Phenotype of HLA Antibodies in Patients with Antibody Mediated Rejection

Thesis submitted for MD(Res)

Alexander Gueret-Wardle

Faculty of Medicine

Imperial College London

June 2019

Acknowledgements

This work would not have been possible without the unwavering support of family and friends. In particular, I would like to thank my mother and my partner for their patience and encouragement.

My “transplant office” family; Dr Sevda Hassan, Dr Gaetano Lucisano and Dr Anamika Adwaney whose knowledge, kindness and comradery I shall always remember and cherish.

The clinical scientists of the Histocompatibility and Immunogenetics laboratory at Hammersmith Hospital, in particular; Eva Santos-Nunez, Rachel Wilson, Nicola Firmin, Fiona Powell, Nicola Gunby and Corinna Steggar for all help “HLA”.

To Candice Roufosse for her guidance in sound methodological practice, transplant biopsy scoring and pastoral support.

I could not have started nor ended this work without the support of Dr Michelle Willicombe, who guided my initial interest in transplant immunology.

Finally, I would like to thank The National Institute for Health Research (NIHR) Biomedical Research Centre at Imperial College London for financial support in multiple projects.

Section of Renal Medicine and Vascular Inflammation

Division of Immunology and Inflammation

Department of Medicine

Imperial College, London

Hammersmith Campus

Imperial College Renal and Transplant Centre

Hammersmith Hospital

Du Cane Road

London W12 0NN

Under the supervision of

Dr Michelle Willicombe

Professor Charles Pusey

DECLARATION

I declare that the work in this thesis, which I submit for examination in consideration of the award of an MD(Res) is my own personal effort. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law and has not been taken from other sources except where such work has been cited and acknowledged within the text.

COPYRIGHT

Abstract

Renal transplantation is the treatment of choice for patients with end stage kidney disease. Not only is there a survival benefit but there is a significant improvement in quality of life when compared to patients that remain on dialysis.

Although short term graft survival has improved in the last 60 years, late graft loss remains a problem. Antibody mediated rejection (AMR) is a major determinant of kidney allograft failure, with chronic AMR being the leading cause of late graft loss. Donor specific anti-HLA antibodies (DSA) have been reported to be strongly associated with an increased risk of rejection and allograft failure. Although the presence of anti-HLA DSAs is thought to be one of the most important biomarkers in predicting allograft failure there is no consensus on their pathogenicity and no standardised protocol on how to approach their clinical management.

Whilst most DSAs are of immunoglobulin (Ig) G isotype Anti-HLA DSAs can also exist in IgM, IgE and IgA subtypes, although their roles are less well understood.

The value of phenotyping these antibodies has not been fully established. The principal aim of this work is to determine whether more sophisticated assays help predict outcomes for those most at risk of allograft rejection.

The underlying hypothesis of this work is that phenotyping of DSAs helps stratify the risk of poor outcomes according to the phenotype of rejection. Patients were recruited from Imperial College Renal and Transplant centre. The results are presented in several related studies.

1. IgG anti-HLA donor specific antibody subclass phenotyping in chronic antibody mediated rejection (cAMR)
2. C1q Binding anti-HLA donor specific antibodies in patients with cAMR
3. What is the role of IgM anti-HLA donor specific antibodies in renal transplantation
4. Anti-HLA IgE: good, bad or indifferent?

Summary of Results

1. Patients with de novo IgG DSAs in the setting of histological features of cAMR have poorer graft survival than those without. In particular, patients with class II IgG2 and IgG3 DSAs present with more severe clinical phenotypes of cAMR, with a dramatically increased risk of allograft loss.
2. C1q binding class II DSAs are associated with reduced allograft survival, however, graft loss in cAMR is unlikely to be a complement dependent process.
3. Whether alone or in the presence of an IgG DSA, IgM DSAs do not add any cumulative risk with regards to allograft loss.
4. Patients who have developed a de novo IgG allo-immune response often have a co-existing IgE DSA. This antibody may indeed be functional and have pathologic implications. The presence of IgG4 DSA correlates with the development of IgE DSA.

TABLE OF CONTENTS

List of Tables

List of Figures

ABBREVIATIONS

AMR Antibody mediated rejection

aAMR Acute antibody mediated rejection

BCR B cell receptor

Bregs Regulatory B cells

cAMR Chronic antibody mediated rejection

CDC Complement dependent cytotoxic

CNI Calcineurin inhibitor

CSR Class switch recombination

DCD Donor after cardiac death

DGF Delayed graft function

dn De novo

DSA Donor Specific Antibody

DTT Dithiothreitol

ESKD End stage kidney disease

EM Electron microscopy

ENDAT Endothelial cell associated transcripts

Fab Antigen binding fragment

Fc Fragment crystallisable region

FcR Fc receptor

FCXM Flow cytometric crossmatch

FK Tacrolimus

g Glomerulitis

GFR Glomerular filtration rate

HLA Human leucocyte antigen

IF Immunofluorescence

Ig Immunoglobulin

IFN Interferon

MAC Membrane attack complex

MBL Mannose binding lectin

MFI Mean fluorescence intensity

MHC Major histocompatibility complex

MI microcirculatory inflammation

MMF Mycophenolate mofetil

PBT Pathogenesis based transcript

PE Phycoerythrin

PRA Panel reactive antigen

PTC Peritubular capillaritis

PTCBML Peritubular capillary basement membrane multilayering

SAB Single antigen bead

TCMR T cell mediated rejection

TCR T cell receptor

TG Transplant glomerulopathy

TGF Transforming growth factor

Th1 Type 1 helper

Th2 Type 2 helper

TLR Toll like receptor

Tregs Regulatory T cells

Oral national and international presentations

British Transplant Society Annual Congress, Brighton 2018:

Medawar Medal presentation

Class II anti-HLA IgG2 and IgG3 predict poorer outcomes in chronic antibody mediated rejection

British Transplant Society Annual Congress, Brighton 2018:

IgM Donor Specific Antibodies Do Not Affect Graft Survival in Renal Transplantation

American Transplant Congress, Seattle 2018:

Class II anti-HLA IgG2 and IgG3 predict poorer outcomes in chronic antibody mediated rejection

American Transplant Congress, Seattle 2018:

IgM Donor Specific Antibodies Do Not Affect Graft Survival in Renal Transplantation

Introduction

For those with end-stage kidney disease (ESKD), renal transplantation remains the treatment of choice[1]. Transplantation provides not only a survival benefit but a significant improvement in quality of life when compared to patients who remain on dialysis[2,3].

An awareness of anti-HLA antibodies occurring after transplantation has existed for almost fifty years[4]. Despite this, it has only been in the last decade that consistent evidence has built up linking these antibodies with allograft rejection[5-8]. There is now a consensus that antibody mediated rejection (AMR) is the leading cause of late allograft failure[9]. Consequently, a greater understanding of the characteristics and the early identification of these antibodies is essential to the progress of clinical management and graft survival in renal transplantation.

Exposure to non-self HLA antigens pre or post-transplant increases the risk of anti-HLA antibody production. The common routes of sensitisation include; blood product transfusion, pregnancy and solid organ transplantation. Whether sensitised or not, all transplant recipients have a significant risk of developing de novo donor specific antibodies (dnDSA)[10]. Importantly, 15% of patients considered at low immunogenic risk will still produce dnDSA within approximately five years post-transplant[8]. These DSAs can produce a wide variety of immune injuries, whether arising early or late post-transplant. Although acute AMR tends to be more severe in its presentation, chronic AMR (cAMR) presents no less of a clinical challenge, with late diagnoses and a lack of treatment options. Pre-formed DSA is more commonly associated with early AMR with higher degrees of glomerulitis and overall microcirculatory inflammation (MI) compared to dnDSA AMR which presents later with increased proteinuria, lower glomerular filtration rates (GFR) and more chronic lesions[11]. Importantly all forms of AMR have a profound effect on graft survival[7] with one study showing a 4.73 fold higher risk of allograft failure in patients with AMR (95% confidence interval, 1.57-

14.26; p=0.006)[12]. Distinct phenotypes of alloimmune injury have been correlated with varying subclasses of IgG DSA and so the importance of phenotyping antibodies beyond their HLA specificity is becoming more apparent[13,14].

As with any disease, the optimal management of AMR is prevention, however immune risk is still unavoidable in the modern day, with high organ need and the practical impossibility of perfect matching in allogeneic transplantation[15]. Thus, understanding the future pathological implications of antibody phenotype will be one of the cornerstones in improving renal transplant outcomes.

Chapter 1

1.1 The Major Histocompatibility Complex

In humans, the products of the major histocompatibility complex (MHC) are termed human leucocyte antigens (HLA) and are essential components of the immune system. They allow recognition of pathogenic proteins by T-cells by binding and displaying antigen on cell surfaces. Ultimately, their role is to recognise foreign pathogens however this extends further in transplantation as donor HLA provokes allorecognition in the recipient, which is the common pathway leading to graft rejection. All vertebrates have MHC genes with great heterogeneity. In humans, this is found on chromosome 6p21 and is comprised of 224 genes across 3.6 megabase pairs. Within are contained three separate regions, class I, class II and class III. The MHC is responsible for encoding the most polymorphic proteins in humans principally, the class I and class II human leucocyte antigens including more than 200 different allelic variations, evolving over thousands of years through interaction of our immune systems with pathogens[16]. Whilst the class III region does not include HLA genes, it encodes important proteins in the inflammatory response, including components of the complement system, three components of the TNF family cluster and heat shock proteins[17].

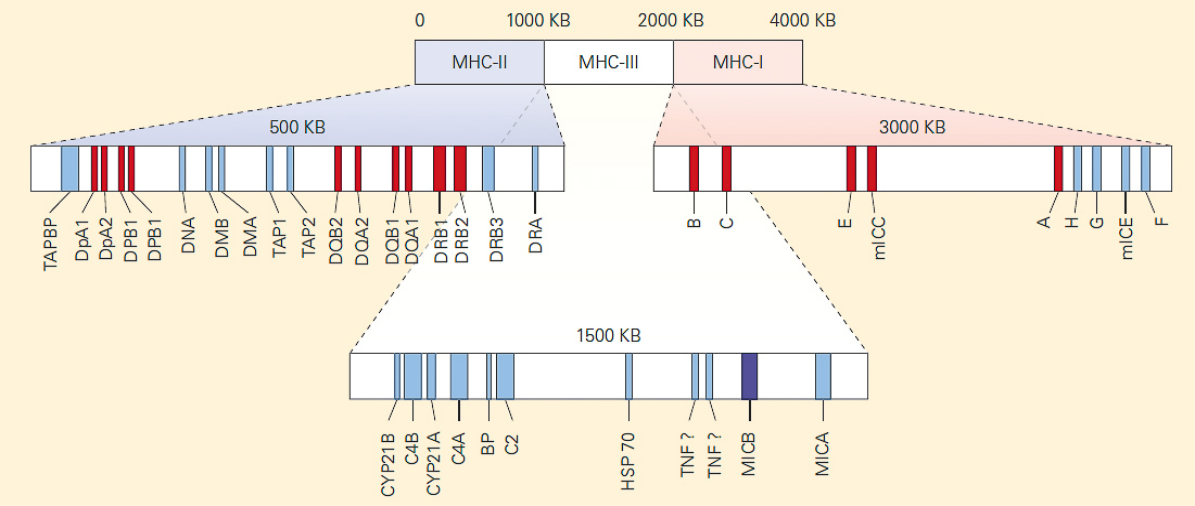


Figure 1.1. Genetic map of the MHC regions on chromosome 6. Image from Bellanti. Immunology IV: Clinical Applications in Health and Disease. I Care Press, Bethesda, MD, 2012]

1.1.1 Classes of MHC

The distinction between HLA classes is not only based on their molecular structure but also their expression. The most well-known class I antigens include HLA-A, -B and –Cw but also include –E, -F and –G. Structurally, class I MHC are made up of a polymorphic α chain bound non-covalently to a β2 microglobulin molecule. The tail of the α chain is the single transmembrane domain. Folding of the polymorphic α1 and α2 domains form the antigen binding site. The α3 subunit will bind CD8 molecules on cytotoxic T cells[18]. These molecules are present on all nucleated cells and are responsible for presenting intracellular peptides including viral proteins, broken down in proteasomes, to cytotoxic (CD8+) T cells leading to apoptosis of the infected cell.

Class II antigens, include; HLA-DR, -DQ and –DP but also –DM and –DO, are principally expressed on antigen presenting cells (APCs) such as dendritic cells, B lymphocytes, macrophages and activated T-cells. In contrast to class I MHC, class II molecules consist of an α and a β chain both of which have transmembrane domains. Folding of polymorphic residues

at the α1 and β1 domains form the antigen binding site. The β2 region binds CD4 molecules on T helper cells[18]. Class II HLA molecules present extracellular peptide following lysosomal degradation after phagocytosis to T helper cells. This, in turn, may result in downstream B-cell activation leading to antibody production.

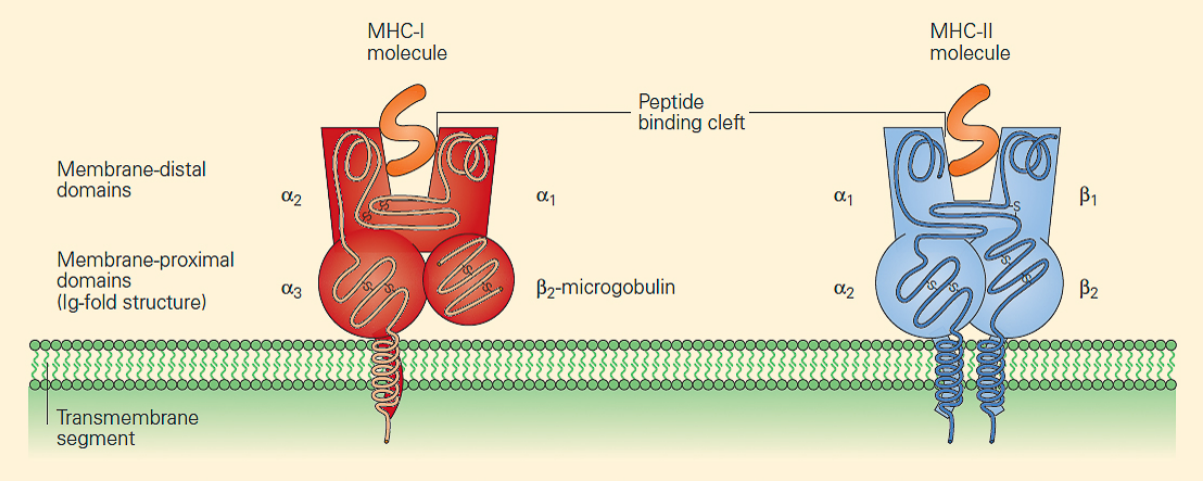


Figure 1.2. MHC-I and MHC-II molecules. Image taken from Bellanti, JA. Immunology IV: Clinical Applications in Health and Disease. I Care Press, Bethesda, MD, 2012

1.2 Mechanisms of Allorecognition in Transplantation

1.2.1 T-Cell Stimulation

Allorecognition is the rate limiting step that eventually results in clinical rejection of allografts. T cells, both CD4+ and CD8+, recognise only specific single peptide antigen complexed on the MHC of APCs. There are two main pathways of allorecognition, the direct and indirect pathways. These are not mutually exclusive of one another and likely have a more discrete interplay than their names imply. Each pathway, does however produce different types of allospecific T-cell clones.

The direct pathway requires host T cells to recognise allo-MHC of donor APCs via the T-cell receptor (TCR). Donor MHC will present complexed antigens from endogenous peptides. This pathway does not occur outside alloimmunity and is thus considered the main pathway required for an early alloimmune response. Interestingly, unlike presentation by self-antigen presenting APCs, the resulting number of T-cell clones produced is much higher[19]. Passenger APCs from the donor have high levels of alloantigen bound to MHC and cause direct recipient T-cell activation[20].

In contrast, the indirect pathway recognises alloantigen from host APC. Donor antigen is released from the allograft and taken up by host APCs. Exogenous peptides bind self-MHC on APCs, resulting in the stimulation of allo-reactive T-cells.

Brown et al discovered a third mechanism in vivo by which recipient T-cells can be stimulated by recipient APCs presenting intact donor antigen-MHC complexes following contact with passenger donor APCs[21].

Co-stimulation is required in all three pathways with specific ligands on the APC and other T-cell surface receptors. CD28 on T cell surfaces complexes with B7-1 and B7-2 to prevent apoptosis that is normally induced after TCR signalling[22].

1.2.2 T-cell Activation

Following TCR signalling, phosphatidylinositol 4,5 bisphosphate (PIP) hydrolyses to become inositol triphosphate (IP3) and diacylglycerol (DAG). With the production of IP3, calcium is released from the endoplasmic reticulum, which forms complexes with calmodulin. Protein kinases are activated, including phosphatase calcineurin. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), upon which it binds to the nuclear IL-2 promoter sequence allowing IL-2 mRNA transcription. Calcineurin provides a major target in transplant

immunosuppression for the calcineurin inhibitors (CNI), cyclosporine and tacrolimus. Cyclosporine and tacrolimus bind the immunophilins cyclophilin and FK506 binding protein respectively, inhibiting the dephosphorylation nuclear factor of activated T-cells (NFAT) by calcineurin. DAG activation of nuclear factor κB (NFκB) results in transcription of genes which include MHC class I, Ig and IL-2. Corticosteroids exert their anti-inflammatory effect by inhibiting the expression of pro-inflammatory transcription factors such as NFAT[23].

1.2.3 Effector T-cell Functions in Rejection

Although strongly linked a two-pathway paradigm has been suggested in the effector mechanisms of T-cells involving both antigen dependent and antigen independent factors[24]. Damage independent of immunity such as that which occurs in infection and ischaemia results in upregulation of MHC class II, cytokines as well as the release of soluble HLA. Indirect allorecognition by CD4+ T-cells occurs. Proliferation into subgroups of type 1 helper (Th1) and type 2 helper (Th2) cells occur depending co-stimulatory cytokines. Th1 cells produce IL2 and IFNγ, which result in cellular responses by the immune system as well as further MHC expression in grafts[25]. Th2 cells stimulate B-cells to produce cytokines and alloantibody. CD8+ T-cells are antigen dependent, following an encounter with MHC class I they induce cell mediated cytotoxicity which can induce apoptosis through the Fas ligand on cells. Cytotoxic mechanisms are thought to play a greater role in acute rather than chronic forms of allograft rejection[26].

1.3 B Lymphocytes in Transplantation

1.3.1 Development

B lymphocytes derive their name from the bursa of Fabricius, a lymphoid organ originally discovered in birds in by Chang and Glick in 1955[27]. In humans, the majority of B-cells develop in the bone marrow, whilst a minority develop in pleural and peritoneal cavities during foetal life. Lymphoid progenitors (Pro-B cells) express CD45 as well as CD19; the latter will be retained throughout the life of the B-cell until the point of differentiation into a plasma cell. CD-19 will also form complexes with MHC class II molecules necessary for antigen presentation. Interaction with stromal cells is required in the early stages along with transcription factors including Pax-5, terminal deoxytransferase (TdT), RAG-1 and RAG-2[28]. Expression of surrogate light chain proteins, $\lambda 5$ and VpreB as well as DNA rearrangement of Ig heavy chains allows the formation of the pre-B cell receptor (pre-BCR) at the point of pre-B-cell development[29]. Successful light chain rearrangement and combination with now formed heavy chains allows IgM to be formed, which is expressed at the cell surface forming the immature B-cell. Two types of selection then occur, either positive selection in which the pre-BCR and BCR bind their ligands or negative selection in which the BCR binds self-antigen[30]. Negative selection leads to either clonal deletion or further rearrangement of light chains[31]. The fate of positively selected pre-B cells results in down regulation of adhesion molecules which bound the immature B-cells to stromal cells allowing migration to secondary lymphoid organs. Those cells that do not encounter their respective pre-BCR and BCR ligands undergo apoptosis resulting in less than 10% of immature B-cells leaving the bone marrow to the periphery.

Peripheral B-cells are divided into two groups follicular and marginal zone B-cells. Both have undergone mRNA transcription allowing the expression of membrane bound IgM and IgD. At

this point the B-cell is naïve. Failure of the B-cell to encounter their specific antigen results in cell death within days. Follicular B-cells are located in lymph node follicles and require T-cell dependent activation to produce a humoral response through peptides associated with MHC class II. Marginal zone B-cells interface at blood-lymphoid borders producing low affinity IgM antibody in response to blood borne pathogens in a T-cell independent manner. These do not undergo class switch and will not mature into memory cells. B-cell activation and Ig isotype switch are described later.

Memory B-cells form in germinal centres following T-cell dependent activation and differentiation of naïve B-cells into both short-lived plasma cells and long lived (years) memory cells. Following re-exposure to antigen they proliferate rapidly into plasma cells creating a more robust secondary humoral response.

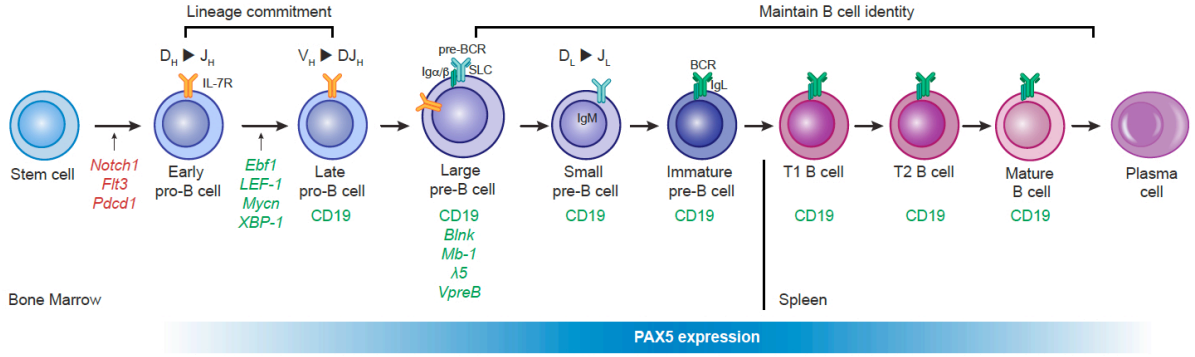


Figure 1.3: B cell differentiation and Pax5. Development progress from stem cells through the pro-B cell, pre-B cell and immature pre-B cell stages. The blue bar below the diagram designates the timing of Pax5 expression. Factors activated by Pax5 are in green, while factors inhibited by Pax5 are in red. Image taken from Ann Murray, Beutler B. Record for glacier, updated Jul 18, 2018. MUTAGENETIX ™, B. Beutler and colleagues, Center for the Genetics of Host Defence, UT Southwestern Medical Center, Dallas TX. (URL: mutagenetix.utsouthwestern.edu)

1.3.2 Roles of B Lymphocytes in Transplant Rejection

B lymphocytes have multiple roles in transplant rejection and are therefore a focus of targeted therapies. Traditionally they produce donor specific anti-HLA antibodies which have been shown to cause allograft injury[32] as well as pro-inflammatory cytokines IL-6, interferon-γ (IFN-γ) and lymphotoxin-α. They are also potent APCs able to activate alloreactive T helper cells through presentation of donor antigen, thus leading to T-cell mediated damage of the graft[33]. Further evidence of alloimmune graft injury caused by B-cells has been demonstrated by the presence of B-cells and their gene transcripts on allograft biopsies[34,35]. Conversely, the pathogenic role of B-cells is not as simple as once thought, studies in mouse models have shown that B-cells can exert an immunoregulatory effect, suppressing pathogenic T-cell responses, upregulating regulatory T-cells (Tregs) and downregulating the inflammatory response, through IL-10 and transforming growth factor β (TGF-β)[36,37]. These regulatory B-cells (Bregs) are thought to be important for tolerance in murine inflammatory models including infection, auto-immunity and neoplasms. Bregs have been shown to increase the survival of skin grafts in mice; however, there is a significant gap to bridge in answering whether Bregs have a role to play in human alloimmunity[36,38].

1.3.3 Immunoglobulin

Immunoglobulins are cell surface and soluble proteins responsible for recognition and adhesion of cells. They consist of four polypeptide chains, two identical heavy chains and two identical light chains linked by disulphide bridges. Each chain folds into variable and constant domains. The variable domain contains the antigen-binding fragment (Fab) and contains the paratope which is highly specific for binding a particular epitope on the target antigen. The constant domain defines the isotype of the immunoglobulin. Recognition also occurs at the

fragment crystallisable region (Fc region) located within the constant domain. Here antibody can bind complement and other effector cells via Fc receptors (FcRs). The main functions of antibodies are as follows. Neutralisation of pathogens occurs when antibody binds their surface and thus inhibit entry into host cells. Opsonisation by antibody identifies pathogen for phagocytosis through interactions with FcRs. Antibody activation of complement induces cell lysis and inflammation via chemotactic accumulation of inflammatory cells and degranulation of basophils, mast cells and eosinophils. The variability of Fc regions across isotypes and subclasses are mostly responsible for the different effector functions of antibodies.

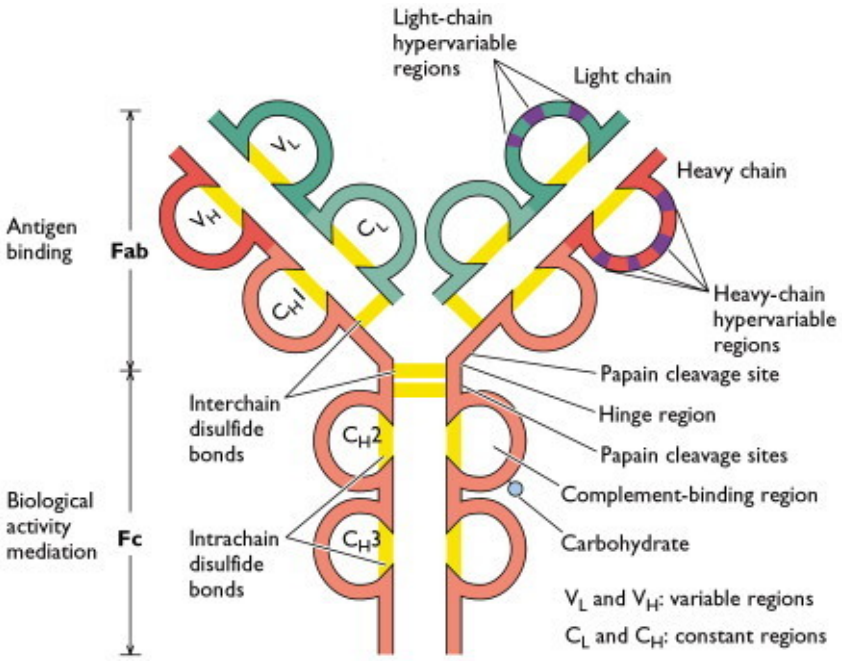


Figure 1.4. Structure of Immunoglobulin.

Image taken from (URL: https://microbiologyinfo.com/antibody-structure-classes-and-functions)

1.3.4 Immunoglobulin Isotypes and Subclasses

The heavy chains are designated as alpha, gamma, delta, epsilon and mu. These define the five main isotypes IgM, IgD, IgG, IgA and IgE. In humans, there are four subclasses of IgG; IgG1, IgG2, IgG3 and IgG4 as well as two subclasses of IgA; IgA1 and IgA2 respectively. This is relevant as the immunological properties of both isotypes and subclasses differ in their pathology.

1.3.5 Immunoglobulin Class Switching

Immunoglobulin isotype switching requires the replacement of the heavy chain constant region (C_H) within the antibody. This region influences the isotype properties influencing affinity for antigen binding, efficacy of effector functions and ability to transcytose across cell membranes, which in turn influences tissue penetration. Naive B-cells initially produce IgM and IgD. Mature B-cells will circulate until they encounter specific antigen either through infection or immunisation. At this point class switch recombination (CSR) occurs, a DNA recombination event, which allows the replacement of μ and δ C_H region exons with the required γ, ε or α heavy chain exons in the daughter B-cells. This DNA deletion recombination occurs in switch (S) regions upstream of the C_H genes[39]. Isotype and subclass production depends on the type of antigen stimulation. These are split into thymus-dependent (TD) and thymus independent (TI) antigens.

TI antigens do not require CD4+ T helper cells to activate B lymphocytes and produce an antibody response. They are further split into TI-1 and TI-2 antigens. TI-1 antigens bind toll-like receptors (TLR) expressed on B-cells and result in low affinity IgM and IgG2 production. This activation however does not result in the development of memory B-cells. TI antigens include bacterial DNA and lipopolysaccharide and are known as B-cell mitogens. TI-2 antigens,

whilst not requiring T-cell help to activate B-cells, do require co-stimulation of cytokines such as IL2, IL3 and IFNγ produced by NK cells and T lymphocytes. They act by cross linking B-cell receptors (BCR) leading to differentiation and production of antibody. Highly repetitive epitopes of encapsulated bacteria are examples of T2 antigens.

