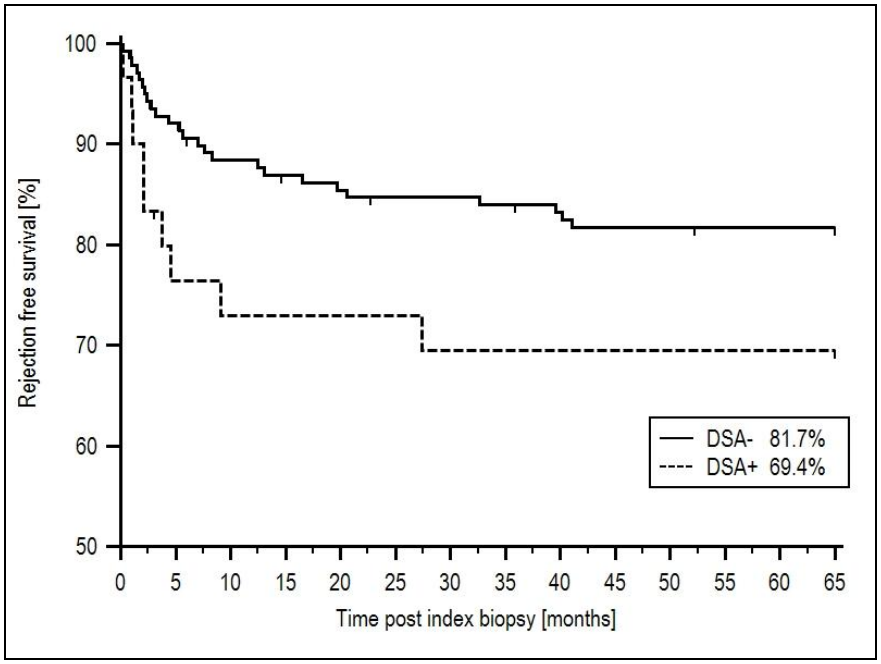
Considering the DSA status at the time of index biopsy alone, there was no difference in allograft loss, however there was a tendency to increased incidence of rejection with a significant risk of subsequent AMR in the DSA+ group as shown in figure 3.7.

**Figure 3.7 Allograft outcomes by DSA status**

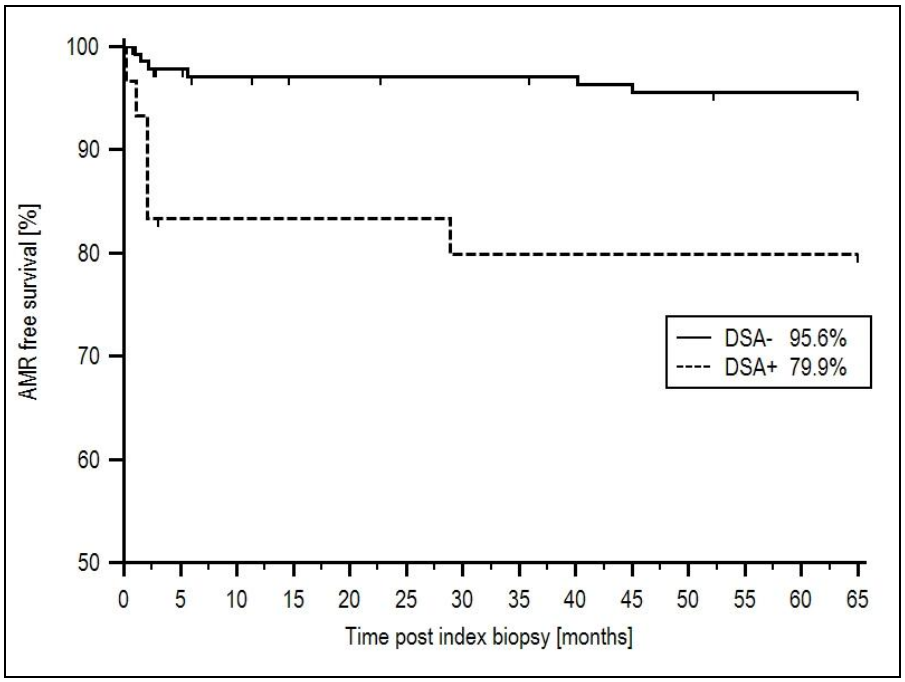
**3.7.1 Allograft survival by DSA status at the time of index biopsy.** There was no difference in allograft loss between the DSA+ and DSA- groups with a 86.4% and 92.1% allograft survival respectively, p=0.33.



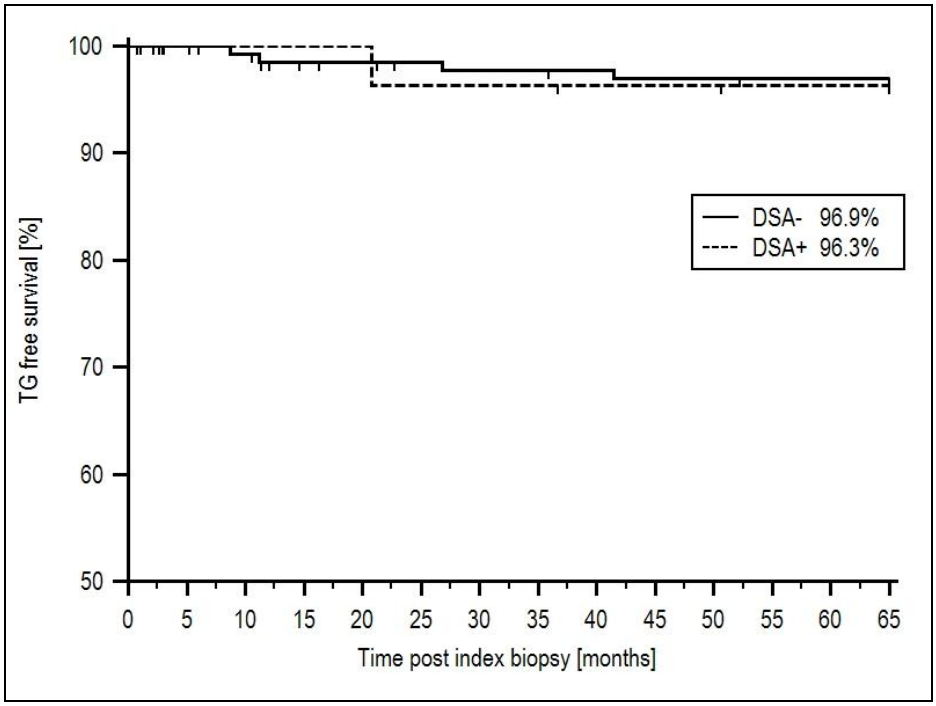

**3.7.2 Rejection free survival by DSA status at the time of index biopsy.** There was a tendency to increased rejection episodes in the DSA+ group compared with the DSA- group with a 69.4% and 81.7% rejection free survival, p=0.093.



**3.7.3 AMR free survival by DSA status at the time of index biopsy.** DSA+ patients are at higher risk of subsequent AMR than patients with no DSA at the time of index biopsy, with an AMR free survival of 79.9% and 95.6% respectively, p=0.0016.




**3.7.4 Transplant glomerulopathy free survival post index biopsy by DSA status.** There was no difference in TG free survival which was 96.3% and 96.9% in the DSA+ and DSA- groups respectively at the end of follow up, p=0.21.



**Outcomes of C4d positive ATN with DSA**

The final analysis involved a comparison of the outcomes of patients with C4d+DSA+ATN compared with C4d+DSA-ATN or C4d-DSA+ATN in those patients with morphological features of ATN alone on allograft biopsy.

101 patients were C4d-/DSA-, 22 patients were C4d-/DSA+, 40 patients were C4d+/DSA- and 8 patients were C4d+/DSA+ at the time of index biopsy.

**Risk of allograft failure**

There was not a significant difference in allograft loss between the different groups as shown in figure 3.8.1. Allograft survival being 92.0%, 81.3%, 92.5% and 100.0% in the C4d-/DSA-, C4d-/DSA+, C4d+/DSA- and C4d+/DSA+ groups respectively, p=0.32.

**Risk of subsequent rejection**

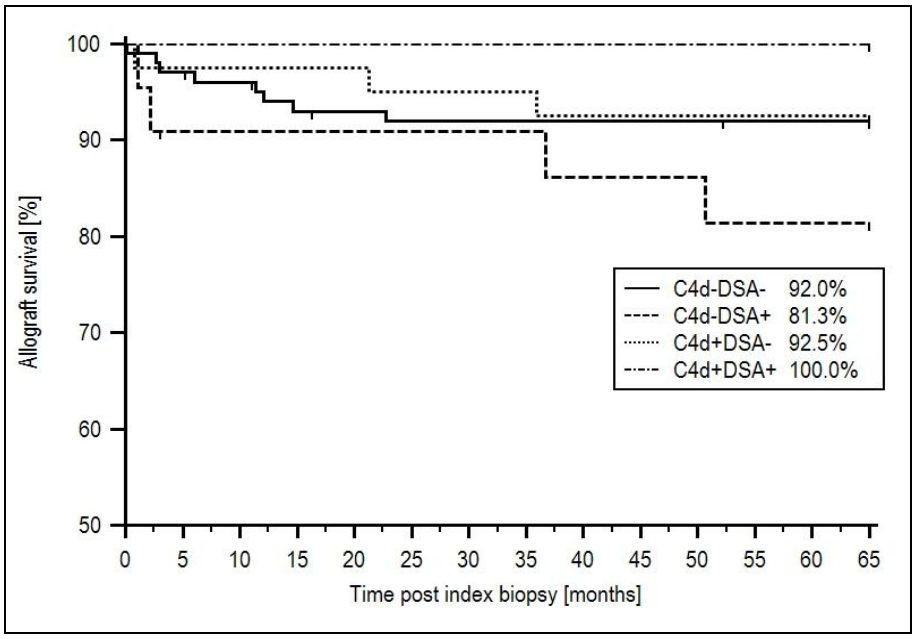
Only the C4d-/DSA+ group showed a tendency to increased rejection as shown in table 3.3. Whilst there was no difference in the occurrence of ACR as seen in figure 3.8.2, with an ACR free survival of 83.5%, 79.5%, 82.0% and 87.5% [p=0.94], C4d-/DSA+ patients were at significant risk of AMR as seen in figure 3.8.3, p=0.0004.

**Table 3.3 Rejection free survival by C4d and DSA status**

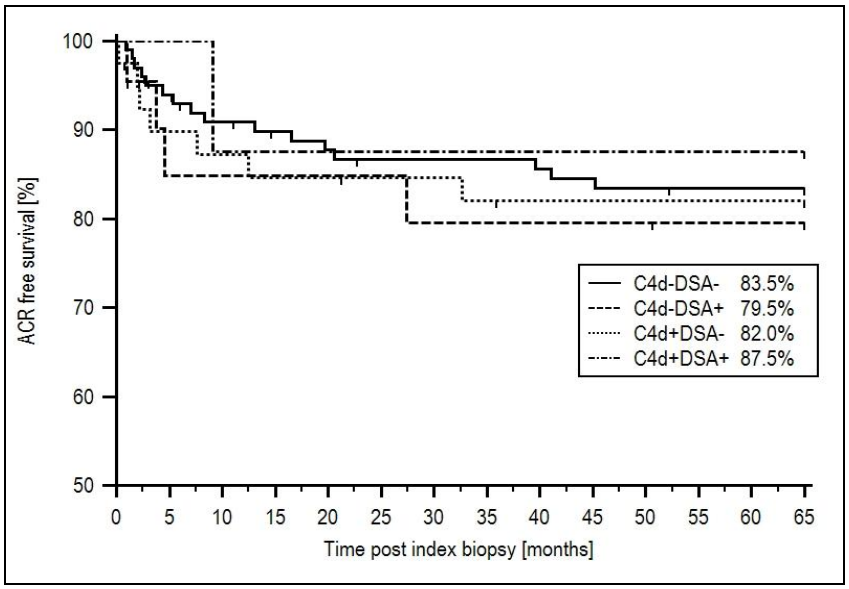
| Group | Rejection free survival [%] | p value |
|---|---|---|
| C4d-DSA- | 82.5% | 0.11 |
| C4d-DSA+ | 62.8% | |
| C4D+DSA- | 79.5% | |
| C4D+DSA+ | 87.5% | |

**Figure 3.8 Allograft outcomes by C4d and DSA status**

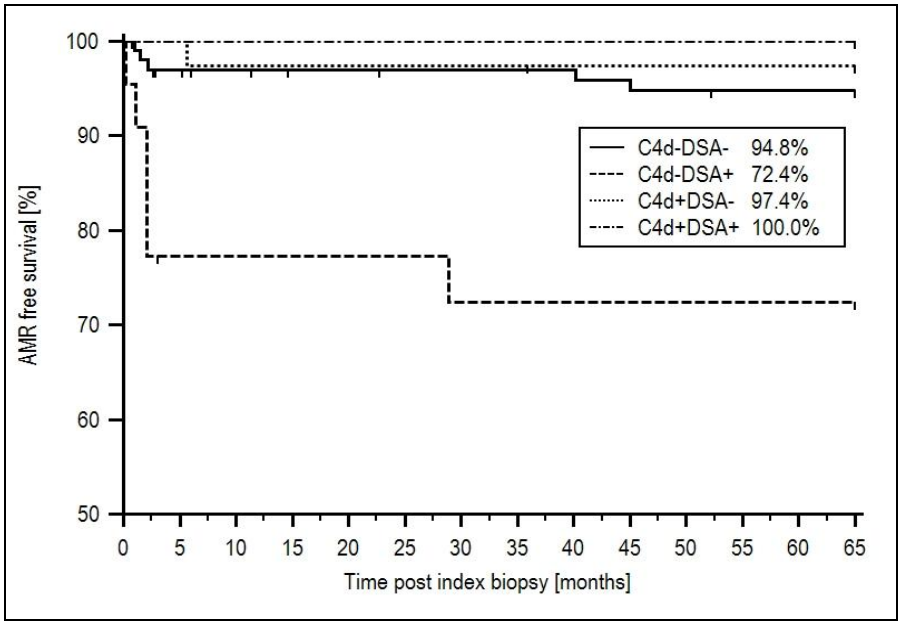
**3.8.1 Allograft survival by C4d and DSA status at the time of index biopsy.** There was no difference in allograft loss between the C4d-/DSA-, C4d-/DSA+, C4d+/DSA- and C4d+/DSA+ groups with a 92.0%, 81.3%, 92.5% and 100.0% graft survival, p=0.32.



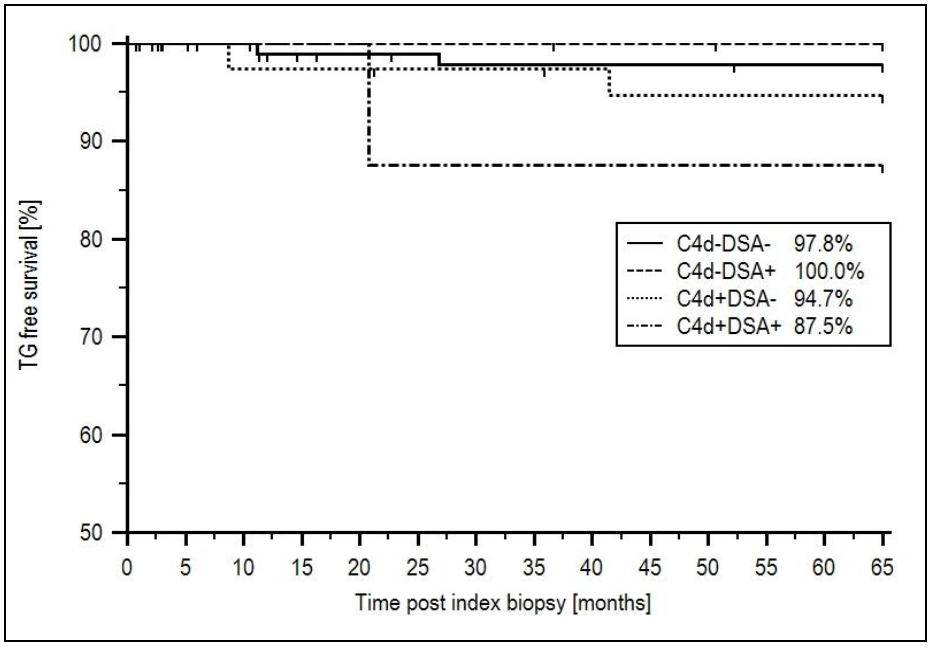

**3.8.2 ACR free survival by C4d and DSA status at the time of index biopsy.** ACR free survival in the C4d-/DSA-, C4d-/DSA+, C4d+/DSA- and C4d+/DSA+ groups was 83.5%, 79.5%, 82.0% and 87.5% respectively, p=0.11.



**3.8.3 AMR free survival post index biopsy by C4d and DSA status at time of index biopsy.** AMR free survival in the C4d-/DSA-, C4d-/DSA+, C4d+/DSA- and C4d+/DSA+ groups was 94.8%, 72.4%, 97.4% and 100.0% respectively, p=0.0004.




**3.8.4 Transplant glomerulopathy free survival post index biopsy by C4d and DSA status**. There was no difference in TG free survival which was 100.0%, 97.8%, 94.7% and 87.5% in the C4d-/DSA-, C4d-/DSA+, C4d+/DSA- and C4d+/DSA+ groups respectively, p=0.29.



**Risk of transplant glomerulopathy**

At the end of follow up, there was no trend in any group towards progression to TG (the apparent difference in the C4d+/DSA+ group [n=8] is only a single case). It will be seen in section 5.3 that AMR itself carries a significant risk of subsequent TG.

By Cox regression analysis, the DSA status at the time of index biopsy, regardless of C4d status is associated with subsequent AMR with typical morphological features, p=0.007.

## 3.4 Discussion

Evidence of the outcomes of patients with C4d positive ATN who have not received augmented immunosuppressive therapy is lacking and a retrospective study was undertaken to determine the natural history of such patients. It was found that C4d+ ATN was not associated with progression to AMR with the classical histological features of rejection when left untreated. However, patients with DSA+ ATN are at increased risk of AMR, with C4d- patients being at greatest risk. Focal C4d+ ATN was not associated with DSA however C4d was associated with DSA when co-existing with morphological features of rejection (data not shown in this chapter). This study has highlighted some important findings which have considerable management implications. Further studies are required in order to reproduce these findings which might prevent patients receiving an inappropriate immunosuppressive burden involved in the treatment of AMR.

There is a large evidence base on the relevance of C4d staining in renal allograft biopsies, however the majority of C4d deposition occurs in renal allografts with co-existing classical features of rejection [Murata et al, 2009; Kayler et al, 2008; Coillins et al, 1999; Satoskar et al, 2008]. The clinical significance of C4d positivity in biopsies otherwise showing a histological diagnosis of ATN only has not been studied in depth. Such biopsies have been included as a subgroup in a few studies, which includes one by Mauyyedi and colleagues, who amongst their analysis of 20 patients with diffuse C4d staining found 2 patients with a histological diagnosis of ATN [Mauyyedi et al, 2002]. Only one of these patients subsequently developed features of AMR. Lederer's group in their study of the relevance of C4d found 19 patients with ATN of which 11 had focal or diffuse C4d but they did not

address the outcomes in these patients [Lederer et al, 2001]. Nickelait and colleagues included in their analysis 11 patients with ATN of which 3 were C4d positive which was too small a number to determine significance; however they did highlight overall a group of C4d positive patients with histological findings other than rejection who had good outcomes despite not receiving augmented therapy [Nickelait et al, 2002]. Finally, from the same group Dickenmann went on to look at 22 patients with C4d positive ATN and found that left untreated there was no rapid progression to conventional histology associated with AMR, although intervening by enhancing the immunosuppression in some resulted in improvement of allograft function [Dickenmann et al, 2006]. Once again the number studied was small and the major limitation of the study was the lack of DSA data. One study looking at patients receiving HLA incompatible grafts also showed that patients with indicative allograft biopsies demonstrating C4d positivity with minimal histological changes had superior outcomes when compared with those with classical morphological features of rejection [Rafiq et al, 2009].