TD antigens can only produce a humoral response in the presence of T-cell help. BCRs bind TD antigen bound to MHC-II molecules and present it to T-cells. Co-stimulation between CD40 on the B-cell and CD40 ligand on the T-cell is required to activate the B-cell and produce antibody. Here, antibody production takes longer than the response to TI antigens, however it results in higher affinity antibody which is more functionally versatile[18]. Ultimately short and long-lived plasma cells are produced as well as memory B-cells, which produce anamnestic secondary antibody responses in both T-cell dependent and independent pathways.

1.3.6 IgG Subclasses

IgG1 represents the most abundant subclass in humans, constituting approximately 65% of total serum IgG. IgG2, IgG3 and IgG4 make up the remaining 25%, 10% and 5% respectively[40,41]. Subclasses of IgG are differentiated by variation in their gamma heavy chains. Whilst, there is almost 90% homology between constant regions, the 10% difference can result in distinct differences in affinity for antigen and effector function[42]. Within the heavy chain constant region 2 (CH2) there is significant variation in the N-linked glycan[43]. These include the Fc regions where immunoglobulin binds complement and FcγRs leading to much of the heterogeneity in subclass effector function.

IgG1 and in particular IgG3 have long hinge regions which give these subclasses greater flexibility when forming intercellular bridges at the Fc region. This is thought to increase the affinity by which they bind effector molecules through the FcR[44]. Therefore, IgG1 and IgG3 have the greatest affinity for binding complement and activating the classical pathway[45]. By comparison, IgG2 and IgG4 have more rigid shorter hinge regions which are likely to contribute to their weaker complement binding properties[13,45,46]. In fact, IgG2 is only capable of binding complement at high titre and IgG4 is unable to bind complement at all. It has been suggested that although IgG4 has a great affinity for binding antigen, its limited effector function may be a mechanism for reducing antibody responses following chronic antigen exposure[47]. IgG subclass switch begins with IgG3 then IgG1 followed by IgG2 and finally IgG4, this occurs in one direction, such that B-cells cannot switch back following change to a downstream subclass[42]. It is important also to consider non-complement fixing anti-HLA subclasses in rejection. Their presence may simply indicate class switch, however studies have shown that non-complement fixing subclasses can act synergistically with complement fixing subclasses[48]. Activation of NK cells, macrophages and neutrophils can occur via antibody interaction with Fc receptors. This results in the production of pro-inflammatory and proliferative cytokines[49].

1.4 Complement

First described by Jules Bordet[50], the complement system consists of cell surface and serum proteins that result in proteolytic enzyme system cascade that acts to complement antibody function. The role of complement after activation is to opsonise pathogens, produce anaphylatoxins and induce cell lysis through the membrane attack complex. Complement products become active when covalently bound to antibody or foreign antigen. Alongside the

cascades regulatory components, this helps to safeguard against complement activation in the absence of foreign peptides.

1.4.1 Complement Pathways

There are three main pathways to activate complement. The classical, alternative and the mannose binding lectin pathway (MBL).

In the classical pathway, C1 complex made up of C1q, C1r and C1s bind to the constant regions of IgM and IgG heavy chains. Binding occurs at the Ig Fc region to C1q. Since activation can only occur if two or more Fc regions are bound, at least two IgG-antigen complexes are required for C1 activation. IgM exists as pentamers; hence, a single IgM-antigen complex can activate the classical pathway. Once bound to C1q, Ig Fc regions undergo conformational change leading to activation of the serine proteases C1r and C1s. C4 is cleaved into two active components C4a, C4b as well as the inactive C4d. Although inert, C4d binds the membranes of endothelial cells and when detected on biopsy specimens suggests activation of complement and possible rejection. C4b binds cleaved fragments of C2 (C2a) forming C4b2a also known as C3 convertase. C3 is subsequently cleaved by C3 convertase releasing C3a and C3b, two potent anaphylatoxins. These can lead to positive feedback with further generation of C3 convertase through the recruitment of factor B when bound on cells. C5 convertase is generated as C4b complexes with C3 convertase producing C5 convertase. Cleavage of C5 to C5a and C5b begins the formation of the membrane attack complex (MAC). Functional MAC binds to cells forming transmembrane channels across lipid bilayers perforating cell membranes triggering osmotic lysis.

Similar to the classical pathway, the MBL pathway replaces the function of C1r and C1s with MBL associated serine proteases (MASPs). MBL binds sugar residues on the surface of

pathogens producing lectin-sugar complexes or MASPs. Activation of complement then follows in the same manner as the classical pathway. The sugars required to bind MBL do not exist on mammalian cells and meaning this pathway is not associated with allograft rejection. Finally, the alternative pathway forms part of the innate immune system and predates adaptive immunity[51]. Small amounts of C3 exist in an auto-activated state responsible for continually monitoring for foreign or damaged cells. Activated C3 binds factor B, mediated by factor D resulting in the alternative pathway C3 convertase C3bBb which ultimately leads to production of the MAC after stabilisation of C5 convertase. C3b production by both the classical and MBL pathways can amplify the alternative pathway.

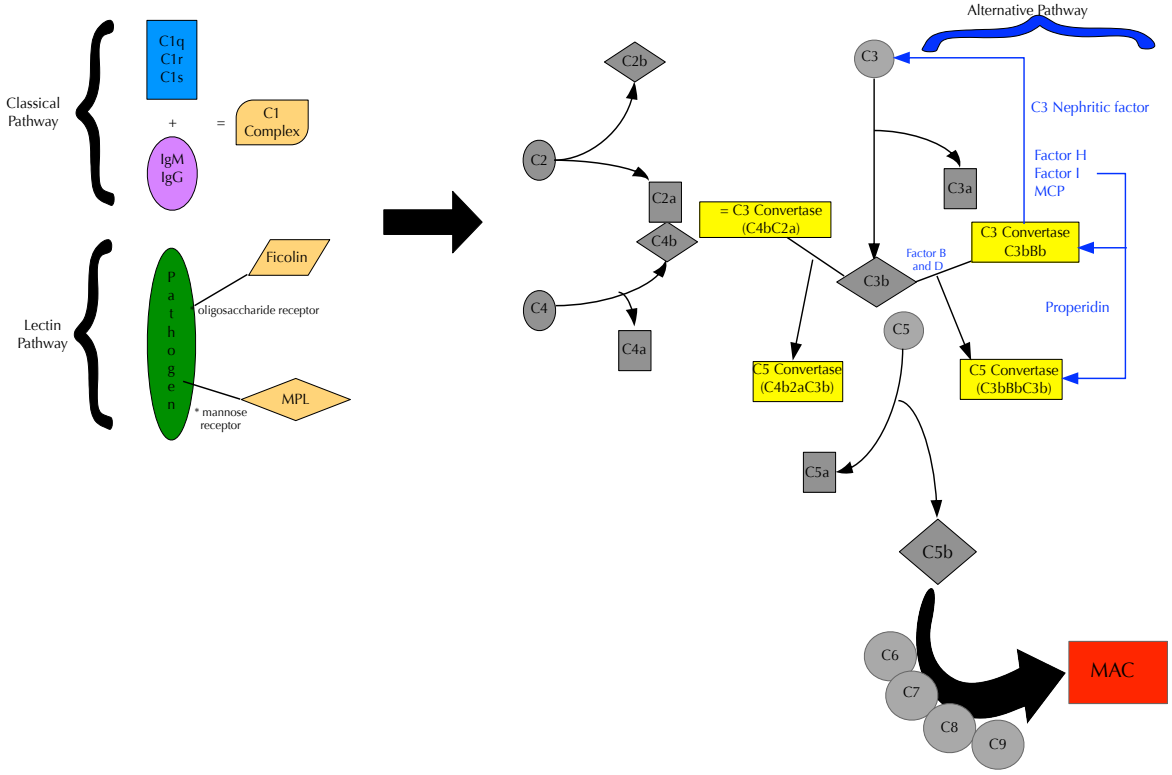


Figure 1.5 The three complement pathways resulting in the formation of the MAC. (Figure produced in powerpoint)

1.4.2 The Role of Complement in Rejection

Complement plays an important role in AMR and this mostly involves the classical pathway. Anti-HLA antibody binding of the C1 complex leads to formation of the MAC and direct injury to endothelial cell membranes[52]. Cleavage fragments C3a and C5a are potent anaphylatoxins and amplify the innate immune response through recruitment of phagocytic cells and further inflammation[53]. Feucht et al demonstrated the deposition of the split products of complement, C4d and C3d in allograft biopsies in 1991[54]. This confirmed an important association of complement in the mechanisms of AMR and as a result led to the introduction of C4d into the Banff criteria of allograft rejection in 2001[55].

1.5 Alloantibody

1.5.1 Anti-HLA Antibody Characteristics

Anti-HLA antibodies have been strongly associated with an increased risk of rejection and allograft loss[56,57]. HLA antibodies are not naturally occurring and arise after exposure to non-self HLA[58]. This process is termed sensitisation. These antibodies may or may not be donor specific, depending on shared expression of HLA epitopes between recipient and donor. DSAs are either “pre-formed” or “de novo”. Pre-formed antibody develops when recipients T-cells have already encountered non-host HLA. Pregnancy, blood product transfusion and previous transplantation are the main modes of development[59,60]; a fourth mode is possible through exposure to bacteria derived antigens which share cross-reactivity with certain HLA epitopes[61]. De novo DSA is defined as occurring after transplantation, often assuming that the recipient has not had prior exposure to non-self MHC molecules. This does not however take into account prior exposure with only a memory B-cell remaining and no circulating DSA at the time of transplantation. Practically speaking, if previously undetectable DSA were to form

within days of transplant, this is highly likely to represent an anamnestic response to alloantigen.

1.5.2 DSA Pathogenesis

The presence of DSA has been shown to be an effective biomarker in the determination of transplant outcomes. Higher incidences of allograft rejection, allograft dysfunction and graft loss are well documented in the presence of either pre-formed or dnDSA[62,63]. Most series have estimated the development of dnDSA to be between 15-30% in previously non-sensitised cohorts[8,64,65]. Common causes for the development of DSA include; a high degree of HLA mismatch particularly at the DQ locus, non-therapeutic levels of immunosuppression, non-adherence and any cause of graft inflammation[8]. With graft tissue injury, there is an increase in expression of HLA antigens on endothelial cells which are prime targets for allorecognition by T-cells and other APCs leading to B-cell activation and DSA production[66].

DSA may cause alloimmune injury through three potential pathways. Direct Antibody-antigen complexes on vascular endothelium results in classical activation of complement[67,68]. Alternatively, damage may occur independent of complement through the innate immune system. DSA binding of Fc receptors on NK cells, neutrophils and macrophages results in antibody dependent direct cellular cytotoxicity triggering degranulation and release of cytokines and lytic enzymes inducing tissue injury, further inflammatory cell recruitment and cell death[69,70]. The second complement independent pathway allows DSA to directly engage endothelial cells and lead to the production of endothelial growth factors and fibroblast growth receptor factor. Resulting endothelial proliferation and fibrosis are thought to contribute to vascular lesions including transplant glomerulopathy, arterial intimal thickening

and interstitial fibrosis and tubular atrophy[49]. Complement independent mechanisms provide a plausible pathway for C4d negative phenotypes of AMR.

Anti-HLA antibodies are mostly reported as being of IgM or IgG isotype[58] with the majority of DSA screening concentrating on IgG anti-HLA DSA. IgM anti-HLA antibodies are easily detected in serum, although their role is less well understood in renal transplantation[71-73]. It is unclear whether IgM DSAs represent a distinct pathological entity in AMR or if their presence simply heralds a potential for isotype switch to IgG. Part of this thesis will attempt to address this question.

IgA is mostly bound to mucosal membranes but does exist in a dimerised form in serum, albeit in smaller amounts than IgM and IgG. IgA anti-HLA antibodies have been reported in the sera of patients with allograft rejection. Although IgA can activate complement via the MBL pathway[74] a causal link to their pathogenicity towards renal allografts is yet to be proven. Originally their presence in serum was associated with positive transplant outcomes[75,76], however a recently published report by Arnold et al suggests significantly worse outcomes in sensitised recipients undergoing re-transplantation, particularly in the presence of anti-HLA IgG[77]. They demonstrated concurrent anti-HLA IgA and IgG in 27% of 694 patients undergoing re-grafting. This group of patients had significantly reduced allograft survival compared to those with anti-HLA IgG alone.

IgE anti-HLA have also been reported in both liver and renal allograft recipients[78,79]. Effector mechanisms for IgE mediated alloimmune injury have also been postulated through activation of basophil and mast cell FcεR1 present in allografts[79]. Part of this thesis will explore the association of IgE DSA in broadly sensitised patients.

Since IgD is predominantly a membrane bound B cell antigen receptor antibody, only trace amounts are ever detected in serum and there have been no studies demonstrating their significance in renal allograft rejection.

1.5.3 Mean Fluorescence Intensity

Mean fluorescence intensity (MFI) expresses the titre of anti-HLA antibodies measured by the Luminex solid-phase assay. An association exists between increased titre of DSA and the degree of potential endothelial injury and complement fixation[80-82]. The degree of tissue injury is not reflective of MFI. Often antibodies of similar MFIs will not fix complement and many patients with seemingly high titre DSA do not experience clinical rejection[68]. Thresholds of MFI significance are not standardised worldwide, across HLA classes or even between different organ allografts. Limitations of the solid phase assay include; in vitro differences in HLA antigen binding on endothelial cells compared to beads, falsely high titres when binding of denatured HLA occurs and the binding of shared epitopes can lead to dilution of MFI. The "prozone" effect may conversely report false negatives or low titre DSA. In patients with multiple DSAs of high titre, antibody-bead binding can be inhibited[83,84]. Serial dilutions have been shown to help reduce this effect.

1.5.4 Non-HLA Antibodies

Non-HLA antibodies are also thought to play an important role in allograft rejection. Through the study of 4000 recipients of HLA-identical sibling donor transplants, Opelz et al found that increased panel reactive antigen (PRA) was strongly associated with graft loss[85]. Since patients cannot form antibody against their own HLA, non-HLA antibodies were highlighted as potentially responsible.

Much like HLA antigens, non-HLA target antigens are expressed on endothelial and epithelial cell surfaces. These may be separated into non-HLA alloantigens and tissue specific autoantigens[86]. Recent work has started to shed some light on the role played by these antigens in allograft rejection. Following a genome wide analysis of 477 pairs of deceased donors, Reindl-Schwaighofer et al demonstrated that non-HLA genetic mismatch resulted in a significant increase risk in graft loss amongst patients with cAMR[87]. Delville et al found an association between preformed IgG antibodies targeting glomerular endothelial cells in 38 patients with early antibody mediated vascular rejection[88]. Such data may prompt greater consideration for non-HLA matching in the future.

1.5.5 Anti-MICA Antibodies

Near the HLA –B locus on chromosome 6 lies an HLA-related polymorphic gene expressing the product MHC class-I related chain A (MICA) and chain B (MICB)[89]. Monocytes, fibroblasts and endothelial cells express MICA and thus provide a non-HLA target on allografts[90]. Although testing for anti-MICA antibodies is not widely adopted in many tissue typing laboratories, there is significant evidence demonstrating their pathogenicity. Terasaki et al published two reports in 2005 and 2007, following recipients up over 10 and 4 year periods respectively. These patients had higher levels of anti-MICA antibodies at the point of graft loss than those that retained their grafts[91,92]. Zou et al confirmed MICA antibodies in 217 recipients of well-matched grafts significantly lowered one-year allograft survival[93].

1.5.6 Anti-Endothelial Cell Antibodies

Although the exact immunological repercussions of anti-endothelial cell antibodies (AECAs) are unknown, there are multiple reports supporting their involvement in hyperacute, acute

and chronic allograft rejection[94-99]. Multiple antigens on the endothelial cells are the targets of AECAs which have been detected in IgG and IgM isotypes[100]. Interestingly IgM AECAs have not correlated with rejection, although IgG, particularly IgG2 and IgG4 have[101,102]. This further supports the framework of this thesis, promoting an increase in the phenotyping of alloantibody.

1.5.7 Angiotensin II Type 1 Receptor Antibodies

Angiotensin II type 1 receptor (ATR1) antibodies target AT1R on glomeruli, proximal tubular cells and arterioles. These antibodies were first reported by Dragun et al in 2005 in 16 HLA antibody negative patients with severe steroid-refractory vascular rejection and malignant hypertension[103]. Alongside classical AMR treatment with plasma exchange and IVIg, allograft survival was improved with the use of angiotensin receptor blockade using losartan. The pathogenicity of ATR1 antibodies has also been shown in cardiac allograft recipients[104]. Further evidence of a sub-phenotype of allograft injury has been demonstrated recently by the Paris group. 233 patients with post-transplant AT1R antibodies had an increased prevalence of intimal arteritis (39%) and reduced prevalence of C4d (17%) when corrected for HLA-DSAs[105].

1.5.8 Tissue Specific Antibodies

Other non-HLA antibodies with varying association with AMR include; anti-vimentin, anti-perlecan, anti-collagen V and anti-Kα antibodies. These can be tested with relative ease with the introduction of solid phase mixed bead arrays. It will be interesting to see their incidence and effect on outcomes in an upcoming multicentre UK based study of DSA negative AMR.

1.6 HLA Screening Techniques

1.6.1 Complement-Dependent Cytotoxic Crossmatch

Lymphocyte crossmatch testing is usually the rate limiting step to transplantation. The traditional method used is the complement-dependent-cytotoxic (CDC) crossmatch, first described by Patel and Terasaki in 1969[106]. They identified that patients with preformed DSA in serum at transplantation had higher rates of hyperacute rejection and primary non-function. The assay involves exposing donor lymphocytes to recipient serum in the presence of exogenous complement[107]. In its time, this test revolutionised transplantation significantly reducing episodes of hyperacute rejection, facilitating increased graft survival. The test is however limited, as donor specific antibodies need to be present in the recipient serum at sufficient titres, as well as have the ability to fix complement for the test to be positive. Positive CDC crossmatches only give an indication to the risk of hyper-acute rejection, which may occur during surgery itself. A disadvantage of this assay is that it cannot distinguish between different iso-types of anti-HLA antibodies such as IgM and IgG. Also, it simply detects those antibodies that fix complement, often only at a high titre. Consequently, DSAs that may not fix complement are not revealed. Auto-antibodies, non-specific IgM and non-HLA antibodies may result in a false positive result. To avoid the confounding by IgM, dithiothreitol (DTT) can be added, reducing intermolecular disulphide bonds and denaturing IgM[108]. The test depends on lymphocytes lysed by the MAC to take up a fluorescent dye which is visualised down the microscope; consequently, human error can also play a part in both false negative and false positive results. The CDC crossmatch was further refined with the addition of anti-human globulin (AHG) which is an antibody against human immunoglobulin and will activate complement upon encountering any DSA bound to lymphocytes. This helps identify traditionally non-complement binding DSA increasing the sensitivity of the test. Despite this,

the CDC crossmatch remains a blunt instrument in predicting allograft failure when used alone.

1.6.2 Flow Cytometric Crossmatch

Introduced by Bray and Gebel in 1989, the flow cytometric crossmatch (FCXM) provides a greater sensitivity compared to CDC in the detection of alloantibody[109]. Non-complement fixing antibodies of a lower titre can be identified. Recipient serum is incubated with donor lymphocytes. In the presence of IgG DSA these lymphocytes will bind immunoglobulin and can be detected by a fluorescent anti-human IgG antibody. This technology can concurrently differentiate between different HLA class I and II antibodies across a broad range of specificities. Also, the test can be specific for either IgG or IgM depending on the conjugating secondary antibody used. Depending on the number of channel shifts of mean fluorescence above baseline, the test can be considered positive or negative. Historically a channel shift of $\geq$200 indicates a positive result. Whilst still highly informative, the FCXM cannot distinguish anti-HLA specificities in the recipient; thus, the introduction of solid phase assays in combination with CDC and FCXM testing has significantly advanced the stratification of risk in potential donor-recipient pairs of allograft rejection. The reliability of the T cell crossmatch is well established however, due to the conflicting reports regarding outcomes following a positive B-cell flow crossmatch, the technique is not used at Imperial college histocompatibility and immunogenetics laboratory.

1.6.3 Solid Phase Assays

The specificity of anti-HLA antibody detection has greatly increased with the introduction of solid phase assays[110]. HLA molecules are bound to polystyrene beads, 5-6μm in diameter.

Upon incubation with the beads, anti-HLA antibodies in the recipient serum binds to the antigen present on the beads. This is then detected using a phycoerthrin-labelled anti-human IgG antibody. The Luminex machine is essentially a flow cytometer which can identify the HLA specificity of each bead according to specific proportions of red and infrared dye on each bead. Phycoerythrin (PE) binding is then detected by a green laser indicating the presence of anti-HLA antibody and a read out of MFI is recorded. Luminex technology has provided a rapid and highly sensitive method for detecting HLA antibodies[110,111]. Whilst highly sensitive, it must be noted that each laboratory employs an arbitrary cut off MFI which even varies between the type of organ being transplanted, leading to a difficulty in comparing methodology of testing between different centres. Also, what may be considered a significant DSA in one department may be disregarded in another, changing the provision of organs to each patient population.

IgM and IgG subclass analysis is not standard in most tissue typing laboratories; the pan-IgG assay cannot distinguish between IgG subclasses without changes to the assay. The Luminex single antigen test can be adjusted by replacing the PE conjugate with alternative monoclonals specific for isotype and subclass. Whilst the importance of this is becoming clearer; cost and time are considerable factors in the clinical setting, which can provide a barrier to these informative tests.

1.6.4 Virtual Crossmatch

Solid phase assays have allowed the omission of prospective “wet” crossmatching in carefully selected patient cohorts[112]. Using a computer algorithm, the result of a wet crossmatch can be predicted based on the donor and recipient’s HLA type as well pre-existing DSA in the recipient’s serum. In an attempt to increase the donor pool, the last ten years has seen a

substantial rise in the number of extended criteria organs and donors after cardiac death (DCD)[113]. Virtual crossmatching has enabled the acceleration of the crossmatch process, reducing cold ischaemic times (CIT) and resulting in a reduced incidence of delayed graft function (DGF). Stringent criteria are required in the selection of recipients eligible for a virtual crossmatch as on the one hand a false positive result can preclude the use of an organ in a patient that may have had a negative CDC/FCXM and on the other a false negative result would put a recipient at increased risk of hyperacute rejection. At our centre, patients who have had sensitising events such as prior organ transplantation, pregnancy (which may be known or not) and recent blood transfusions are excluded. Ultimately this tends to leave only unsensitised males and until technology advances with regards to improved antibody phenotyping and risk modelling, highly sensitised individuals will remain disadvantaged.

1.7 Antibody Mediated Rejection

1.7.1 C4d Deposition

C4d will bind at sites of complement activation and can be demonstrated by immunohistochemistry on allograft biopsies for many weeks. Immunofluorescence (IF) and immunoperoxidase identify C4d deposition using monoclonal antibodies on either frozen or paraffin embedded tissue respectively. C4d deposition is only deemed of significance in peritubular capillaries, although often seen in glomeruli, it is considered a non-specific finding as it is commonly positive on IF and is not indicative of AMR. Multiple groups have investigated the diagnostic value of C4d. Following their initial demonstration of complement split product deposition in peritubular capillaries, Feucht et al studied C4d staining in a cohort of 93 patients with early graft dysfunction. The majority (N=51) demonstrated C4d deposition with one year allograft survival rates differing markedly; 63% in the C4d positive group and

90% in the C4d negative group[114]. This finding was later correlated with anti-HLA antibody in acute and chronic humoral rejection in two papers by Mauiyyedi et al[115,116]. C4d is now accepted universally as a poor prognostic indicator for ABO-compatible graft survival[117,118]. C4d deposition however, is not an absolute requirement for a diagnosis of AMR. C4d negative AMR was introduced into the Banff classification in 2013 following studies by the Paris and Cedars-Sinai groups. Loupy et al demonstrated that even in the absence of C4d, capillaritis was predictive of future TG in 3-month protocol biopsies[119]. Haas et al showed that patients with ultrastructural features of MI who did not receive treatment for AMR, despite C4d negative biopsies, had a significant progression to TG compared to those who were treated[120]. Whilst C4d is a useful biomarker of AMR it is not quite the magic bullet it was once hoped to be. Work into measuring gene transcripts is a potentially exciting replacement once techniques have been optimised.

1.7.2 Microcirculatory Inflammation

The vascular endothelium expresses the target antigens involved in AMR. This is demonstrated by microcirculatory inflammation; glomerulitis and peritubular capillaritis. The combination of glomerulitis (g) and peritubular capillaritis (ptc) scores into a composite microcirculatory inflammation (MI) score was proposed by Sis et al in 2012[121]. Increasing scores strongly associated with the presence of circulatory DSA and prediction of future graft failure independently of C4d. While MI has been widely accepted as the hallmark feature of AMR[122-124], C4d deposition was mandated by Banff for its diagnosis. The introduction of MI scores into the Banff criteria in 2013 permitted the inclusion of C4d negative AMR into the classification[125]. An MI score of $\geq$2 provides sufficient evidence of recent or ongoing antibody interaction with the vascular endothelium in the absence of linear C4d deposition in

peritubular capillaries. Gupta et al validated this threshold of MI≥2 using microarrays to examine the expression of pathogenesis-based transcripts (PBT) associated with AMR[126,127]. Expression of PBTs was not found to differ in biopsies with MI≥2 with or without C4d staining[126].

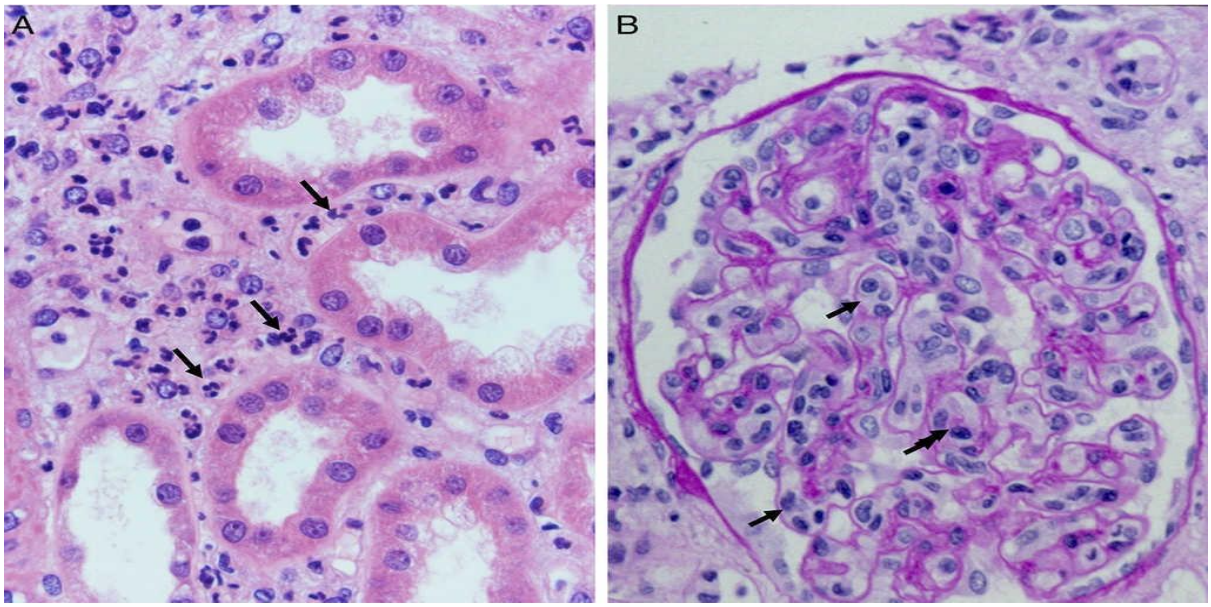


Figure 1.6. Light micrograph of (A) peritubular capillaritis, with the presence of polymorphonuclear leucocytes in peritubular capillaries indicated by black arrows. (H&E)

(B) Glomerulitis, with the presence of macrophages in glomerular capillaries indicated by black arrows (PAS).

Image taken from, Gonzalez-Molina et al, (2016). Immune response and histology of humoral rejection in kidney transplantation. Nefrología (English Edition). 36. 354-367. 10.1016/j.nefroe.2016.08.002.

1.7.3 Chronic Active Antibody Mediated Rejection

In the last 10 years the importance of cAMR has become more apparent. It is increasingly understood to be a significant cause of late kidney allograft failure[128]. Whilst persistent and late AMR often translates into cAMR, the definition “chronic” does not represent time

according to the Banff 2007 classification[129], rather it is a structural representation of ongoing endothelial damage secondary to alloreactive antibody. Continued and persistent capillaritis is thought to lead to fibrosis and remodelling. Within glomeruli, mesangial cell interpositioning, matrix expansion and basement membrane duplication can be visualised on light and electron microscopy (EM) as TG. Within peritubular capillaries, basement membranes become multi-layered and can only be accurately assessed with electron microscopy. Both TG and peritubular capillary multilayering are features consistent with chronic antibody mediated rejection. In 2005 chronic active AMR (CAAMR) was included in the Banff criteria[130]. Histological evidence of chronic endothelial injury alone, as described by TG and peritubular capillary basement membrane multilayering (PTCBML), would be described as chronic inactive AMR. The presence of microcirculatory inflammation and or C4d positivity is required to make a diagnosis of chronic active AMR. Strictly speaking, either diagnosis still requires the presence of DSA in the patient serum.