A caveat of the interpretation of C4d positivity, regardless of the histopathological features, will depend upon the method of staining used, with immunofluorescence having a higher sensitivity than immunoperoxidase [Solez et al, 2008]. Controversy also exists between the relevance of focal C4d when compared with diffuse C4d staining. However there have been two studies which have looked at the outcomes of focal C4d determined by immunoperoxidase. They both showed that focal C4d was associated with circulating DSAs with one also showing a negative impact on allograft survival [Kayler et al, 2008; Haririan et al, 2009]. This latter study by Haririan and colleagues also showed that DSA status independent of C4d staining predicted allograft survival [Haririan et al, 2009] .

It should also be considered that ATN, a common histological finding especially in the early transplant period, is associated with inferior allograft outcomes in its own right [Gwinner et al, 2008]. This is thought to be due in part to the increased risk of acute rejection in histologically proven ATN. However ATN without subsequent rejection does not result in inferior allograft outcomes [Lederer et al, 2001]. Halloran et al. proposed the mechanism behind this association is that the ‘injury response’ triggers the ‘immune response’. It has been demonstrated that ischaemic injury leads to upregulation of MHC expression and an immune response [Halloran et al, 1997]. In cardiac transplantation, C4d is often seen early after transplant and is associated with ischaemic reperfusion injury and the diagnosis of AMR in cardiac allografts requires morphological features of rejection [Baldwin et al, 1999; Fedson et al, 2008; Moseley et al, 2010].

An intriguing finding in the study is that C4d-DSA+ ATN patients are at greatest risk of subsequent AMR. This might be explained by considering the postulated stages of AMR described by Colvin [Colvin, 2007] and shown in figure 3.9. Circulating alloantibody is considered the first stage of AMR and only when the antibody forms a complex with its endothelial target is complement activated and the 'footprint marker' C4d detectable. So the 'C4d-DSA+' status might represent an early stage of AMR and such patients require close monitoring with repeat allograft biopsy if there is no improvement clinically.

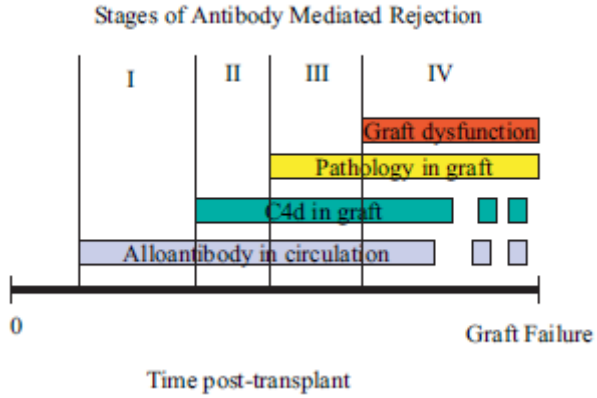



**Figure 3.9** Postulated stages of antibody mediated rejection. [Taken from Colvin, 2007]

Whilst these findings suggest that the presence of C4d staining alone does not predict acute antibody mediated rejection, the risk of chronic antibody mediated damage is not clear, especially in those patients with documented donor specific antibodies, chronic antibody mediated rejection being recognised as one of the leading causes of allograft loss [El-Zoghby et al, 2009]. However even with chronic antibody mediated rejection the relevance of C4d positivity has also recently been questioned. Gloor and colleagues reported that in a study of 55 patients with transplant glomerulopathy (TG), less than a third were C4d positive, despite a strong association between the development of TG and class II anti-HLA [Gloor et al, 2007]. More recently Einecke et al. studied the phenotype of patients undergoing indication biopsies for allograft dysfunction and concluded that the presence of histological changes such as glomerulitis, capillaritis and glomerulopathy, along with anti-HLA antibodies predicited graft loss, whereas C4d staining did not [Einecke et al, 2009]. The same group also hypothesised that antibody mediated damage against antigens on the endothelium could alter gene expression, so they studied endothelial cell transcriptome expression using

microarrays and found that higher scores were significantly associated with the presence of donor specific antibody and morphological AMR whilst only 40% of these grafts were C4d+[Sis et al, 2009].

The limitations of this study are its retrospective nature, the relatively short term follow up and the lack of electron microscopic [EM] findings. Further follow up of these patients will be required to assess the long term outcomes. A multicentre prospective study of C4d positive ATN would be the most appropriate next step to establish the true significance of such lesions. However, until further evidence is available this study shows that, in the absence of morphological features of rejection by light microscopy (with or without DSA), the presence of C4d staining does not predicts progression to the classical histopathological findings of acute AMR despite unaltered immunosuppression.

## Chapter 4 Relevance of patients with preformed donor specific antibodies detected with single antigen beads in the setting of a negative crossmatch

### 4.1 Introduction

The development of single antigen bead assays has facilitated the identification of anti-HLA antibodies in sera with high sensitivity and specificity [El-Awar et al, 2005; Pei et al, 2003; Zachary et al, 2008]. Although previous reports suggested that these solid phase technologies are too sensitive for routine clinical use [Gupta et al, 2008; Vaughan et al, 2008; Patel et al, 2007], there are now an increasing number of studies demonstrating that, even in the circumstance of negative complement dependent cytotoxic [CDC] and flow cytometry crossmatches [FCXM], preformed donor specific antibodies are associated with inferior renal allograft outcomes [Patel et al, 2007; Amico et al, 2009; Gibney et al, 2006]. However there is no general consensus as to how these patients are best managed. The recently issued British Society of Histocompatibility and Immunogenetics/British Transplant Society [BSHI/BTS] guidelines state they should be managed no differently from other low risk patients, with no augmentation of immunotherapy [bshi.org.uk/BTS/BSHI]. This reflects the continued controversy over the clinical relevance of DSAs detected by solid phase techniques only, which is shared by transplant immunologists worldwide [Opelz et al, 2009].

From the literarture there are no studies describing the risk of preformed DSAs in patients receiving antibody induction and tacrolimus monotherapy without the use of mycophenolate mofetil or more than one week of corticosteroids. Evidence published to date is taken from protocols using standard triple immunosuppressive regimens, with and without the use of induction agents [Gupta et al, 2008; Vaughan et al, 2008; Patel et al, 2007; Amico et al, 2009]. The use of induction agents in renal transplantation has been shown to reduce the incidence of acute rejection, with T cells being the primary target for commonly used polyclonal and monoclonal antibodies [Nashan et al, 1997; Charpentier et al, 2003; Mourad et al, 2001]. There is now increasing evidence that B cells play a pivotal role in graft rejection [Zarkhin et al, 2008; Tarlinton et al, 2008]. However a randomised control trial using B cell directed induction therapy alone had to be prematurely terminated due to high rates of rejection [Clatworthy et al, 2009] and it is now believed that allograft survival is dependent upon both T and B cell immunomodulation [Parsons et al, 2009].

Alemtuzumab is a humanised anti-CD52 monoclonal antibody that has a rapid and profound depleting effect on both T and B lymphocytes [Bloom et al, 2006] (although not plasma cells), which may provide some protection in the context of crossmatch negative preformed DSAs. Alemtuzumab has been shown to be an effective induction agent in low risk patients [Calnet et al, 1998]. Furthermore, in high immunological risk patients, a randomised control trial comparing alemtuzumab against thymoglobulin induction showed equivalent outcomes [Thomas et al, 2007]. Vo and colleagues also demonstrated that alemtuzumab induction could be used in conjunction with desensitisation therapy in highly sensitised patients with excellent allograft survival and function [Vo et al, 2008]. Contradicting these outcomes are reports of an increased risk of antibody mediated rejection (AMR) in patients receiving alemtuzumab (22,23,24), particularly in the absence of a calcineurin inhibitor as maintenance immunosuppression. This described risk of AMR prompts questions about the role of identifiable DSAs.

The aim of this study was to investigate the clinical relevance of preformed DSAs in patients with a negative CDC and FCXM receiving tacrolimus maintenance monotherapy with alemtuzumab induction.

## 4.2 Methods

480 patients who received a kidney alone transplant between 2005 and 2009 were analysed retrospectively. All patients had a negative crossmatch and received alemtuzumab induction. Baseline demographics are shown in table 4.1. Histological and immunological methods are described in chapter 2.

**Table 4.1 Patient demographics**

| | DSA+ n=45 (%) | DSA- n=435 (%) | p value |
|---|---|---|---|
| **Gender** | | | |
| **Female** | 24 (53.3%) | 141(32.4%) | p=0.008 |
| **Male** | 21 (46.7%) | 294 (67.6%) | |
| **Age at transplant (years)** | 47.15 ±11.85 | 47.44 ±13.14 | NS* |
| **Type of graft** | | | |
| **DD†** | 17 (37.8%) | 196 (45.1%) | NS |
| **LD‡** | 25 (55.6%) | 197 (45.3%) | |
| **SPK** | 3 (6.7%) | 42 (9.7%) | |
| **Mean HLA mismatch** | 3.96 ± 1.33 | 3.26 ± 1.64 | p<0.01 |
| **HLA mismatch summary¶** | | | |
| **000** | 0 (0%) | 42 (9.7%) | 0.02 |
| **0DR and 0/1B** | 3 (0.7%) | 52 (12.0%) | NS |
| **[0DR and 2B] or [1DR and 0/1B]** | 18 (40%) | 172 (39.5%) | NS |
| **[1DR and 2B] or [2DR]** | 24 (53.3%) | 169 (38.9%) | NS |
| **Graft number** | | | |
| **1st** | 26 (57.8%) | 399 (91.7%) | p<0.0001 |
| **2nd** | 15 (33.3%) | 32 (7.4%) | |
| **3rd** | 4 (8.9%) | 4 (0.9%) | |
| **Ethnicity** | | | |
| **Caucasian** | 19 (42.2%) | 219 (50.3%) | NS |
| **Asian** | 16 (35.6%) | 145 (33.3%) | |
| **Black** | 7 (15.6%) | 55 (12.6%) | |
| **Other** | 3 (6.7%) | 16 (3.7%) | |
| **Mean follow up (years)** | 1.89 ± 0.94 | 1.96 ± 0.97 | NS |

†DD deceased donor, ‡LD living donor

*NS= p>0.05

¶ NHS Blood and Transplant mismatch level

## 4.3 Results

DSAs were found in 45/480 [9.4%] of patients at the time of transplantation. A further 75/435 [17.2%] were HLA sensitised and had non-donor specific anti-HLA antibody detected pre-transplantation. 24/45 [53.3%] of DSA+ patients were female compared with 141/435 [32.4%] of the DSA- group [p=0.008]. 19/45 [42.2%] of patients with preformed DSAs were receiving a regraft compared with 36/435 [8.3%] of DSA- patients [p<0.0001]. There was no difference in DSA status between patients receiving transplants from live donors and from deceased donors. Overall the mean HLA mismatch was higher in the patient group with preformed DSAs [3.96 ± 1.33 and 3.26 ± 1.64 in the DSA+ and DSA- group respectively, p<0.01]. Table 3.1 shows that 42/435 [9.7%] of the DSA- group had a 000 mismatch compared with 0/45 [0%] of the DSA+ group, p=0.02. A higher proportion of DSA+ patients [24/45 (53.3%)] had a non-favourable mismatch, which is classified as a [1DR and 2B] or [2DR] mismatch by the NHS Blood and Transplant organ allocation criteria in the UK [www.organdonation.nhs.uk], compared with 169/435 [38.9%] of DSA- patients, although this failed to reach statistical significance [p=0.08].

Twenty three patients [23/45 (51.1%)] had HLA class I [CI] DSAs alone, 14 patients [14/45 (31.1%)] had class II [CII] DSAs alone and 8 patients [8/45 (17.8%)] had both class I and II [CI+CII] DSAs. Twenty nine patients [29/45 (64.4%)] had DSAs with a summed MFI > 1000 (see section 2.2 in chapter 2).

**Table 4.2 List of the specificities of the DSA and corresponding MFI**

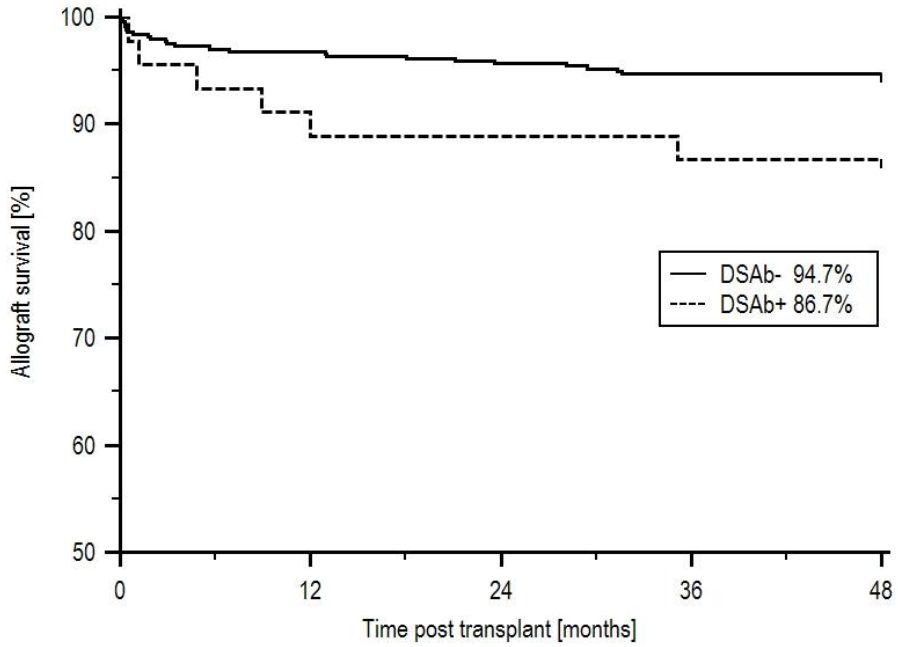
| Patients with Class I DSAs alone [n=23] | | Patients with Class II DSAs alone [n=14] | |
|---|---|---|---|
| **Specificities** | **MFI** | **Specificities** | **MFI** |
| **Cw5** | 4680 | **DQ7** | 1723 |
| **Cw1** | 979 | **DQ8, DQ7** | 2349, 600 |
| **Cw7** | 1174 | **DR53, DQ5** | 2200, 2300 |
| **B35, B7, A3** | 2600, 841, 300 | **DQ6** | 2472 |
| **B57** | 591 | **DQ8** | 1018 |
| **A2** | 800 | **DR53, DQ2, DQ7** | 3943, 700, 370 |
| **A2, Cw1** | 1750, 1312 | **DR1, DQ5** | 1605, 2297 |
| **B55, A3** | 1000, 900 | **DR12** | 2240 |
| **B37** | 549 | **DQ2** | 1581 |
| **B35** | 3200 | **DQ5** | 6500 |
| **A30, A2** | 2256, 1000 | **DQ7** | 906 |
| **B27** | 927 | **DR1, DR51** | 2000, 4454 |
| **B53** | 756 | **DQ6** | 900 |
| **A11,A29,B44,Cw12,Cw16** | 2000,1000,1000,3500, 3500 | **DQ6** | 772 |
| **B62** | 430 | **Patients with both CI and CII DSAs [n=8]** | |
| **A11** | 500 | | |
| **A2** | 364 | **A31, DQ2** | 1616, 300 |
| **A26** | 600 | **A11, A33, DQ9** | 1300, 1200, 500 |
| **B41** | 900 | **B60, DQ7** | 1166, 2549 |
| **Cw5** | 1000 | **A24, DR11, DQ7** | 800, 4540, 2500 |
| **B7** | 900 | **A2,Cw10,Cw5,DQ7,DQ5** | 600,600,550,9955,2374 |
| **A26** | 3237 | **A2, B52, DR17, DR52** | 2900, 450, 3084, 547 |
| **B62, A2, B37** | 1136, 750, 380 | **Cw1, DR13, DQ6** | 3300, 2088, 4806 |
| | | **A30, DR52, DQ6** | 4200, 750, 370 |

**Patient and allograft survival**

Overall patient survival at 12 and 36 months in the DSA negative and positive groups was similar at 98.2% and 97.5% in the DSA- and 97.8% and 97.8% in the DSA+ groups respectively [p=0.89 (log rank)].

Allograft survival however was significantly inferior in the DSA+ group [p=0.047 (logrank)] as shown in Figure 4.1. Six DSA+ patients (6/45 [13.3%]) lost their grafts and five of these were as a result of rejection. 1 was lost due to donor related factors (the graft was received from a marginal donor). Twenty five DSA- patients (25/435 [5.7%]) lost their grafts. Twelve of these patients (12/25 [48.0%]) lost their graft as a consequence of rejection and eight of these patients had developed de novo DSA after transplantation. 13 grafts were lost due to a combination of surgical complications (2), donor related factors (4), renal artery stenosis (1), renal vein thrombosis (2), recurrent disease (2), pyelonephritis (1), haemorrhage after biopsy (1) and non-compliance (1).

**Figure 4.1 Comparison of allograft survival in DSA positive versus DSA negative patients.** Kaplan-Meier curve demonstrating allograft survival in the DSA+ and DSA- groups. At 12 and 36 months allograft survival was 91.1% and 86.7% (DSA+) and 96.3% and 94.3% (DSA-) respectively [p=0.047 (logrank)].

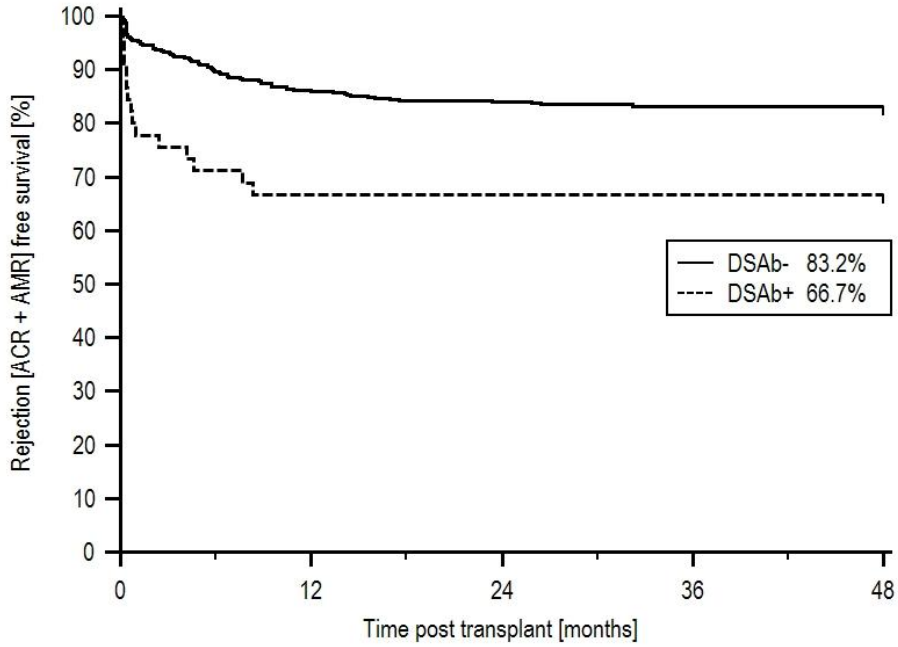


**Rejection Episodes**

The incidence of rejection in the DSA+ patients was significantly higher than in DSA- patients.

Acute rejection [ACR and AMR] occurred in fifteen DSA+ patients (15/45 [33.3%]) and seventy seven DSA- patients (77/435 [17.7%], p=0.019), [figure 4.2]. AMR was significantly more frequent in patients who were DSA+ at time of transplant [p<0.0001 (logrank)] as shown in figure 4.3. Twelve DSA+ patients (12/45 [26.7%]) compared with thirty eight DSA- patients (38/435 [8.7%]) developed AMR. On multivariant analysis there was no association of AMR with patients receiving regrafts [p=0.81] or a non-favourable mismatch [p=0.96], whereas the presence of preformed DSA was associated with development of AMR [p=0.006]. The mean time to the episode of AMR in patients with preformed DSAs was 7.4 ± 7.0 days which was earlier than the AMR occurring in DSA- patients [mean 198.6 ± 286.1 days, p=0.03]. The histological features and subsequent outcomes of the AMR episodes in the DSA+ patients are shown in table 4.3. 7/45 [15.6%] of DSA+ patients proceeded to develop TG compared with 12/435 [2.8%] of the DSA- patients, p<0.001.

**Figure 4.2 Rejection (ACR and AMR) free survival by DSA status.** Kaplan-Meier curve showing all rejection episodes. Patients with preformed DSAs had a rejection free survival of 66.7% compared with 82.3% in DSA- patients, p=0.0028.



**<u>Figure 4.3</u> Antibody mediated rejection free survival by DSA status.**

Kaplan-Meier curves comparing AMR free survival in DSA+ and DSA- groups. At 1 month AMR free survival equals 80.0% and 96.3% in the DSA+ and DSA- patients respectively, [p=0.0002 (log rank)].

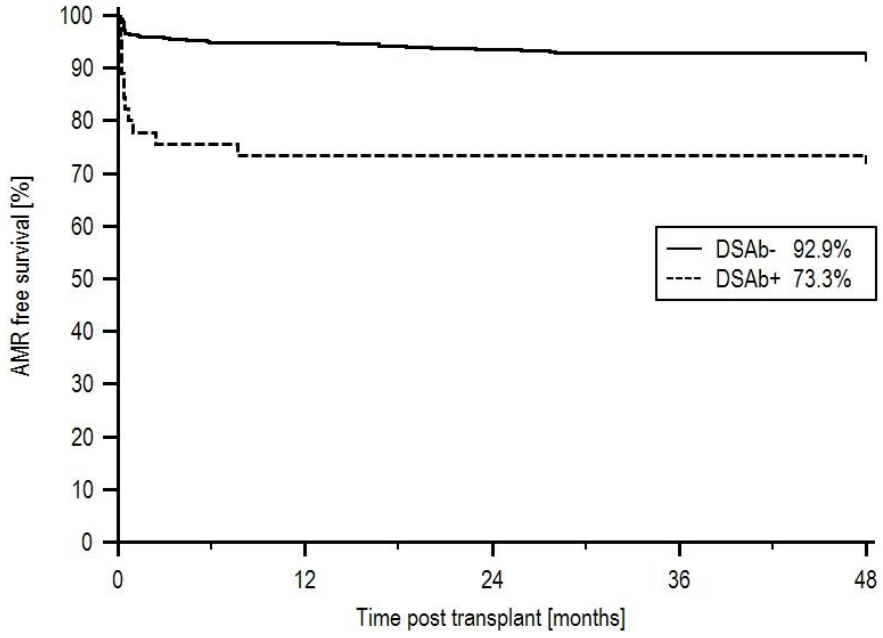


**Table 4.3 Histological features of AMR by DSA specificities and associated outcomes.**

| Preformed DSA specificities | Preformed DSA MFIs | De novo DSA specificites | Histology¤ | C4d | TG§ | Allograft loss |
|---|---|---|---|---|---|---|
| **A31, DQ2** | 1616, 300 | No | AMR II | Diffuse | Y | Y |
| **A11, DQ9** | 1300, 500 | A33 | AMR I | Focal | N | N |
| **B53** | 3200 | Cw4 | AMR II | Diffuse | N | N |
| **A30, A2** | 2256, 1000 | DQ4, DQ2 | AMR II | Diffuse | Y | N |
| **B7** | 927 | A1, DQ7 | AMR II/ACR IIA | Focal | N | N |
| **DQ5, DR1** | 2297, 1605 | A11 | AMR II [suspicious] | Negative | Y | N |
| **A24, DR11, DQ7** | 800, 4540, 2500 | B35, A3, DQ6, DR51 | AMR III | Diffuse | N | Y |
| **B62** | 430 | B44, A11, A2, DQ1, DR51 | AMR I | Diffuse | N | N |
| **A2, B52, DR17, DR52** | 2900, 450, 3084, 547 | No | AMR II | Diffuse | Y | N |
| **Cw1, DQ6, DR13** | 3300, 4806, 2088 | No | AMR II/ACR IB | Diffuse | N | Y |
| **A30, DQ6, DR52** | 4200, 370, 750 | No | AMR II | Diffuse | Y | Y |
| **DQ6** | 772 | No | AMR I | Focal | N | N |

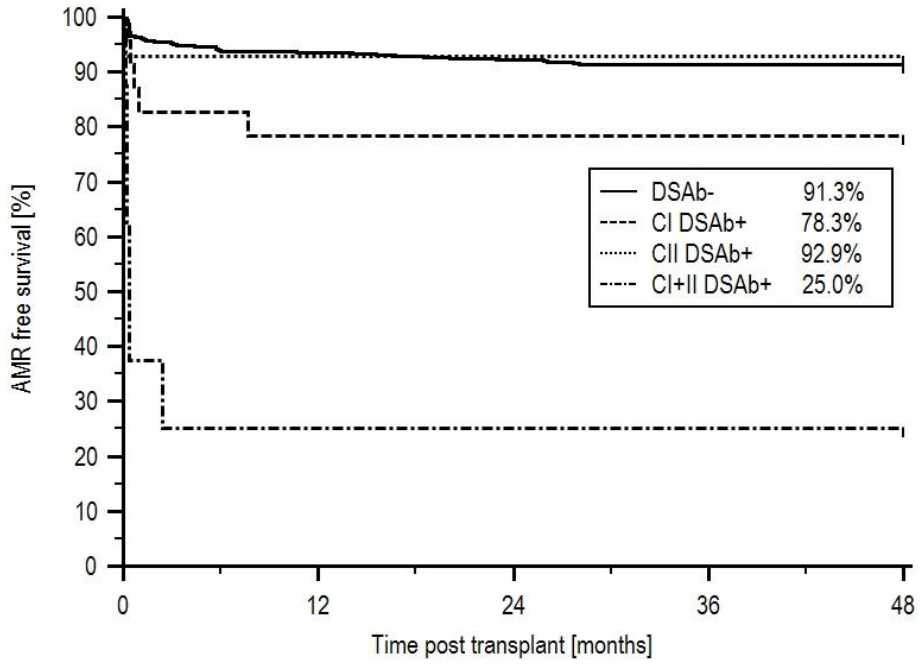
¤Banff 07; AMR I [ATN-like minimal inflammation], AMR II [Capillary ± glomerular inflammation ± thromboses], AMR III [arterial v3]
§ Transplant glomerulopathy

The incidence of ACR was similar in both groups of patients [ACR free survival being 88.9% and 87.8% in the DSA+ and DSA- patients respectively, p=0.86 (logrank)]. There was a significant difference in the absolute lymphocyte count at the time of AMR compared with ACR in both the DSA+ patients [AMR: 0.28 ± 0.21, ACR: 0.77 ± 0.06, p=0.002] and the DSA- patients [AMR: 0.44 ± 0.56, ACR: 0.82 ± 0.57, p=0.02]. However, there was no difference in lymphocyte count between the DSA+ and DSA- groups at the time of ACR [0.77 ± 0.06, 0.82 ± 0.57 respectively, p=0.87] and AMR [0.28 ± 0.21, 0.44 ± 0.56, p=0.35]. There was no difference in the tacrolimus levels at the time of rejection, which were 7.6 ± 4.1 in the DSA+ group, compared with 7.0 ± 3.0 in the DSA- group [p=0.53]. 383/480 [79.6%] of all patients remained on tacrolimus monotherapy at the end of follow up.

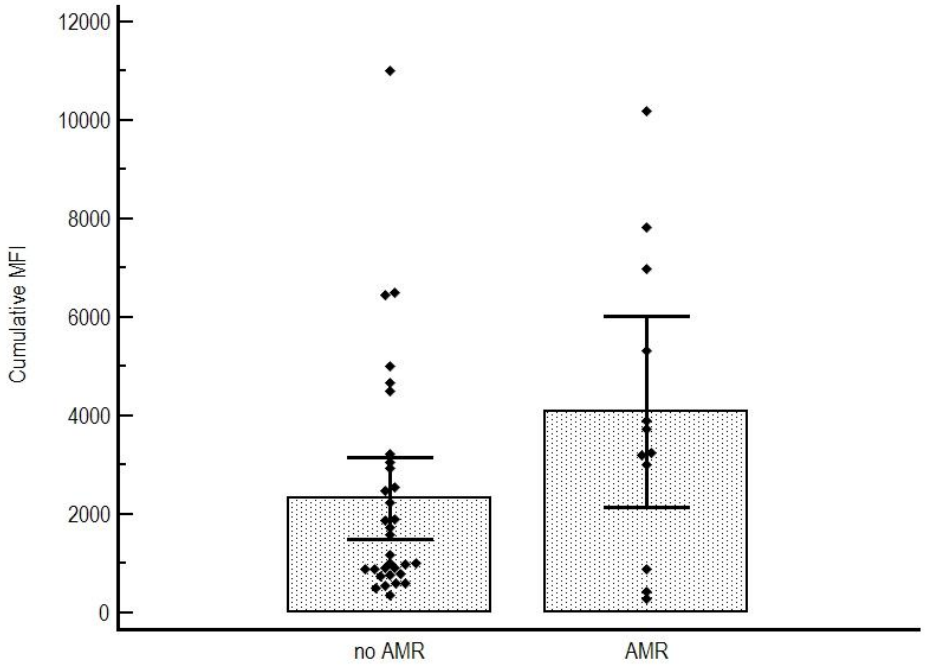
The HLA class of the DSA associated with AMR was analysed. It was found that when compared with the DSA- cohort, only patients with HLA class I (CI) DSA, alone or in combination with class II DSAs (CI+CII) were significantly at risk of acute AMR [Figure 4.4; AMR free survival in the DSA- group was 92.9% compared with 78.3%, 92.9% and 25% in patients with CI DSAs alone, CII DSAs alone or both CI+CII DSAs respectively (p=0.0072, p=0.969 and p<0.0001].

Figure 4.5 shows that patients who developed AMR had a higher mean MFI [4089 ±3045] compared with patients who did not develop AMR [2328 ±2332], p=0.046.

**<u>Figure 4.4</u> AMR free survival according to HLA class of DSA.** Kaplan-Meier curves comparing AMR free survival in patients with no DSA, CI DSA, CII DSA and both CI+CII DSA [92.9%, 78.3%, 92.9% and 25%], with a significance difference of p=0.0072, p=0.9685 and p<0.0001 in CI, CII and CI+CII DSA when compared with DSA- patients.



**<u>Figure 4.5</u> Relationship of MFI to AMR** Patients who developed AMR had a significantly higher MFI than patients who did not have AMR [p=0.046]. The mean MFI in AMR+ patients was 4089 ±3045 and in AMR- patients was 2328 ±2332.

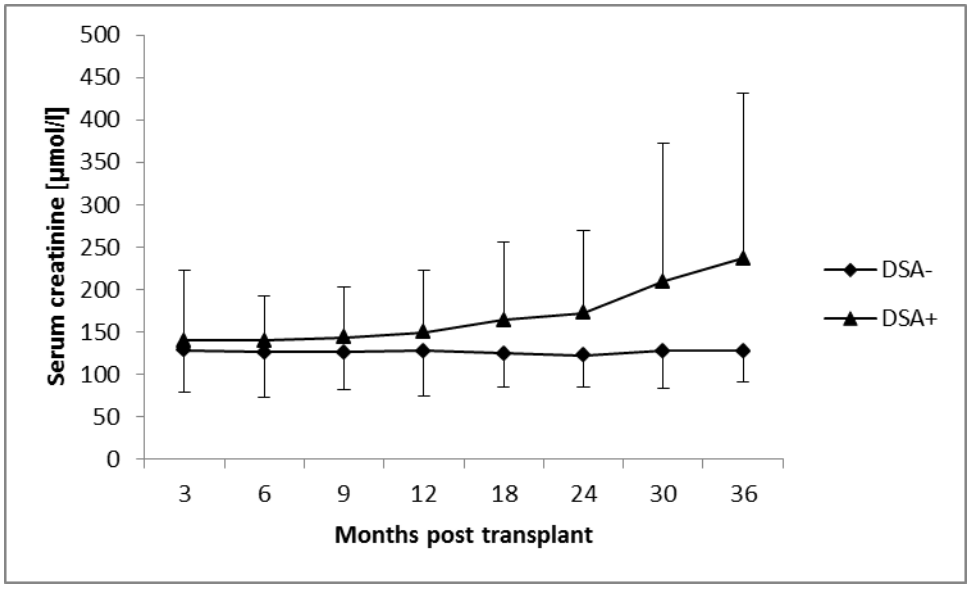



## <u>Allograft Function</u>

Figure 4.6 shows that the mean allograft function was significantly inferior after 12 months after transplant in the DSA+ patients when compared with the DSA- patients.

**Figure 4.6: Comparison of allograft function in DSA+ and DSA- patients.**

Graph showing allograft function post-transplant measured by serum creatinine [µmol/l]. DSA+ patients had significantly inferior function when compared with DSA- patients. Serum creatinine in the DSA+ group was 149.56 ± 72.56, 172.14 ± 97.75 and 236.33 ± 195.43 µmol/l at 1 year, 2 years and 3 years after transplant compared with 127.71 ± 53.82 (p=0.06), 122.02 ± 37.62 (p<0.01) and 127.34 ± 36.14 (p<0.01) µmol/l in the DSA- patients.




**Infection**

There was no difference in the incidence of BK nephropathy between the DSA+ and DSA- groups [3/45 (6.7%) and 12/435 (2.8%),respectively, p=0.16]. Incidence of CMV disease was low at 5/480 [1.0%] overall with no cases in the DSA+ group.

## 4.4 Discussion

This study shows that patients with crossmatch negative preformed DSAs receiving tacrolimus monotherapy following alemtuzumab induction have a high risk of AMR, impaired allograft function and graft loss. The occurrence of a greater degree of HLA mismatch in the DSA+ group presumably reflects the fact that, in patients with HLA antibodies, the greater the degree of mismatch, the more likely it is that the HLA antibody is donor specific. Despite alemtuzumab depleting both B and T cells, patients with DSAs are more likely to have early AMR when compared with those patients with no DSAs at the time of transplant. AMR is more common in patients with class I DSAs, whether alone or particularly when in combination with class II DSAs, and in patients with a higher level of antibody as judged by MFI.

This study is not without precedent in patients receiving conventional immunosuppressive regimens. There is increasing evidence that preformed DSAs detected by single antigen [SAg] beads are associated with inferior allograft outcomes [Gupta et al, 2008; Patel et al, 2007; Amico et al 2009]. Patel et al. demonstrated that the presence of DSAs pre-transplant significantly increased the risk of AMR in their analysis of 20 patients [$p=0.02$] [Patel et al, 2007]. Gupta et al. did not find a significant difference in AMR rates in 16 patients, although there was a higher risk of graft loss in the DSA positive group (relative risk 6.5, $p<0.05$) [Gupta et al, 2008]. Both of these studies included patients receiving triple immunosuppressive regimens and although the clinical relevance of the different regimens cannot be determined by this study, it is an area for consideration in future work. The largest study to date describing the relevance of pre-transplant DSAs was published by Amico et al. who analysed 67 DSA+ patients and found that the rate of rejection in the positive group was 55% [$p<0.0001$] [Amico et al, 2009]. A major caveat to this study is that these patients did not have a flow cytometry crossmatch [FCXM] pre-transplant. T-cell FCXM positive patients have historically been shown to have an increased risk of AMR, although the relevance of a positive B-cell FCXM alone remains controversial [Delgado et al, 2008; Lefaucheur et al, 2009; Amico et al, 2009, Gebel et al, 2003]. SAg bead assays have been shown to compare more closely with the FCXM than the CDC crossmatch in the ability to detect anti-HLA, although SAg bead assays are more sensitive and specific for anti-HLA antibody [Riethmuller et al, 2010; Amico et al, 2009]. This study is the largest to include patients with preformed DSAs not only with a negative CDC, but also a negative T cell FCXM.

Importantly, whilst it was found patients with preformed DSAs had an increased risk of rejection, two-thirds of the patients did not develop clinical evidence of antibody mediated damage at the end of follow up. This suggests that the immunological risk of these crossmatch negative alloantibodies

need to be further refined. Determinants of the pathogenicity of preformed DSAs include the level of antibody, the HLA class and the complement fixing ability, which is largely dependent upon the immunoglobulin class and subclass [Gebel et al, 2003]. It was demonstrated that patients with DSAs with lower MFIs and antibody against HLA class II alone were less likely to develop acute AMR, which would be in keeping with published reports of patients with preformed DSA in the setting of a positive crossmatch [Burns et al, 2008].

Complement fixation has been shown to be associated with AMR and it follows that the presence of preformed DSA able to fix complement, irrelevant of its level, will be associated with increased risk of rejection, which is the basis of the CDC crossmatch [Terasaki, 2003; Patel et al, 1969]. The major drawback of the CDC cytoxicity assay is the relative lack of sensitivity [Lefaucheur et al, 2009]. The flow cytometry crossmatch is more sensitive at detecting alloantibody but fails to differentiate between complement and non-complement fixing antibodies [Garovoy et al, 1983]. Wahrmann et al. have developed a technique for detecting complement fixing alloantibodies using microparticles [Wahrmann et al, 2009; Bohmig et al, 2008]. Applying this method to preformed DSAs may help with the identification of patients at higher immunological risk [Wahrmann et al, 2009]. An indirect approach to establishing the complement fixing ability of these low level DSAs will be to determine the immunoglobulin class or subclass of these DSAs [Bindon et al, 1988; Bartel et al, 2007]. Within the 4 subclasses of IgG, $IgG_3$ is the most effective at fixing complement followed by $IgG_1$ then $IgG_2$ and lastly $IgG_4$ which does not fix complement [Griffiths et al, 2004; Arnold et al, 2008]. It has been shown that $IgG_1$, which is the most common of the subclasses, when present in presensitised patients, is associated with inferior allograft outcomes in those patients who have proportionately higher $IgG_1$ levels than the other subclasses [Griffiths et al, 2004]. Although far less commonly found, $IgG_3$ is only required in extremely low levels to effectively fix complement [Bartel et al, 2007]. It is therefore hypothesised that clarifying immunoglobulin subclass of the DSA will help further quantify risk.