1.7.4 Transplant Glomerulopathy

First described by Porter et al in 1968, TG is often a hallmark of chronic alloimmune injury. Whilst it may occur acutely, it often signifies irreversible endothelial damage heralding an increased likelihood of future allograft failure. The prevalence of TG is described as between 5-20% at five years in most publications[131]. This can increase significantly in cohorts with pre-formed DSA[132-134]. TG has a poor prognosis, with graft failure occurring in up to 70% of patients within 2 years of diagnosis[135,136]. Proteinuria occurs as a result of TG and increases in quantity as the lesion progresses. Evidence shows that >2.5g/day of proteinuria is associated with increased graft loss (92% vs 33%, $p<0.005$)[132]. The early stages of TG are not always

visible on light microscopy and in order to fully exclude glomerulopathy scores (cg) of 0 ultrastructural examination with electron microscopy is advised[137].

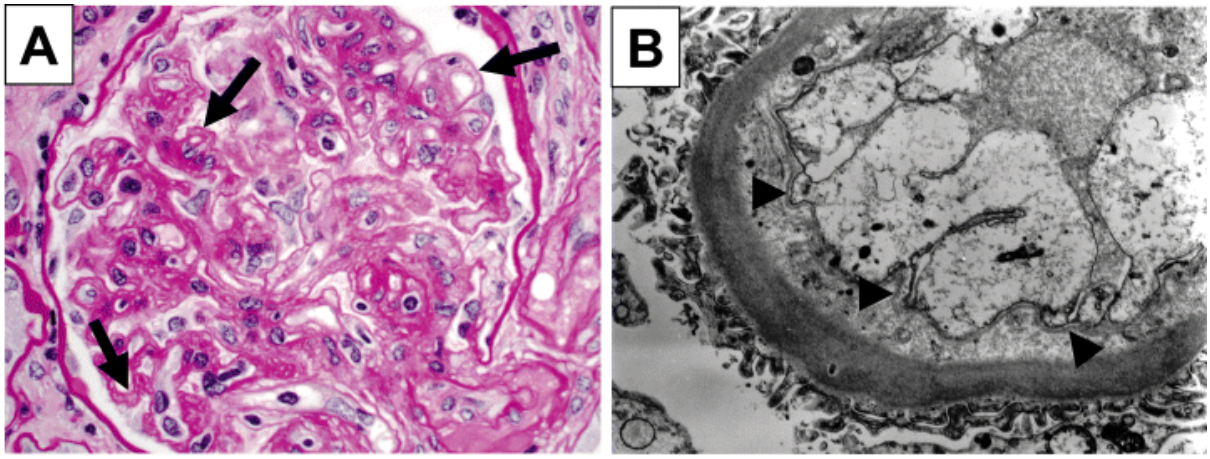


Figure 1.7. (A) Transplant glomerulopathy by light microscopy with double contours indicated by black arrows (PAS). (B) Transplant glomerulopathy by electron microscopy: There is electron lucent widening of the subendothelial space and formation of new basement membrane layers as indicated by black arrowheads.

Image adapted from American Journal of Transplantation, Volume: 7, Issue: 7, Pages: 1743-1752, First published: 09 June 2007, DOI: (10.1111/j.1600-6143.2007.01836.x)

1.7.5 Peritubular Basement Membrane Multilayering

PTCBML was originally associated with cAMR in 1990 and has been added as a defining characteristic of cAMR to the Banff classification[129,138]. Similar to TG, PTCBML represents recurrent endothelial injury and repair by alloantibody and can occur early after transplantation[139]. Whilst often occurring in concert with TG, PTCBML may occur in isolation. PTCBML can be a useful predictor of TG in patients with dnDSA and therefore an early predictor of cAMR[140]. This lesion is only seen on EM and further underlies the importance of tissue analysis beyond light microscopy.

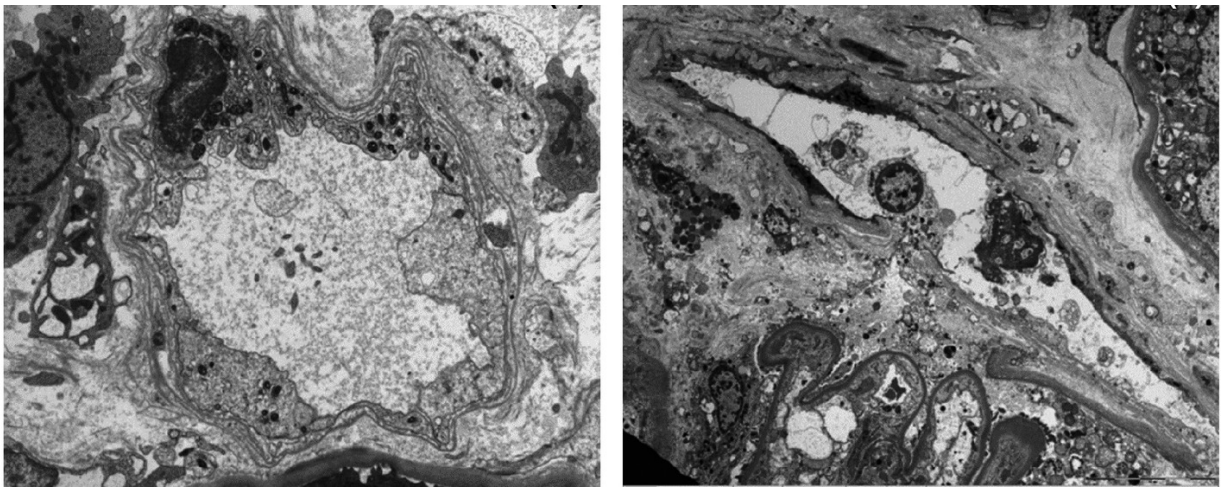


Figure 1.8. Electron micrograph showing the ultrastructural features of peritubular capillary basement membrane multilayering (PTCBML). Left image, four circumferential layers of PTCBML. Right image more than 10 circumferential layers of PTCBML with an activated endothelial cell and inflammatory cell infiltration.

Image adapted from Transplant International, Volume: 30, Issue: 4, Pages: 398-409, First published: 21 January 2017, DOI: (10.1111/tri.12921)

1.7.6 DSA negative AMR

The Banff classification has strict criteria for the diagnosis of AMR. All three of the criteria must be present in order for a formal diagnosis of AMR to be made; histological evidence of acute tissue injury, recent evidence of antibody interaction with the vascular endothelium in the form of C4d deposition or moderate microvascular inflammation and finally serological evidence of circulating DSA[141]. Within clinical practice, it is important to consider these criteria are often not fully met. DSA negative cases that fulfil histological criteria for AMR are quite common; here Banff only allows the description “suspicious for AMR”. Categorising patients into this subgroup has potential implications on the subsequent management whereby outcomes may be significantly different but as yet undefined. An attempt has been

made by Banff in the 2017 update, to include these cases using C4d positivity as a surrogate marker, supporting the presence of a circulating DSA[141]. This schema has not been shown to be without problems. DSA positive AMR has been demonstrated to have significantly worse outcomes on allograft function with a greater tendency towards chronicity and graft loss when compared to AMR in the absence of DSA[142-144]. As such, if C4d binding is to be considered a surrogate for circulating DSA, this does not address the difference found in outcomes by Senev et al, whereby 123 patients with DSA negative AMR had similar 10-year allograft survival with and without C4d (72.5% C4dpos vs 78.9% C4dneg, p=0.91)[144].

It remains unclear what the pathophysiology of DSA negative AMR is, however a number of theories exist. Whilst DSA may not be found in the serum; the allograft has been identified as a reservoir for antibody. Allo-antibody may remain bound to donor vasculature, preventing detection in circulating serum. Martin et al demonstrated bound anti-HLA antibodies in the elutes of 70.6% of recipients with cAMR following graft nephrectomy[145]. They also identified that following transplantectomy 31.6% of cases developed detectable circulating DSA, further supporting this theory. Non-donor specific HLA antibodies acquired through prior alloimmunisation as well as memory B-cells specific for HLA, have been shown to be deleterious and detectable in allografts despite the absence of circulating DSA[146,147]. Anti-HLA antibodies that are not donor specific may not be considered significant against matched HLA at the allelic level, however cross-reactivity on the HLA epitope is described[148-150]. HLA antigens do share many epitopes, thus expanding our consideration of antibody reactivity at the epitopic level increases the potential for non-donor specific HLA to cause tissue damage[151]. Soluble HLA can also confound testing methods by binding HLA-antibody in the serum preventing detection[152], although solid phase testing methods have likely reduced this confounder. Most tissue typing laboratories do not screen for non-IgG HLA antibodies and

although the pathogenicity of other isotypes remains in question, IgM, IgA and IgE DSAs may in fact have a role to play in IgG DSA negative AMR. It should also be considered that non-HLA antibodies directed against the vascular endothelium may also play a role[153]. Whilst mostly reported in smaller case series, larger testing arrays have been developed for a host of antibodies not directed against HLA, which may elicit specific phenotypes of AMR. Finally, MI is not necessarily an entirely antibody-dependent process. Work into gene transcript has shown upregulation of cytotoxic T-cell associated transcripts in patients with DSA negative and C4d negative transplant glomerulopathy in the absence of transcripts associated with AMR[154].

1.7.7 The Molecular Microscope

Despite the high specificity of C4d and effectiveness of circulating anti-HLA antibodies as biomarkers for AMR, many cases remain under dispute when other features of endothelial cell injury remain absent. A new method for assessing alloimmune endothelial damage is required. Sets of endothelial cell associated transcripts (ENDATs) were identified by Sis et al in 2009, demonstrating antibody mediated injury on allografts biopsied for cause with or without C4d positivity. High ENDAT expression correlated well with AMR lesions and DSA but not the hallmark lesion of TCMR tubulitis[155]. Further transcript work from the same group identified a novel role for antibody dependent cell mediated cytotoxicity through direct interaction with the endothelium modulated by natural killer (NK) cells rather than lymphocytes or complement[70]. Increased ENDAT expression was included as a marker of active endothelial cell/antibody interaction in 2013, allowing the inclusion of C4d-negative AMR into the schema. With the advent of NanoString technology, access to RNA has been made easier as previously, further sample cores were required for transcript analysis,

increasing the potential risks of allograft biopsies. Relatively stable RNA can be extracted from paraffin blocks allowing validation of molecular diagnostics on both prospective and retrospective samples[156]. Potential applications of transcript analysis may be seen in the future with less invasive testing of blood and urine. The additional information provided by the molecular microscope alongside conventional structural assessment provides exciting opportunities to improve diagnostics.

1.8 Cellular Rejection

T-cell mediated rejection (TCMR) is the dominant rejection phenotype early in the lifespan of a renal allograft[157]. Whilst it may occur later on, it is most common within the first 12 months and usually does not present beyond five years after transplantation[158]. The hallmark feature of TCMR is infiltration of the interstitium with T-cells and macrophages. Ultimately T-cells cross the basement membrane and enter the epithelium of tubules resulting in the diagnostic lesion, tubulitis[129]. Typically, TCMR has a better prognosis than AMR however, the two are not mutually exclusive and can exist together. Willicombe et al confirmed in 147 cases of TCMR, that mixed rejection in 55/147 cases had inferior outcomes than TCMR alone, particularly in cases with positive C4d and circulating DSA (OR=4.9, 95% CI=2.0-12.0, $p<0.1$)[159].

1.9 Vascular Rejection

Infiltration of the vascular endothelium by mononuclear cells has previously been thought to be a solely T-cell mediated process[160]. Intimal arteritis or v-lesions are no longer believed to be a distinct feature of TCMR having been correlated with circulating DSA[155,161,162]. Although often antibody mediated, vascular rejection is believed to be different from pure TCMR and AMR both prognostically and pathologically[163].

Updated 2015 Banff classification categories

Category 1: Normal biopsy or nonspecific changes

Category 2: Antibody-mediated changes

Acute/active ABMR

All three features must be present for diagnosis. Biopsies showing histological features plus evidence of current/recent antibody interaction with vascular endothelium or DSA, but not both, may be designated as suspicious for acute/active ABMR. Lesions may be clinically acute or smoldering or may be subclinical; it should be noted if the lesion is C4d-positive or C4d-negative, based on the following criteria:

1 Histologic evidence of acute tissue injury, including one or more of the following:
- Microvascular inflammation (g >0 in the absence of recurrent or *de novo* glomerulonephritis, and/or ptc >0)
- Intimal or transmural arteritis (v >0)
- Acute thrombotic microangiopathy in the absence of any other cause
- Acute tubular injury in the absence of any other apparent cause

2 Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
- Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections or C4d >0 by IHC on paraffin sections)
- At least moderate microvascular inflammation ([g + ptc] ≥2), although in the presence of acute TCMR, borderline infiltrate, or infection; ptc ≥2 alone is not sufficient, and g must be ≥1
- Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated

3 Serologic evidence of DSAs (HLA or other antigens)
- Biopsies suspicious for ABMR on the basis of meeting criteria 1 and 2 should prompt expedited DSA testing

Chronic active ABMR[2]

All three features must be present for diagnosis. As with acute/active ABMR, biopsies showing histological features plus evidence of current/recent antibody interaction with vascular endothelium or DSA, but not both, may be designated as suspicious, and it should be noted if the lesion is C4d-positive or C4d-negative, based on the criteria listed:

1 Histologic evidence of chronic tissue injury, including one or more of the following:
- TG (cg >0), if no evidence of chronic thrombotic microangiopathy; includes changes evident by EM only (cg1a)
- Severe peritubular capillary basement membrane multilayering (requires EM)
- Arterial intimal fibrosis of new onset, excluding other causes; leukocytes within the sclerotic intima favor chronic ABMR if there is no prior history of biopsy-proven TCMR with arterial involvement but are not required

2 Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
- Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d >0 by IHC on paraffin sections)
- At least moderate microvascular inflammation ([g + ptc] ≥2), although in the presence of acute TCMR, borderline infiltrate, or infection, ptc ≥2 alone is not sufficient and g must be ≥1
- Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated

3 Serologic evidence of DSAs (HLA or other antigens):
- Biopsies suspicious for ABMR on the basis of meeting criteria 1 and 2 should prompt expedited DSA testing

Figure 1.9. 2015 Banff criteria for the diagnosis of acute/active AMR and chronic active AMR. Image adapted from American Journal of Transplantation, Volume: 17, Issue: 1, Pages: 28-41, First published: 10 November 2016, DOI: (10.1111/ajt.14107)

Banff lesion grading system

Lesions	
Quantitative criteria for inflammation: i score	
i0	No inflammation or in <10% of unscarred cortical parenchyma
i1	Inflammation in 10–25% of unscarred cortical parenchyma
i2	Inflammation in 26–50% of unscarred cortical parenchyma
i3	Inflammation in >50% of unscarred cortical parenchyma
Quantitative criteria for tubulitis: t score	
t0	No mononuclear leukocytes in tubules
t1	Foci with one to four leukocytes per tubular cross-section (or 10 tubular cells)
t2	Foci with five to 10 leukocytes per tubular cross-section (or 10 tubular cells)
t3	Foci with >10 leukocytes per tubular cross-section or the presence of two or more areas of tubular basement membrane destruction accompanied by i2/i3 inflammation and t2 elsewhere
Quantitative criteria for intimal arteritis: v score	
v0	No arteritis
v1	Mild to moderate intimal arteritis in at least one arterial cross-section
v2	Severe intimal arteritis with at least 25% luminal area lost in at least one arterial cross-section
v3	Transmural arteritis and/or arterial fibrinoid change and medial smooth muscle necrosis with lymphocytic infiltrate in vessel
Quantitative criteria for glomerulitis: g score	
g0	No glomerulitis
g1	Glomerulitis in <25% of glomeruli
g2	Segmental or global glomerulitis in 25–75% of glomeruli
g3	Glomerulitis in >75% of glomeruli
Quantitative criteria for peritubular capillaritis: ptc score	
ptc0	At least one leukocyte in <10% of cortical PTCs and/or maximum number of leukocytes <3
ptc1	At least one leukocyte cell in ≥10% of cortical PTCs with three or four leukocytes in most severely involved PTC
ptc2	At least one leukocyte in ≥10% of cortical PTCs with five to 10 leukocytes in most severely involved PTC
ptc3	At least one leukocyte in ≥10% of cortical PTCs with >10 leukocytes in most severely involved PTC
Quantitative criteria for total inflammation: ti score	
ti0	No or trivial interstitial inflammation (<10% of total cortical parenchyma)
ti1	10–25% of total cortical parenchyma inflamed
ti2	26–50% of total cortical parenchyma inflamed
ti3	>50% of total cortical parenchyma inflamed
Quantitative criteria for inflammation in area of interstitial fibrosis and tubular atrophy: i-IFTA score	
i-IFTA0	No inflammation or <10% of scarred cortical parenchyma
i-IFTA1	Inflammation in 10–25% of scarred cortical parenchyma
i-IFTA2	Inflammation in 26–50% of scarred cortical parenchyma
i-IFTA3	Inflammation in >50% of scarred cortical parenchyma
Quantitative criteria for C4d score	
C4d0	No staining of PTCs (0%)
C4d1	Minimal C4d staining (>0 but <10% of PTCs)
C4d2	Focal C4d staining (10–50% of PTCs)
C4d3	Diffuse C4d staining (>50% of PTCs)
Quantitative criteria for double contour: cg score	
cg0	No GBM double contours by light microscopy or EM
cg1a	No GBM double contours by light microscopy but GBM double contours (incomplete or circumferential) in at least three glomerular capillaries by EM, with associated endothelial swelling and/or subendothelial electron-lucent widening
cg1b	Double contours of the GBM in 1–25% of capillary loops in the most affected nonsclerotic glomerulus by light microscopy; EM confirmation is recommended if EM is available
cg2	Double contours affecting 26–50% of peripheral capillary loops in the most affected glomerulus
cg3	Double contours affecting >50% of peripheral capillary loops in the most affected glomerulus
Quantitative criteria for mesangial matrix expansion: mm score	
mm0	No more than mild mesangial matrix increase in any glomerulus
mm1	At least moderate mesangial matrix increase in up to 25% of nonsclerotic glomeruli
mm2	At least moderate mesangial matrix increase in 26–50% of nonsclerotic glomeruli
mm3	At least moderate mesangial matrix increase in >50% of nonsclerotic glomeruli

Lesions	
Quantitative criteria for arteriolar hyalinosis: ah score	
ah0	No PAS-positive hyaline arteriolar thickening
ah1	Mild to moderate PAS-positive hyaline thickening in at least one arteriole
ah2	Moderate to severe PAS-positive hyaline thickening in more than one arteriole
ah3	Severe PAS-positive hyaline thickening in many arterioles
Alternative quantitative criteria for hyaline arteriolar thickening: aah score	
aah0	No typical lesions of calcineurin inhibitor–related arteriolopathy
aah1	Replacement of degenerated smooth muscle cells by hyaline deposits in only one arteriole, without circumferential involvement
aah2	Replacement of degenerated smooth muscle cells by hyaline deposits in more than one arteriole, without circumferential involvement
aah3	Replacement of degenerated smooth muscle cells by hyaline deposits with circumferential involvement, independent of the number of arterioles involved.
Quantitative criteria for vascular fibrous intimal thickening: cv score	
cv0	No chronic vascular changes
cv1	Vascular narrowing of up to 25% luminal area by fibrointimal thickening
cv2	Vascular narrowing of 26–50% luminal area by fibrointimal thickening
cv3	Vascular narrowing of >50% luminal area by fibrointimal thickening
Quantitative criteria for interstitial fibrosis: ci score	
ci0	Interstitial fibrosis in up to 5% of cortical area
ci1	Interstitial fibrosis in 6–25% of cortical area (mild interstitial fibrosis)
ci2	Interstitial fibrosis in 26–50% of cortical area (moderate interstitial fibrosis)
ci3	Interstitial fibrosis in >50% of cortical area (severe interstitial fibrosis)
Quantitative criteria for tubular atrophy: ct score	
ct0	No tubular atrophy
ct1	Tubular atrophy involving up to 25% of the area of cortical tubules (mild tubular atrophy)
ct2	Tubular atrophy involving 26–50% of the area of cortical tubules (moderate tubular atrophy)
ct3	Tubular atrophy involving in >50% of the area of cortical tubules (severe tubular atrophy)

aah, hyaline arteriolar thickening; ah, arteriorlar hyalinosis; cg, glomerular double contours; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular fibrous intimal thickening; EM, electron microscopy; g, glomerulitis; GBM, glomerular basement membrane; i, inflammation; i-IFTA, interstitial inflammation in areas of interstitial fibrosis and tubular atrophy; mm, mesangial matrix expansion; PAS, periodic acid–Schiff; ptc, peritubular capillaritis; PTC, peritubular capillary; t, tubulitis; v, intimal arteritis.

Figure 1.10. Banff 2015 lesion grading system. Image taken from American Journal of Transplantation, Volume: 17, Issue: 1, Pages: 28-41, First published: 10 November 2016, DOI: (10.1111/ajt.14107)

Chapter 2: Materials and Methods

2.1 Patient Selection

All the patients investigated were transplanted at Imperial College London Renal and Transplant Centre; Imperial College NHS Trust between June 2005 and November 2016. All patients that received a kidney alone or simultaneous pancreas kidney transplant that had a negative CDC and flow cytometric crossmatch were included. Patients transplanted against an incompatible blood group or with a positive CDC or flow cytometric crossmatch were excluded. These patients were defined as antibody incompatible (ABOi or HLAi). Inclusion criteria and numbers of patients for each study differ according to the dates of investigation. These will be described separately for each.

2.2 Immunosuppression Regimens

Patients received a steroid sparing tacrolimus maintenance regime with monoclonal antibody induction. Patients received either anti-CD52; alemtuzumab (Campath-1H®, Genzyme, UK) or an IL-2R-antagonist including either basiximab (Simulect®, Novartis Pharma Corp, NJ) or daclizumab (Zenax®, Roche Inc, NJ). Those patients receiving alemtuzumab were maintained on a tacrolimus (FK) based monotherapy with the aim to achieve a trough level of 5-8ng/ml as measured by the liquid chromatography and mass spectroscopic (LCMS) method. In the first year, those receiving IL2R-antagonists were maintained on a combination of FK and mycophenolate mofetil (MMF) aiming at trough levels of 8-12ng/ml and 1.2-2.4mg/l respectively. In the second year, tacrolimus levels were reduced to 5-8ng/ml. All patients received a steroid sparing protocol, consisting of 500mg of IV methylprednisolone preoperatively, followed by oral prednisolone 30mg twice a day reduced to once daily at day 4. Steroids were then discontinued at day 7.

2.3 Treatment of Rejection

Biopsy proven episodes of acute T cell mediated rejection (TCMR) and acute antibody mediated rejection (aAMR) were treated with the addition of mycophenolate mofetil to achieve 12-hour pre-dose mycophenolic acid levels of 1.2-2.4mg/l measured by HPLC and pulsed methylprednisolone 500mg over three consecutive days. Prednisolone was then started at 30mg per day and weaned to 10mg daily over 3 months. Tacrolimus dosing was adjusted to achieve levels of 8-12ng/ml. Acute antibody mediated rejection episodes were also treated with 10 plasma exchanges with an exchange volume of 50mls/kg with a maximum of 4.5L and 4g/Kg intravenous immunoglobulin (IVIg) (Vigam®; Bio products, Hertfordshire UK) divided in two doses after 5 exchanges.

2.4 Histopathology

All graft biopsies were assessed and graded by the histopathology department at Imperial College NHS Trust. Only those with histological evidence of AMR activity as defined in Banff 2015 were retained. Biopsies were fully scored according to the Banff 2015 criteria[164]. The following histologic factors were graded 0-3: glomerulitis (g), peritubular capillaritis (PTC), tubulitis (t), interstitial inflammation (i), intimal arteritis (v), total inflammation (ti), glomerular double contours (cg), arteriolar hyaline (ah), arterial intimal thickening (cv), interstitial fibrosis (ci), tubular atrophy (ct). Interstitial fibrosis/tubular atrophy (IFTA) was estimated to the nearest 10%. Microcirculatory inflammation (MCI) was defined as the sum of g+ptc (range 0-6). C4d staining was carried out by immunoperoxidase on paraffin sections using polyclonal rabbit anti-C4d antibody at 1/40 (Oxford Biosystems, BI-RC4D). C4d staining in peritubular capillaries was classified as negative/minimal (C4d0/C4d1 <1% and 1%–10% of

peritubular capillaries, respectively), focal (C4d2, 11%–50% of peritubular capillaries), or diffuse (C4d3, >50% of peritubular capillaries).

2.5 Detection of HLA Antibodies

Sera from patients are routinely tested for IgG anti-HLA antibodies pre-transplant and at months 1, 3, 6 and 12. Following this annual screening is performed or at times of allograft dysfunction. Patients were typed for HLA-A, -B, -Cw, -DR, -DQ but not routinely –DP antigens. Patients are initially screened using LABScreen mixed beads (One Lambda, Canoga Park, CA). Positive mix screens undergo further testing with LABScreen single antigen class I and II beads in order to identify the specificity of their anti-HLA antibody. The Luminex fluoroanalyser detects signal intensity and records this as mean fluorescence index (MFI). As per previous published reports from our centre an MFI of >500 on two separate occasions was considered positive[165,166]. Patients with more than one DSA detected will be analysed according to their class I or II immunodominant antibody.

2.6 Statistical Analysis

Data for continuous variables are reported as mean ± standard deviation or median (interquartile range) as appropriate. Student's T test, ANOVA or chi-squared tests were used to compare normally distributed variables. Mann-Whitney U-test and Kruskal-Wallis tests were used to compare non-parametric data. Kidney allograft survival was plotted using Kaplan-Meier curves with significance assessed using log rank testing. Outcome and cumulative hazard functions were determined using Cox regression analysis. Statistical and graphical analyses were performed with IBM SPSS Statistics Ver. 20.0 (SPSS, Chicago, IL, USA). The two-sided level of significance was set at $p<0.05$.

Chapter 3: IgG anti-HLA donor specific antibody subclass phenotyping in chronic antibody mediated rejection

3.1 Introduction

A large proportion of renal transplant recipients will progress to late graft failure and end stage kidney disease[9]. Chronic antibody mediated rejection (cAMR) is well understood to be the leading cause of late allograft failure[5,128] and there is a clear association between the development of anti-HLA donor specific antibodies (DSAs) and poor outcomes[167]. This is demonstrated by microcirculatory inflammation (MCI) in glomeruli and peritubular capillaries[168] which progress to transplant glomerulopathy (TG) and peritubular basement membrane multilayering (PTCBML)[133,169]. Although acute AMR tends to be more severe in its presentation, cAMR presents no less of a clinical challenge, diagnosed late, with a poor prognosis and without any robust treatment strategies[170].

The incidence of cAMR is poorly described in the literature although histological evidence in the form of TG is seen in up to 20% of allografts biopsied at 5 years[131,133]. A recent single centre retrospective review demonstrated these poor outcomes, with 76% of patients losing their grafts within a median of 1.9 years following diagnostic biopsy[135].

IgG anti-HLA DSAs are an effective biomarker in predicting graft outcomes[7,8,14]. In acute AMR, the pathogenicity of IgG DSAs has been shown to differ across subclasses. In particular IgG3 and C1q-binding are associated with more severe forms of acute AMR[13,82]. IgM is the first antibody isotype to be produced. Following this, with the maturation of the immune response, there is a class switch to IgG, initially IgG3 then IgG1, IgG2 and finally IgG4[42]. Each subclass of IgG has different functional properties such as the ability to fix complement and bind Fc receptors with varying degrees of affinity. Phenotyping the properties of IgG DSAs has

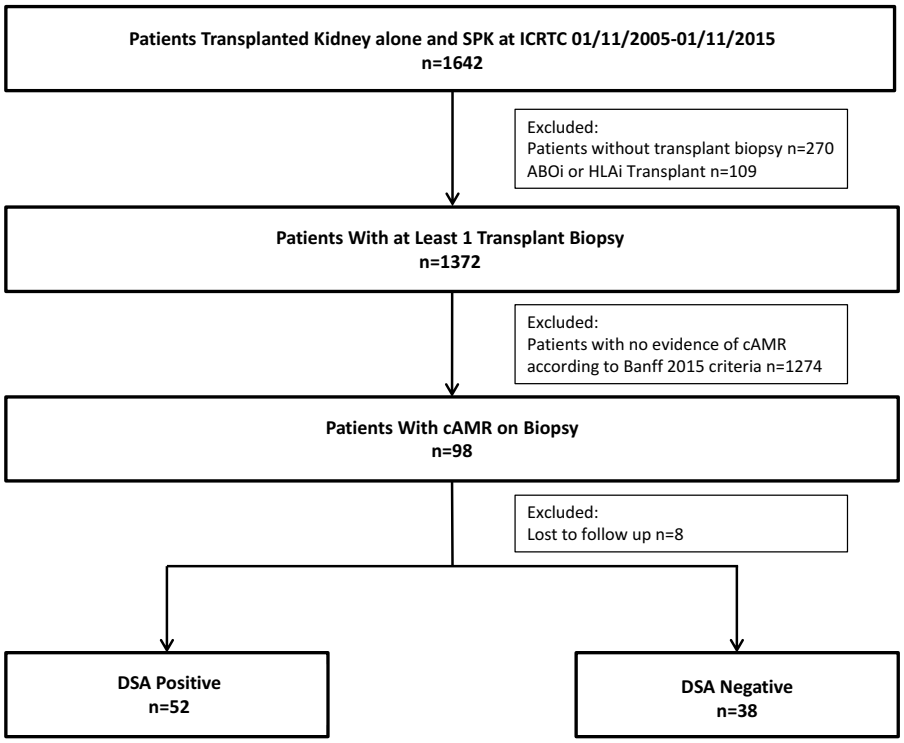
great potential in risk stratifying patients with AMR not only in terms of improved understanding of clinical outcomes but also to help guide the use of emerging greatly needed novel therapies. There are studies that investigate the roles of different antibody characteristics in certain phenotypes of AMR[13,72]. None of these however, have specifically assessed the distribution of IgG DSA profiles in a cohort of patients with cAMR. This is urgently required to improve our understanding and provide a treatment for cAMR, which is now one of the main barriers to improving long-term allograft survival.