This study shows that patients with crossmatch negative preformed DSAs receiving alemtuzumab induction and tacrolimus monotherapy are at significant risk of developing AMR. This is common to other immunosuppressive regimens and although it is not possible to draw direct comparisons, the use of alemtuzumab certainly does not mitigate the risk involved. Further studies are required in order to define the DSA characteristics associated with immunological pathogenicity as not all patients subsequently developed AMR. However, these results have important management implications having demonstrated that patients with preformed DSAs carry a significant

immunological risk and it is proposed that such patients should be considered for augmented immunosuppression.

# Chapter 5 Antibody mediated rejection: incidence, risk factors and predictors of poor outcome

## 5.1 Introduction

Over the past decade the clinical, serological and pathological diagnosis of antibody mediated injury has been improving. AMR was first included as a distinct entity in the Banff classification as late as 2001 [Racusen et al, 2003]. The current criteria include morphological evidence of tissue injury, C4d positivity and the detection of donor specific antibody [Sis et al, 2010]; this will continue to be adapted with ongoing work into the molecular markers of AMR [Reeve et al, 2009]. The ability to correctly diagnose acute AMR is important in order to deliver appropriate therapy and reduce subsequent allograft loss [Humar et al, 1999; Lefaucheur et al, 2007], acute AMR being a significant risk factor for the development of chronic rejection and TG [El-Zoghby et al, 2009; Issa et al, 2008]. There have been few studies analysing factors predictive of poor outcomes following AMR in immunologically low risk patients.

The aim of this study is to analyse the incidence, time course, risk factors and outcomes of acute AMR in patients receiving a homogenous minimal immunosuppressive regimen. The recognition of factors associated with poor allograft survival will help identify a subgroup of patients who might benefit from novel therapeutic options.

## 5.2 Methods

Immunosuppression regimen, treatment of rejection along with the histological and immunological methods are described in chapter 2.

## 5.3 Results

462 patients were retrospectively studied who received an ABO compatible, crossmatch negative renal transplant. Overall, 93/462 patients [20.1%] experienced rejection, 52/462 [11.3%] developed ACR alone and 41/462 [8.9%] of patients had biopsy proven acute AMR (Type II or III, see table 1.2 and section 2.4). 29/41 [70.7%] of the cases fulfilled the Banff criteria for *Definite AMR* (both C4d and DSA positive), whilst 12/41 [29.3%] were categorised as *Suspicious for AMR* (either C4d+ or DSA+ but not both). The 369/462 [79.9%] patients who remained rejection free [no AMR or ACR] were used as the control group. The baseline demographics are shown in table 5.1, this shows that patients with preformed DSAbs and those receiving a kidney with a higher mean HLA or –DR locus mismatch are at increased risk of developing AMR. 11/41 [26.8%] of AMR patients had preformed DSAbs compared with 33/369 [8.9%] of non rejectors [NR], p=0.0012. The mean HLA mismatch [A,B,DR] in the AMR group was 3.98 ± 1.19 which was significantly higher than the non rejectors who had a mean mismatch of 3.13 ± 1.61, p=0.001.

The median time to rejection in the AMR group was 2.06 months [0.2 - 31.7 months] compared with 5.77 months [0.4-44.57 months] in the patients with ACR, p=0.42. The mean tacrolimus [FK] levels at the time of AMR and ACR were 7.12 ± 2.97 and 6.55 ± 2.30 respectively, p=0.34. The absolute lymphocyte count was significantly higher at the time of ACR [0.8 ± 0.59 x $10^9$/ml] than AMR [0.55 ± 0.53 x $10^9$/ml], p=0.034.

**Table 5.1. Patient demographics**

| | | Non rejectors n=369 (%) | AMR n=41 (%) | ACR n=52 (%) | p value |
|---|---|---|---|---|---|
| **Gender** | Male<br>Female | 239 (64.8)<br>130 (35.2) | 25 (61.0)<br>16 (39.0) | 39 (75.0)<br>13 (25.0) | 0.28 |
| **Age at transplant** | Years | 48.54 ± 13.10 | 48.64 ± 12.11 | 45.64 ± 14.85 | 0.30 |
| **Ethnicity** | Caucasian<br>Asian<br>Afrocarribean<br>Other | 175 (47.4)<br>131 (35.5)<br>47 (12.7)<br>16 (4.3) | 15 (36.6)<br>17 (41.5)<br>6 (14.6)<br>3 (7.3) | 27 (51.9)<br>15 (28.8)<br>6 (11.5)<br>4 (7.7) | 0.68 |
| **Cause ESRF**[§] | Glomerulonephritis<br>Diabetes<br>APKD<br>Urological<br>Other<br>Unknown | 87 (23.6)<br>58 (15.7)<br>55 (14.9)<br>28 (7.6)<br>28 (7.6)<br>113 (30.6) | 11 (26.8)<br>6 (14.6)<br>4 (9.8)<br>3 (7.3)<br>1 (2.4)<br>16 (39.0) | 18 (34.6)<br>6 (11.5)<br>8 (15.4)<br>2 (3.8)<br>4 (7.7}<br>14 (26.9) | 0.73 |
| **Time at ESRF** | Years | 4.45 ± 6.0 | 4.38 ± 3.94 | 4.33 ± 5.56 | 0.99 |
| **Type of graft** | DD*<br>LURT[†]<br>LRT[‡] | 185 (50.1)<br>76 (20.6)<br>108 (29.3) | 23 (56.1.)<br>7 (17.1)<br>11 (26.8) | 27 (51.9)<br>10 (19.2)<br>15 (28.8) | 0.96 |
| **Graft number** | First<br>≥Second | 329 (89.2)<br>40 (10.8) | 33 (80.5)<br>8 (19.5) | 46 (88.5)<br>6 (11.5) | 0.26 |
| **Sensitisation** | Non-sensitised<br>Sensitised<br>Preformed DSAbs | 285 (77.2)<br>51 (13.8)<br>33 (8.9) | 23 (56.1)<br>7 (17.1)<br>11 (26.8) | 37 (71.2)<br>10 (19.2)<br>5 (9.6) | 0.0057 |
| **HLA mismatch** | Mean | 3.13 ± 1.61 | 3.98 ± 1.19 | 3.13 ± 1.84 | 0.0059 |
| **HLA mismatch level**[¶] | 000 mismatch<br>0DR and 0/1B<br>[0DR + 2B] or [1DR + 0/1B]<br>[1DR + 2B] or [2DR] | 38 (10.3)<br>52 (14.1)<br>151 (40.9)<br>128 (34.7) | 0 (0)<br>1 (2.4)<br>20 (48.8)<br>20 (48.8) | 8 (15.4)<br>3 (5.8)<br>21 (40.4)<br>20 (38.5) | 0.025 |
| **Follow up** | Mean | 2.20 ± 1.25 | 2.01 ± 1.17 | 2.36 ± 1.22 | 0.40 |

§ End stage renal failure

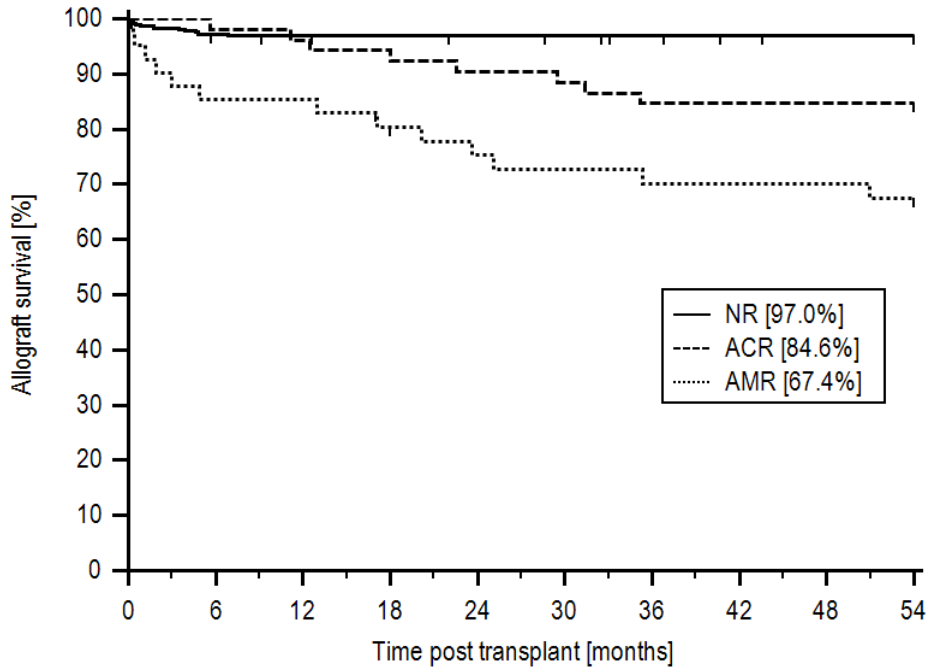
*Deceased donor, †‡ Live related/unrelated renal transplant

¶ HLA mismatch level by NHS Blood and Transplant allocation criteria

There was no difference in patient survival between the non rejectors, AMR group and ACR group, with patient survival being 95.9%, 92.7% and 96.2% respectively, p=0.61. Allograft survival however was inferior in the patients who developed AMR as shown in figure 5.1, when compared with the NR group; allograft survival being 97.0% in the NR group and 67.4% in the AMR group, p<0.0001. Allograft survival in the ACR group was 84.6%, which was not statistically different from allograft survival in the AMR group, p=0.07. Of the 13/41 [31.7%] patients with AMR who lost their grafts; 3 were lost acutely during the first episode of AMR, 4 were lost due to transplant glomerulopathy [TG] and 6 grafts were lost due to resistant or ongoing rejection with or without complications [2 pyelonephritis, 1 renal artery stenosis].

**Figure 5.1 Effect of AMR on allograft survival.**

Comparison of patients with AMR versus those patients with no rejection [NR] and acute cellular rejection only [ACR]. Allograft survival being 97.0%, 84.6% and 67.4% in the NR, ACR and AMR respectively, p<0.001



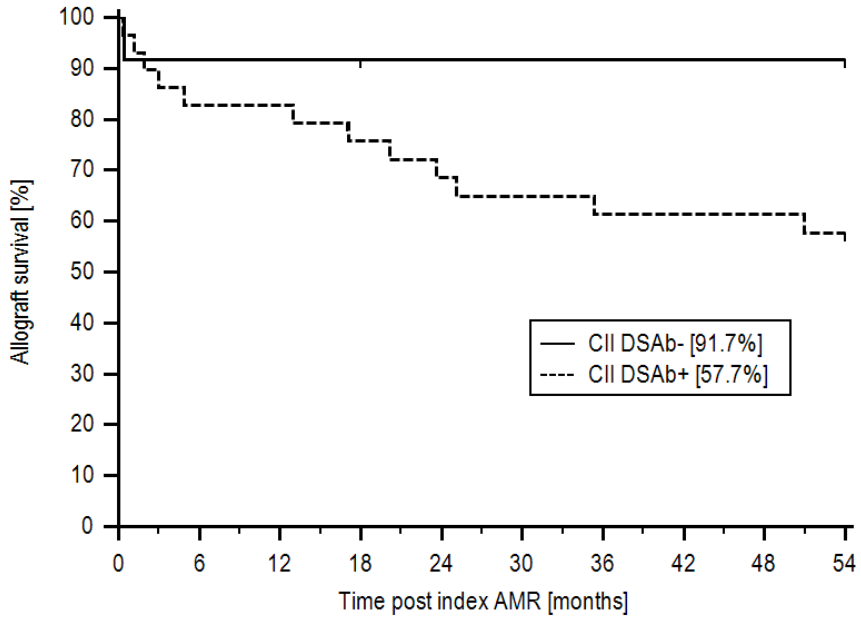
**Anti-HLA DSAs**

There was a highly significant association between the presence of anti-HLA DSAs and the development of acute AMR. 35/41 [85.4%] of patients with acute AMR had detectable DSAs compared with 68/369 [18.4%] of patients who did not develop acute AMR during follow up but who were anti-HLA DSA positive on at least one occasion, OR: 25.82 (10.44-63.84), p<0.0001. 24/35 [68.6%] of DSAb positive AMR cases had developed their DSA de novo after transplantation, 11/24 [45.8%] of these DSA positive cases had their antibody detected at the time of stable allograft function, at a median time of 5.2 months [0.53 – 17.7 months] pre rejection episode. The risk of developing acute AMR following the detection of de novo DSA in the setting of stable function was high [OR: 3.75 (1.61-8.72), p=0.0022].

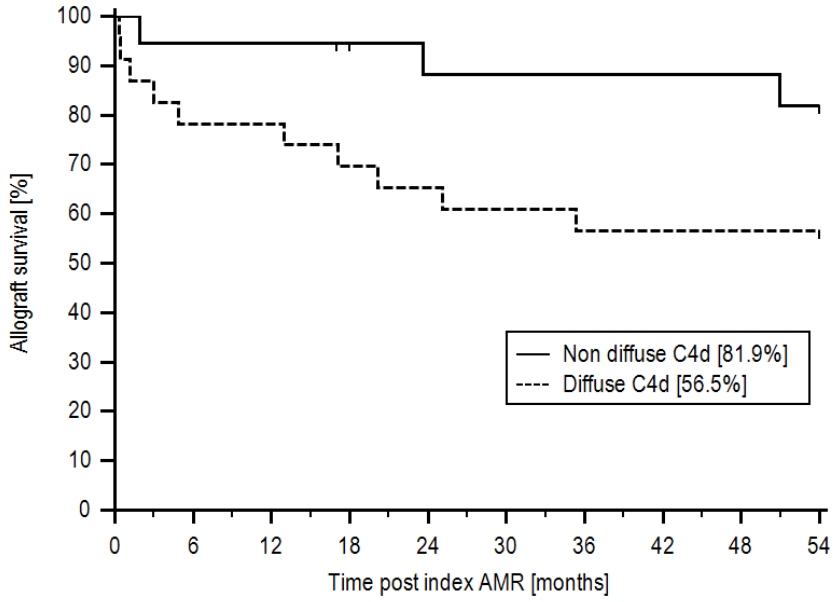
At the time of rejection, 6/41 [14.6%] of patients had no DSA [DSA had become undetectable in one of the patients with preformed DSA], 6/41 [14.6%] had class I [CI] DSA alone, 11/41 [26.8%] had class II [CII] DSA alone and 18/41 [43.9%] had both CI and CII DSAs. Patients with CII DSAs at the time of AMR, whether alone or in combination with CI DSAs were had a tendency to increased risk of graft loss as shown in figure 5.2, p=0.061.

**Figure 5.2. Allograft survival according to diagnostic features.**

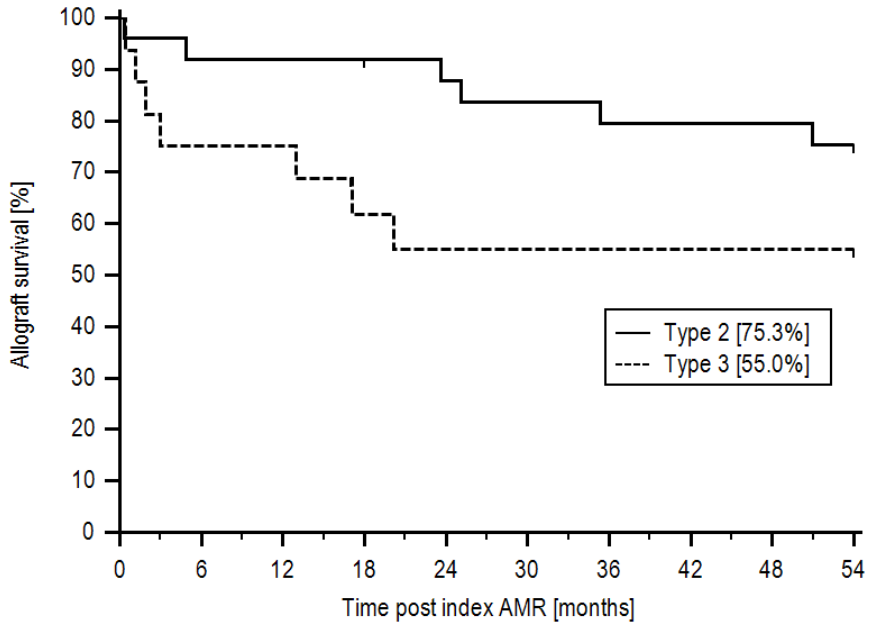
**5.2.1** Allograft survival by presence or absence of class II DSA. Patients with HLA CII DSAs at the time of index biopsy showed a trend to increased risk of graft loss [p=0.061]




**5.2.2** Allograft survival by C4d staining. Patients with diffuse C4d had a tendency to inferior outcome [p=0.07]

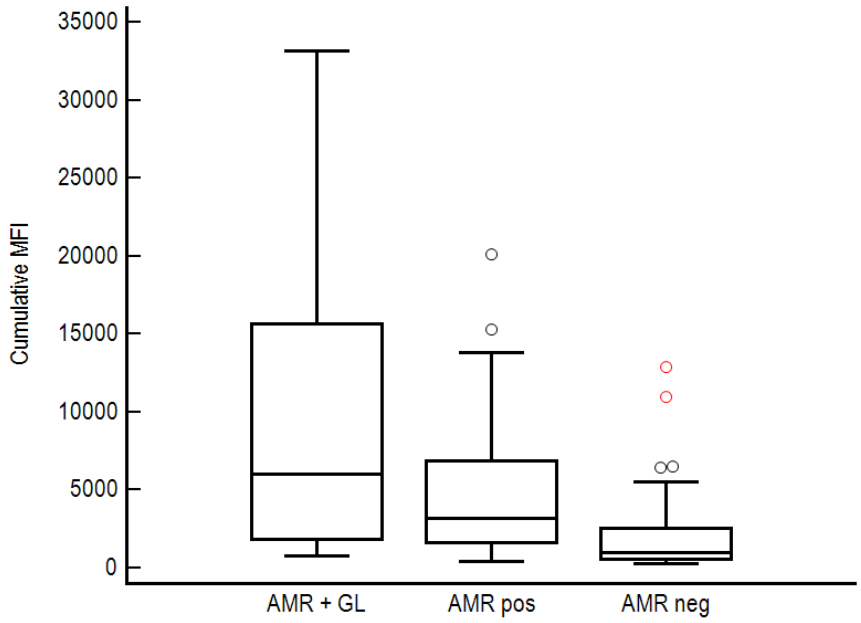


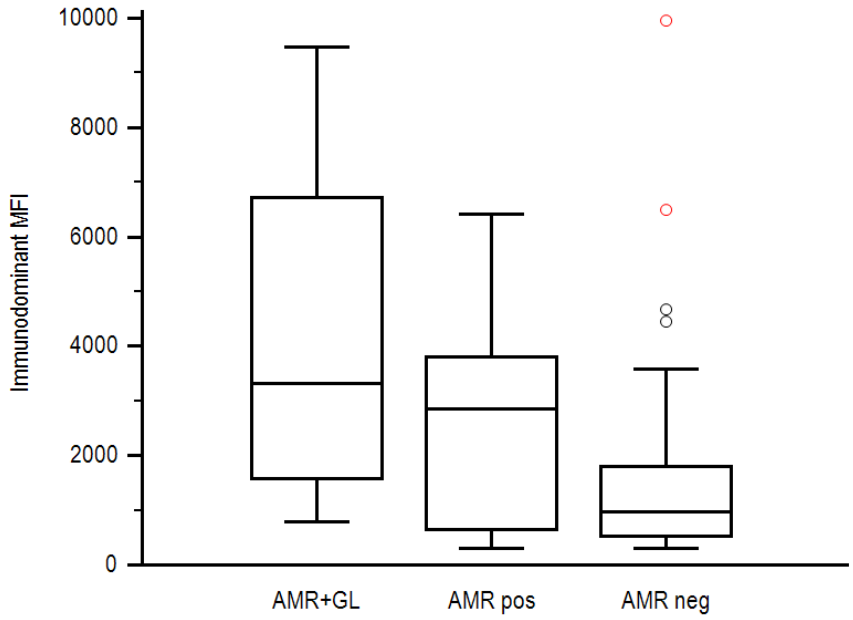
**5.2.3** Allograft survival by histological grade of AMR. There is a tendency to inferior outcome with histological features of vascular involvement [p=0.11] when compared with those patients with features of glomerular or peritubular inflammation only. [Type II AMR = glomerular/capillary inflammation, Type II AMR = arterial lesion].




Analysis of the impact of MFI, using both the cumulative and the immunodominant value, revealed a significant association with outcomes as shown in figure 5.3. The mean cumulative MFI in the group without rejection, in the AMR with graft survival group and in the AMR with allograft loss group were 1944 ± 2378, 5304 ± 5591 and 9278 ± 9382 respectively, p<0.001. Similarly, the MFI of the immunodominant DSAb in each group was 1512 ± 1623, 2711 ± 1912 and 4108 ± 2794 respectively, p<0.001. The presence of persistent DSAs after treatment was also associated with a trend to higher risk of allograft failure. 11/13 [84.6%] graft losses had persistent DSAs after treatment, OR: 3.14 (0.55-17.89), p=0.20.

**Figure 5.3 Outcome by cumulative MFI and the MFI of the immunodominant DSA** Comparison of patients with DSAs and no rejection [AMR-], AMR with DSAs and graft survival [AMR+] and AMR with DSAs and graft failure [AMR+GL].



**C4d staining**

34/41 [82.9%] of patients treated for AMR had focal C4d or diffuse C4d. 37/140 [26.4%] of non rejectors had C4d on an indication biopsy, therefore C4d was significantly associated with acute AMR, OR: 13.52 (5.52-33.13), p<0.0001. Of the AMR patients, 11/41 [26.8%] had focal C4d and 23/41 [56.1%] had diffuse C4d, whilst only 11/140 [7.9%] and 26/140 [18.6%] of the non rejectors had diffuse or focal C4d respectively. Diffuse C4d was highly significantly associated with AMR, OR: 14.98 (6.27-35.82), p<0.0001, whilst focal C4d was not OR: 1.61 (0.71-3.62), p=0.25.

The presence of diffuse C4d staining on the index AMR biopsy was associated with a tendency to inferior allograft survival, p=0.061 as shown in figure 5.2. There was no difference in outcome in those with focal C4d staining when compared with negative C4d, p=0.80.

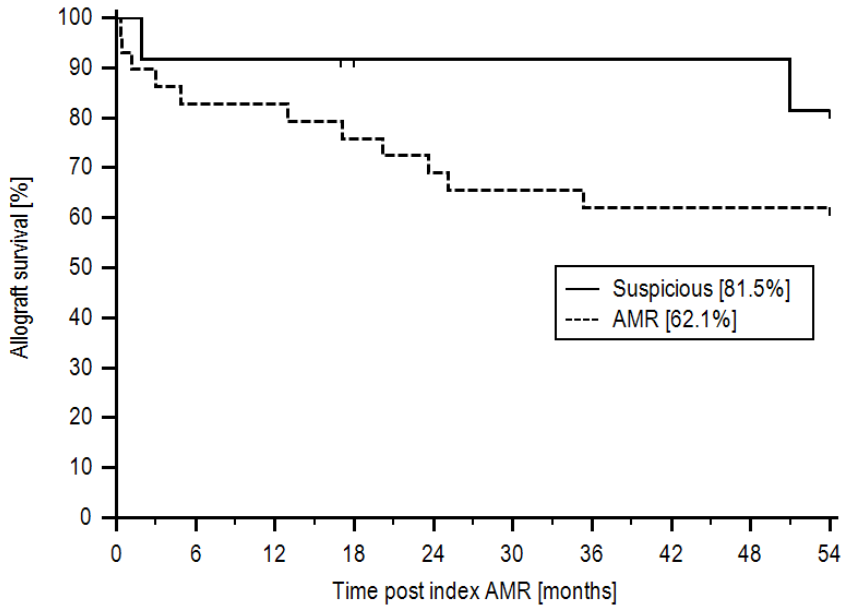
**Histological features**

25/41 [61.0%] of patients had capillary and/or glomerular inflammation and/or thrombosis [type II AMR] and 16/41 [39.0%] of patients had arterial involvement [type III AMR]. There was a trend to inferior outcomes in patients with Type III AMR (arteritis) with 7/16 experiencing allograft failure, compared with 6/25 patients with Type II AMR, giving an allograft survival rate of 55.0% and 75.6% respectively, p=0.17 as shown in figure 5.2.3.

Patients with all 3 Banff criteria for AMR; DSA, C4d positivity and morphological features of rejection did not have inferior allograft survival compared with those cases which were suspicious for AMR only, having morphological evidence with either C4d or DSA as shown in figure 5.4. 11/29 [37.9%] of patients with definite AMR lost their graft compared with 2/12 [16.7%] of patients with suspicion only.

**<u>Figure 5.4.</u> Allograft survival by full Banff criteria of AMR versus 'suspicious for AMR'.**

Suspicious for AMR is defined as histological evidence of tissue injury with either C4d or DSAs. Allograft in the full AMR criteria compared with suspicious for AMR criteria was 62.1% and 81.5% respectively, p=0.23.




**Other poor prognostic factors**

**Allograft function**

5/41 [12.2%] patients were dialysis dependent at the time of the diagnosis of AMR, 2 of these patients subsequently lost their grafts, p=0.64. Comparison of allograft function at the time of diagnosis showed no difference in the patients whose graft subsequently failed or survived [serum creatinine in those with graft loss: 314.4 ± 148.0, no graft loss: 273.8 ± 99.0 µmol/l, p=0.34]. However, patients who lost their grafts had inferior function after treatment: serum creatinine after treatment in those with subsequent graft loss was 364 ± 151.1, compared with 197.6 ± 116.2 µmol/l in patients whose grafts were still functioning at the end of follow up, p=0.0015.

**Further AMR episodes**

10/41 [24.4%] patients had more than one episode of acute AMR, of which 6/10 [60.0%] subsequently lost their graft. 6/31 [19.4%] of patients with a single episode of AMR lost their graft, and allograft survival in these patients was superior to those with ≥2 episodes of AMR, p=0.04.

**Transplant glomerulopathy**

16/41 [39.0%] of patients with acute AMR developed TG, which was significantly higher than the patients with no rejection, of whom only 5/369 [1.4%] patients had TG, OR: 46.2 (15.65-136.47), p<0.0001. As yet, there is no difference in allograft survival between the AMR patients who subsequently developed TG when compared with those with no TG following AMR, OR: 0.97 (0.25-3.73), p=0.96. However, longer follow up will be required to establish the full effects.

## 5.4 Discussion

In this large study, an analysis has been performed of the risk factors and outcomes of acute AMR in patients receiving a homogenous minimal immunosuppressive regimen. AMR is not uncommon and is associated with inferior allograft survival.

The incidence of AMR has been reported to be between 4-8% in immunologically low risk patients [Kirk et al, 2003; Singh et al, 2009]. There have been concerns that there is an increased incidence of rejection with a humoral component in patients receiving alemtuzumab induction [Knechtle et al, 2003; Flechner et al, 2005]. However, these findings appear to be limited to patients receiving CNI free immunosuppressive regimens. Knechtle et al. in a pilot study of 29 patients using alemtuzumab induction and sirolimus monotherapy reported 25% of the patients experiencing rejection which was predominantly antibody mediated [Knechtle et al, 2003]. Flechner et al. then introduced MMF along with sirolimus in their study of 22 patients, however there was still an unacceptable rate of rejection [8/22 (36.8%)] and 2 of these episodes were antibody mediated [Flechner et al, 2005]. Reassuringly, randomised control trials [RCT] involving alemtuzumab induction with maintenance CNI, have failed to show a higher incidence of rejection than other standard regimens. Three RCT trials which include CNI monotherapy following alemtuzumab induction have demonstrated excellent medium term outcomes [Margreiter et al, 2008; Vathsala et al, 2005; Thomas et al, 2007]. Watson et al. have

further shown that such protocols also produce good long term outcomes, with no increase in rejection episodes [Watson et al, 2005].

The development of de novo DSAs has been shown to be associated with AMR and allograft loss, and therefore monitoring for DSAs after transplant can help with the early identification and diagnosis of AMR [Zhang et al, 2005]. The reported incidence of de novo DSAs after renal transplant is highly variable, ranging from 5.5-24.2%, in cohorts not only receiving different immunosuppressive regimens but also using different screening methods [Hourmant et al, 2005, Akalin et al, 2006]. There are few studies describing the development of DSAs in patients receiving alemtuzumab induction. Cai et al. in a study into anti-HLA antibody production with alemtuzumab induction and sirolimus maintenance, showed 6/24 [25%] patients developed DSAs of which 4 had biopsy proven AMR [Cai et al, 2004]. Shapiro et al.used a protocol for weaning tacrolimus monotherapy whilst monitoring for the development of DSA. They found that 15% of stable patients developed DSAs on weaning the dose of tacrolimus [Shapiro et al, 2008]. The data showing that 17.7% of patients develop DSAs after transplant and that 52.7% of these patients develop AMR are consistent with these reports, with an overall incidence of AMR of 10.2%.

DSAs are not only important in predicting AMR but they may also be used to guide the management of rejection. This study has shown that higher levels of DSA [as judged by MFI] at the time of AMR are associated with graft loss and that their persistence may be associated with graft failure and these findings are consistent with those of other authors [Everly et al, 2010; Everly et al, 2009]. Patients with high and persisting DSAs may benefit from either more aggressive therapy or novel agents.

Whilst diffuse C4d staining has been found to be an indicator of AMR [Cai et al, 2011; Mauiyyedi et al, 2002], the significance of focal C4d has been more controversial [Magil et al, 2006; Nickeleit et al, 2002]. Staining is dependent upon the technique used, with immunohistochemistry being less sensitive than immunofluroscence [Solez et al, 2008]. Lefaucheur and colleagues did not find an association between C4d and poor outcome in AMR [Lefaucheur et al]. A strong correlation of diffuse C4d staining with AMR was found. It should be stressed however that the majority of cases of AMR were associated with DSAs, and therefore likely to produce C4d positivity.

Histologically, it was found that patients with vascular involvement have a tendency to inferior outcome, similar to Rafiq et al, 2009.

Overall, it has been demonstrated that patients with the full Banff criteria of AMR have a poor prognosis. The lack of randomised control trials in the treatment of acute AMR means that the most effective management protocol is unknown. Treatments usually used include a combination of antibody removal with plasmapharesis [Houmant et al, 2005], intravenous immunoglobulin [Jordan et al, 1998], rituximab [Pescovitz et al] and optimising tacrolimus, MMF and corticosteroids [Sun et al, 2007]. Those patients with poor predictive factors may benefit from enhanced therapy or alternative agents such as bortezomib or eculizumab [Locke et al, 2009].

39.0% of patients who experienced AMR developed TG and approximately a third of these patients have subsequently lost their grafts. Gloor et al. have described how the incidence of TG increases over time [Gloor et al, 2007], so with longer follow up the number of patients affected in this cohort might increase. TG has a poor prognosis being progressive in nature and is associated with reduced allograft survival [Cosio et al, 2008; Cosio et al, 2005; Rostaing et al, 2009].

In conclusion, this study shows that acute AMR is not uncommon and has an important consequential effect on allograft survival. Patients who have diffuse C4d, histological features showing vascular involvement, HLA CII DSAs, DSAs of high MFI and persistent DSAs after treatment are at highest risk of graft loss. Such patients might benefit from alternative therapies which are now available, which could be answered in the form of a multicentre randomised control trial.

# Chapter 6 DQ donor specific antibodies: incidence, risk factors and association with antibody mediated rejection and transplant glomerulopathy

## 6.1 Introduction

The importance of HLA matching on the outcome of renal transplantation has been recognised for over 30 years with studies showing greater benefit of compatibility at the DR locus than matching at either the A or B locus [Ting et al, 1980; Gilks et al, 1987]. In recent years the evidence for this has been strengthened by studies showing that the development of de novo donor specific antibodies [DSAs] after transplant is associated with antibody mediated injury and allograft failure, with class II DSAs having a greater effect than class I [Terasaki et al, 2007]. Anti-DQ DSAs are increasingly recognised as the most frequently occurring class II DSA and this extends beyond renal transplantation to include other solid organ transplants [Worthington et al, 2003; Issa et al, 2008; Musat et al, 2011; Smith et al, 2011; Palmer et al, 2002]. However, the clinical relevance of de novo DQ DSAs has not been extensively described.

The effect of DQ matching has not been examined using all the recent advances in histocompatibility and immunogenetics, historical studies having shown conflicting results [Freedman et al, 1997; Hais wt al, 1993; Bushell et al, 1989]. It is known that DR and DQ demonstrate a strong linkage disequilibrium and it was thought that matching at the DR locus would include co-existing compatibility at the DQ locus [Navarrete et al, 1985]. The incidence for this not being the case is not known. However it is recognised that DQ expression may be upregulated on endothelial cells and as such may be a target for DQ antibodies and their subsequent actions [Gibbs et al, 1985]. With chronic rejection being the leading cause of allograft loss, mechanisms which minimise immunological injury should be sought [El-Zoghby et al, 2009]. It can be hypothesised that consideration of DQ matching may improve long term outcomes.

The aim of this study was to determine the incidence of de novo DQ DSAs and the effect on allograft outcomes following their development in patients transplanted at a single centre with a homogenous immunosuppressive regimen. It can be questioned whether matching at the –DR locus

is sufficient to assume simultaneous –DQ matching and whether it risks higher levels of subsequent de novo DQ DSAs than previously suspected.

## 6.2 Methods

An observational study was carried out of 505 patients who received a kidney alone transplant, the demographics of which are shown in table 5.1. All patients were included who had a negative B and T cell CDC crossmatch, negative T cell flow cytometry crossmatch and those who had no detectable DSA detected by single antigen beads pre-transplant. Only patients who received alemtuzumab induction were included in the analysis. Mean follow up after transplant was 30.0 ± 17.0 months.

Immunosuppression regimen and rejection treatment along with histological and H&I methods are described in chapter 2.

**Table 6.1. Patient demographics**

| | | DQ DSA+ [N=50 (%)] | DQ DSA- [N=455 (%)] | p value |
|---|---|---|---|---|
| **Gender** | Male<br>Female | 35 [70.0]<br>15 [30.0] | 313 [68.8]<br>142 [31.2] | 0.99 |
| **Age at transplant** | Years | 47.62 ± 14.53 | 48.43 ± 13.33 | 0.33 |
| **Ethnicity** | Caucasian<br>Asian<br>Afrocaribbean<br>Other | 19 [38.0]<br>23 [46.0]<br>5 [10.0]<br>3 [6.0] | 217 [47.7]<br>158 [34.7]<br>59 [13.0]<br>21 [4.6] | 0.39 |
| **Aetiology of renal failure** | Glomerulonephritis<br>Diabetes<br>APKD<br>Urological<br>Other<br>Unknown | 13 [26.0]<br>11 [22.0]<br>3 [6.0]<br>3 [6.0]<br>3 [6.0]<br>17 [34.0] | 108 [23.7]<br>78 [17.1]<br>67 [14.7]<br>29 [6.4]<br>36 [7.9]<br>137 [30.1] | 0.36 |
| **Time receiving dialysis** | Years | 3.79 ± 5.13 | 3.85 ± 6.10 | 0.94 |
| **Type of allograft** | Deceased donor<br>Living related<br>Living unrelated | 26 [52.0]<br>15 [30.0]<br>9 [18.0] | 243 [53.4]<br>126 [27.7]<br>86 [18.9] | 0.94 |
| **Graft number** | $1^{st}$<br>≥2nd | 45 [90.0]<br>5 [10.0] | 373 [93.0]<br>32 [7.0] | 0.63 |
| **Sensitisation pre-transplant** | Non-sensitised<br>Sensitised | 44 [88.0]<br>6 [12.0] | 373 [82.0]<br>82 [18.0] | 0.38 |
| **HLA MM** | Mean | 3.86 ± 1.23 | 3.17 ± 1.63 | 0.004 |

## 6.3 Results

92/505 [18.2%] patients developed de novo DSAs after transplantation. 28/92 [30.4%], 41/92 [44.6%] and 23/92 [25.0%] developed HLA class I DSAs alone, HLA class II DSAs alone or both HLA class I+II DSAs respectively. 50/92 [54.3%] of DSA positive patients (that is 50/505 [9.9%] of the overall cohort) developed a DQ DSA. 26/50 [52.0%] developed DQ DSAs along with DSAs specific for other loci, whilst 24/50 [48.0%] developed a DQ DSA in isolation. The distribution of DSA per HLA class is shown in table 6.2.

**Table 6.2. Distribution of DSAs**

| | DSAs detected | Number of patients N=92 [%] |
|---|---|---|
| **Class I DSAs alone** | A | 7 [7.6%] |
| | B | 9 [9.8%] |
| | Cw | 6 [6.5%] |
| | A and B | 2 [2.2%] |
| | A and Cw | 2 [2.2%] |
| | B and Cw | 2 [2.2%] |
| **Class II DSAs alone** | DR | 11 [12.0%] |
| | DQ | 25 [27.2%] |
| | DR and DQ | 5 [5.4%%] |
| **Class I+II DSAs** | A and DQ | 3 [3.3%] |
| | A and DR | 1 [1.1%] |
| | A and DQ,DR | 1 [1.1%] |
| | A,B and DQ | 3 [3.3%] |
| | A,B and DQ,DR | 2 [2.2%] |
| | A,B,Cw and DQ,DR | 2 [2.2%] |
| | A,Cw and DQ,DR | 2 [2.2%] |
| | B and DQ | 2 [2.2%] |
| | B and DR | 2 [2.2%] |
| | B and DQ,DR | 1 [1.1%] |
| | B,Cw and DQ | 1 [1.1%] |
| | B,Cw and DQ,DR | 1 [1.1%] |
| | Cw and DQ | 1 [1.1%] |
| | Cw and DQ,DR | 1 [1.1%] |

**Allograft outcomes in DQ DSA positive patients**

35/505 [6.9%] of patients experienced allograft failure. 12/50 [24.0%] of DQ DSA positive patients lost their graft, compared with 2/42 [4.8%] of the non-DQ DSA positive patients and 21/413 [5.1%] of DSA negative patients. Patients with DQ DSAs were at significant risk of allograft loss when compared to patients with non-DQ DSAs [OR 6.15 (1.29-29.30), p=0.023] or no DSAs [OR 5.89 (2.69-12.91), p<0.0001].