The aim of this retrospective study was to describe the clinical significance of IgG DSA subclass phenotyping in a group of patients with anti-HLA IgG DSA positive biopsy-proven cAMR. This work identifies which antibody characteristics predict poor outcomes in cAMR and ascertain which groups of patients are at the highest risk of graft loss.

3.2 Methods

This study was approved by the Imperial College Transplant Research Study Group. All patients that received a complement dependent cytotoxic (CDC) and flow cytometric crossmatch (FCXM) negative kidney transplant at the Imperial College Kidney and Transplant Centre between March 2005 and November 2015 (n=1,642) were retrospectively analysed. Kidney alone (deceased and living donors) and simultaneous pancreas and kidney recipients were included. All patients with antibody incompatible transplants, namely those with pre-formed DSA detected by Luminex assay and those receiving an ABO-incompatible transplant were excluded. Cases with either the presence of double contours (TG) on light microscopy (cg1b and above) and/or the presence of significant multilayering of peritubular capillary basement membranes on electron microscopy (PTCL; at least 3 capillaries with 5 or more layers of basement membrane, Banff 2005 definition) were selected. 90 cases of biopsy-proven cAMR according to Banff 2015 criteria[164] were identified. 52 patients with an IgG DSA detectable within 3 months of the diagnosis of cAMR were included as shown in figure 3.1.

Figure 3.1: Flow chart



The DSA positive patients were further separated into 2 groups; those that were DSA positive with identifiable subclasses (DSA+/SC+) and those that were DSA positive but no identifiable subclass when tested (DSA+SC-). In each patient, the DSA with the highest MFI was termed the immunodominant (ID) DSA. Each subclass mixture pertains to the subclasses making up the immunodominant pan IgG DSA.

The standard LABScreen single antigen test was adjusted replacing the anti-human pan IgG phycoerythrin conjugate with anti-human monoclonal antibodies specific for IgG1-4 subclasses (IgG1 clone HP6001, IgG2 clone 31-7-4, IgG3 clone HP6050, IgG4 clone HP6025; Southern Biotech). The IgG subclass assay was performed using the LABscreen single antigen beads alongside the same protocol used for standard anti-HLA IgG antibodies. The positive

threshold MFI for each subclass was calculated by testing 10 negative control sera from healthy, non-sensitised males. Calculation of the mean positive threshold MFI levels was similar to the method used by Khovanova et al[171]. The mean positive threshold MFI levels of each IgG subclass was 3 times greater than the most positive bead from each of the control assays. This gave a cutoff MFI for each subclass; IgG1 482, IgG2 79, IgG3 88 and IgG4 40.

3.3 Results

Of the 1,642 patients who were transplanted between March 2005 and November 2015 at Imperial College Renal and Transplant Centre, 90 (5.4%) had glomerular double contours and/or significant PTCL on biopsy. Of these, 52 had an identifiable de novo IgG DSA at the time of cAMR diagnosis (3.1%). The median follow-up after transplant was 65.5 (39.1-89.1) months and a range of 20.4-152.6 months. The incidence of cAMR in the biopsied cohort was similar to other centres[172]. The vast majority fulfilled criteria required for chronic active antibody mediated rejection with features of activity (glomerulitis, peritubular capillaritis). 30 cases had an MI score of $\leq$1. 21 had MI scores of 1. Of these 8 had g1 with C4d>1, 10 had ptc1 with C4d>1 in the absence of tubulitis. 3 cases had ptc1 with peritubular C4d however there was moderate tubulitis (t2). 9 cases had only features of chronic antibody mediated rejection in the absence of activity, MI=0. The cohort was separated into patients that were DSA negative (n=38 42.2%), DSA positive without an identifiable subclass (DSA+/SC-) (n=15 16.7%) and DSA positive with an identifiable subclass (DSA+/SC+) (n=37 41.1%). The baseline patient characteristics for each group are shown in Table 3.1. DQ mismatch was significantly greater in the DSA+/SC+ group compared to both the DSA negative and DSA+/SC- groups; median mismatch 0 (0-1), 1 (0-2) and 1 (1-2) respectively, p=0.021. Allograft failure was more common in the DSA+/SC+ group [28/37 (75.7%)] compared to all groups, p=0.004 by log rank. Interestingly, the prevalence of allograft failure in the DSA+/SC+ group was also greater compared to the DSA+/SC- group [5/15 (33.3%)], p=0.006. Finally, at diagnosis eGFR was significantly lower and uPCR greater in the DSA+/SC+ patients compared to both the DSA negative patients and DSA+/SC- patients, p=0.007 and p0.038 respectively.

Table 3.1: Group demographics

	Total (n=90)	**DSA neg (n=38)**	**DSA+ / SC- (n=15)**	**DSA+ / SC+ (n=37)**	**p**
Female, n (%)	34 (37.8)	15 (39.5)	3 (20.0)	16 (43.2)	0.282
Age at Tx, years	48 ± 12	53 ± 11	46 ± 11	44 ± 12	0.083
Donor age, years	47 ± 14	50 ± 13	37 ± 10	47 ± 14	0.323
Time to diagnosis, months	28.3 (15.1-45.5)	28.8 (14.7-46.0)	32.5 (14.7-44.8)	32.9 (14.4-50.6)	0.402
Ethnicity					
-Caucasian	43 (47.8)	16 (42.0)	7 (46.7)	20 (54.1)	
-Asian	35 (38.9)	18 (47.4)	6 (40.0)	11 (29.7)	0.501
-Afro-Caribbean	6 (6.7)	2 (5.3)	0 (0.0)	4 (10.8)	
-Other	6 (6.7)	2 (5.3)	2 (13.3)	2 (5.4)	
Diabetes mellitus, n (%)	24 (26.7)	13 (34.2)	2 (13.4)	9 (24.3)	0.276
Live donor, n (%)	44 (48.9)	14 (36.8)	8 (53.3)	22 (59.5)	0.137
Pre-emptive, n (%)	10 (11.1)	1 (2.6)	3 (20.0)	6 (16.2)	0.084
HLA-A/B MM	2 (2-3)	2 (1-3)	2 (2-3)	2 (2-3)	0.793
HLA-DR MM	1 (1-2)	1 (1-2)	1 (1-2)	1 (1-2)	0.552
HLA-DQ MM	1 (1-1)	0 (0-1)	1 (0-2)	1 (1-2)	0.021
Total MM	4 (3-4)	4 (2-4)	4 (3-5)	4 (3-5)	0.846
Induction, n (%)					
-Anti-CD52	70 (77.8)	30 (78.9)	14 (93.3)	26 (70.3)	
-Anti-IL-2R	20 (22.2)	8 (21.1)	1 (6.7)	11 (29.7)	0.189
Graft. No. ≥2 n (%)	6 (6.7)	2 (2.2)	3 (3.3)	1 (1.1)	0.069
Graft loss, n (%)	50 (55.6)	17 (44.7)	5 (33.3)	28 (75.7)*	0.004
eGFR at diagnosis, ml/min	35 (27-48)	34 (26-41)	50 (37-64)	26 (19-46)	0.007
uPCR at diagnosis, ml/mmol	75 (19-160)	91 (46-160)	21 (1-56)	77 (14-211)	0.038

*DSA+/SC- vs DSA+/SC+, p=0.006

3.3.1 DSA Characterization

In total 33 (63.5%) patients were class I DSA positive, 45 (86.5%) were class II DSA positive and 27 (51.9%) had both class I and II DSAs. Patients with class II DSAs had higher median MFI than those with class I DSAs on the pan-IgG assay (1,655 (723-3,054) vs 2,060 (1,350-4,800), p=0.008). The characteristics according to DSA and subclass group can be seen in table 3.2. The mean number of DSAs in the DSA+/SC+ group was slightly higher although this did not reach statistical significance. Significantly fewer patients with only a class I DSA had an identifiable subclass 1/37 compared to those in the DSA+/SC- group 7/15, p<0.001. Pan IgG MFI did not affect whether a subclass was identifiable (p =0.88).

Table 3.2: Characteristics of anti-HLA DSAs according to detectable subclasses

	DSA+ / SC-	**DSA+ / SC+**	**p value**
Number of DSA, mean	1.8 ± 1.0	2.68 ± 1.83	0.09
HLA class			
- Class I, n (%)	7 (46.7)	1 (2.7)	<0.001
- Class II, n (%)	4 (26.7)	17 (45.9)	0.17
- Both classes, n (%)	4 (26.7)	19 (51.4)	0.09
Cumulative MFI (IQR)	11290 (2970-14710)	8345 (4960-14208)	0.88

3.3.2 Subclass Distribution

The subclass distribution for patients with class I DSAs revealed IgG1 to be positive in 9 (27.3%) patients, IgG2 in 0 (0%) patients, IgG3 in 1 (3.0%) patient and IgG4 in 3 (9.1%) patients. Subclass profiles included 8 (24.2%) IgG1 only, 1 (3.0%) IgG1+4, 1 (3.0%) IgG3+4 and 1 (3%) IgG4. The subclass distribution for class II DSAs revealed IgG1 to be positive in 34 (75.6%) patients, IgG2 in 21 (46.7%) patients, IgG3 in 7 (15.6%) patients and IgG4 in 14 (31.1%) patients. Subclass profiles were a combination of 14 (31.1%) IgG1 only, 4 (8.9%) IgG1+2, 2 (4.4%) IgG1+4, 3 (6.7%) IgG1+2+3, 8 (17.8%) IgG1+2+4, 3 (6.7%) IgG1+2+3+4, 1 (2.2%) IgG2 only, 1 (2.2%) IgG2+3 and 1 (2.2%) IgG2+4. In total, of the 78 DSAs identified in the cohort, 27 (22 class I and 8 class II) had no detectable IgG subclass. The median MFI of class I immunodominant DSA (ID DSA) without an identifiable subclass was 1,170 (1,170-3,385) and 800 (540-1,340) for class II ID DSA. The subclass profiles are presented in table 3.3.

Subclass Permutations	**Class I n = 33**	**Class II n = 45**
IgG 1	8	14
IgG 1+2	-	4
IgG 1+4	1	2
IgG 1+2+3	-	3
IgG 1+2+4	-	8
IgG 1+2+3+4	-	3
IgG 2	-	1
IgG 2+3	-	1
IgG 2+4	-	1
IgG 3+4	1	-
IgG 4	1	-
No subclasses	22	8

Table 3.3: Subclass mixture permutations according to HLA class of immunodominant DSA

3.3.3 Kidney Allograft Survival According to IgG Subclass

There was no difference in the mean graft survival from cAMR diagnosis according to whether a class I or a class II DSA was detected; class I: 55.2 months (95%CI 38.1-72.2) vs 43.3 months (95%CI 26.9-59.8) without, p=0.656; class II: 47.2 months (95%CI 34.0-60.5) vs 82.4 months (95%CI 46.0-118.7) without, p=0.121).

Importantly, DSA+/SC+ patients had significantly reduced allograft survival compared to DSA negative and DSA+/SC- groups; DSA+/SC+ 46.3 months (95% CI 31.2-61.3) vs DSAneg 75.5 months (95% CI 56.1-94.9) and DSA+/SC- 65.6 months (95% CI 49.3-82.0), p=0.027 and p=0.019 respectively by log rank. (figure 3.2)

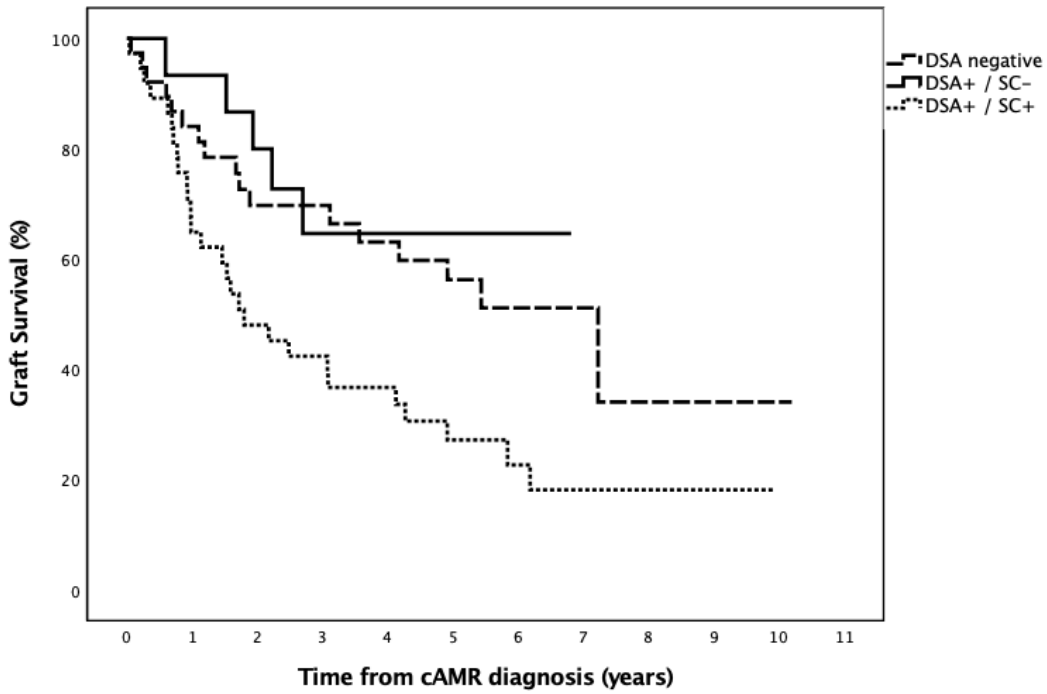


Figure 3.2: Allograft survival according to DSA group status.

DSA+/SC+ vs DSA negative, p=0.027, log rank

DSA+/SC+ vs DSA+/SC-, p=0.019, log rank

The hazards of graft loss for each subclass according to HLA class are shown in Table 3.4. Class I subclass hazards cannot be commented on since subclasses were not detectable in 22/33 patients with class I DSA.

Table 3.4: Hazard ratio of graft loss for each class II IgG subclass.

IgG subclass	**Class I**			**Class II**		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
IgG 1	1.53	0.63-3.72	0.351	2.29	0.79-6.63	0.126
IgG 2	-	-	-	2.91	1.36-6.2	0.006
IgG 3	-	-	-	5.04	1.98-12.8	0.001
IgG 4	2.35	0.68-8.15	0.178	2.10	0.96-4.49	0.064

Patients with class II IgG1 had a mean graft survival of 48.6 months (95% CI 32.5-64.8) vs the DSA+/SC- group, 65.6 months (95% CI 49.3-82.0), p= 0.028. Class II IgG2 mean allograft survival was 31.8 months (95%CI 18.6-44.9) vs the DSA+/SC- group, 65.6 months (95% CI 49.3-82.0), p=0.003. Patients with class II IgG3 had a mean graft survival of 13.6 months (95%CI 6.6-20.5) vs the DSA+/SC- group, 65.6 months (95% CI 49.3-82.0), p<0.001. Mean graft survival in those with class II IgG4 was 30.2 months (95% CI 13.4-47.0) vs the DSA+/SC- group, 65.6 months (95% CI 49.3-82.0), p=0.011 (Figures 3.3-3.6).

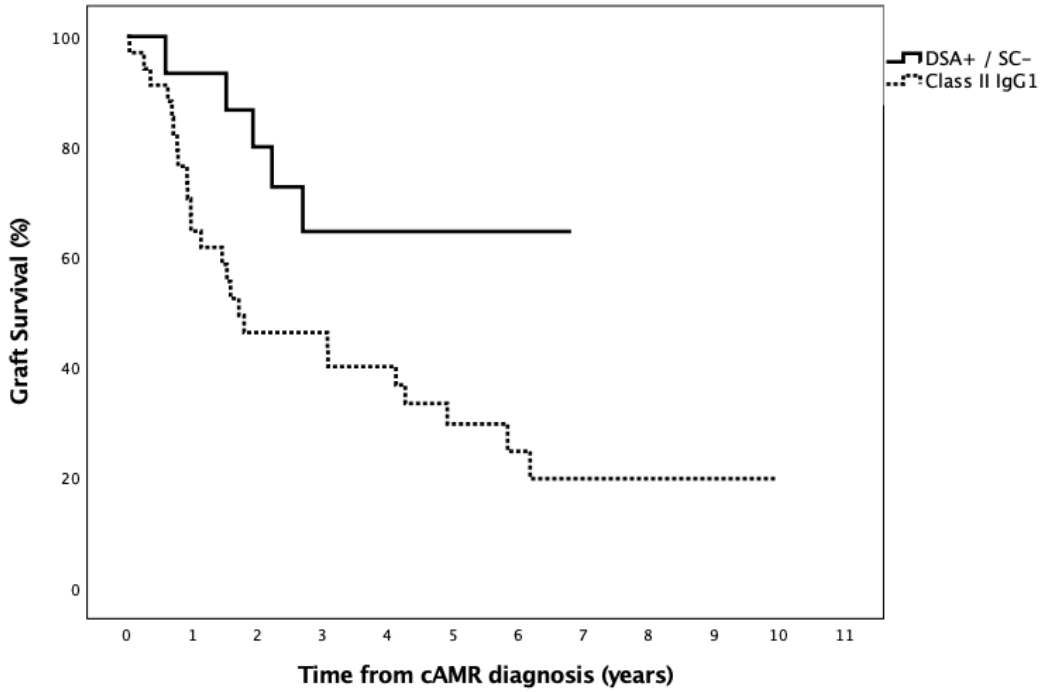


Figure 3.3: Allograft survival comparing class II IgG1 vs DSA+/SC- groups, p=0.028, log rank

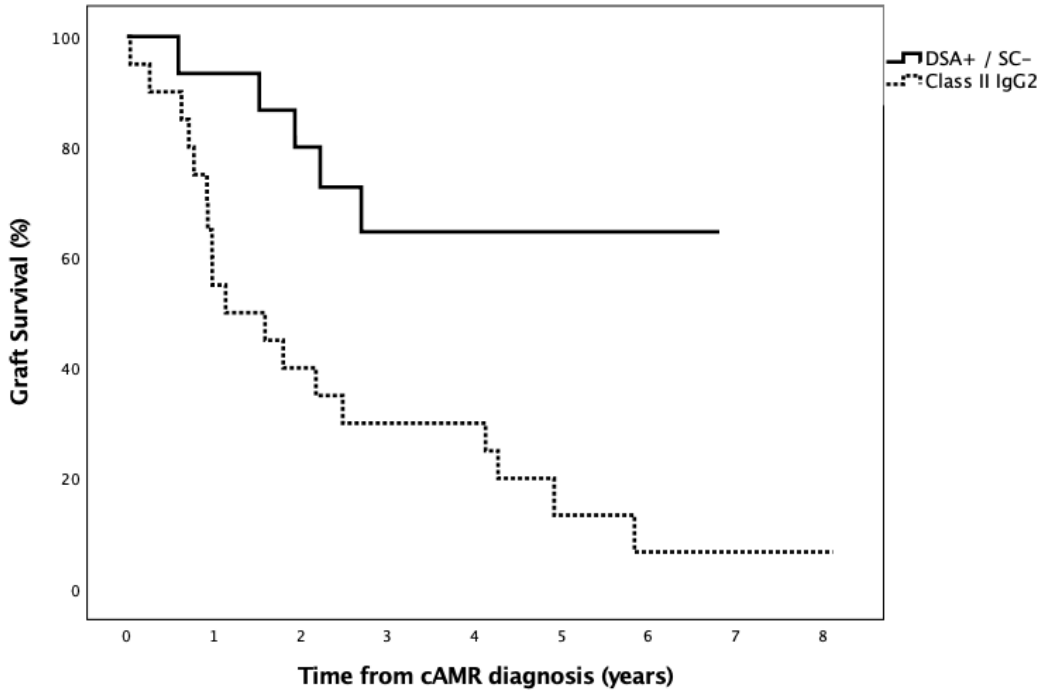


Figure 3.4: Allograft survival comparing class II IgG2 vs DSA+/SC- groups, p=0.003, log rank

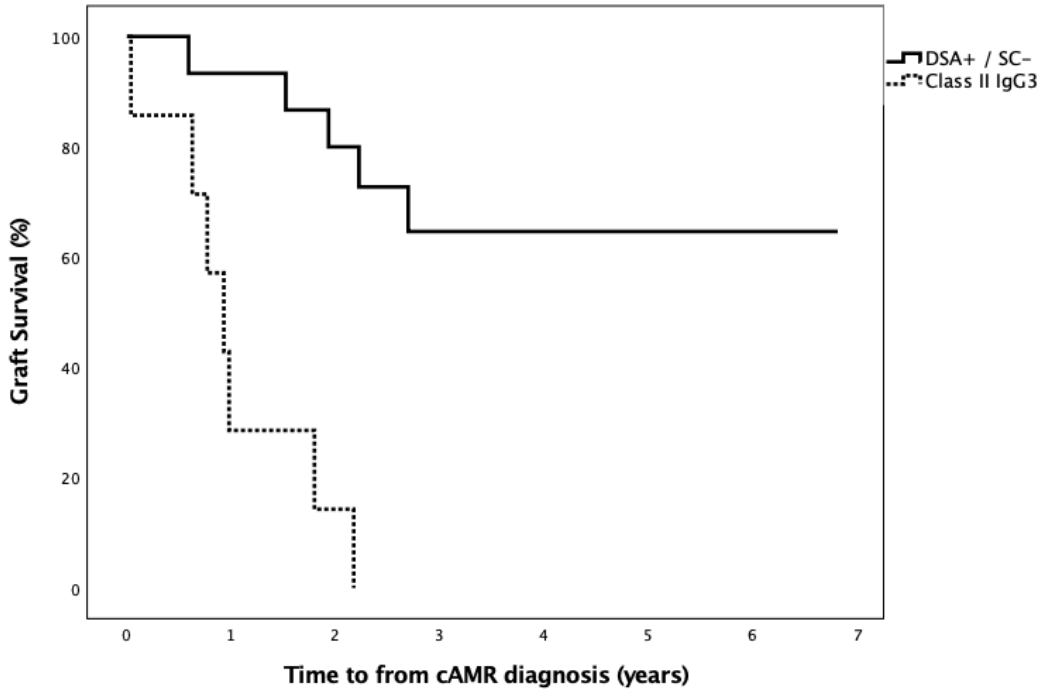


Figure 3.5: Allograft survival comparing class II IgG3 vs DSA+/SC- groups, p<0.001, log rank

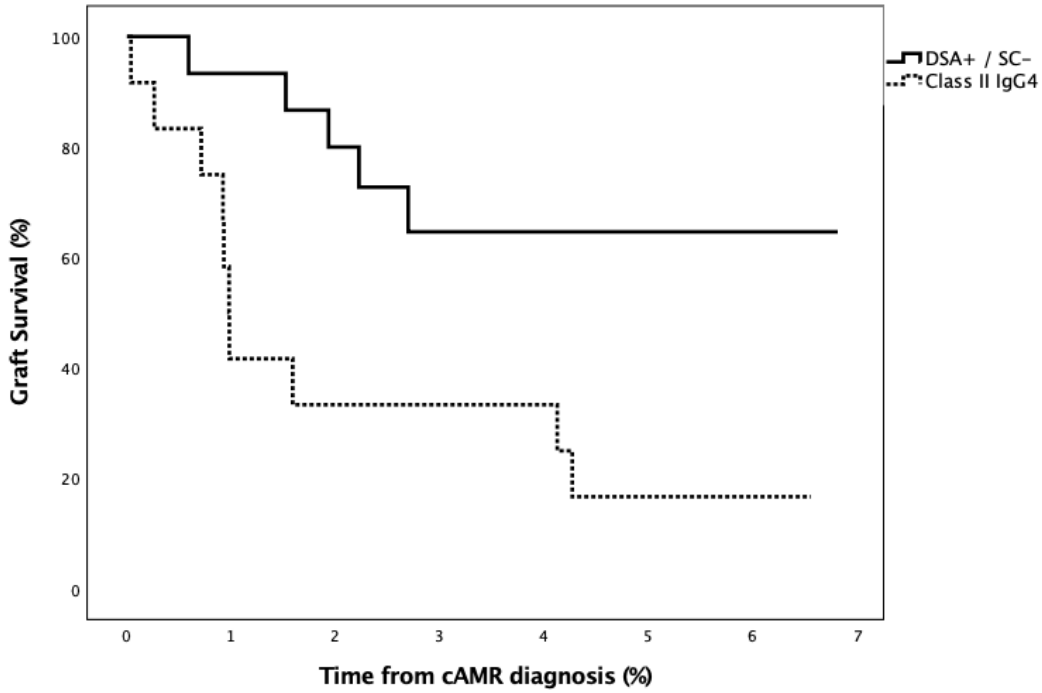


Figure 3.6: Allograft survival comparing class II IgG4 vs DSA+/SC- groups, p=0.011, log rank

Looking in particular at the class II IgG2 and IgG3 positives, the risk of graft failure was almost 3 and 5-fold greater in these groups respectively (IgG2 positive: HR2.91 (95%CI 1.36-6.20), p=0.006; IgG3 positive: HR 5.04 (95%CI 1.98-12.8), p=0.001).

Considering the effect of having any class II IgG subclass was significantly detrimental to allograft survival, comparison was made of patients with more than one of any of the class II subclasses versus those with only a single subclass (Figure 3.7).

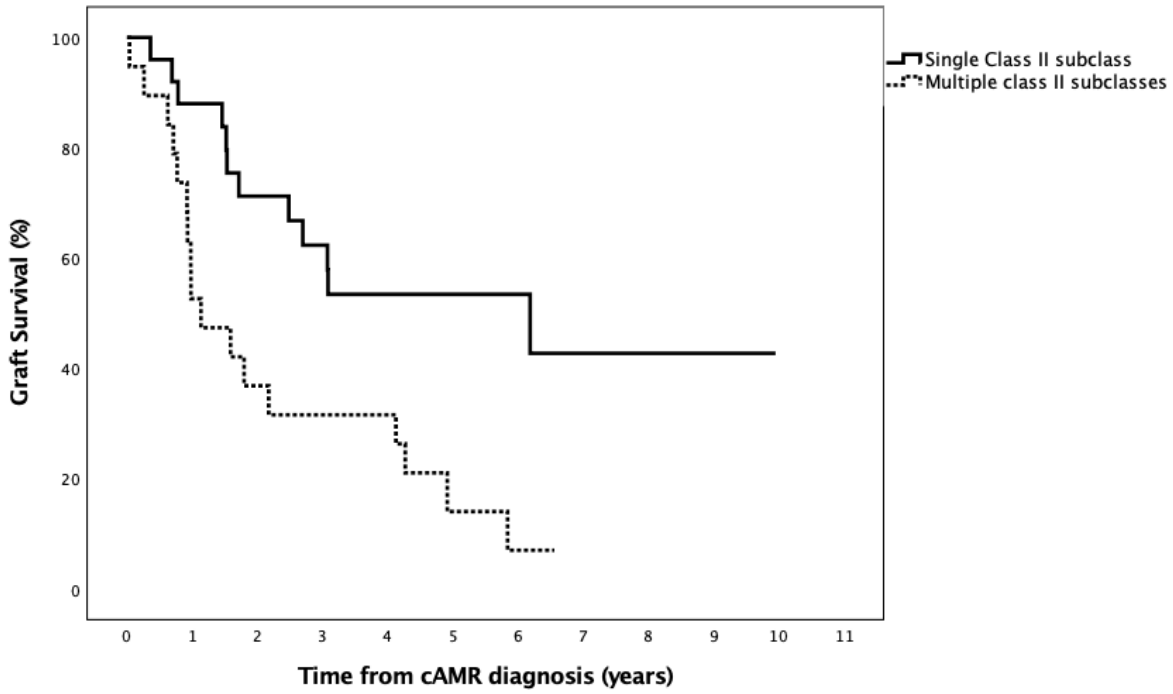


Figure 3.7: Allograft survival of single class II subclass vs Multiple class II subclasses, p=0.005 by log rank.

Patients who had more identifiable class II subclasses tended to have a higher MFI of the ID DSA on the pan IgG assay. Single subclass MFI 5500 (2085-8970) vs multiple classes MFI 7500 (6000-15400), p=0.021 (figure 3.8).

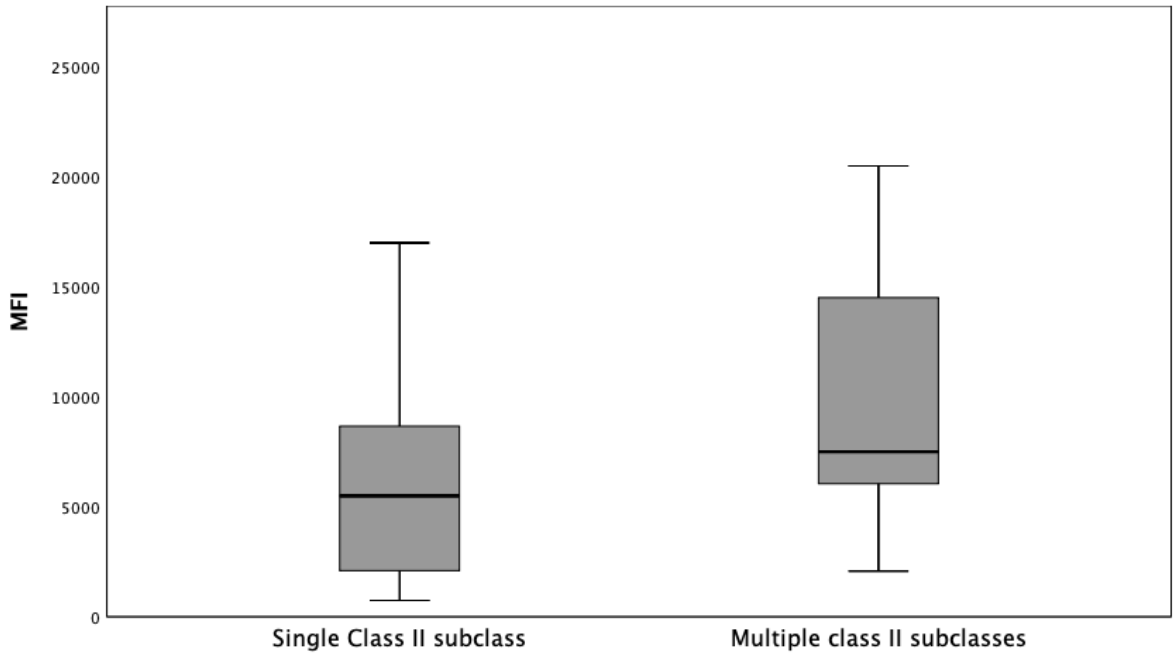


Figure 3.8: Relationship of ID DSA MFI to single vs multiple class II subclasses.

Comparison was made between the patient with and without both class II IgG2 and IgG3 given these were the most hazardous subclasses. The mean allograft survival from cAMR diagnosis was 70.3 months (95%CI 52.1-88.5) for group IgG2 neg/IgG3 neg, 38.3 months (95%CI 22.0-54.7) for the group IgG2 pos/IgG3 neg and 12.5 months (95%CI 6.1-18.9) for the group IgG2 pos/IgG3 pos. The group IgG2 pos/IgG3 pos had the worst allograft survival compared to both IgG2 pos/IgG3 neg and IgG2 neg/IgG3 neg groups (p= 0.016 and p<0.001 respectively), whereas the group IgG2 pos/IgG3 neg showed only a trend towards a worse outcome

compared to the group IgG2 neg/IgG3 neg (p= 0.069) (Figure 3.9). All patients with both class II IgG2 and IgG3 lost their grafts within 2.2 years.

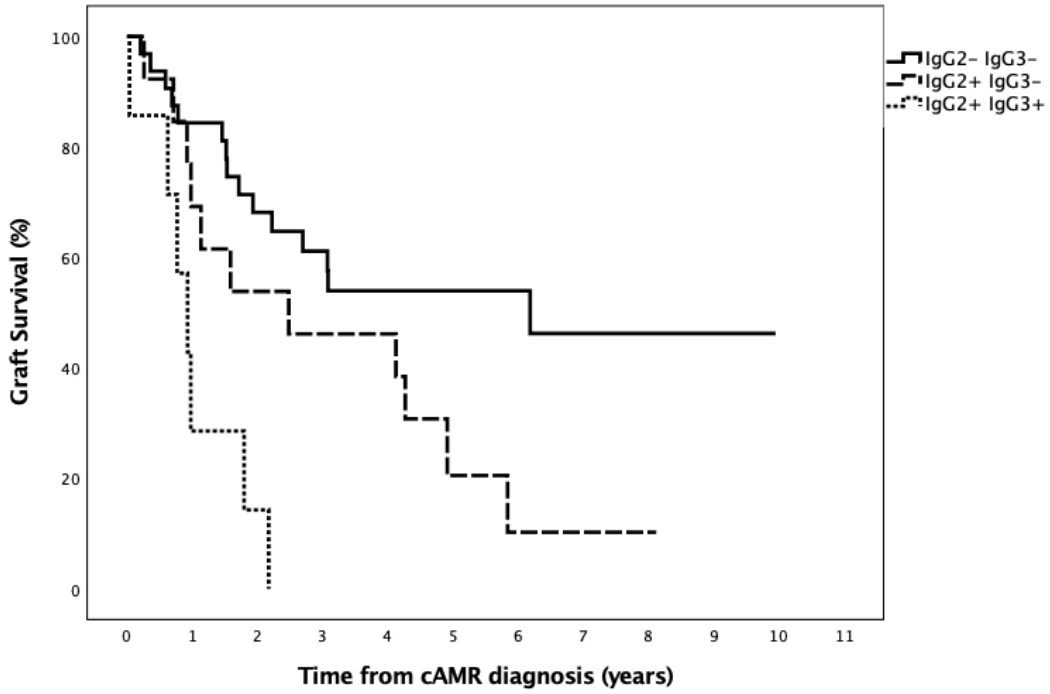


Figure 3.9: Allograft survival comparing Class II IgG2 and IgG3 positivity.

IgG2+/IgG3+ vs IgG2+/IgG3-, p=0.016, log rank

IgG2+/IgG3+ vs IgG2-/IgG3-, p<0.001, log rank

3.3.4 Histological and Clinical Characteristics with Class II IgG Subclass

The histological and clinical characteristics were compared across the entire cohort of cAMR patients and split according class II IgG subclass positivity. Class II IgG1 was associated with increased peritubular capillaritis (p=0.003) and total microcirculatory inflammation (p=0.042). There was a trend towards a shorter time to allograft failure (p=0.056). Class II IgG2 was associated histologically with increased chronicity in the form of transplant glomerulopathy (p=0.034) and microcirculatory inflammation in both glomeruli (p=0.043),

peritubular capillaries ($p<0.001$) and the aggregate mi score ($p<0.001$). C4d deposition tended to be increased in class II IgG2 positive patients ($p=0.052$). Clinically the time to allograft failure was significantly reduced ($p=0.035$). There was also an association towards a lower eGFR at diagnosis ($p=0.06$). Class II IgG3 demonstrated increased peritubular capillaritis ($p=0.001$) and total microcirculatory inflammation ($p=0.004$). Clinically there was a reduced time to allograft failure and a higher degree of proteinuria at diagnosis, $p=0.007$ and $p=0.030$ respectively. Class II IgG4 histologically was associated with increased glomerulitis ($p=0.05$), peritubular capillaritis ($p=0.002$) and hence total microcirculatory inflammation ($p=0.002$). There was also a trend towards increased allograft glomerulopathy ($p=0.063$). There was no significant difference in the clinical characteristics. Interestingly a combination of class II subclasses had more histological consequences with high significance. These included allograft glomerulopathy ($p=0.007$), all forms of microcirculatory inflammation g score ($p=0.034$), ptc score ($p<0.001$) and mi score ($p<0.001$). There was a trend towards greater scaring and C4d deposition, $p=0.061$ and $p=0.065$ respectively. There was a shorter time to allograft failure and lower eGFR at diagnosis, $p=0.029$ and $p=0.038$ respectively. (See tables 3.4-3.8)

Tables 3.5-3.9: Histological and clinical characteristics according to class II subclass positivity

	IgG1 positive (n=34)	**IgG1 negative (n=56)**	**p value**
Banff scores			
Glomerulopathy (cg)	1 (1-3)	1 (1-2)	0.332
Glomerulitis (g)	1.5 (1-2)	1 (0-2)	0.748
Peritubular capillaritis (ptc)	1 (0-2)	1 (0-1)	**0.003**
mi (g+ptc)	3 (2-4)	1 (0-1)	**0.042**
C4d	2 (1-2)	1 (0-2)	0.089
IFTA % - nearest 5%	15 (10-20)	10 (5-20)	0.051
Clinical characteristics			
Time to diagnosis (months)	22.3 (15.6-49.1)	28.8 (13.8-44.8)	0.934
Time to failure (months)	21.5 (11.5-65.8)	40.7 (20.3-44.8)	0.056
eGFR at diagnosis (ml/min)	34 (26-44)	37 (27-50)	0.133
uPCR at diagnosis (mg/mmol)	67 (11-226)	76 (22-155)	0.991

	IgG2 positive (n=20)	**IgG2 negative (n=70)**	**p value**
Banff scores			
Glomerulopathy (cg)	1.5 (1-3)	1 (0-2)	**0.034**
Glomerulitis (g)	2 (1-2)	1 (0-2)	**0.043**
Peritubular capillaritis (ptc)	1 (1-2)	1 (0-1)	**<0.001**
mi (g+ptc)	4 (3-4)	2 (1-3)	**<0.001**
C4d	2 (1-3)	1 (0-2)	0.052
IFTA % - nearest 5%	15 (10-30)	15 (5-20)	0.294
Clinical characteristics			
Time to diagnosis (months)	24.6 (14.0-56.8)	28.3 (15.2-44.8)	0.965
Time to failure (months)	17.7 (10.5-55.0)	40.8 (19.4-71.6)	**0.035**
eGFR at diagnosis (ml/min)	32 (23-40)	37 (28-50)	0.060
uPCR at diagnosis (mg/mmol)	98 (56-195)	60 (14-157)	0.137

	IgG3 positive (n=7)	IgG3 negative (n=83)	p value
Banff scores			
Glomerulopathy (cg)	1 (1-3)	1 (0-2)	0.375
Glomerulitis (g)	2 (1-2)	1 (0-2)	0.196
Peritubular capillaritis (ptc)	2 (2-3)	1 (0-2)	**0.001**
mi (g+ptc)	4 (3-4)	1 (1-2)	**0.004**
C4d	2 (1-2)	1 (0-2)	0.421
IFTA % - nearest 5%	15 (10-50)	15 (10-20)	0.563
Clinical characteristics			
Time to diagnosis (months)	15.8 (9.4-62.3)	(15.2-44.8)	0.839
Time to failure (months)	12.1 (8.1-23.4)	40.5 (15.5-71.1)	**0.007**
eGFR at diagnosis (ml/min)	32 (22-40)	37 (27-50)	0.214
uPCR at diagnosis (mg/mmol)	184 (96-238)	70 (17-152)	**0.030**

	IgG4 positive (n=12)	IgG4 negative (n=78)	p value
Banff scores			
Glomerulopathy (cg)	1 (1-3)	1 (0-2)	0.063
Glomerulitis (g)	1 (1-2)	1 (0-2)	**0.05**
Peritubular capillaritis (ptc)	2 (1-2)	1 (0-2)	**0.002**
mi (g+ptc)	3 (2-4)	2 (1-3)	**0.002**
C4d	2 (1-2)	1 (0-2)	0.149
IFTA % - nearest 5%	20 (10-45)	15 (5-20)	0.939
Clinical characteristics			
Time to diagnosis (months)	25.9 (12.0-46.6)	28.3 (15.2-46.0)	0.686
Time to failure (months)	12.7 (9.9-55.0)	40.0 (19.4-71.2)	0.071
eGFR at diagnosis (ml/min)	29 (21-42)	37 (27-50)	0.654
uPCR at diagnosis (mg/mmol)	87 (6-186)	72 (19-160)	0.201

	Multiple IgG SC (>1) (n=19)	Single IgG SC (n=71)	p value
Banff scores			
Glomerulopathy (cg)	2 (1-3)	1 (0-2)	**0.007**
Glomerulitis (g)	2 (1-2)	1 (0-2)	**0.034**
Peritubular capillaritis (ptc)	2 (1-2)	1 (0-1)	**<0.001**
mi (g+ptc)	4 (3-4)	2 (1-3)	**<0.001**
C4d	2 (1-2)	1 (1-2)	0.061
IFTA % - nearest 5%	15 (10-30)	15 (5-20)	0.065
Clinical characteristics			
Time to diagnosis (months)	22.1 (15.2-59.9)	28.4 (14.7-44.8)	0.886
Time to failure (months)	14.8 (10.0-55.5)	40.5 (19.5-71.6)	**0.029**
eGFR at diagnosis (ml/min)	31 (22-40)	37 (28-50)	**0.038**
uPCR at diagnosis (mg/mmol)	96 (46-198)	61 (17-156)	0.263

3.3.5 Risk Factors for Allograft Loss According to Immunodominant DSA subclass Composition

The factors affecting allograft loss according to the ID DSA characteristics in univariate and multivariate cox regression analyses are shown in table 3.9. Although class II IgG2, IgG3, IgG4 and a combination of at least >1 subclass were all significant on a univariate cox regression analysis, when included in a multivariate model class II IgG3 positivity was the only factor to be independently associated with allograft loss (adjusted HR 3.91 (95% CI 1.36-11.23), p=0.011.

Table 3.10: Characteristics of immunodominant DSA associated with graft loss

	N	Failure	HR	95% CI	p value
Univariate Cox regression	52	33			
Specificity of ID DSA					
- Class I	10	6	1.17	0.48-2.84	0.73
- Class II	42	27	1.21	0.46-3.13	0.70
MFI	52	33	1.00	0.99-1.00	0.16
Class II IgG1					
- No	18	9			
- Yes	34	25	2.29	0.79-6.64	0.13
Class II IgG2					
- No	32	2			
- Yes	20	18	2.91	1.36-6.20	0.006
Class II IgG3					
- No	45	0			
- Yes	7	7	5.04	1.98-12.82	0.001
Class II IgG4					
- No	40	2			
- Yes	12	10	2.08	0.96-4.50	0.06
Multiple class II SC					
- No	25	12			
- Yes	19	17	2.77	1.31-5.83	0.007
Multivariate Cox regression	52	33			
Class II IgG3					
- No	45	0			
- Yes	7	7	3.91	1.36-11.23	0.011

3.4 Discussion

The leading cause of late allograft failure is chronic antibody mediated rejection[5]. This study has defined significant associations between identifiable class II IgG subclass and the clinical and histological outcomes of a large cohort of patients with cAMR. There has been limited investigation of the outcomes of patients with cAMR with and without anti-HLA DSA. This study is the first to show in renal transplantation, significantly reduced allograft survival amongst all class II IgG subclasses, with class II IgG2 and IgG3 being the most discriminatory. It has also been demonstrated that class II IgG2 and IgG3 correlate with the most severe phenotypes of chronic active antibody mediated rejection both clinically and histologically.

DQ mismatch was highest in the DSA+/SC+ cohort. Wiebe et al demonstrated in 2012 that the majority of de novo DSA in AMR develops against class II mismatches[8]. In particular, Anti-DQ DSAs have been shown to be the most common de novo DSA produced[173]. Gloor et al showed a higher prevalence of class II DSAs versus class I DSAs in a cohort of patients with cAMR particularly with transplant glomerulopathy[133] which, reflects our findings. It is not clear why class II DSA predominates in more chronic lesions. This phenomenon is not exclusive to renal transplantation. In cardiac and lung transplantation, class II DSAs lead to chronic rejection phenotypes such as cardiac allograft vasculopathy[174] and bronchiolitis obliterans syndrome[175]. In 2010 Walsh et al not only showed that class II DSA predominated in late AMR but also demonstrated a greater resistance to treatment with anti-B cell and plasma cell therapies[176]. No mechanism has been proven to explain the increased pathogenicity of class II DSA in AMR. Ongoing study is needed to establish the immunogenicity of class II epitopes and whether greater consideration needs to be given to matching at the HLA-DQ locus[177].

Although all class II subclasses had deleterious impacts on allograft failure, the combination of class II IgG2pos/IgG3pos in particular, had the greatest impact on allograft survival of all

the subclasses. This effect was independent of other donor/recipient characteristics. Whilst IgG3 has been shown to be associated with a high risk of allograft failure in kidney transplantation[72], this has been restricted to cases with acute AMR[13]. Lefaucheur et al investigated 186 patients who developed de novo DSA within one year of transplant. 87 patients developed AMR defined as acute (n=51) and subclinical (n=36). IgG3 strongly correlated with more severe phenotypes of rejection with lower GFRs at diagnosis[13]. Khovanova et al demonstrated de novo development of IgG3 was associated with early graft failure although, the cause of graft loss was not indicated[171]. O'Leary et al observed inferior graft and patient survival in 53 liver allograft recipients that were IgG3 positive with rejection (HR=2.1, p=0.004), though this was a preformed antibody cohort. Importantly, none of these studies distinguish the HLA class of the immunodominant DSA subclass. Similarly, Kaneku group, in their analysis of 39 liver transplant recipients with chronic rejection also identified IgG3 with a three-fold increased risk of graft loss, in contrast to 69 matched controls without rejection (HR=3.35, 95% CI 1.39-8,05, p=0.007)[178]. Thus, this is the first study revealing that the combination of de novo class II IgG2 and IgG3 DSA has been shown to be injurious to renal allografts with cAMR, with class II IgG3 in particular representing an independent hazard of allograft loss. The pathogenicity of IgG3 is well described. IgG3 has a superior affinity to binding complement compared to all other IgG subclasses. IgG3 also binds a broad spectrum of Fc receptors with wide tissue and serum distribution[44]. Humans possess two types of Fcγ receptors that are either inhibitory or activating, with broad but subtly differing distribution within amongst immune cells[179]. FcγRI has broad myeloid distribution and has the greatest affinity for binding IgG, with the principal ligands being IgG1 and IgG3[180]. FcγRII is further split into FcγRIIa and FcγRIIb, the former having a role in phagocytosis, while the latter a more inhibitory function[179]. FcγRIII is principally found on NK cells and participates in cell mediated

cytotoxicity[181]. Subclass affinity for specific Fc receptors is an important means of understanding their function. Besides the affinity of IgG3 for Fc receptors, the structure of the subclass is also important. The extra length of the hinge region allows more efficient bridge formation between allo-antigen on endothelial cells and Fc receptors on host immune cells[182]. Antibody dependent cell mediated cytotoxicity is thus facilitated more efficiently.

IgG2 can still bind complement although not with the same prolonged affinity as IgG1 or IgG3. Expansion to non-complement binding subclasses has been considered to indicate a chronic triggering of the humoral immune system mediated by prolonged T-cell activation[42]. Arnold et al demonstrated that expansion into non-complement binding subclasses in 11/17 patients with DSA did not result in poorer graft survival when compared to the presence of purely complement fixing subclasses[183]. However, the presence of IgG2 in combination with IgG3 might result in a synergistic effect of complement activation if these antibodies bind the same HLA molecule on different epitopes[184,185].

Class II IgG4 was, on an uncorrected analysis, associated with reduced allograft survival. It is important to note that class II IgG4 was never found alone, always existing in combination with other subclasses. IgG4 has been considered to be a possible immunomodulatory subclass with the potential to inhibit the effects of more injurious subclasses by potentially through competition allosteric binding of the HLA molecules as well as steric inhibition on Fc receptors of complement[186,187]. This phenomenon is not restricted to alloimmune injury alone, but also membranous nephropathy[188]. Whether this study's findings represent previous data that IgG4 is predicative of chronicity in AMR[13] or if it has a similar effect as IgG2 in synergy with complement binding subclasses, is not yet clear and beyond the scope of this work.

IgG1 was the most frequent subclass identified. This was expected as it is the most abundant IgG subclass in humans, with the longest half-life[42]. This does not however explain why IgG1

did not result in independently significant allograft damage. Prior to this work IgG1 appearing alone has only been shown to associated with allograft rejection in patients with preformed DSA[171,189]. IgG2,3 and 4 tend to develop as part of a mixture of subclasses, mostly including IgG1 so whilst there may not be a direct pathogenic role, the presence of IgG1 may be necessary prior to expansion into more diverse subclass combinations.

As in previous studies a proportion of DSAs did not have an identifiable subclass. This was particularly evident for class I DSAs. Prior studies have suggested this may reflect the assay's inability to detect subclasses with low pan-IgG MFIs[190] however, in this study MFI did not discriminate whether a subclass was identified.

Increased MCI in particular, peritubular capillaritis was associated with all class II subclasses but in particular with IgG2 and IgG3. This provides further evidence for the pathogenicity of these class II DSA phenotypes. Interestingly the degree of C4d staining was not significantly associated with any phenotype. Loupy et al demonstrated in 55% of C4d negative biopsies from 80 DSA positive patients, that the presence of MCI and class II DSA are risk factors for the progression to cAMR, independent of C4d deposition[119]. This suggests that the immunological processes occurring in cAMR may lean towards complement independence with endothelial injury through direct antibody cytotoxic effects rather than the classical pathway of complement activation. I will go on to discuss the role of complement in cAMR in the following chapter.

Redfield et al showed the poor outcomes of cAMR in a large cohort of transplant recipients with allografts having a median survival of 2 years post diagnosis[135]. Characterisation of IgG DSA helps to predict outcome in cAMR and highlights those at the greatest risk of allograft failure. The finding of 100% allograft loss in patients with class II IgG2 and IgG3 DSA phenotypes at 2.2 years further supports the need to stratify patients beyond a histological

diagnosis. If future therapies are to be given the greatest opportunities for success, we need predictive models of allograft loss, whereby serological and histological phenotypes identify groups that would benefit most from tailored individualised immunosuppressive therapies. Exciting results are expected from upcoming trials of the IL-6R antibody, tocilizumab as well as the SYK inhibitor fostamatinib are expected in the coming years. If DSA phenotyping can help predict allograft loss, these characteristics should be incorporated into recruitment into future trials as a "one fits all" approach is clearly not effective.

The retrospective design of this study is one of its limitations. Even though the purpose of this study was to investigate whether a specific subclass at the time of cAMR diagnosis could be a predictor of allograft loss, the single time point serum samples do not allow monitoring of the kinetics of these DSAs as patients progress to graft failure. It was also not possible to compare the effect of class II IgG3 alone as it was not present in the absence of class II IgG2 in this cohort. Despite the great improvement of solid phase assays in the last 10 years, the subclass assay still needs optimising in order to detect low pan-IgG MFI DSAs.

In conclusion, this study has shown the importance of DSA phenotyping in cAMR.

For the first time in cAMR, the deleterious effect and clinical significance of de novo class II IgG DSA subclasses particularly IgG2 and IgG3 in a well categorised cohort of patients in renal transplantation has been demonstrated.

Chapter 4: C1q binding anti-HLA donor specific antibodies in patients with chronic antibody mediated rejection

4.1 Introduction

C1q binding of DSAs has been used to evaluate the risk of graft loss across solid organ transplantation in pre and post-transplant sera[191-193]. C1q fixing anti-HLA DSA have been associated with increased rates of acute episodes of AMR, increased risk of graft failure and increased risk of progression to TG[82,193-195]. Alongside subclass phenotyping, understanding the complement fixing ability of DSAs has added significantly to the hazard modelling of allograft rejection in renal transplantation[13,14]. There is however a paucity in the literature with regards to the long-term outcomes in patients with and without complement binding antibodies, particularly in cohorts of established cAMR.

Disappointing results from trials into terminal complement pathway inhibition have left the transplant community wondering, to what extent complement plays a role in cAMR[196]? Clearly, with the addition of C4d negative AMR into the Banff classification and the development of humoral rejection in DSA negative patients, other mechanisms for microcirculatory injury must exist[197].

The C1q bead assay has brought the sensitivity and specificity of flow solid phase assays to further delineate the function of DSA. Aside from IgG3 and C3d it is felt to be particularly informative when establishing the pathogenicity of DSA[84,198], although controversy still exists in its application. C1q is the inaugural component of the classical pathway prior to the formation of the membrane attack complex which in turn leads to the destruction of endothelial cells and deposition of the complement split product C4d in glomeruli and peritubular capillaries. Undoubtedly the story does not end here as not all DSAs have

complement fixing characteristics and repeatedly cAMR can be present in the absence of active inflammation. The presence of non-complement fixing (NCF) subclasses in elutes of rejected kidney allografts further supports alternative mechanisms to complement mediated cytoxocity[199]. The old schema defining putative stages of antibody mediated injury following the development of DSA and later allograft dysfunction are no longer so clear cut[200]. In fact, in those with chronic persistent DSA, damage can be far more insidious alongside cyclical repair, leaving smouldering rejection often undetectable by clinical assessment alone[15]. Therefore, there is a clear demand for further risk stratification based on antibody phenotyping.

This study was devised to evaluate the clinical significance of C1q binding DSA alongside IgG subclass phenotyping on long term outcomes in patients with biopsy-proven cAMR.

4.2 Methods

4.2.1 Patients

The same patient cohort used in chapter 2 was analysed. In this study only the 52 patients with an IgG DSA detectable within 3 months of the diagnosis of cAMR were included. DSA positive patients were defined according to whether their immunodominant DSA had identifiable subclasses; SCpos or SCneg. If DSA positive patients had a C1q binding antibody this was defined as C1qpos or C1q neg. This provided 3 group combinations of SCneg, SCpos/C1qneg and SCpos/C1qpos.

4.2.2 C1q Testing Protocol

Testing was performed using the C1qScreen™ kit (OneLambda Inc, Canoga Park, CA.). Sera stored at -20˚C was thawed and then centrifuged at 10,000 RPM for 5 minutes. 50µL of serum was then heat-inactivated on a hot plate at 56˚C for 30 minutes. This serum was then stored once again in the freezer until needed.

C1q (PEPC1q) is diluted in a 1:5 dilution with 10mM Hepes buffer pH7.2. In V-bottomed wells, 2.5µL of C1q solution is spiked in the base. 10µL of heat inactivated serum is then added to each well. The plate is then vortexed. 2.2µL of LABscreen® single antigen beads (One Lambda, Inc) are added to each testing well, depending on the class of anti-HLA antibody being tested. 0.25µL of C1qScreen positive control beads are finally added prior to incubation on a shaker at room temperature for 30 minutes protected from light. The plate is then centrifuged at 10,000 RPM for 5 minutes. Following this, 2.5µL of phycoerythrin-labelled anti-C1q antibodies are added to each well and the mixture is vortexed once more. The plate is then incubated for another 30 minutes. At this point, the solution requires transfer onto a filter bottomed plate. To facilitate transfer 100µL of wash solution is added and the entire contents of the V-

bottomed wells is transferred by micropipette to a pre-wetted by wash solution, filter bottomed plate. Each well is then washed with 100µL of wash solution and then vacuumed. 80µL of phosphate-buffered solution (PBS) is added to re-suspend the beads prior to analysis on the Luminex machine.

Filter plates are not used from the beginning as the filter bottoms can in fact promote complement activation and give false positive results. The Luminex probe at Hammersmith hospital H&I laboratory is calibrated to filter plates only, thus transfer is necessary. All assays had bead counts of >100 beads per well indicating that transfer to filter-bottomed plates did not result in significant bead loss.

C1q-SAB binding was expressed as normalised MFI using HLA Fusion software (v3.2; One Lambda). MFI of >500 over background was considered positive.

4.3 Results

Of the 1,642 patients who were transplanted between March 2005 and November 2015 at Imperial College Renal and Transplant Centre, 90 (5.4%) had glomerular double contours and/or significant PTCL on biopsy. Of these, 52 had an identifiable de novo IgG DSA at the time of cAMR diagnosis (3.1%). The median follow-up after transplant was 65.5 (39.1-89.1) months. The cohort was split according to C1q binding into three groups; DSA positive but subclass negative (SCneg) n=15 (28.8%), subclass positive without C1q binding (SCpos/C1qneg) n=16 (30.8%) and subclass positive with C1q binding (SCpos/C1qpos) n=21 (40.4%). The baseline characteristics are shown in table 4.1.