There was a significant association between DQ DSAs and AMR. 23/50 [46.0%] of DQ DSA+ patients had biopsy proven AMR compared with 6/42 [14.3%] of the non-DQ DSA+ group and 7/413 [1.7%] of DSA- patients, giving an OR of 5.11 (1.83-14.28), p=0.0019 and 49.41 (19.47-125.40) respectively, p<0.0001. ACR occurred in 15/50 [30.0%] of DQ DSA+ patients, 8/42 [19.0%] of non-DQ DSA+ patients and 56/413 [13.6%] of DSA- patients. Therefore whilst DQ DSAs were not significantly associated with ACR when compared with non-DQ DSA+ patients [OR 1.82 (0.68-4.85), p=0.23], there remained a positive association with ACR when compared with the DSA- group [OR 2.73 (1.40-5.32), p=0.0032]. 15/50 [30.0%] DQ DSA+ patients had biopsy proven transplant glomerulopathy [TG] compared with 3/42 [7.1%] of non-DQ DSA+ patients and 6/413 [1.5%] of DSA- patients giving an OR of 5.57 (1.49-20.88), p=0.011 and 29.07 (10.61-79.64), p<0.0001 respectively.

Table 6.3 shows a univariant analysis of the association of allograft outcomes with DSAs of different specificities. DSAs of all specificities are associated with AMR , TG and allograft loss. Only DQ DSAs were associated with ACR. On multivariant analysis, DQ DSAs were the only specificity associated with all of ACR, AMR, TG and allograft failure, as shown in table 6.4.

**Table 6.3 Univariant analaysis of association of DSA specificities and allograft outcomes**

| DSA | Graft loss | | AMR | | ACR | | TG | |
|---|---|---|---|---|---|---|---|---|
| | OR (95% CI) | p value | OR (95% CI) | p value | OR (95% CI) | p value | OR (95% CI) | p value |
| **A** | 3.56 (1.26-10.22) | 0.017 | 13.31 (5.55-31.98) | <0.0001 | 1.67 (0.65-4.30) | 0.29 | 10.01 (3.71-27.02) | <0.0001 |
| **B** | 5.6 (2.19-14.42) | 0.0003 | 15.13 (6.38-35.87) | <0.0001 | 1.97 (0.81-4.84) | 0.14 | 12.16 (4.63-31.90) | <0.0001 |
| **Cw** | 3.05 (0.83-11.18) | 0.09 | 20.75 (7.38-58.34) | <0.0001 | 1.69 (0.54-5.34) | 0.37 | 7.20 (2.15-24.07) | 0.0013 |
| **DR** | 4.02 (1.52-10.65) | 0.005 | 13.29 (5.71-30.96) | <0.0001 | 1.13 (0.42-3.06) | 0.81 | 10.95 (4.22-28.46) | <0.0001 |
| **DQ** | 5.93 (2.74-12.85) | 0.0001 | 28.96 (13.23-63.39) | <0.0001 | 2.62 (1.35-5.07) | 0.004 | 21.24 (8.68-51.99) | <0.0001 |

**Table 6.4 Multivariant analysis of association between DSA specificities and allograft outcomes**

| Outcome | DSA | 95% CI | p value |
|---|---|---|---|
| **Graft loss** | DQ | 1.83-8.59 | 0.0005 |
| | B | 0.98-6.17 | 0.056 |
| **AMR** | DQ | 6.10-27.49 | <0.0001 |
| | Cw | 1.46-7.34 | 0.004 |
| | A | 1.04-4.89 | 0.04 |
| ACR | DQ | 1.40-4.30 | 0.0015 |
| **TG** | **DQ** | 4.28-28.56 | <0.0001 |
| | **DR** | 0.89-6.29 | 0.087 |
| | B | 1.14-7.81 | 0.027 |

**Risk factors for development of DQ DSAs**

From the patient demographics it was seen that patients who developed de novo DQ DSAs received a graft with a higher mean HLA mismatch. Analysing in more detail the antigen mismatches associated with DQ DSAs, there was no association on univariant analysis between mismatch at the A locus and DQ DSAs, p=0.10 [log rank] or mismatch at the B locus and DQ DSAs, p=0.68 [log rank]. However patients mismatched at the DR locus were at significant risk of developing DQ DSAs, p=0.0021 [log rank].

Analysing the effect of linkage disequilibrium between DR and DQ, it was found of the 505 patient studied, 108 [21.4%] were matched at both DR and DQ [DR+DQ+] whilst 284 [56.2%] were mismatched at both the DR and DQ loci [DR-DQ-]. However, 38 [7.5%] of patients were matched at DR but mismatched at DQ [DR+DQ-] and 75 [14.9%] were matched at DQ but mismatched at DR [DR+DQ-]. Therefore a total of 113 [22.4%] did not display the compatibility expected with a strong linkage disequilibrium.

**Allograft outcomes by DR and DQ mismatch**

There was no difference in censored allograft survival between the DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ- groups which was 92.5%, 97.4%, 93.2% and 92.5% at 5 years respectively, p=0.74. Overall there was no significant difference in the total rejection free survival between the subgroups which was 84.3%, 86.8%, 79.3% and 76.4% in the DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ- groups respectively, p=0.23 which is shown graphically in figure 6.1. Similarly analysing acute cellular rejection [ACR] there also was no difference with a 84.3%, 89.5%, 80.7% and 83.5% ACR free survival amongst the DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ- groups, p=0.73. However as shown in figure 6.2, antibody mediated rejection [AMR] occurred at a significantly higher incidence in the group mismatched at both DR and DQ [DR-DQ-], with a AMR free survival of 88.5%, compared with 98.0%, 97.4% and 98.6% in the DR+DQ+, DR+DQ- and DR-DQ+ groups, p=0.001.

**<u>Figure 6.1.</u> All rejection free survival by DR, DQ and DR-DQ mismatch**

Rejection free survival in the DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ- groups was 84.3%, 86.8%, 79.3% and 76.4% respectively, p=0.23

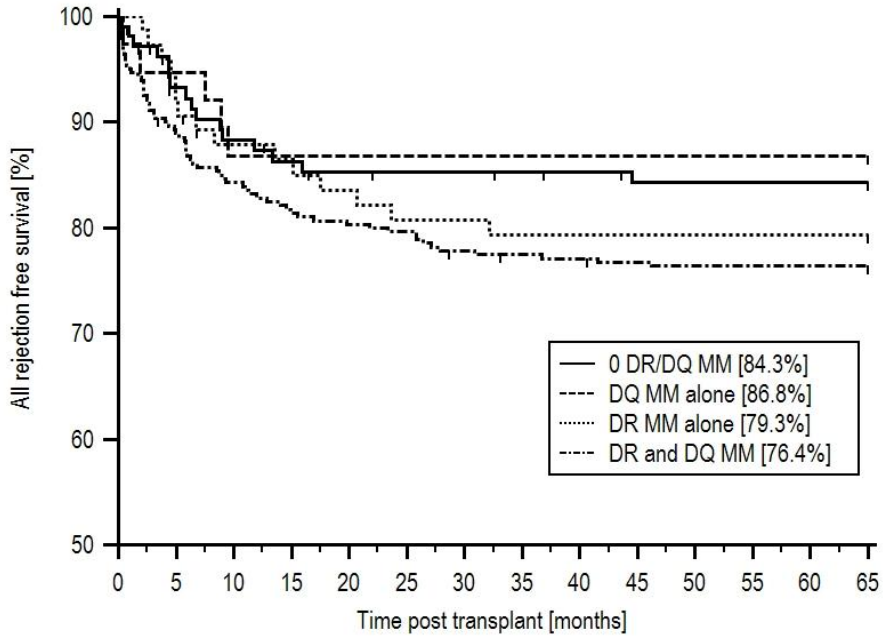
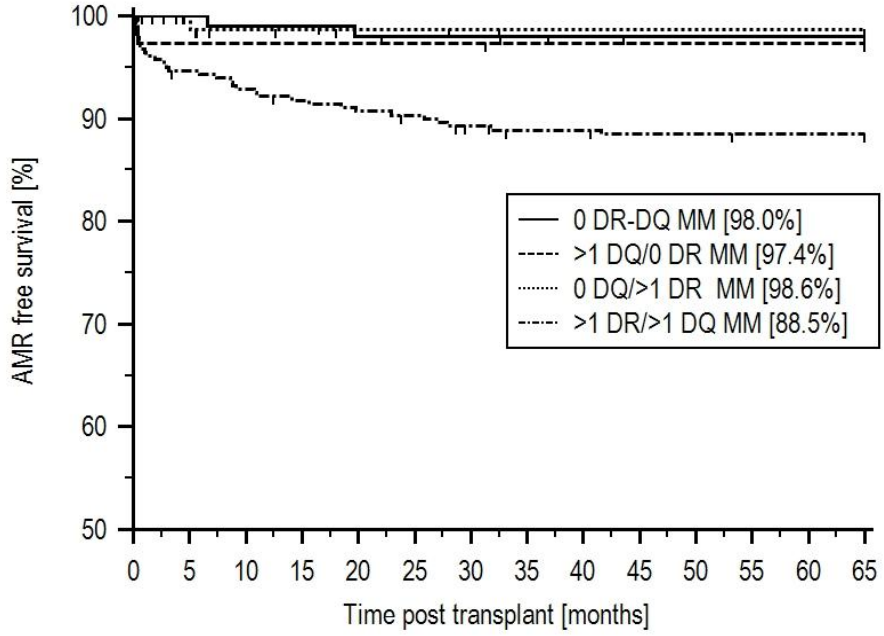



**Figure 6.2. AMR free survival by DR, DQ and DR-DQ mismatch**

AMR free survival in the DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ- groups was 98.0%, 97.4%, 98.6% and 88.5% respectively, p=0.001



Given the highly significant risk of AMR in the patients mismatched at both DR and DQ [DR-DQ-] the next analysis was of the incidence of DSAs in each of the subgroups [DR+DQ+, DR+DQ-, DR-DQ+ and DR-DQ-]. The results are shown in table 6.5 and there was an strong association with the production of DSAs in patients who are mismatched at both the DR and DQ loci [DR-DQ-] when compared to those mismatched at either DQ [DQ-DQ+] oor DR [DR+DQ-] alone, p=0.001.

**Table 6.5. Analysis of DQ and DR mismatching and corresponding DSA production**

| | | Number of patients [%] | Corresponding DSAs [%] |
|---|---|---|---|
| **DQ mismatch** | 0 DQ MM | 184 [36.4%] | 0 |
| | 1 DQ MM | 250 [49.5%] | 35 [70.0%] |
| | 2 DQ MM | 71 [14.1%] | 15 [30.0%] |
| **DR mismatch** | 0 DR MM | 146 [28.9%] | 0 |
| | 1 DR MM | 263 [52.1%] | 20 [69.0%] |
| | 2 DR MM | 96 [19.0%] | 9 [31.0%] |
| **DR-DQ mismatch** | 0 DR-DQ MM | 108 [21.4%] | 0 |
| | 0 DR/≥1 DQ MM | 38 [7.5%] | 2 [3.1%] |
| | ≥1 DR/0 DQ MM | 75 [14.9%] | 2 [3.1%] |
| | ≥1 DR/≥1DQ MM | 284 [56.2%] | 60 [93.8%] |

## 6.4 Discussion

This study has revealed several important findings. Firstly, there is a >20% discordance between matching at the DR and DQ locus. Secondly, there appears to be an enhanced immunogenicity with mismatching at both the DR and DQ loci, which is associated with increased risk of development of DSAs. Finally, DQ DSAs are the most frequently occurring HLA DSA post-transplant and are associated with antibody mediated rejection, transplant glomerulopathy and allograft loss. Thus, the study highlights the importance of DQ typing and matching in renal transplantation in defining immunological risk.

Ting and Morris were the first to show that mismatching at the DR locus correlated with poor allograft survival in 1978 [Ting et al, 1980]. At that time the DQ locus had not been formally defined, which occurred after the 9th Histocompatibility workshop in 1984 [Albert et al, 1984]. This was only possible with the introduction of molecular methods of HLA typing, which had previously been undertaken using serological and cellular methods which were unsatisfactory in distinguishing DQ from DR given their juxtaposition and linkage disequilibrium [Navarrete et al, 1985; www.ihwg.org]. Following this finding, a few studies addressed the impact of DQ matching on allograft survival. Bushell et al in a small study compared 25 DQ mismatched patients in a cohort of 62 patients who were DR matched and found no effect of independent DQ mismatching [Bushell et al, 1989]. Contradictory to this report, Hsia and colleagues reviewed the outcomes of 63 patients and found DQ matching did provide a survival benefit [Hsia et al, 1993]. However, the largest study was carried out in 1997 by Freedman et al; they analysed 12,050 cadaveric transplants which were serologically typed and found that DQ compatibility when considered in isolation did improve allograft survival [10]. However once adjusted for other factors known to be associated with poor outcome, including DR mismatch, there was no overall benefit of independent matching at the DQ loci [Freed man et al, 1997].

The progressive refinement in the methodology of antibody identification after transplant, with the advent of single antigen beads, has revealed substantial evidence that the development of de novo donor specific antibodies is associated with inferior allograft survival [Terasaki et al, 2007]. Only in the past few years has it been demonstrated that the presence of class II DSAs is more detrimental to allograft survival [Gloor et al, 2007; Campos et al, 2006] than class I DSAs. The relevance of DQ DSAs has been less well studied, which in part might reflect the fact that typing for DQ is not required for allocation purposes. Of the published reports, Worthington et al showed that DSAs in the 112 patients studied were highly predictive of graft failure and antibodies to DQ were the commonest DSA detected [Worthington et al, 2003]. Three other studies have shown that DQ DSAs are the predominant DSA detected post-transplant, however they failed to show a correlation with either chronic antibody mediated rejection or allograft function [Kobayashi et al, 2011; Iniotaki-Theodoraki et al, 2003; Zhu et al, 2008].

The detrimental consequences following detection of DQ DSAs are not restricted to renal transplantation. In a similar evolution of understanding, it has been established that class II DSAs and especially DQ DSAs are associated with graft failure in cardiac, lung and liver transplantation [Musat et al, 2011; Smith et al, 2011, Palmer et al, 2002, Levine et al, 2009]. Cardiac transplantation has been the most extensively studied of the other solid organ transplants. In a recent publication, Smith

et al followed 243 cardiac recipients after transplant and found that 57 patients developed de novo DSAs of which 41 were directed against DQ [Smith et al, 2011]. A new study has also shown that liver transplants, which are believed to be the most 'tolerogenic' of the solid organs transplanted, can also be damaged in association with DQ DSAs [Musat et al, 2011]. Applying the diagnostic criteria of tissue injury, C4d positivity and circulating DSA to liver recipients undergoing indication biopsies, Musat and colleagues found that over half of biopsies demonstrating rejection had a humoral component. Of the 43 patients studied, 27 patients were found to have DSAs of which 22 included DQ specificities [Musat et al, 2007].

Now there is increasing acceptance of a potential role of DQ DSAs in antibody mediated injury, it will be necessary to establish the pathogenesis. It is known that mismatching a donor and recipient for class II HLA antigens enhances allorecognition [Afzali et al, 2007]. The beneficial role of T regulatory cells [Tregs] in transplantation has been the focus of major work over the past decade and it has been shown that Tregs specific for a renal transplant may protect it against rejection. Rodriguez et al showed a strong correlation between donor specific regulation and degree of DR matching, which was even greater in the setting of simultaneous DR and DQ matching [Rodriguez et al, 2004].

To conclude, the use of screening for DSAs both routinely and when indicated using sensitive Luminex beads, has enabled a fuller appreciation of the immunological consequences of current HLA matching schemes. It certainly has highlighted the previously underestimated consequences of mismatching at the DQ locus. A full Registry analysis is now required and if findings are reproduced, DQ matching could be considered during the allocation process of deceased donor kidneys with the potential to increase allograft survival.

# Chapter 7 Outcomes of T cell mediated rejection with a humoral component

## 7.1 Introduction

Allograft survival has not significantly changed over the past few decades [Lamb et al, 2011], but although rejection remains the leading cause of allograft failure [El-Zoghby et al, 2009], there has been a change in the type of rejection associated with graft loss [Stegall et al, 2011], possibly reflecting improvements in diagnosis and subclassifications of rejection.

Historically, rejection has been categorised in many different ways, most commonly by its timing following transplantation (hyperacute, acute or chronic), the immunological mechanisms thought to be involved (cellular or antibody mediated) or whether there is allograft dysfunction or not (subclinical or clinical). Through the Banff classification, rejection treatments are defined depending on interpretation of the grade of rejection and whether predominantly cellular and antibody rejection is seen. However cellular and antibody mediated rejection are not mutually exclusive (the adaptive immune response results in both B and T cell activation [Janeway]).

Allograft survival following antibody mediated rejection has been found to be inferior to cellular rejection [Figure 5.1]. Outcomes following 'mixed' rejection have not been extensively reported and consequently evidence on how such patients should be treated is lacking. The aim of this study is to determine if patients found to have cellular rejection with a humoral component, when treated in a conventional manner have inferior outcomes to those patients with pure cellular rejection by Banff criteria.

## 7.2 Methods

There was a retrospective review of the biopsies and medical notes of all patients who received a kidney or simultaneous kidney and pancreas transplant at Imperial Renal and Transplant Centre between October 2005 and May 2011. All patients who had an indicative renal biopsy which had histological features of cellular rejection who subsequently received directed therapy were identified. Patients were excluded if receiving an antibody incompatible transplant, defined as either blood group incompatibility or those patients with a positive B cell CDC or T cell flow crossmatch who underwent desensitisation pre-transplant. Patients with Luminex positive donor specific antibodies [DSA] in the context of a negative crossmatch were included. Only first rejection episodes were analysed and separated into 4 groups as follows:

1. Group 1. Pure TCMR
2. Group 2. Histological TCMR with C4d positivity
3. Group 3. Histological TCMR with DSA
4. Group 4. Histological TCMR with C4d positivity and DSA

Groups 2 to 4 are collectively referred to as TCMR with a humoral component or mixed TCMR. Patients found to have evidence of BK on their index biopsy were further excluded from the analysis. The patient demographics are shown in table 7.1. Patients were followed from the time of index biopsy to determine if there was a difference in subsequent incidence of TCMR, AMR, TG and allograft loss.

Immunosuppression regimen, treatment of rejection along with histological and H&I methods are described in chapter 2.