Table 4.1: Demographics

	Total (n=52)	**SC neg (n=15)**	**SCpos/C1qneg (n=16)**	**SCpos/C1qpos (n=21)**	**p**
Female, n (%)	19 (36.5)	3 (20.0)	9 (56.3)	7 (33.3)	0.103
Age at Tx, years	45 ± 12	46 ± 11	47 ± 11	41 ± 12	0.234
Donor age, years	44 ± 14	38 ± 10	47 ± 13	48 ± 16	0.027
Time to diagnosis, months	23.4 (14.8-46.9)	32.4 (14.7.-44.8)	18.2 (9.1-42.3)	29.8 (15.5-56.8)	0.469
Ethnicity					
-Caucasian	27 (51.9)	7 (46.7)	10 (40.0)	10 (51.9)	
-Asian	17 (32.7)	6 (40.0)	3 (18.8)	8 (38.1)	
-Afro-Caribbean	4 (7.7)	0 (0.0)	1 (4.0)	3 (14.3)	0.322
-Other	4 (7.7)	2 (13.3)	2 (12.5)	0 (0.0)	
Diabetes mellitus, n (%)	11 (21.2)	2 (13.3)	2 (12.0)	6 (28.6)	0.523
Live donor, n (%)	30 (57.7)	8 (53.3)	11 (44.0)	11 (21.2)	0.560
Pre-emptive, n (%)	9 (17.3)	3 (20.0)	4 (25.0)	2 (9.5)	0.443
HLA-A/B MM	2 (2-3)	2 (2-3)	2 (1-3)	2 (2-3)	0.891
HLA-DR MM	1 (1-2)	2 (1-2)	2 (2-2)	1 (1-2)	0.797
HLA-DQ MM	1 (1-2)	1 (1-2)	1 (1-2)	2 (1-2)	0.017
Total MM	4 (3-4)	4 (2-4)	4 (3-5)	4 (3-4)	0.678
Induction, n (%)					
-Anti-CD52	40 (76.9)	14 (93.3)	11 (68.8)	15 (76.9)	
-Anti-IL-2R	12 (23.1)	1 (6.7)	5 (31.3)	6 (28.6)	0.198
Graft. No. ≥2 n (%)	4 (23.1)	3 (20.0)	1 (4.0)	0 (0.0)	0.082
Graft loss, n (%)	33 (63.5)	5 (33.3)	9 (36.0)	19 (90.5)	0.002
eGFR at diagnosis, ml/min	40 (29-50)	50 (37-64)	44 (34-53)	32 (23-40)	0.002
uPCR at diagnosis, ml/mmol	52 (8-176)	21 (21-56)	33 (8-214)	96 (40-211)	0.102

Patient characteristics were comparable throughout all groups. Patients with identifiable subclasses had transplants from older donors, p=0.027. DQ mismatch was significantly greater in the DSA+/SC+ group compared to both the DSA negative and DSA+/SC- groups; median mismatch 2 (1-2), 1 (1-2) and 1 (1-2) respectively, p=0.017. No patients with C1q positive DSAs were re-transplanted by the time of the analysis. Allograft failure was more common in the SCpos/C1qpos group [19/21 (90.5%)] compared to all other groups, p=0.002. At diagnosis, eGFR was significantly lower in the SCpos/C1qpos group 32 (23-40) compared to both SCpos/C1qneg 44 (34-53) and SCneg 50 (37-64) groups, p=0.002. Finally, there was a trend towards increased proteinuria at diagnosis in the SCpos/C1qpos group, p=0.102

4.3.1 DSA Characterisation

In total 33 (63.5%) patients were class I DSA positive, 45 (86.5%) were class II DSA positive and 27 (51.9%) had both class I and II DSAs. The characteristics according to C1q binding can be seen in table 4.2.

Table 4.2: Characteristics of anti-HLA DSAs according to C1q binding status

	SCpos / C1qneg n=16	**SCpos / C1qpos n=21**	**p value**
Number of DSA, mean	1.94 ± 0.9	3.2 ± 2.2	0.078
HLA class			
- Class I only, n (%)	1 (6.3)	1 (4.8)	0.432
- Class II only, n (%)	10 (62.5)	6 (28.5)	0.076
- Both classes, n (%)	5 (31.2)	14 (66.7)	0.035
ID MFI (IQR)	5211 (1702-7950)	9780 (6050-13985)	0.018

There was trend towards a higher mean number of DSAs and Class II HLA in the SCpos/C1qpos group, p=0.078 and p=0.076 respectively. Significantly more patients with a C1q binding DSA had coexisting class I and II antibodies, p=0.035. The median MFI of the ID DSA was significantly higher in the SCpos/C1qpos group (figure 4.1).

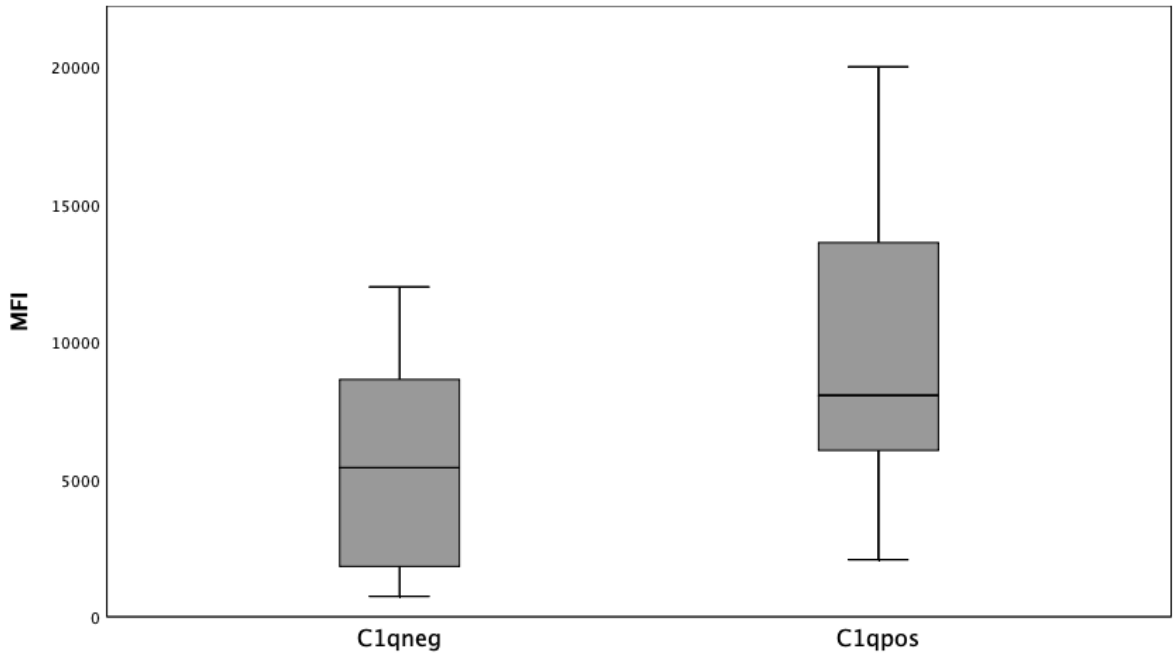


Figure 4.1: Box and whisker plot comparing the MFI of the Immunodominant DSA according to C1q positivity

4.3.2 Subclass Distribution

In total 23 (44.2%) patients had a DSA that bound C1q; 3 (13.0%) were class I, 16 (69.6%) were class II and 4 (17.4) were both class I and II DSA positive. The subclass profiles for these patients was: IgG1 only (n=4, 17.4%), IgG1+2 (n=3, 13.0%), IgG1+3 (n=1, 4.3%), IgG1+4 (n=1, 4.3%), IgG1+2+3 (n=2, 8.7%), IgG1+2+4 (n=5, 21.7%), IgG1+2+3+4 (n=3, 13.0%), IgG2+3 (n=1, 4.3%) and IgG2+4 (n=1, 4.3%). Of the 23 patients with a C1q-binding DSA only one had a

subclass profile that was not expected to bind complement (IgG2 and/or IgG4). Of note 2 (8.7%) patients had a C1q binding DSA without any identifiable subclass.

4.3.3 Allograft Survival

Allograft survival was poorest in the SCpos/C1qpos group vs SCneg and SCpos/C1qneg groups overall; mean allograft survival 33.1 months (95% CI 19.7-46.5) vs SCneg 65.6 months (95% CI 49.3-82.0) and SCpos/C1qneg 65.5 months (95% CI 38.3-92.6), p=0.008. There was however, only a trend towards significance comparing C1q positivity in patients with identifiable subclasses (p=0.079) (Figure 4.2).

Given 2 cases of C1q binding occurred in the SCneg group, overall binding was analysed. C1q binding irrespective of HLA class once again only showed a trend towards significance. C1qpos mean allograft survival 39.9 months (95% CI 25.4-54.4) vs C1qneg 73.1 months (95% CI 52.6-93.8), p=0.058 (Figure 4.3).

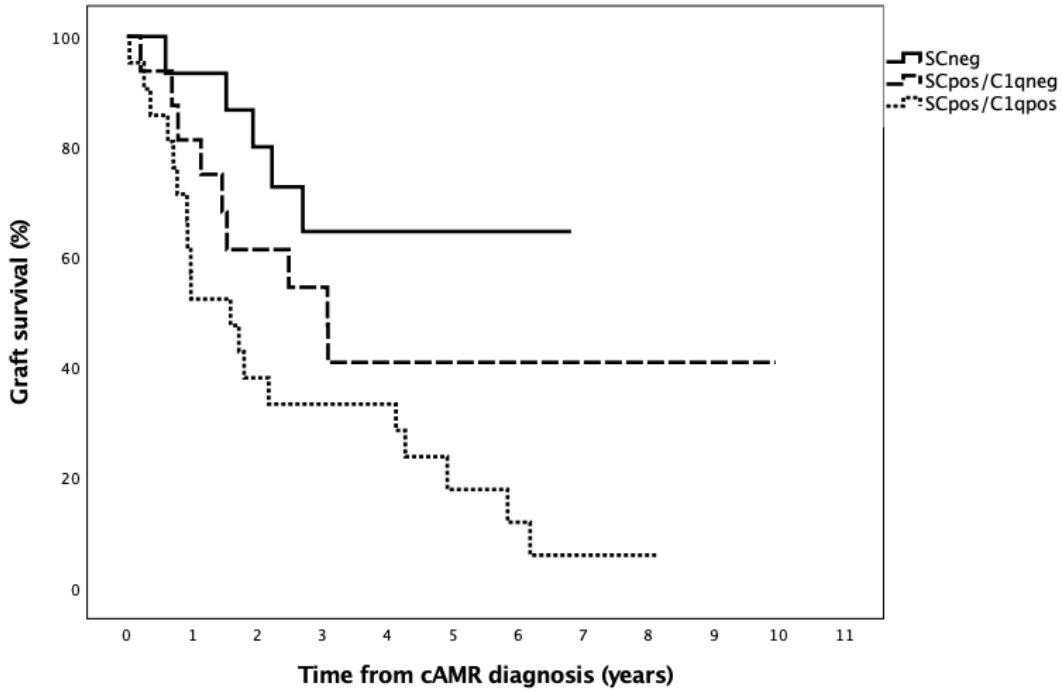


Figure 4.2: Allograft survival according to SC and C1q positivity

SCpos/C1qpos vs SC neg, p=0.002, log rank

SCpos/C1qpos vs SCpos/C1qneg, p=0.079, log rank

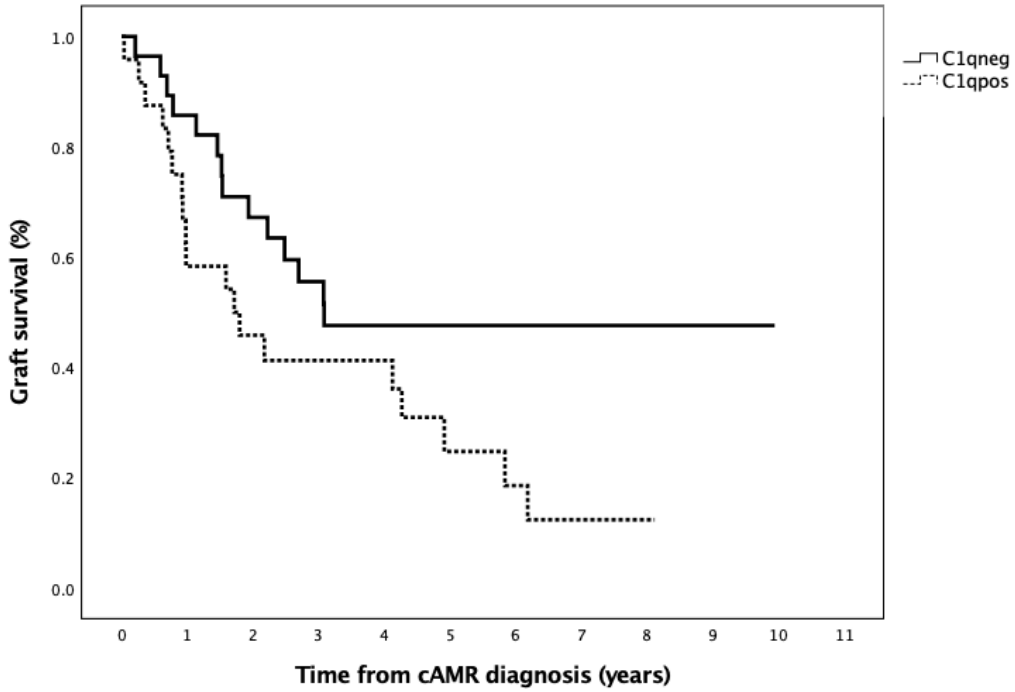


Figure 4.3: Allograft survival according to C1q status p=0.058

There was no difference in allograft survival in patients with class I C1q binding DSA alone compared with those that were C1q negative (p=0.385). The majority of C1q binding DSA were of class II specificities (16/21), in this group there was a significant difference in allograft survival compared to C1q negative patients, with a 50% survival difference of 3 years; class II C1qpos mean allograft survival 35.7 months (95% CI 21.2-50.2) vs C1qneg 71.9 months (95% CI 52.4-91.4), p=0.024 (Figure 4.4).

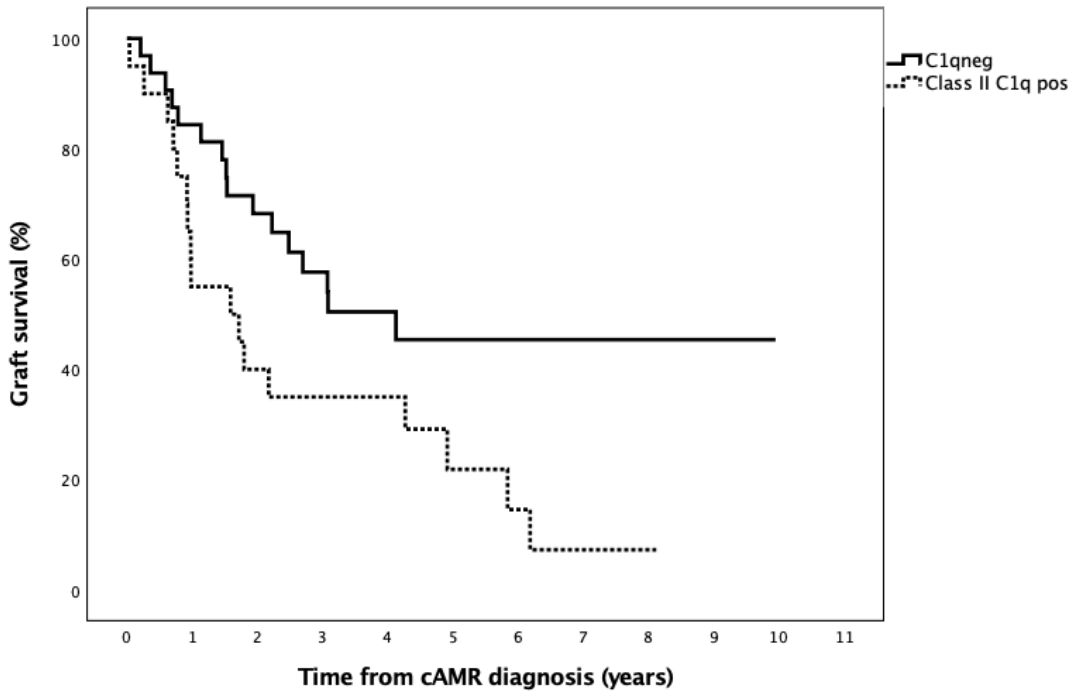


Figure 4.4: Allograft survival according to class II C1q positivity, p=0.024, log rank.

Patients with strongly complement fixing (CF) subclasses had inferior allograft survival than those without identifiable subclasses however, when present in combination with NCF subclasses allograft survival was poorest, p=0.014 (Figure 4.5).

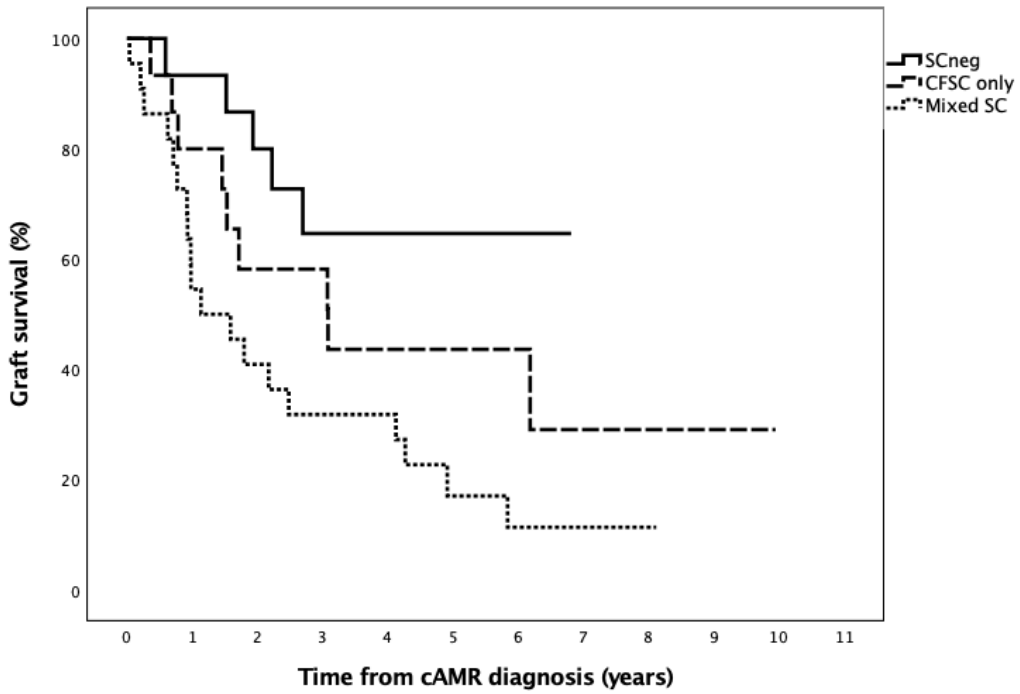


Figure 4.5: Comparison of allograft survival according to IgG subclass profile, p=0.014.

4.3.4 Clinical and Histological Characteristics of Class II C1q Binding DSA

Bearing in mind class II C1q binding was the most discerning of complement binding DSAs for allograft loss, the clinical and histological characteristics were more closely investigated. These are shown in table 4.3. Patients with class II complement fixing DSAs had lower GFRs at diagnosis (p=0.04) with more severe transplant glomerulopathy (p=0.017) and microcirculatory inflammation (p=0.006), predominantly peritubular capillaritis (p=0.017).

Table 4.3: Characteristics of class II DSAs according to C1q binding

	Class II C1q pos (n=20)	Class II C1q neg (n=32)	p value
Clinical characteristics			
eGFR at diagnosis (ml/min)	33 (23-40)	45 (34-54)	0.04
uPCR at diagnosis (mg/mmol)	86 (20-195)	24 (3-152)	0.356
Time to failure, months	21.5 (10.5-57.9)	37.5 (19.1-68.0)	0.144
Histological characteristics			
Glomerulopathy (cg)	2 (1-3)	1 (0-2)	0.017
Glomerulitis (g)	2 (1-2)	1 (0-2)	0.092
Peritubular capillaritis (ptc)	2 (1-2)	1 (0-2)	0.019
Microcirculatory inflammation (mi)	2 (2-4)	2 (1-3)	0.006
C4d	2 (1-2)	1 (0-2)	0.189
IFTA %, nearest 5%	15 (10-20)	15 (10-20)	0.775
Class II IgG subclass composition			
IgG1, n (%)	18 (90.0)	16 (50.0)	0.003
IgG2, n (%)	17 (85.5)	3 (9.4)	<0.001
IgG3, n (%)	7 (35.0)	0 (0.0)	0.001
IgG4, n (%)	10 (50.0)	2 (6.3)	0.002
>1 IgG subclass, n (%)	17 (85.0)	3 (9.4)	<0.001

4.3.5 Risk Factors for the Development of C1q Binding DSA

In a univariate model analysing the risk factors for the development of a C1q binding DSA, HLA-DQ mismatch, number of DSAs, CF subclasses and a mixture of CF and NCF subclasses were all significant. These, including HLA-DR mismatch were combined in a multivariate model in which only combinations of CF and NCF subclasses were significant OR=13.28 (95% CI 3.40-95.20), p=0.001. See table 4.4.

Table 4.4: Univariate and multivariate analyses predicating the development of C1q binding

	Development of C1q binding DSA n=21		
	Odds ratio	**95% CI**	**p value**
Univariate analysis			
Female	2.00	0.42-9.49	0.38
Recipient age	0.97	0.92-1.02	0.21
Donor age	1.01	0.98-1.07	0.35
HLA-A/B mismatch	0.91	0.53-1.57	0.73
HLA-DR	1.01	0.91-1.84	0.09
HLA-DQ	1.9	1.42-2.34	0.02
No. DSAs	1.54	1.02-2.33	0.04
Induction	1.22	0.34-4.45	0.76
CF Subclasses	5.21	3.61-8.92	0.001
Mixture of CF and NCF subclasses	18.0	3.40-95.20	0.001
Multivariate analysis			
Mixture of CF and NCF subclasses	13.28	2.39-73.73	0.003

4.3.6 Risks Factors of Graft Loss

In a multivariable cox regression model including; microcirculatory inflammation, eGFR at diagnosis, C1q binding, age of the donor, DQ mismatch and the presence of CF and NCF subclasses, C1q binding was not an independent risk factor for allograft loss. A higher eGFR at diagnosis was a protective factor. See table 4.5.

Table 4.5: Multivariable cox regression analysis of graft loss

	Hazard ratio	**95% CI**	**p value**
Microcirculatory inflammation	1.16	0.82-1.65	0.39
eGFR at diagnosis	0.92	0.89-0.97	0.001
C1q positive	1.33	0.40-4.45	0.64
Age donor	0.99	0.97-1.02	0.89
HLA-DQ MM	0.46	0.15-1.38	0.16
CF+NCF subclasses	0.83	0.25-2.80	0.77

4.4 Discussion

DSAs that fix complement are well understood to strongly associate with poor outcomes, more severe phenotypes of AMR and progression to TG. The aims of this study were to evaluate the long-term outcomes of patients with established cAMR with C1q positive DSAs. The main finding of this study suggests that C1q binding was only associated with reduced allograft survival if the DSA was directed against class II HLA. However, on multivariate analysis it is not an independent risk factor for graft loss when considered against other variables in cAMR.

The incidence of C1q binding DSA was comparable to other studies[65,201], with the majority being of class II specificity. In particular, DQ DSAs associate with C1q binding and once again, highlight the importance of the –DQ locus. Whilst the pathogenicity of DQ positive DSA has been independently reported by multiple centres[202,203], there has only recently been a change to the UK national matching algorithm. Freitas et al found increased numbers of IgG1/IgG3 combinations (51%, p=0.01) and C1q positivity (63%, p=0.001) in 34 recipients with de novo DQ only DSA, that developed AMR compared to those that did not, as well as increased risk of allograft loss and a 30% reduction in 5-year allograft survival[177]. In a cross-sectional study of 55 sensitised patients awaiting kidney transplant, Arreloa-Guerra et al demonstrated DQ positive HLA antibodies were an independent risk for C1q binding (OR 9.82, 95% CI 5.4-17.6, p<0.001)[204]. Why the majority of C1q binding DSA are of class II specificity is unclear. This may in part be associated with the fact class II DSAs tend to have higher MFIs than class I. Since the clinical introduction of the C1q solid phase assay in 2011, sceptics have claimed that C1q binding only represents a function of MFI. Reports exist showing a correlation between high MFI and in vitro C1q positivity[205,206]. Similar to these, the median MFI of the C1qpos DSAs in this study were significantly higher than the C1qneg. Sufficient density is required for IgG to

form hexamers that cluster and bind complement through non-covalent Fc binding[207]. If MFI is a semi quantitative measure of antibody titre this may explain why more class II DSA can fix complement. This relatively simple paradigm does not elucidate why some DSA with low pan IgG MFI still fix complement, though consideration of the presence of IgM DSA or interference from NCF subclasses may provide some explanation[208]. Conversely, not all DSAs with high MFIs are positive for C1q. Clearly, in vivo antibody-complement interactions are not quite so simple, meaning that results should be interpreted with caution and a negative C1q assay does not exclude complement activating potential[209].

To complicate matters, only 5/21 patients in the SCpos/C1qpos group had subclass profiles consisting of only complement binding subclasses (IgG1/IgG3). The remainder consisted of a mixture of CF and NCF. Survival was poorest in the mixed group when compared to the SCneg and CF fixing cohorts. Combinations of CF and NCF were also the single independent risk factor for C1q positive DSA development on multivariate analysis. As described in my previous chapter, sustained T-cell stimulation results in prolonged triggering of B cells to produce NCF subclasses, which enhances the diversity of the immune response[210]. Evidence suggests that when competing for the same HLA epitope NCF subclasses are able to inhibit the effects of CF subclasses. Honger et al investigated complement activation with mixtures of IgG subclasses with identical V-regions against HLA DRB1*_07:01_. IgG2 and IgG4 were found to inhibit the C1q binding effects of IgG1 and IgG3 in a dose dependent fashion[185]. Importantly, the same group also demonstrated that if targeting a different epitope on the same HLA molecule, NCF subclasses, in particular IgG2, could synergistically amplify complement activity. HLA-DQ provides greater complexity given both the alpha and beta chains are polymorphic[211], thus the number and pathogenicity of HLA-DQ epitopes are broad[165,212]. Considering the majority of de novo DSA is against DQ, this may, in part, explain why the

majority of CF DSAs in this cohort were class II and why mixtures of subclasses seemed to be most significant in determining C1q positivity. Why some patients will develop NCF subtypes is not understood, though Arnold and colleagues postulated that high panel reactive antibody (PRA) values may represent large numbers of activated B-cells which have undergone somatic hypermutation and produce different antibody isotypes[183]. In this cohort, there were 2 C1qpos cases of NCF only subclasses present. It was therefore not possible to investigate the effects of NCF subclasses alone, considering it is unusual for these to exist in the absence of CF IgG, particularly IgG1[209].

This is the one of the first reports showing the correlation of DSA phenotyping with allograft survival and histological findings in a single-centre cohort of patients with cAMR and detectable IgG DSAs, at the time of diagnosis. Biopsies of patients in the SCpos/C1qpos had significantly greater degrees of chronic injury and microcirculatory inflammation, though not C4d deposition. Importantly, complement deposition is no longer a required histological finding for the diagnosis of acute or chronic antibody mediated rejection[197]. C4d deposition is not always antibody dependent. During periods of infection, non allo-antibody associated pathways may also be responsible, including the lectin pathway, which can still result in formation of MAC and C4d as a by-product[213]. Importantly, many of these patients have experienced prior rejection episodes before the diagnosis of cAMR, leading to augmentation of immunosuppression. Thus, the C1q binding ability of DSAs may have been significantly reduced, yet prior complement activation and its deposition remains. These factors may help explain why C4d was of no significance on any group analysis.

In this study C1q binding was not found to be independently associated with graft loss following the diagnosis of cAMR, however there was a difference in mean allograft survival of 3 years between the SCpos/C1qneg and SCpos/C1qpos groups. Although the ability of DSAs

to fix complement has been associated with reduced allograft survival[82], the function of complement in cAMR is still not well understood. C4d-negative AMR still has the potential to result in chronic rejection[214]. Thus, complement dependent cytotoxicity may have a greater role in acute AMR which is also indicative of the more severe clinical manifestations seen. Loupy et al demonstrated in 157 protocol biopsies from 80 DSA positive patients that the presence of microcirculatory inflammation (HR=4.0, 95% CI 1.0-16, p=0.05) and class II DSA (HR=6, 95% CI 1.8-20, p=0.01) were risk factors for progression to cAMR, independent of C4d deposition[119]. This illustrates that the immunological processes occurring in cAMR possibly lean towards complement independence. Interestingly, MCI was not a significant risk factor for allograft loss in this cohort. This could suggest that, whilst ongoing inflammation is important in the development of cAMR, the degree of glomerulopathy, interstitial fibrosis and tubular atrophy might play a predominant role leading to allograft loss. It has been suggested that early complement activation is responsible for the infiltration of immune cells into allografts[7,215], however the development and maintenance of MCI in cAMR may occur discrete from classical pathway complement activation[52]. Hidalgo and colleagues determined that macrophages, NK cells and NK cell transcripts were elevated in peritubular capillaries in biopsies of patients with TG and late antibody mediated injury[70]. Complement independent mechanisms of endothelial cell injury are clearly of significance in most rejection phenotypes. Following diagnosis of AMR, despite differences in the degree of MI and C4d in patients, with and without DSA irrespective of C1q fixing ability, a common pathway exists typically resulting in increased interstitial fibrosis, tubular atrophy and TG, prior to graft loss[64].

There are limitations to this study which must be addressed. It is retrospective in its design resulting in a degree of confounding. The cross-sectional observations did not allow assessment of whether complement fixing ability changed with time, nor if changes in

subclass profiles had an effect on outcome. Sera were not serially diluted in an attempt to eliminate prozone effects which may have affected the interpretation of the assay in some cases, particularly in those in which C1q binding may have been borderline. Sera were also not exposed to DTT to abrogate any IgM binding which may help to explain why two cases of C1q binding had no identifiable subclass.

Evidently defining the presence of C1q binding antibodies is a useful marker for increased risk of allograft rejection and early graft failure however, in the longer term the issue is more complex. In conclusion, this long-term follow up study has demonstrated that the presence of C1q fixing DSA does have a moderate effect on allograft survival though cannot prove that complement activation alone is an independent cause for allograft loss. With regards to successful future treatments of cAMR assuming the pendulum swings in only one direction is unwise.

Chapter 5: The role of IgM anti-HLA donor specific antibodies in renal transplantation

5.1 Introduction

IgG anti-HLA DSAs are associated with allograft rejection and graft loss[5-8], however, the role of IgM anti-HLA DSAs is still not clear. Whilst antigen-specific IgM antibodies against viral and bacterial pathogens are established in the primary immune response, their significance in antibody mediated disease remains debatable[216].

Not all positive crossmatches exclude the possibility of transplantation. IgM auto-antibodies can produce false positives which can be easily discriminated against using DTT. DTT will reduces disulphide bonds within the IgM pentamer whilst not interfering with the interchain disulphide bonds of IgG[217,218]. In the non-sensitised individual the majority of IgM antibodies are auto-antibodies, although it is estimated that up to 99% of IgM in sensitised patients is in fact allo-antibody[219]. The use of DTT will not identify between auto and allo-IgM, therefore it may be important to distinguish the latter.

Multiple conflicting reports exist investigating the possible role of IgM anti-HLA antibodies not only with respect to renal allografts but also in liver and cardiac transplantation[71,73,220-222]. Some studies have even shown IgM anti-HLA as potentially beneficial. Kerman et al described in 78 renal transplant recipients with a positive IgM FCXM, significantly reduced rates of rejection (13% vs 26%, $p<0.02$), in the first year as well as improved 1 year allograft survival (100% vs 81%, $p<0.02$), compared to IgM FCXM negative patients[221]. Despite these contradictions, there remains a paucity of evidence assessing outcomes of transplant recipients with proven IgM anti-HLA that is donor specific.

IgM appears first in the humoral response and is potent at agglutinating and binding antigen as well as having a strong affinity to fix complement. Whether IgM alloantibodies undertake a direct harmful role against grafts or simply herald the potential of future class switching to IgG is unclear.

The Banff criteria require evidence of circulating DSA in order to classify rejection as antibody mediated. C4d staining or evidence of increased ENDAT expression can in some cases replace the DSA criterion[141]. In clinical practice, many cases with histological features of rejection do not completely fulfil Banff criteria. This is often due to the absence of a DSA and thus many cases of AMR are defined as DSA negative for practical management purposes. Most tissue typing laboratories do not routinely screen for IgM DSA and therefore, isolated IgM DSA may help to define presumed DSA negative AMR episodes[72]. Whether IgM also plays a role in ameliorating downstream IgG DSA related tissue injury or in synergy with IgG leading to more severe phenotypes of rejection is unknown.

In this study, the significance of de novo IgM HLA DSAs in isolation as well as in the presence of IgG anti-HLA DSAs is investigated in patients across the main phenotypes of allograft rejection including TCMR, acute AMR and cAMR.

5.2 Methods

This study retrospectively analysed 1667 CDC/FXCM negative renal transplant recipients transplanted between June 2005 and August 2016. Indicative biopsies were studied identifying patients into four cohorts; 50 with TCMR, 50 with acute AMR (aAMR) 57 with cAMR and 50 controls. The diagnosis of rejection was based on Banff 2015 criteria[164]. Patients were screened at the time of diagnostic biopsy for IgG anti-HLA DSA using LABScreen single antigen beads (SAB). Serum from the time of biopsy was also screened for IgM anti-HLA DSA using LABScreen SABs. The IgG assay was adjusted by replacing the anti-human pan-IgG PE conjugating antibody with an anti-human IgM PE conjugate.

Controls included an unsensitised cohort of 50 individuals with no evidence of rejection on surveillance biopsies and negative for IgG DSA on single antigen testing, both historically and at the time of biopsy. Each cohort was further split depending on IgG and IgM DSA positivity and categorised as follows; IgG-/IgM-, IgG+/IgM-, IgG-/IgM+ and IgG+/IgM+. The categories were chosen in an attempt to distinguish the effect of IgM and IgG in isolation as well as together on allograft outcomes.

5.3 Results

5.3.1 Patient Characteristics

The demographics of all the groups are demonstrated in tables 5.1 and 5.2. The aAMR and cAMR groups are presented in separate tables due to the risk of patient overlap when comparing the groups against TCMR and controls. Amongst the acute AMR, TCMR and control groups there were no significant differences with regards to donor type, type of induction, mismatch at class I or II, total mismatch, graft number and whether or not the transplant was pre-emptive. Compared to the aAMR and TCMR groups, controls had significantly fewer females (6 vs 24 and 18, $p<0.001$), a greater number of Caucasians (35 vs 19 and 26, $p=0.027$) and fewer cases of graft loss (1 vs 21 and 19, $p<0.001$). The TCMR group were slightly younger compared to the aAMR and control groups (46.3±12.3 years vs 51.5±12.2 years and 53±8.7 years, $p=0.009$). The median follow-up for the group was 5.95 (2.95-8.28) years.

Between the cAMR, TCMR and control groups there was no difference regarding donor type, mismatch at class I or II and total mismatch. The control group had fewer females (6 vs 23 and 18, $p=0.003$) and a slightly increased age (53±8.7 vs 45.3±11.8 and 46.3±12.3, $p= 0.001$). The TCMR group differed slightly in ethnicity defined as other (non-Caucasian, non-Afro-Caribbean, non-Asian) (6 vs 1 and 0, $p=0.035$). The cAMR group included fewer pre-emptive transplants (11 vs 6 and 21, $p=0.038$), more patients with a previous graft (11 vs 4 and 0, $p=0.016$) and a higher percentage of graft loss (68.4% vs 38% and 2%, $p<0.001$). There was also significantly greater use of IL2RA at induction in the cAMR cohort (31.6% vs 14% and 2%, $p<0.001$). The median follow-up was 6.6 (3.5-9.0) years.

The median time to diagnosis was 2.7 (1.6-4.7) years for the cAMR group, 0.63 (0.18-1.48) years for the TCMR group and 0.36 (0.07-1.41) years for the aAMR group.

Table 5.1: Demographics of AMR, TCMR and Control groups

	aAMR (n= 50)	**TCMR (n= 50)**	**Controls (n= 50)**	**p-value**
Female, n (%)	24 (48.0)	18 (36.0)	**6 (12.0)**	**<0.001**
Age at Tx, years	51.5 ± 12.2	**46.3 ± 12.3**	53 ± 8.7	**0.009**
Ethnicity, n(%):				
-Caucasian	19 (38.0)	26 (52.0)	**35 (70.0)**	
-Asian	17 (34.0)	14 (28.0)	12 (24.0)	**0.027**
-Afro-Caribbean	6 (12.0)	4 (8.0)	3 (6.0)	
-Other	8 (16.0)	6 (12.0)	**0 (0.0)**	
LD, n (%)	21 (42.0)	23 (46.0)	24 (48.0)	0.828
SPK, n (%)	1 (2.0)	2 (4.0)	0 (0.0)	0.360
Pre-emptive, n (%)	10 (20.0)	16 (32.0)	21 (42.0)	0.060
HLA-A/B MM	2.4 ± 1.1	2.6 ± 1.1	2.6 ± 1.0	0.632
HLA-DR MM	1.0 ± 0.8	1.0 ± 0.8	1.1 ± 0.8	0.882
Total MM	3.4 ± 1.4	3.7 ± 1.6	3.7 ± 1.4	0.640
Induction, n (%)				
Anti-CD52	46 (92.0)	43 (86.0)	49 (98.0)	0.087
Anti-IL-2R	4 (8.0)	7 (14.0)	1 (2.0)	
Graft number:				
>1	6 (12.0)	4 (8.0)	0 (0.0)	0.132
Graft loss, n (%)	21 (42.0)	19 (38.0)	**1 (2.0)**	**<0.001**
Graft survival, days	1,132 ± 897	1,581 ± 995	-	0.142

Table 5.2: Demographics of cAMR, TCMR and control groups

	cAMR (n= 57)	**TCMR (n= 50)**	**Controls (n= 50)**	**p-value**
Female, n (%)	23 (40.4)	18 (36.0)	**6 (12.0)**	**0.003**
Age at Tx, years	45.3 ± 11.8	46.3 ± 12.3	**53.0 ± 8.7**	**0.001**
Ethnicity:				
-Caucasian	30 (52.6)	26 (52.0)	35 (70.0)	
-Asian	22 (38.6)	14 (28.0)	12 (24.0)	**0.035**
-Afro-Caribbean	4 (7.0)	4 (8.0)	3 (6.0)	
-Other	1 (1.8)	**6 (12.0**)	0 (0.0)	
LD, n (%)	35 (61.4)	23 (46.0)	24 (48.0)	0.217
SPK, n (%)	0 (0.0)	2 (4.0)	0 (0.0)	0.114
Pre-emptive, n (%)	**11 (19.3)**	16 (32.0)	21 (42.0)	**0.038**
HLA-A/B MM	2.3 ± 1.1	2.6 ± 1.1	2.6 ± 1.0	0.217
HLA-DR MM	1.3 ± 0.7	1.0 ± 0.8	1.1 ± 0.8	0.117
Total MM	3.6 ± 1.6	3.7 ± 1.6	3.7 ± 1.4	0.983
Induction, n (%)				
Anti-CD52	**39 (68.4)**	43 (86.0)	49 (98.0)	**<0.001**
Anti-IL-2R	**18 (31.6)**	7 (14.0)	1 (2.0)	
Graft number:				
>1	**11 (19.3)**	4 (8.0)	0 (0.0)	**0.016**
Graft loss, n (%)	**39 (68.4)**	19 (38.0)	1 (2.0)	**<0.001**
Graft survival, days	1,712 ± 937	1,581 ± 995	-	0.635

5.3.2 IgM DSA Categories

The number of patients with a detectable IgM was 18/57 (31.1%) in the cAMR group, 10/50 (20%) in the acute AMR group, 5/50 (10%) in the TCMR group and 6/50 (12%) in the control group. Table 5.3 shows the distribution of both IgG and IgM DSA across all four groups. Of note, the presence of an IgG DSA was significantly greater in the acute AMR, cAMR and TCR groups compared to controls, ($p<0.01$, $p=0.05$ and $p<0.01$ respectively). There was greater IgM DSA in the acute AMR group compared to TCMR and controls ($p<0.05$ and $p<0.01$). There were also more patients with IgM DSA in the cAMR group compared to TCMR and controls ($p<0.05$ and <0.01).

Table 5.3: DSA numbers according to IgG/IgM category

	Acute AMR n (%)	cAMR n (%)	TCMR n (%)	Controls n (%)
IgG- / IgM-	22/50 (44.0)	18/57 (31.6) *‡	36/50 (72.0)	42/50 (84.0)
IgG+ / IgM-	18/50 (36.0) *‡	21/57 (36.8) *‡	9/50 (18.0) *	2/50 (4.0)
IgG- / IgM+	3/50 (6.0)	6/57 (10.5)	3/50 (6.0)	6/50 (12.0)
IgG+ / IgM+	7/50 (14.0) *‡	12/57 (21.1) *‡	2/50 (4.0)	0/50 (0.0)

*p<0.01 vs controls
†p=0.05 vs controls
‡p<0.05 vs TCMR

5.3.3 IgM DSA Specificities

Of the 207 patients investigated 39 (18.8%) had a detectable IgM DSA at the time of allograft biopsy. The specificities are shown in table 5.4. Of all patients with detectable IgM, distribution of HLA classes was equal; class II IgM DSA alone (n=17, 44%), class I alone, (n=17, 44% and both class I+II (n=5, 13%). The presence of both class I and II IgM DSA was only seen

in the cAMR group. In total 21 (53.8%) cases had an IgM DSA in the presence of an IgG DSA. Of these 15 had an IgM DSA that matched the specificity of their IgG DSA, implying a class switch. The vast majority of isotype switched IgG was from class II IgM 13/15 (86.7%). The median MFI of the IgM DSAs was significantly greater in the cAMR group, p=0.04.

Table 5.4: IgM anti-HLA specificities

Acute AMR (n=10)	IgM DSA Spec	IgM DSA MFI	IgG matched DSA?	IgG DSA Spec	IgG MFI
1	A2, Cw10	8580, 560	Yes	A2	4300
2	A11	1138	No	-	-
3	A11	5652	No	-	-
4	B50	515	No	-	-
5	Cw4	986	No	-	-
6	DR52	678	Yes	DR52	1400
7	DR52, DR17	2090, 1469	Yes	DR52, DR17	450, 2640
8	DQ5	5073	Yes	DQ5	1000
9	DQ6	1280	Yes	DQ6	6200
10	DQ6	4286	No	-	-
Median IQR		1375 (909-4483)			

TCMR (n=5)	IgM DSA Spec	IgM DSA MFI	IgG matched DSA?	IgG DSA Spec	IgG MFI
1	A2	541	No	-	-
2	DQ5, DQ6	666, 1648	No	-	-
3	DQ6	2105	No	-	-
4	DQ7	721	No	-	-
5	DQ7	2861	Yes	DQ7	5840
Median IQR		1184 (680-1991)			

Controls (n=6)	IgM DSA Spec	IgM DSA MFI	IgG matched DSA?	IgG DSA Spec	IgG MFI
1	A2	1241	-	-	-
2	A2	2784	-	-	-
3	A3	6700	-	-	-
4	B7	1098	-	-	-
5	B58	932	-	-	-
6	Cw1	2683	-	-	-
Median IQR		1962 (1134-2759)			

Acute AMR – 5/10 (50%) cases had an IgM DSA that matched the specificity of their IgG DSA
TCMR – 1/5 (20%) cases had an IgM DSA that matched the specificity of their IgG DSA

cAMR (n=18)	IgM DSA Spec	IgM DSA MFI	IgG matched DSA?	IgG DSA Spec	IgG MFI
1	A1	1280	Yes	A1	4000
2	A24	1990	No	-	-
3	A68	530	No	-	-
4	Cw2	3240	No	-	-
5	Cw4	940	No	-	-
6	A26, DQ5	566, 1232	No	-	-
7	B7, DQ5, DQ6	2360, 4585, 6480	Yes	DQ6	1100
8	B51, DQ7	1450, 1800	No	-	-
9	B58, DQ6	1080, 1660	Yes	B58, DQ6	6200, 8000
10	Cw4, DR52	1278, 1647	No	-	-
11	DR53	5080	Yes	DR53	10950
12	DQ2	1567	Yes	DQ2	1200
13	DQ5	710	Yes	DQ5	500
14	DQ6	1585	Yes	DQ6	600
15	DQ7	800	No	-	-
16	DQ7	1426	Yes	DQ7	1125
17	DQ7	7030	No	-	-
18	DR51, DQ6	4125, 4580	Yes	DR51	8765
Median IQR		2361 (1232-3240)			

cAMR - 9/18 (50%) cases had an IgM DSA that matched the specificity of their IgG DSA

5.3.4 Risk of IgM DSA Development

A logistic regression analysis was performed to assess potential factors associated with development of an IgM DSA. Variables included were; race, gender, pre-emptive transplant, HLA mismatch, allograft rejection, transplant type and induction agent. Univariate analysis indicated that female gender and the development of cAMR were associated with the presence of an IgM DSA, whilst Asian ethnicity appeared protective (Table 5.5). On multivariate analysis, the presence of cAMR increased the risk of IgM DSA development by almost three-fold (HR=2.83, 95% CI 2.83-6.05, p=0.007). Interestingly Asian ethnicity seemed to be a protective factor (HR=0.36 95% CI 0.15-0.90, p=0.028).

The next stage involved attempting to predict the likelihood of IgM class switch to IgG. Similar variables were used again in the univariate analysis with the addition of the class of the IgM DSA. This time, mismatch at class II loci (DR and DQ), development of cAMR and class II IgM DSA were factors associated with class switch. On multivariate analysis only the presence of a class II IgM DSA was close to significance. (HR=4.46 95% CI 0.91-21.86, p=0.065).

Table 5.5: Univariate and multivariate analyses of predictors of IgM DSA development

	IgM DSA development (n=39)		Class switch to IgG (n=15)	
Univariate Analysis	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Patient characteristics				
-Female	1.76 (0.87-3.56)	0.08	0.75 (0.22-2.57)	0.45
-Caucasian	1.61 (0.79-3.26)	0.13	1.70 (0.49-5.93)	0.30
-Asian	0.40 (0.17-0.96)	0.02	1.08 (0.26-4.56)	0.60
-Afro-Caribbean	0.89 (0.24-3.24)	0.58	0.50 (0.05-5.24)	0.50
-Pre-emptive	0.81 (0.45-1.46)	0.30	0.43 (0.09-1.89)	0.22
Transplant characteristics				
-HLA-A mismatch >0	1.13 (0.52-2.44)	0.47	2.78 (0.28-27.26)	0.35
-HLA-B mismatch >0	1.27 (0.56-2.88)	0.38	2.32 (0.67-23.34)	0.42
-HLA-DR mismatch >0	1.31 (0.58-2.96)	0.33	4.24 (0.79-22.67)	0.076
-HLA-DQ mismatch >0	1.07 (0.49-2.38)	0.52	5.24 (0.91-22.72)	0.06
-Deceased donor	1.52 (0.86-2.68)	0.103	1.11 (0.33-3.76)	0.56
-Alemtuzumab induction	1.25 (0.61-2.57)	0.35	0.30 (0.061-1.47)	0.14
Post-transplant events				
-Acute AMR	1.25 (0.57-2.72)	0.36	1.24 (0.54-2.87)	0.43
-Chronic AMR	2.69 (1.31-5.51)	0.006	5.24 (1.41-19.52)	0.013
-TCMR	2.23 (0.92-5.38)	0.40	2.05 (0.34-12.32)	0.35
-IgM DSA class II	-	-	4.78 (1.23-18.53)	0.02
Multivariate Analysis				
Female	1.47 (0.70-3.10)	0.31	-	-
Deceased donor	0.78 (0.37-1.63)	0.50	-	-
Asian	0.36 (0.15-0.90)	0.028	-	-
Chronic AMR	2.83 (1.33-6.05)	0.007	2.74 (0.65-11.61)	0.17
IgM DSA class II	-	-	4.46 (0.91-21.86)	0.065
HLA-DQ mismatch >0	-	-	3.92 (0.53-28.87)	0.18
HLA-DR mismatch >0	-	-	0.98 (0.12-8.03	0.99

5.3.5 IgM DSA Allograft Outcomes

Overall graft survival was significantly worse in the cAMR group compared to the TCMR group until approximately the 10-year point (88.2 months, 95% CI 74.6-101.8 vs 97.0 months 95% CI 84.8-109.0, p=0.048), figure 5.1. Interestingly both acute AMR and TCMR groups had no significant difference in graft survival (87.7 months, 95% CI 71.4-104 vs 96.9 months 95% CI 84.8-109.0, p=0.206), figure 5.2. All groups had significantly worse survival as compared to controls.

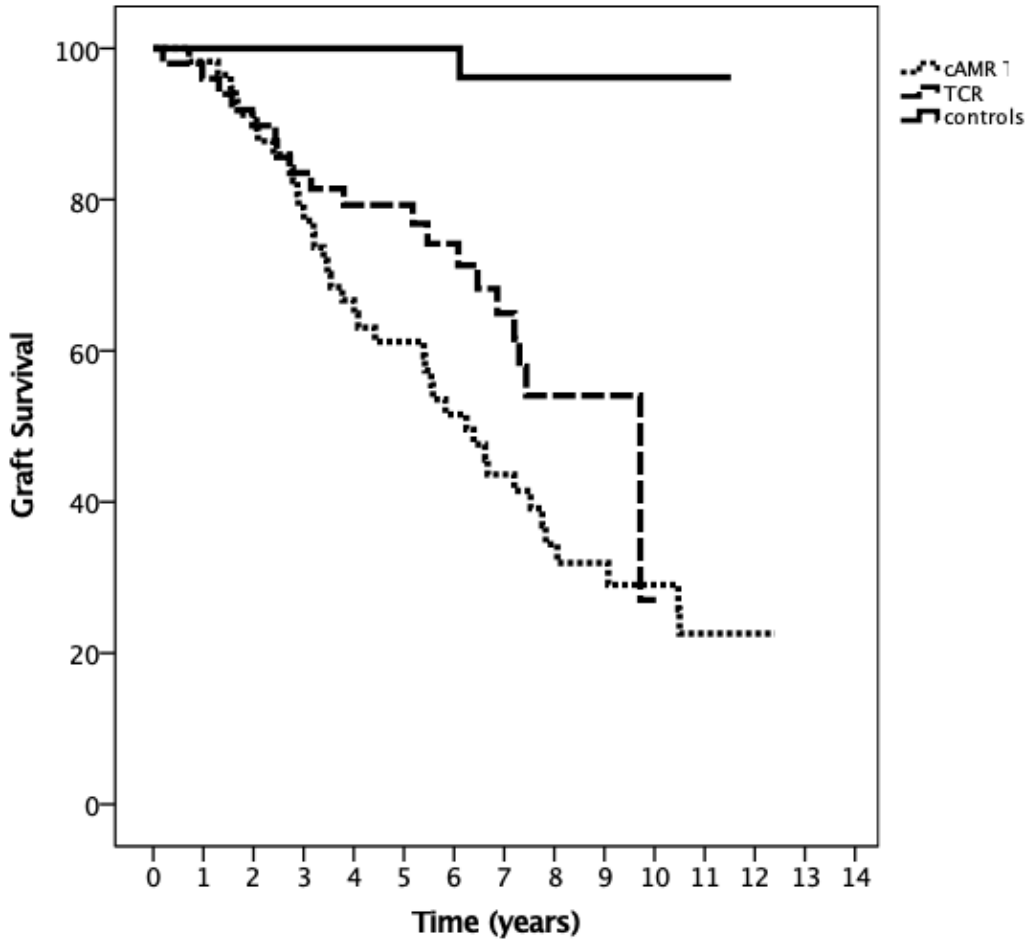


Figure 5.1: Graft survival according to cAMR, TCR and control cohort.

cAMR vs TCMR, log rank, p=0.048

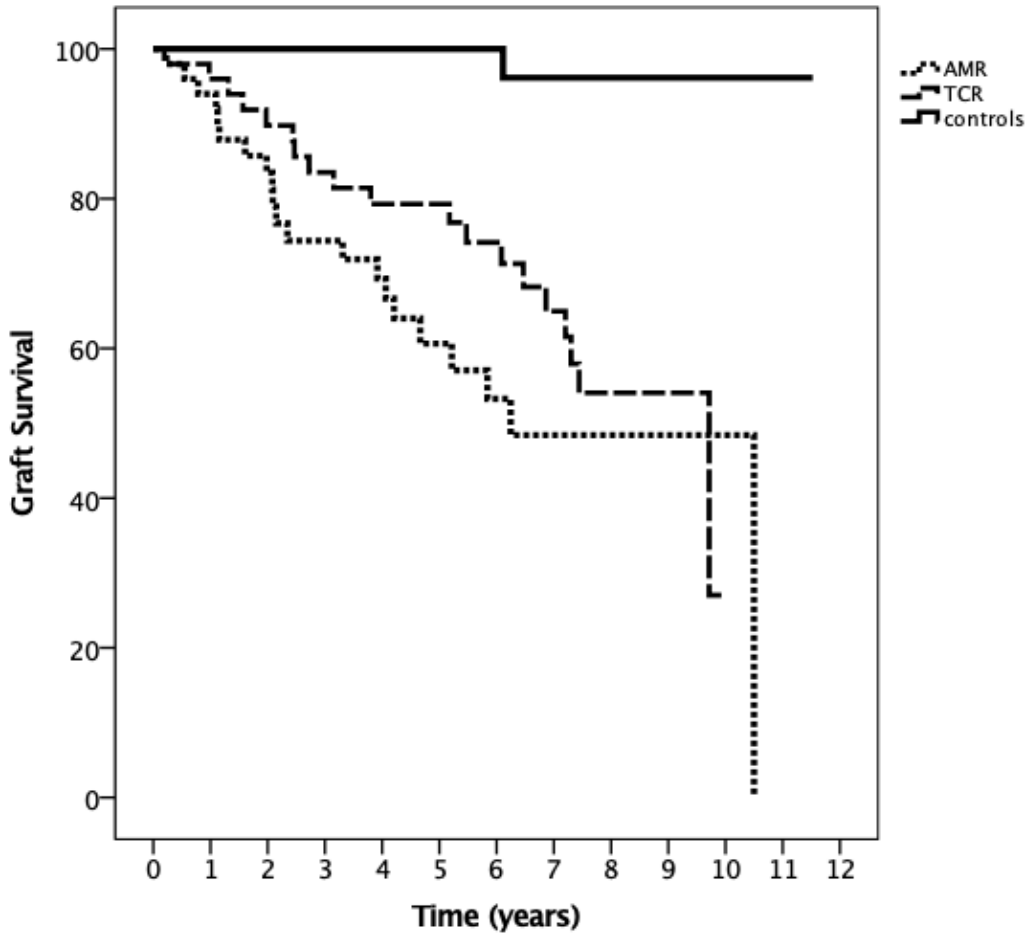


Figure 5.2: Graft survival according to AMR, TCR and control cohorts.

AMR vs TCMR log rank, p=0.206

5.3.6 Acute AMR, TCMR and Controls

Allograft survival was compared according to the category of IgG/IgM DSA. Acute AMR, TCMR and control groups were first compared. Overall graft survival was not significantly reduced in the presence of an IgM DSA alone (IgG-/IgM+) or paired with IgG (IgG+/IgM+). IgG-/IgM+ mean allograft survival 108.0 months (95% CI 89.6 – 127.8) vs IgG+/IgM+ 98.6 months (95% CI 66.6 – 130.7), p=0.268, Figure 5.3. In the AMR group alone, graft survival seemed not to be affected in the presence of an IgM DSA (IgG-/IgM+) alone or together with an IgG DSA (IgG+/IgM+), 50.8 months (95% CI 43.8-57.9) vs 104.0 months (95% CI 57.6-150.3), p=0.668,

figure 5.4. The presence of an IgM initially appeared to affect survival in the TCMR group, p=0.05 (figure 5.5). However, looking more specifically at the combination of IgM and IgG DSA, the greater risk of allograft failure was shown to be as a result of IgG DSA positivity only, IgG-/IgM- 102.0 months (95% CI 88.7-115.8) vs IgG+/IgM+ 62.2 months (95% CI 39.5-84.9), p=0.033, figure 5.6.