**Table 7.1 Table showing patient demographics**

| | | Pure group N=75 (%) | Mixed group N=51 (%) | p value |
|---|---|---|---|---|
| **Gender** | *Male*<br>*Female* | 57<br>18 | 31<br>20 | 0.10 |
| **Age at transplant** | *Years* | 48.48 ± 13.66 | 44.88 ± 12.73 | 0.80 |
| **Ethnicity** | *Afrocaribbean*<br>*Indoasian*<br>*Caucasian*<br>*Other* | 13<br>21<br>36<br>5 | 12<br>15<br>24<br>0 | 0.79 |
| **Aetiology of ESRF** | *Diabetes*<br>*APKD*<br>*GN*<br>*Urological*<br>*Unknown*<br>*Other* | 13<br>6<br>22<br>7<br>21<br>6 | 3<br>4<br>17<br>3<br>9<br>5 | 0.52 |
| **Time at ESRF** | *Years* | 3.76 ± 4.69 | 5.88 ± 6.65 | 0.038 |
| **RRT pre** | *Pre-emptive*<br>*Dialysis* | 19<br>56 | 9<br>42 | 0.42 |
| **Type of donor** | *DD*<br>*LD*<br>*SPK* | 36<br>32<br>7 | 26<br>24<br>1 | 0.76 |
| **Graft number** | *1st*<br>*≥2* | 69<br>6 | 36<br>15 | 0.0035 |
| **Induction** | *Daclizumab*<br>*Alemtuzumab* | 12<br>63 | 17<br>34 | 0.04 |
| **HLA mismatch** | | 3.24 ± 1.84 | 3.53 ± 1.46 | 0.35 |
| **Pre-sensitisation** | *Non-sensitised*<br>*HLA antibodies*<br>*DSA* | 59<br>15<br>1 | 25<br>9<br>17 | 0.0011 |
| **UKT mismatch** | *000*<br>*0DR + 0/1 B*<br>*0DR +2B; 1DR +0/1 B*<br>*1DR + 2B; 2DR* | 10<br>6<br>32<br>27 | 1<br>4<br>26<br>20 | 0.17 |
| **Time to rejection** | *Months* | 10.72 ± 11.36 | 10.98 ± 12.53 | 0.90 |
| **Follow up** | *Months* | 25.38 ± 17.09 | 27.45 ± 16.24 | 0.50 |

## 7.3 Results

149/817 [18.24%] of patients were found to have a histological diagnosis of TCMR. 15 cases occurred following previous biopsy proven AMR and were excluded, as were 8 patients with co-existing BK nephropathy, which resulted in 126 index cases remaining; 75 patients had pure TCMR, 10 cases had C4d+ TCMR, 30 cases had DSA+ TCMR and 11 patients had C4d+DSA+ TCMR. The distribution of patients within the mixed group is shown in table 7.2.

Patients with TCMR with a humoral component were more likely to be sensitised, receiving a re-graft and having spent a longer time at end stage renal failure. 59/75 [78.67%] of the pure group were non-sensitised compared with 25/51 [49.02%] of the mixed group, p=0.0011. The mean time spent at end stage renal failure in the pure group was 3.76 ± 4.69 years compared with 5.88 ± 6.65 years in the mixed group. 69/75 [92.0%] of the pure group but only 36/51 [70.59%] of the mixed group were receiving their first allograft, p=0.0035. A higher proportion of the daclizumab group had mixed rejection [17/29 (58.62%)] than the alemtuzumab induced group [34/97 (35.05%)], p=0.04.

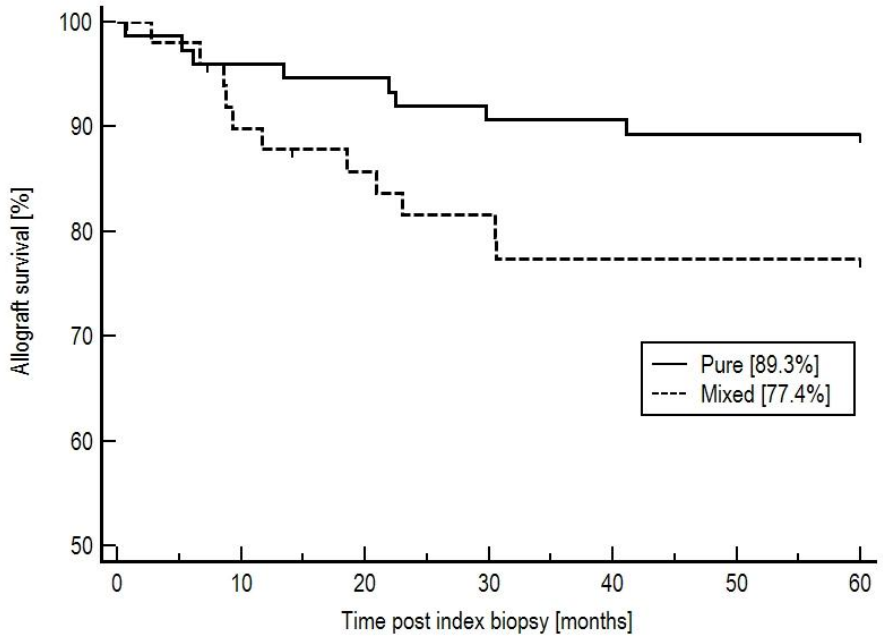
**Table 7.2 Distribution of patients per cellular rejection category**

| Group | Number of patients 126 (%) |
|---|---|
| Pure ACR | 75 (59.5%) |
| TCMR with C4d positivity | 10 (7.9%) |
| TCMR with DSA positivity | 30 (23.8%) |
| TCMR with C4d and DSA positivity | 11 (8.7%) |

**Allograft survival**

There was a tendency to inferior allograft survival in the patients in the mixed group when compared with the pure group. Allograft survival in the pure group was 89.3%, with 8/75 (10.67%) patients subsequently losing their grafts compared with 77.4% allograft survival in the mixed group [11/51 (21.5%) grafts were lost], p=0.072 [Figure 6.1]. Causes of graft loss in the pure group were: 4 were secondary to rejection, defined by recurrent TCMR episodes with progressive scarring, 1 had to undergo immunosuppression withdrawal due to sepsis and subsequently died, 1 had complications of obstruction/urosepsis, 1 had recurrent rejection with de novo glomerulonephritis and only 1 had rejection with signs of chronic antibody mediated rejection. In the mixed group: 2 patients had recurrent rejection with pyelonephritis, 1 had complications following nephrostomy for obstruction, 1 had recurrent disease, 1 had de novo glomerulonephritis and 6 had chronic antibody mediated rejection. Therefore, in the pure group recurrent rejection appeared to evolve as progressive scarring, compared with the mixed group who appeared to develop glomerular involvement and signs of chronic antibody mediated rejection or transplant glomerulopathy.

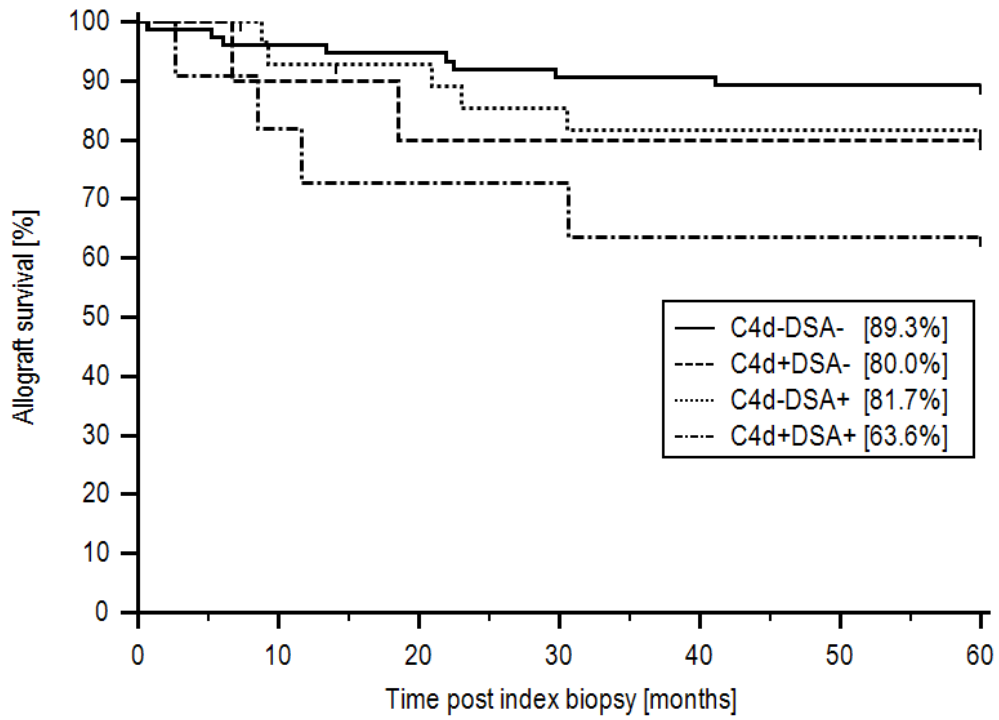
**Figure 7.1. Allograft survival post index biopsy in pure TCMR versus mixed TCMR.** Allograft survival in the pure group was 89.3%, compared with 77.4% allograft survival in the mixed group [p=0.072]



Separating the mixed group by the manifested humoral component (i.e. C4d and/or DSA positive), compared with the pure group, patients with C4d positivity and DSA were more likely to experience graft failure with an allograft survival of 89.3% and 63.6% respectively, p=0.014 as shown in figure 7.2. 4/11 [36.36%] of C4d DSA positive TCMR subsequently lost their grafts, all as a result of rejection.

**<u>Figure 7.2.</u> Allograft survival post index biopsy according to humoral component of TCMR**

Allograft survival in the pure group was 89.3% and 63.6% in the C4d+DSA+ TCMR group, p=0.014.

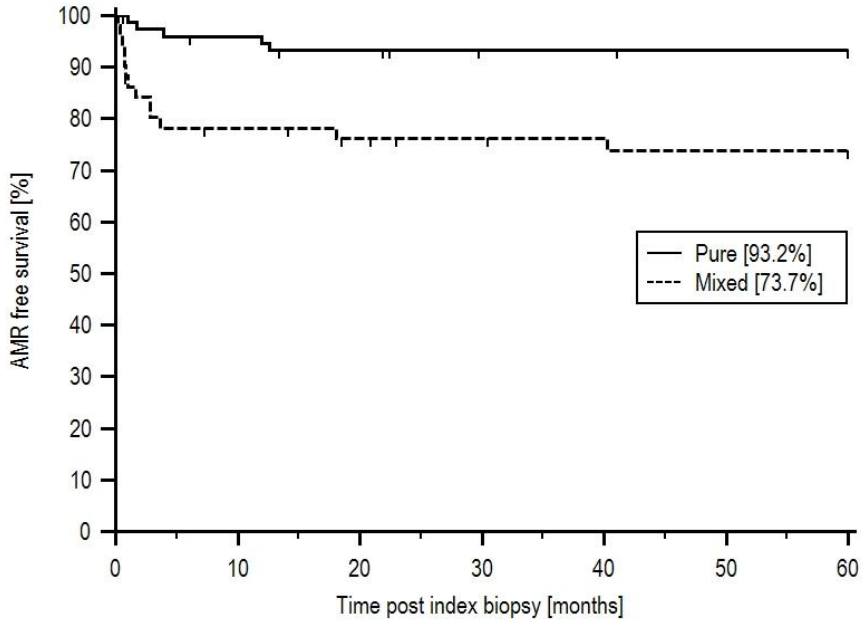


**Recurrent rejection episodes**

There was no difference between the pure and mixed groups with respect to further rejection episodes of all types, total rejection free survival being 52.3% in the pure group and 49.4% in the mixed group, p=0.49. There was also no difference in recurrent TCMR episodes, with a cell mediated rejection free survival of 61.2% in the pure group and 65.5% in the mixed group, p=0.75. However, AMR occurred significantly more in the mixed group, the AMR free survival after index biopsy in the pure group being 93.2% and 73.7% in the mixed group, p=0.0021 as shown in figure 7.3.

**Figure 7.3. AMR free survival post index biopsy in the pure compared with the mixed groups.**

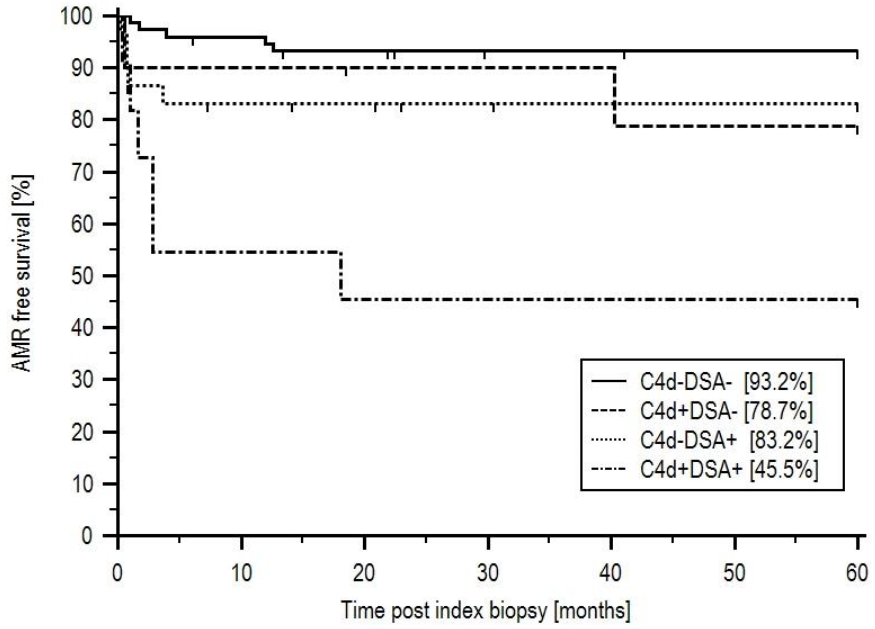
There was a significant risk of subsequent histologically diagnosed AMR in the mixed group when compared with the pure group. AMR free survival after index biopsy was 73.7% in the mixed group and 93.2% in the pure group, p=0.0021.



Patients with C4d and DSA positive TCMR were at highest risk of developing classical AMR, with a AMR free survival of 45.5% compared with 93.2% in the pure group, p<0.0001 as shown in figure 7.4. C4d positive, DSA negative TCMR and C4d negative, DSA positive TCMR had an AMR free survival of 78.7% and 83.2%, p=0.15 and p=0.088 respectively.

**<u>Figure 7.4</u> AMR free survival post index biopsy according to extent of humoral involvement.**

AMR free survival in the pure, C4d+DSA-, C4d-DSA+ and C4d+DSA+ groups were 93.2%, 78.7%, 83.2% and 45.5% respectively. There was a significant risk of AMR in the C4d+DSA+ TCMR when compared with the pure group, p<0.0001.

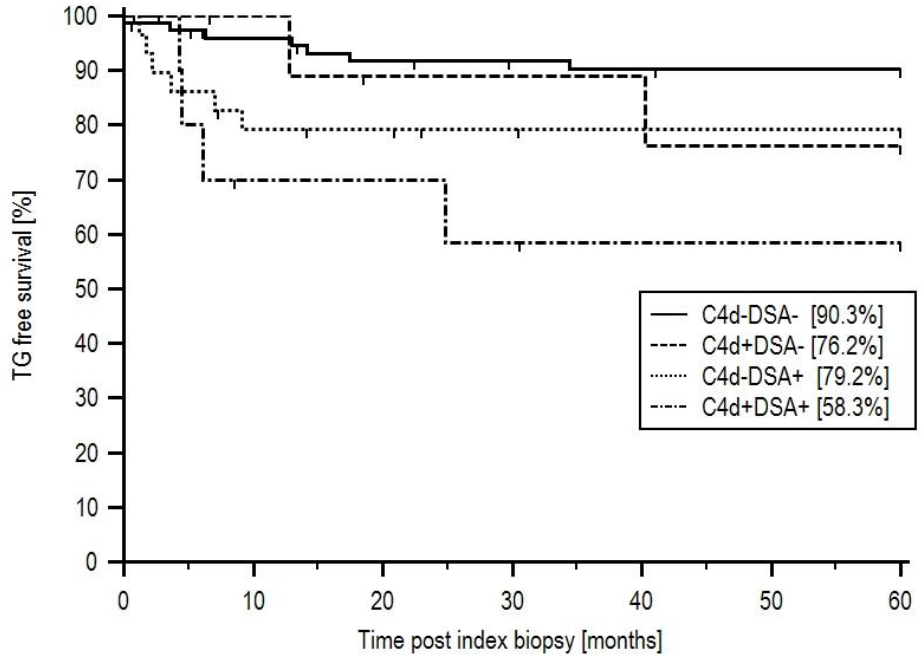



**Transplant glomerulopathy**

Patients with mixed TCMR also had a higher incidence of subsequent transplant glomerulopathy [TG] than the pure group. In this study the diagnosis of TG was only made at indicative biopsy in the setting of allograft dysfunction and/or proteinuria. TG free survival was 90.3% and 74.2% in the pure and mixed groups, p=0.0169. As in the case of AMR, the risk was highest in those patients with both C4d and DSA at the time of index biopsy, as is shown in figure 7.5.

**Figure 7.5. TG free survival post index biopsy by humoral involvement of TCMR.**

Transplant glomerulopathy free survival in the pure, C4d+TCMR, DSA+TCMR and C4d+DSA+TCMR was 90.3%, 76.2%, 79.2% and 58.3% respectively, p=0.0169




**Glomerulitis**

31/126 [24.6%] of TCMR (all cases) had T cell dominant glomerulitis. Glomerulitis was not associated with C4d staining, with only 4/31 (12.90%) of the T cell glomerulitis biopsies being C4d positive, compared with 17/95 (17.89%) TCMR biopsies with no glomerulitis, p=0.59. T cell glomerulitis was also not associated with the presence of DSAs; 11/31 (35.48%) biopsies with T cell glomerulitis had DSA compared with 29/95 (30.53%) biopsies with no glomerulitis, p=0.77.

Glomerulitis on univariant analysis was not associated with subsequent AMR, with an AMR free survival of 80.6% in the patient group with glomerulitis compared with 86.8% in the patient group with no glomerulitis, p=0.38. However, T cell glomerulitis was associated with increased risk of TG, with the TG free survival being 61.3% and 92.0% in the glomerulitis positive and negative groups respectively, p<0.0001. However at the time of calculation, allograft loss was not inferior in the

glomerulitis positive groups compared with the negative group, being 90.3% and 82.7% respectively, p=0.31.

**Multivariant analysis of factors associated with adverse clinical outcomes**

In order to determine the factors at the time of index biopsy associated with AMR, TG and allograft loss, a Cox-proportional hazard model was used considering factors which were significant on univariant analysis, namely C4d positivity, DSA, glomerulitis and patients with C4d positive DSA positive TCMR. Results are shown in table 7.3.

**Table 7.3. Multivariant analysis of humoral features associated with histological TCMR and subsequent outcome.**

| Outcome | TCMR status | HR (95% CI) | p value |
|---|---|---|---|
| **Graft loss** | C4d+DSA+ TCMR | 1.47 (1.02-2.12) | 0.039 |
| **AMR** | C4d+DSA+ TCMR | 1.86 (1.34-2.58) | <0.001 |
| **TG** | DSA+ TCMR | 3.32 (1.37-8.24) | 0.01 |
| | Glomerulitis+TCMR | 5.47 (2.16-13.85) | <0.001 |

## 7.4 Discussion

It was found that TCMR with a humoral component had inferior allograft outcomes when compared with patients with pure TCMR. Patients with C4d positive DSA positive TCMR were at risk of subsequent allograft failure, morphological AMR and TG. These results have important management implications as TCMR with a humoral component might have a better prognosis when treated with immunotherapy directed at both B and T cells.

The Banff classification of TCMR is defined as the presence of interstitial inflammation (i-score ≥2; >25% of parenchyma affected), tubulitis (t-score ≥2; foci of moderate tubulitis) with or without intimal arteritis (v-score ≥0) [Solez et al, 2008]. By immunophenotyping the infiltrating cells consist of either T cells and/or macrophages/monocytes with only a small fraction of B cells [Sementilli et al, 2010]. Historically studies have demonstrated that the prognosis of allografts with TCMR is dependent upon the grade of rejection and arterial involvement. Nickeleit et al, looked specifically at arterial lesions in patients undergoing acute cellular rejection and found that those patients with vascular involvement were more likely to be steroid unresponsive and those with fibrinoid necrosis lost their grafts at 1 year despite augmented therapy with OKT3/ATG [Nickeleit et al, 1998]. Haas et al compared the outcomes of patients with TCMR with mild to moderate arteritis against a group with TCMR with severe arteritis [Banff grade IIA versus IIB TCMR] and found that patients in the latter category had a worse prognosis [Haas et al, 2002]. Bates et al, in their evaluation of the outcomes associated with the different Banff categories, also found prognosis to be correlated with the degree of vascular involvement [Bates et al, 1999]. More up to date studies employing the use of microarrays have shown that 'pure TCMR' can be easily reversed regardless of vascular involvement or timing of rejection episode as long as it is not complicated by AMR [Mueller et al, 2007; Reeve et al, 2009]. Furthermore, molecular methodology is being used to look at the pathogenicity of the isolated vascular lesion (the 'v' lesion), which in the absence of tubulointerstitial inflammation does not display other markers of rejection [Mueller et al, 2007; Sis et al, 2010]. A Banff working group has subsequently been created to try to establish its relevance [Sis et al, 2010].

By Banff criteria acute AMR is defined as morphological evidence of acute tissue injury, C4d positivity and evidence of DSA. It is well established that C4d is strongly associated with DSA and AMR. The histological features of the acute tissue injury considered as AMR lesions include; ATN-like, capillary and/or glomerular inflammation and/or thrombosis or arterial (v3) lesion. The v3 lesion is placed under both the TCMR and AMR category; however glomerulitis although categorised as an

AMR lesion may also be seen in TCMR. Batal et al, in an indepth analysis of glomerulitis, found 47% of DSA negative C4d negative TCMR and 62% of borderline rejection had co-existing glomerulitis [Batal et al, 2011]. Magil et al demonstrated the difference between the glomerulitis associated with TCMR and AMR is the infiltrating cell type, which are lymphocytes in the former and monocytes in the latter.

There have been few studies which have included data on TCMR biopsies with a humoral component. Feucht et al have shown that C4d positivity strongly correlates with allograft loss in their study where the majority of grafts had combined histological features of cellular rejection and what was then described as vascular rejection, and a significant number also had 'cell mediated interstitial' rejection [Feucht et al, 1995]. Nickeleit et al in their analysis of 398 allograft biopsies with the aim to determine the diagnostic relevance of C4d, found that 43% of biopsies with 'interstitial rejection' were C4d positive [Nickeleit et al, 2002]. They treated all cases of C4d positive rejection (regardless of histological features) with enhanced immunosuppressive therapy (OKT3/ATG) and as a result they found no difference in clinical outcomes between the C4d groups. They went further to propose that the term acute cellular rejection should be discontinued as it does not take into account potential co-existing humoral mechanisms and biopsies should be reported according to morphological features modified to include the C4d status [Nickeleit et al, 2002]. Mauiyyedi et al found that the outcomes of patients with C4d positive TCMR and C4d positivity with mixed morphological features of AMR and TCMR correlated closely with pure C4d positive AMR [Mauiyyedi et al, 2002]. In more recent studies, Al-Aly et al showed that it is not uncommon for patients to exhibit mixed morphological features of TCMR and AMR, with inferior outcomes to the pure forms of either category [Al-Aly et al, 2008]. Finally Kayler et al established that even patients with focal C4d and TCMR might have underlying humoral involvement and steroid unresponsiveness occurred significantly more frequently in the focal and diffuse C4d groups irrespective of the underlying histological features, compared with the C4d negative group [Kayler et al, 2007].

It should be highlighted that in this last study as in the one outlined in this chapter, the majority of patients received alemtuzumab induction. It has been reported that rejection episodes following alemtuzumab may consist of a higher proportion of C4d positive cases. Zhang and colleagues in a retrospective descriptive study found that 47.2% of ACR cases following alemtuzumab were C4d positive [Zhang et al, 2007]. In the recently published randomised control trial assessing alemtuzumab compared with conventional induction (basiliximab or ATG) in patients undergoing early steroid withdrawal, there was a tendency to more C4d positive acute rejection episodes in the alemtuzumab treated arm, although this failed to reach significance [Hanaway et al, 2011]. It has

also been reported that the infiltrate following alemtuzumab induction in pure TCMR consists mainly of monocytes, although such cases respond well to corticosteroid therapy [Pearl et al, 2005]. In a further detailed analyses of the cellular infiltrate, patients with CD20+ B cells infiltrates have a worse prognosis, such infiltrates being less frequent in alemtuzumab treated patients [Hippen et al, 2005]. However results from this chapter's study, like others, demonstrate the distinct pathogenesis of the rejection processes in the TCMR episodes that are pure compared with those that have evidence of humoral responses.

It has been shown that patients with TCMR with evidence of a humoral component have inferior outcomes when compared with cases of pure TCMR when treated in a conventional way. Either C4d positive and/or DSA positive TCMR suggests that humoral along with cell mediated immune mechanisms are present, a conclusion that has therapeutic implications. Such patients might benefit from more aggressive therapies such as those used for the treatment of AMR. Given the common interaction of the humoral and cellular components of the immune system, the classic division of the categories of the Banff criteria may be an over simplification. It may be more appropriate for C4d+DSA+ TCMR to be categorised as AMR when deciding on therapeutic measures.

## CHAPTER 8 SUMMARY

Work from this thesis has demonstrated that HLA DSAs are associated with inferior allograft outcomes, whether they are detected pre-transplant or following their development de novo after transplantation. Testing for DSA with sensitive methods such as Luminex beads used in this study creates an opportunity for intervention which in turn might improve outcomes. The following summarises each chapter and how this may impact on clinical practice.

1. ATN is a common finding on indication transplant biopsies. I have shown that C4d positivity in the absence of DSA and morphological features of rejection is not associated with subsequent rejection. Conversely, ATI with detectable DSA in the setting of negative staining for C4d is associated with subsequent rejection. Therefore patients with DSA positive ATI should be considered for interval biopsy.

2. Patients with preformed DSA even with a negative crossmatch are at significant risk of AMR, TG and allograft loss. Therefore such patients should be considered as high immunological risk and should not receive minimalist immunosuppression regimens. How the outcomes compare with positive crossmatch transplants has not been addressed in this thesis.

3. The development of de novo DSAs are associated with AMR, TG and allograft loss. Whilst a proportion of patients will have the DSA detected at the time of or after the rejection episode, the majority will have DSAs detected prior to rejection occurring. Therefore routine screening for DSA after transplant will allow for early detection or prediction of rejection episodes and allow for possible augmentation of baseline immunosuppression. Whether such a protocol for screening and treatment will be of benefit needs formal testing.

4. DQ DSAs are the most frequent DSA to be detected after transplantation and their presence is associated with AMR and TG. Patients mismatched at both DR and DQ alleles are more likely to develop class II DSA. It is important to know the level of DQ mismatch pre-transplant in order to define risk, and its use in organ allocation schemes may help in reducing DQ DSA formation.

5. The outcome of TCMR is dependent upon the DSA and C4d status at the time of rejection. Patients with ‘pure’ TCMR have a good outcome, however those with ‘mixed’ TCMR are more likely to develop histological features of AMR and TG in the absence of antibody

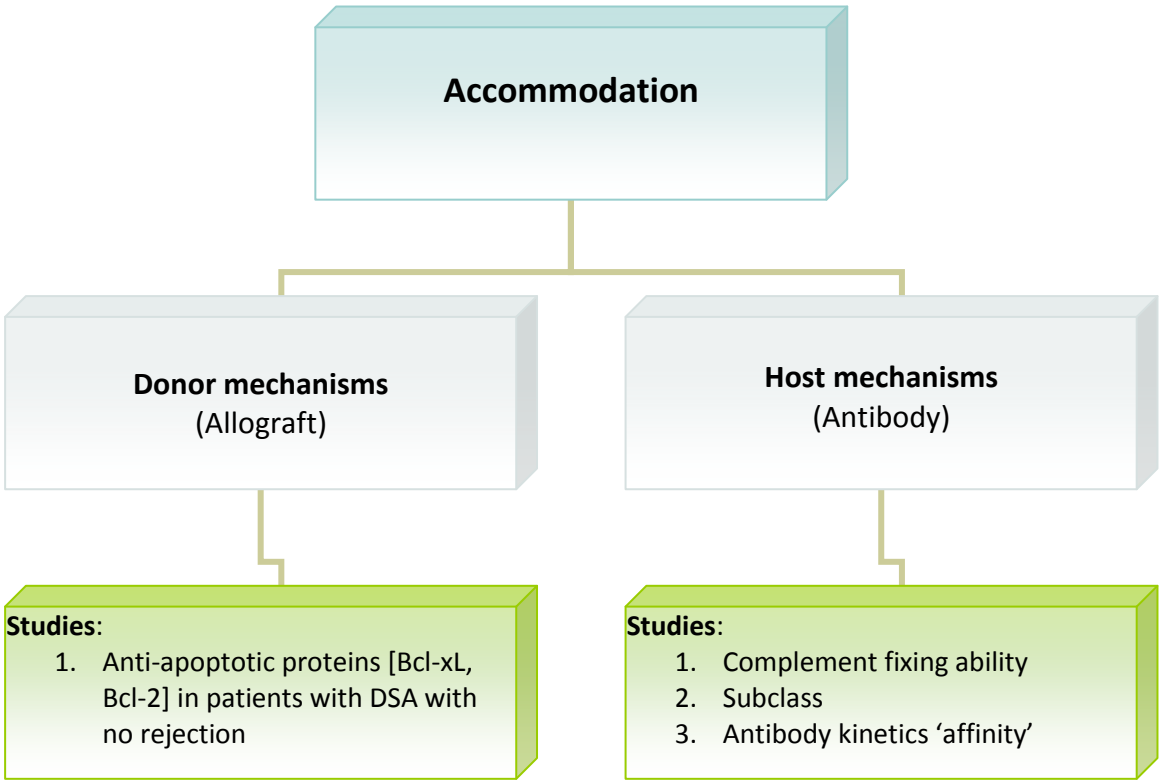
directed therapy. Patients with mixed TCMR might benefit from the more aggressive treatments conventionally used for AMR.

## Chapter 9 Future work

It is postulated that accommodation can occur either by adaption of the allograft or by changes in the characteristics of the donor specific antibody as discussed in chapter 2.

The work in this thesis has shown that HLA donor specific antibodies are associated with AMR and allograft loss. Conversely, there are a proportion of patients who do not experience AMR despite high levels of circulating DSA. Future investigations planned by our department as a result of the work in this thesis will focus on delineating the antibody characteristics associated with pathogenicity and graft properties associated with accommodation as shown in figure below.

**Figure 9.1 Flow diagram of mechanisms of accommodation**



**Host mechanisms of accommodation**: **Pathogenic characteristics of donor specific antibodies**

## 1. Complement fixing DSA

Stemming from this work on HLA donor specific antibodies, an Imperial College post graduate student is currently analysing the complement fixing ability in-vitro of both preformed and de novo DSA. The technique includes incubating patient sera with single antigen Luminex beads and then adding human serum from a recipient with normal C4 levels and CH50 activity. Beads are then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal anti-C4d antibody before being placed in the analyser. This method was first described by Wahrmann and colleagues [Wahrmann et al, 2006]. Anlaysis will then be carried out on the correlation between the complement fixing ability and clinical outcomes.

## 2. DSA affinity (kinetics)

Affinity is most correctly defined as the ratio of rate of association of an antibody-antigen complex to the rate of dissociation and can be determined using a biosensor.

### Blood group antibody kinetics

Preliminary work, by Lee and colleagues at Imperial NHS Trust, has been undertaken on the antibody kinetics of blood group antibodies pre and post ABOi renal transplantation [Lee et al, 2011]. They studied 3 patients who had received ABOi allografts, 1 had experienced early rejection and 2 had an uncomplicated course. Serial serum samples pre and post-transplant were analysed using surface plasmon resonance experiments in order to determine the binding kinetics of antibody to synthetic blood group A and B antigens. The off rate constants [k*off*] were calculated, reflecting how transient binding was. The higher the k*off* the lower the affinity; where affinity equals k*on*/k*off*. They found that the k*off* in the 2 patients with no rejection increased post-transplant, whilst the patient with rejection had a

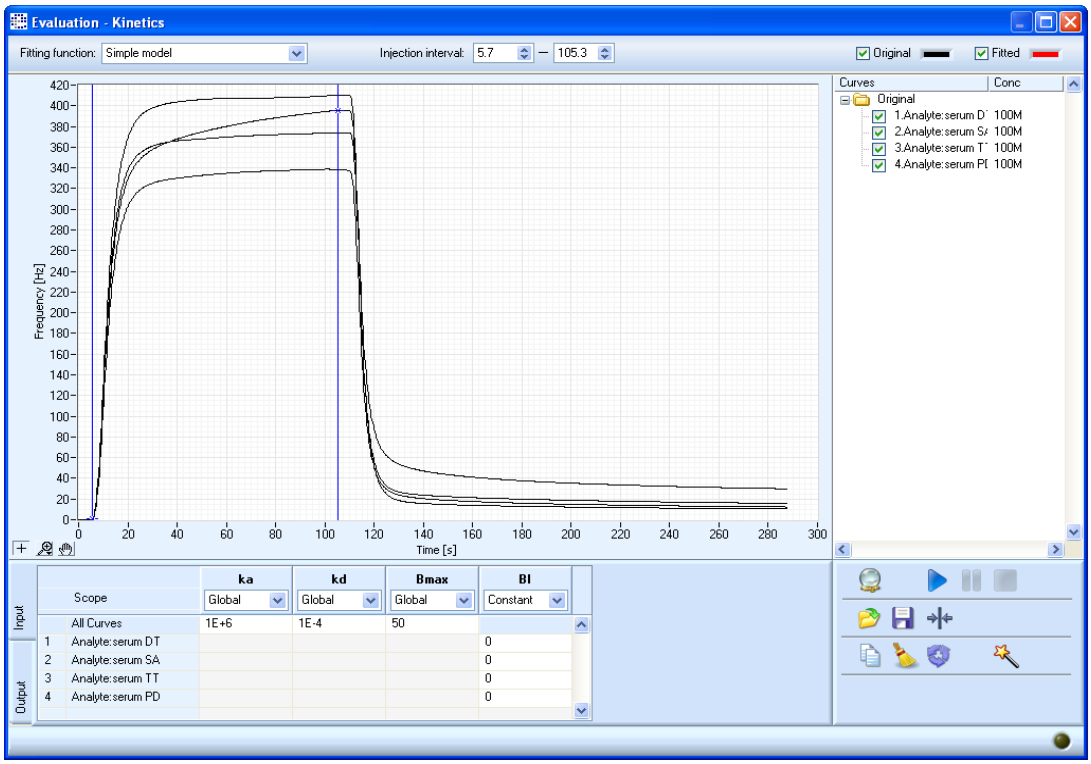
decrease in k*off* . They hypothesise that antibody with a falling affinity, reflected in increased k*off*, protects the graft; whilst increased affinity is associated with rejection [Lee et al, 2011].

Similarly, antibody affinity of anti-GBM antibodies has been shown to correlate with clinical outcomes [Dougan et al, 2002] in work also carried out at Imperial College.

**HLA DSA kinetics**

Dr Lee has been instrumental in the preparatory work for the next study. Using biotinylated HLA pentamers bound to a chip with streptavidin, we were able to develop an assay in which the kinetics of HLA antibody binding to a HLA molecule were determined. We used the Attana® biosensor, which uses a quartz crystal which undergoes mechanical oscillation when an electric potential is passed through it. The magnitude of oscillation depends on how much the crystal is dampened (ie the antibody load). The graph below is a pictorial representation of the kinetics from 4 patients with A2 donor specific antibodies. The up curves represent association, whilst the down curves represent dissociation. Future work will involve refinement of this assay and its subsequent application to characterise the affinity of donor specific antibodies and correlate with clinical outcomes. This potentially could have clinical diagnostic application.

**Figure 9.2 Association and dissociation curves of 4 separate patients with de novo A2 DSA.**




## Donor mechanisms of accommodation: Phenotype of accommodation

During the work carried out in this thesis patients have been identified who might be considered to have accommodated. Notable are the patients who are C4d and DSA positive without the classical morphological features of rejection on biopsy. We plan to carry out immunohistochemical staining of the C4d+DSA+ATI patients looking for expression of anti-apoptotic proteins [Bcl-xL and Bcl-2] and make a comparison of the outcomes. As RNA is extracted from biopsy specimens, the gene expression for the anti-apoptotic proteins can also be tested for. This potentially can be a predictive tool which will enable identification of patients at risk of poor outcome. This study may also be applied to surveillance biopsies which show C4d positivity in patients with DSA.

Other related studies:

1. **Clinical relevance of HLA epitope mismatching**

   HLA matchmaker is a computer based algorithm devised by Duquesnoy and colleagues. The initial aim of the computer programme was to determine HLA compatibility at the epitope level as opposed to the serologically defined antigen level in sensitised patients. It was subsequently shown that previously considered unacceptable HLA mismatches can be compatible at the structural level, which is important as it opens up the opportunity of transplantation for those patients who are highly sensisited [Duquesnoy et al, 2001, 2008, 2009].

   Using the algorithm, Silva and colleagues analysed the outcomes of renal transplantation comparing epitope versus antigen mismatching. Although there was no difference in failure and rejection rates, there was a greater correlation between de novo HLA antibody formation and epitope mismatching compared with HLA antigen mismatching.

   Using the patient cohort studied in this thesis, it would be useful to determine:

   a. The epitope mismatching in the patient group with preformed DSA and correlate with outcomes.

   b. Analyse the epitope matching in the patients who developed de novo DSA and compare an HLA antigen mismatched group who did not develop DSA.

2. **Revelance of non-HLA DSA**

   The H&I laboratory have screened for MICA antibodies in all patients studied in this thesis. Although patients are not typed for MICA, it will be useful to look at the clinical outcomes associated with those patients who develop de novo MICA antibodies.

   During the work of this thesis patients were also identified who had biopsy proven AMR with or without C4d positivity, but had no detectable HLA DSA or non-DSA. The circulating antibody associated with the immune mediated damage therefore remains unknown. In such cases, the stored serum of the patients should be tested for an array of non-HLA

antibodies, including vimentin antibodies, angiotensin II type I receptor antibodies and endothelial cell antibodies].

**3. Variation of DSA over time**

Evidence presented in this thesis has shown that patients with preformed DSA in the setting of a negative crossmatch are at increased risk of AMR and allograft failure. We hypothesise that factors impacting outcomes include: method of sensitisation and variation of MFI over time. The natural history of how HLA antibody detected by solid phase methods varies in terms of MFI is not known. Patients with worse outcomes might have had higher MFIs on historic samples, sufficient even to cause a positive crossmatch. They are also more likely to have a definitive sensitisation event such as previous transplantation, pregnancy or transfusion. Understanding the precipitating event and the subsequent timeline might help us further in terms of defining risk.

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