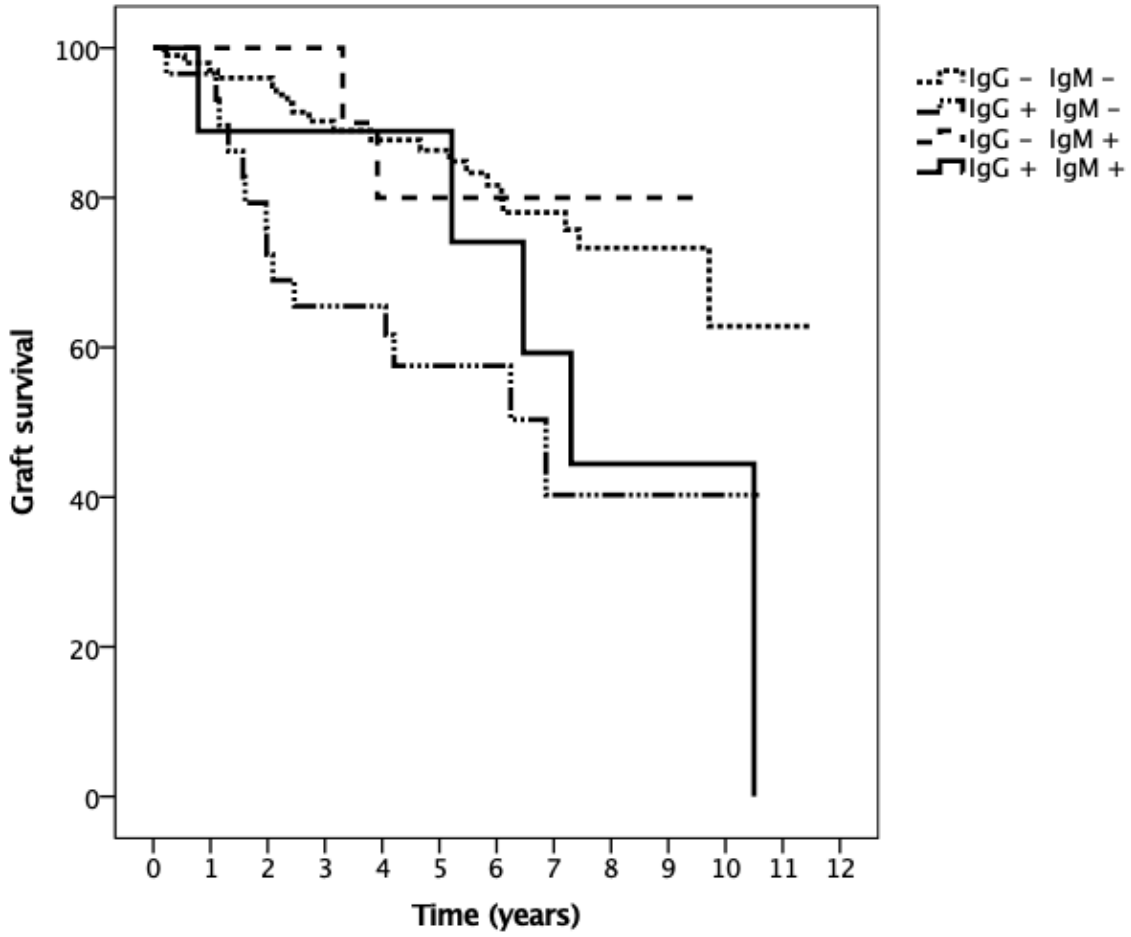


Figure 5.3: Overall graft survival (acute AMR/TCMR/Controls) according to IgG/IgM positivity

IgG-/IgM+ vs IgG+/IgM+ p=0.268, log rank

IgG+/IgM+ vs IgG+/IgM- p=0.688, log rank

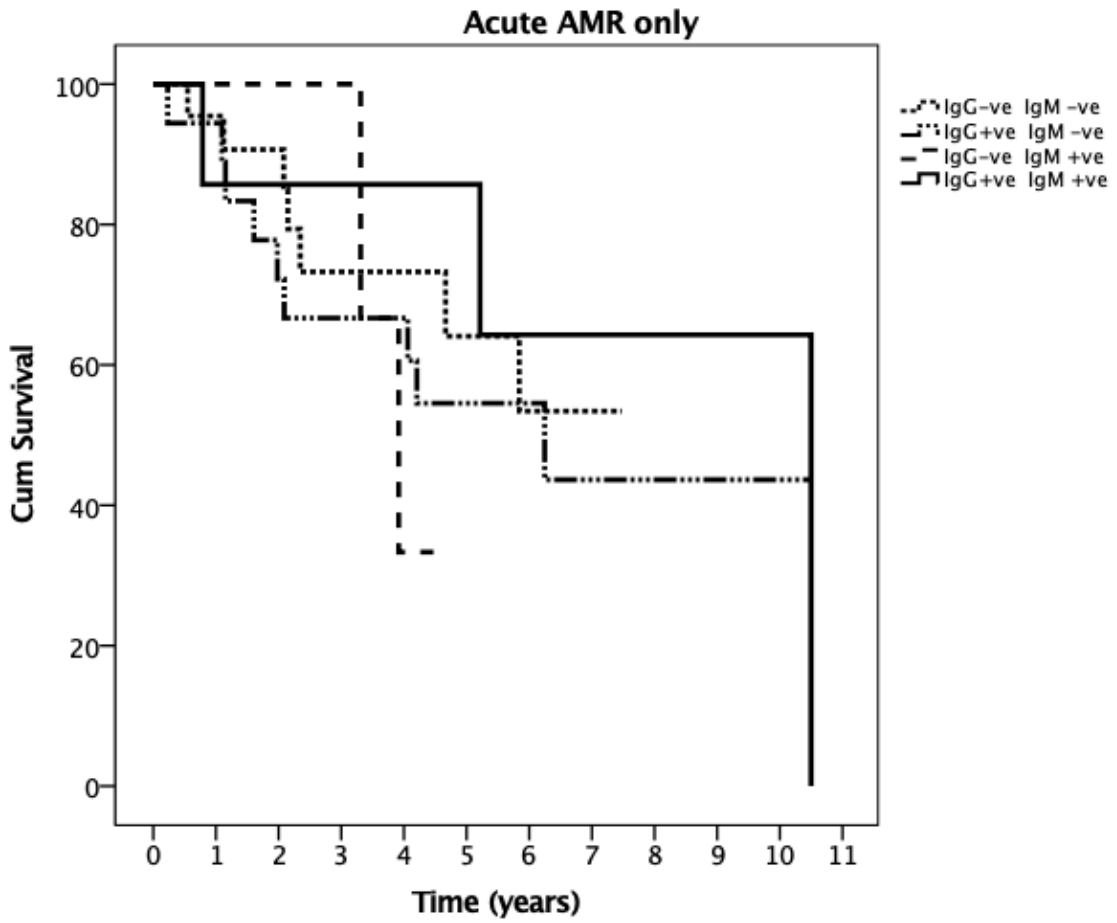


Figure 5.4: Graft survival in patients with acute AMR alone according to IgG/IgM positivity

IgG-/IgM+ vs IgG+/IgM+ p=0.668, log rank

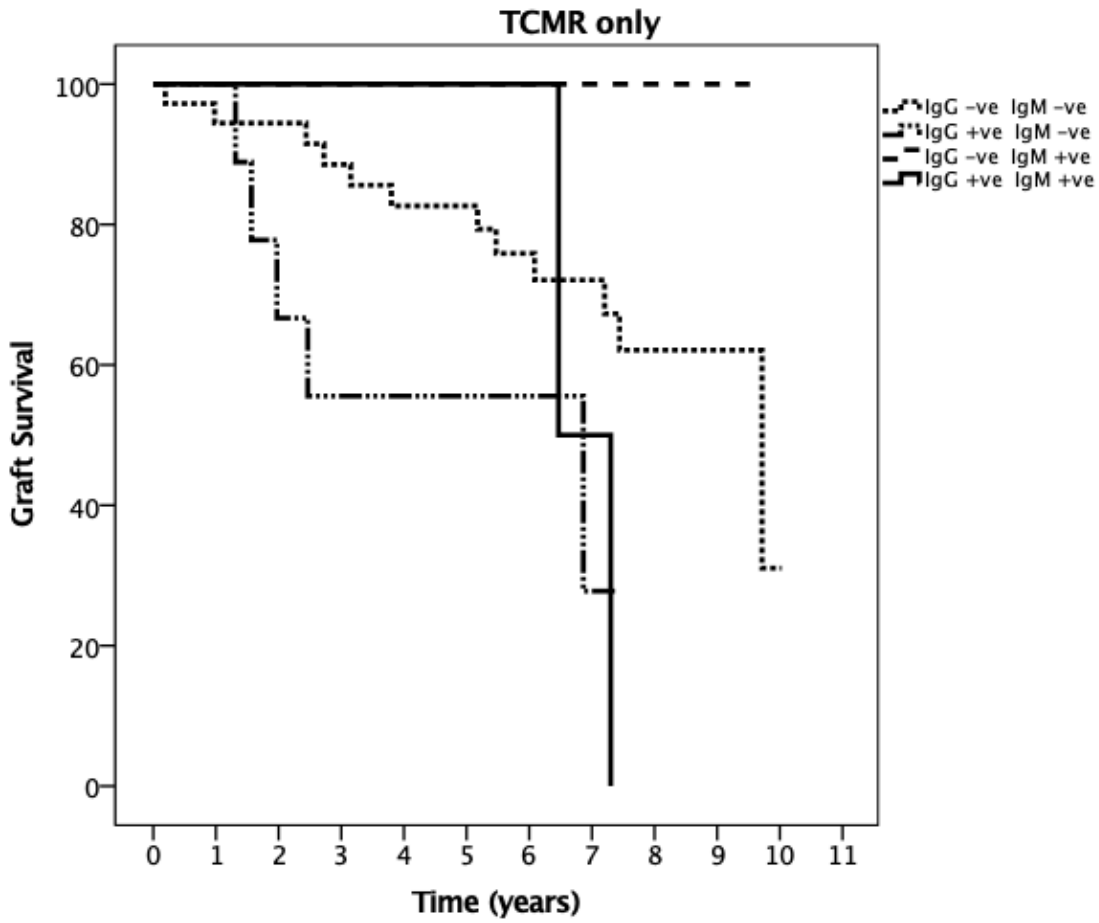


Figure 5.5: Graft survival in patients with TCMR alone according to IgG/IgM positivity, p=0.05

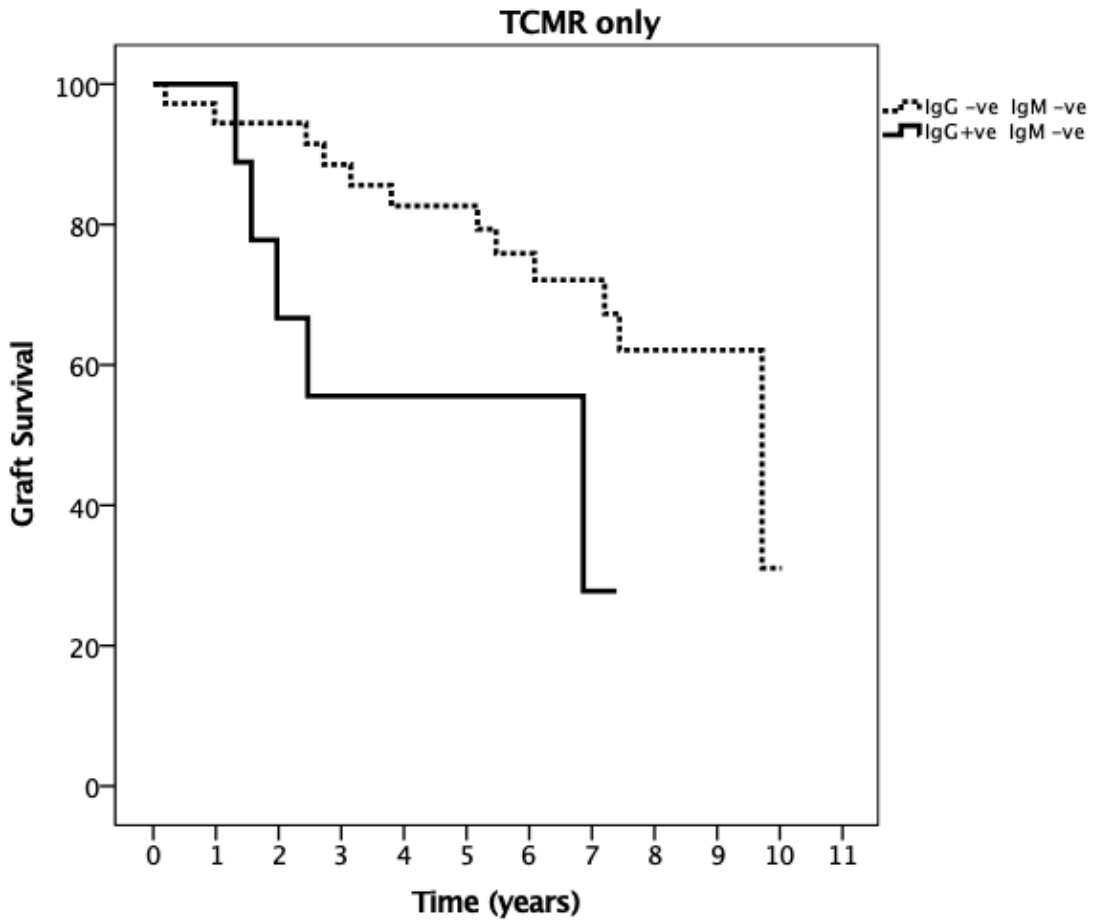


Figure 5.6: Allograft survival in patients with TCMR alone according to IgG DSA

IgG-/IgM- vs IgG+/IgM+ p=0.033, log rank

5.3.7 cAMR, TCMR and Controls

The cAMR group was compared in a separate analysis which did not include the acute AMR group, due to the potential patient overlap across the timeline of humoral rejection. Comparing groups overall, there was a significant reduction in allograft survival in the IgG+IgM- and IgG+/IgM+ categories compared to the IgG-/IgM- and IgG-/IgM+ categories, p<0.001, figure 5.7. When separating the groups and assessing cAMR in isolation across the four antibody categories, IgM positivity did not reach statistical significance in terms of reduction in graft survival, mean allograft survival IgG+/IgM- 77.8 months (95% CI 59.6-96.0)

vs IgG+/IgM+ 58.4 months (95% CI 42.14-74.6), p=0.231, figure 5.8. The categories with significantly poorer outcomes were only those in which there was an IgG DSA. In fact, when comparing patients with cAMR, those that are IgG DSA positive show a a difference in the mean graft survival of almost 3.5 years compared to being IgG DSA negative (mean allograft survival IgG+ 71.8 months, 95% CI 57.9-85.6 vs IgG- 111.2 months, 95% CI 88.8-133.6, p=0.006), figure 5.9. IgM DSA positivity did not add a graft survival disadvantage when present with an IgG DSA p=0.231.

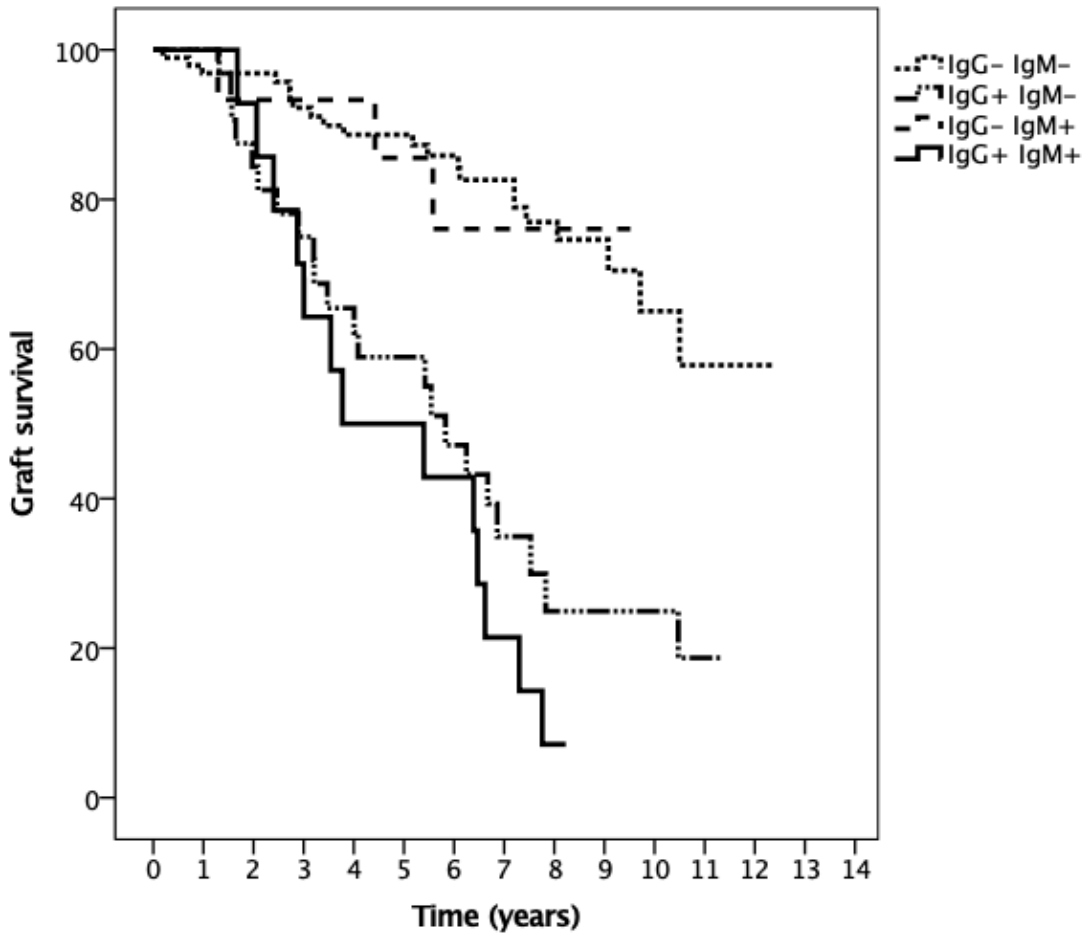


Figure 5.7: Overall graft survival (acute cAMR/TCMR/Controls) according to IgG/IgM positivity, p<0.001

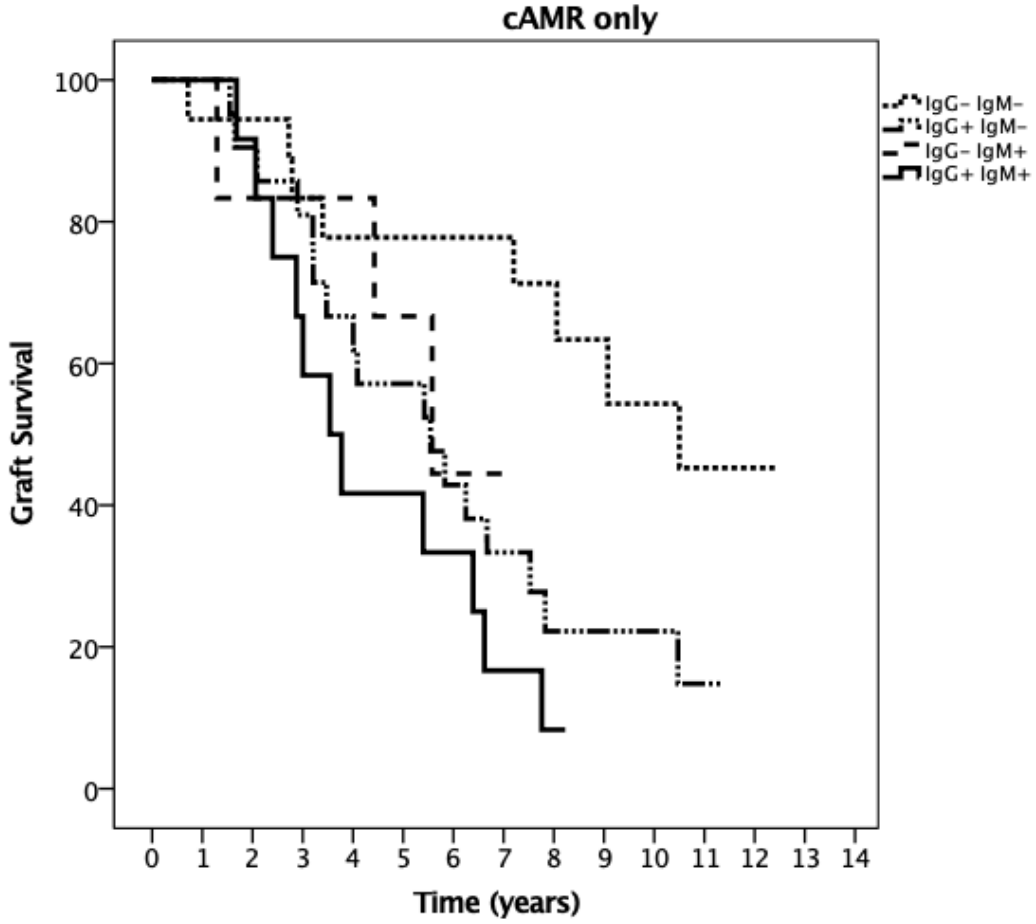


Figure 5.8: Graft survival in patients with cAMR alone according to IgG/IgM positivity.

IgG+/IgM- vs IgG+/IgM+ p=0.231, log rank

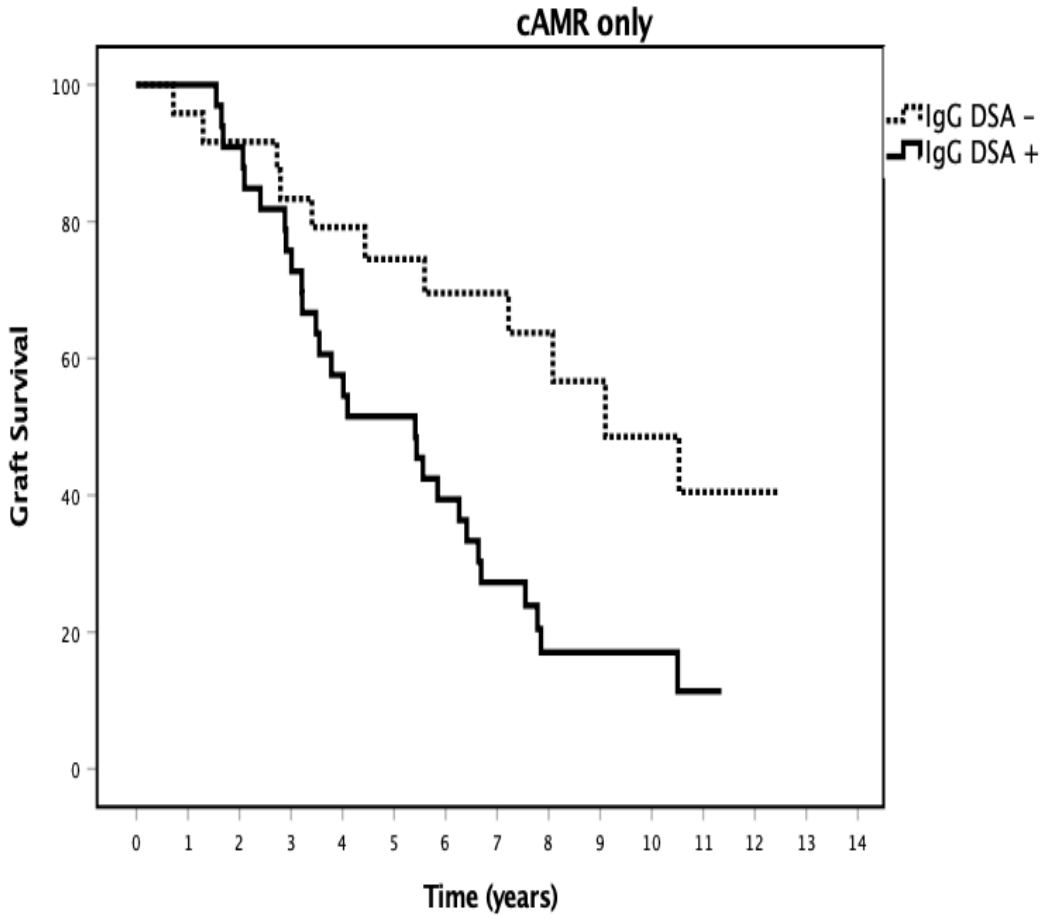


Figure 5.9: cAMR allograft survival according to IgG positivity, p=0.006, log rank

5.3.8 Histological Characteristics According to DSA Category

Given the original hypothesis of this study was to investigate the effect of IgM DSAs in isolation or in combination with IgG DSAs on kidney allografts, all patient diagnostic biopsies were assessed in an attempt to correlate DSA category with histological changes on the allograft. The median score for each lesion as defined by the Banff 2015 criteria was compared for each DSA category and separated according to rejection cohort. No significant differences were found in the acute AMR and TCMR groups. In the cAMR group there was a significant difference in activity of AMR. The IgG+/IgM+ group had significantly greater degrees of

microcirculatory inflammation, demonstrated by increased g, ptc and mi (g+ptc) scores compared to all other categories p=0.028, p=0.002, p<0.001 respectively, table 5.6.

Table 5.6: Histological characteristics (Banff 2015) according to rejection cohort

Lesions (Banff scores 2015)	IgG- / IgM- (n=18)	IgG+ / IgM- (n=21)	IgG- / IgM+ (n=6)	IgG+ / IgM+ (n=12)	p value
ct (median IQR)	1 (1-2)	1 (1-2)	1 (1-1)	1 (1-2)	0.49
ci (median IQR)	1 (1-2)	1 (1-2)	1 (1-1)	1 (1-2)	0.56
i (median IQR)	0 (0-1)	0 (0-2)	1 (1-1)	1 (1-1)	0.68
ti (median IQR)	1 (1-2)	1 (1-2)	1 (1-1)	1 (1-2)	0.73
t (median IQR)	0 (0-0)	0 (0-1)	1 (1-1)	0 (0-0)	0.08
cv (median IQR)	1 (1-2)	1 (1-2)	1 (1-1)	1 (1-2)	0.56
v (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)	0.5
ah (median IQR)	1 (1-2)	0 (0-1)	0 (0-2)	0 (0-1)	0.34
cg (median IQR)	1 (1-2)	2 (2-3)	1 (1-2)	3 (1-3)	0.25
g (median IQR)	1 (1-2)	2 (1-3)	2 (2-3)	3 (2-3)	0.028
ptc (median IQR)	0 (0-1)	1 (1-2)	1 (1-1)	2 (1-2)	0.002
mi (median IQR)	2 (1-3)	4 (2-4)	3 (1-4)	5 (3-5)	<0.001
C4d (median IQR)	1 (1-2)	1 (1-2)	0 (0-1)	2 (1-3)	0.09
IFTA% (median IQR)	15 (15-30)	20 (15-30)	15 (10-25)	2 (1-3)	0.76

cAMR

Lesions (Banff scores 2015)	IgG- / IgM- (n=36)	IgG+ / IgM- (n=9)	IgG- / IgM+ (n=3)	IgG+ / IgM+ (n=2)	p value
ct (median IQR)	1 (1-2)	1 (1-2)	1 (0-1)	1 (1-1)	0.72
ci (median IQR)	1 (1-2)	1 (1-2)	1 (1-1)	1 (1-1)	0.47
i (median IQR)	2 (1-2)	1 (1-3)	3 (2-3)	2 (1-2)	0.21
ti (median IQR)	2 (1-2)	1 (1-3)	3 (2-3)	2 (1-2)	0.39
t (median IQR)	2 (1-2)	2 (2-3)	2 (1-2)	2 (1-2)	0.67
cv (median IQR)	1 (1-2)	1 (1-2)	0 (0-0)	1 (0-1)	0.91
v (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0.29
ah (median IQR)	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-0)	0.66
cg (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0.94
g (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	1 (0-1)	0.51
ptc (median IQR)	0 (0-1)	1 (0-1)	0 (0-0)	1 (0-1)	0.81
mi (median IQR)	0 (0-1)	1 (0-2)	0 (0-0)	1 (0-1)	0.85
C4d (median IQR)	0 (0-1)	0 (0-2)	1 (1-1)	1 (1-1)	0.63
IFTA% (median IQR)	10 (5-30)	10 (0-10)	5 (0-5)	20 (15-20)	0.11

TCMR

Lesions (Banff scores 2015)	IgG- / IgM- (n=22)	IgG+ / IgM- (n=18)	IgG- / IgM+ (n=3)	IgG+ / IgM+ (n=7)	p value
ct (median IQR)	1 (0-1)	1 (0-1)	1 (0-1)	1 (0-2)	0.71
ci (median IQR)	1 (0-1)	1 (0-1)	1 (0-1)	1 (0-2)	0.63
i (median IQR)	1 (0-1)	1 (0-1)	1 (0-1)	0 (0-2)	0.88
ti (median IQR)	1 (1-1)	1 (0-1)	1 (0-1)	0 (0-2)	0.92
t (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)	0.62
cv (median IQR)	1 (1-2)	1 (1-1)	0 (0-0)	1 (0-1)	0.34
v (median IQR)	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-1)	0.23
ah (median IQR)	1 (0-1)	0 (0-2)	0 (0-0)	0 (0-0)	0.37
cg (median IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0.80
g (median IQR)	1 (1-2)	1 (0-2)	1 (0-1)	0 (0-1)	0.32
ptc (median IQR)	1 (1-2)	1 (0-2)	1 (0-1)	2 (1-2)	0.21
mi (median IQR)	2 (1-3)	2 (1-3)	1 (1-1)	2 (1-3)	0.99
C4d (median IQR)	1 (0-2)	2 (0-3)	3 (0-3)	2 (1-3)	0.50
IFTA% (median IQR)	5 (5-15)	5 (0-10)	0 (0-0)	10 (0-30)	0.73

AMR

5.4 Discussion

IgG DSAs are well known biomarkers for anticipating rejection however the screening and management of IgM DSAs remains poorly understood. Previous studies have only concentrated on humoral forms of rejection. This study investigated the clinical impact of IgM DSAs, alone or in conjunction with IgG DSAs across multiple phenotypes of allograft rejection including acute and chronic forms of AMR as well as TCMR. The findings demonstrate that IgM DSAs do not alter the clinical outcomes of renal allografts following rejection.

Although the characteristics are quite well matched across the cohorts there are some differences that should be clarified. A greater number of males and fewer graft losses are to be expected within an unsensitised control group. Importantly these groups were immunologically comparable when considering mismatch, previous grafts and induction in the acute AMR, TCMR and control group. The cAMR group differed in induction in part due to the nature of patients having received augmented immunosuppression, such that some patients with a previous graft could not be re-dosed with alemtuzumab.

Our findings resonate with other studies in the literature[72,73,223], however they also show that even in the presence of an IgG DSA, IgM does not play a cumulative role to worsen outcomes. Everly et al showed in a of study 189 renal transplant recipients, the presence of an IgM DSA in the absence of an IgG DSA did not significantly alter the probability of allograft survival when compared to patients with no detectable DSA at 5 years. The presence of an IgG DSA whether co-existing with IgM or not (n=47) resulted in significantly reduced probability of graft survival compared to IgM DSA positives and DSA negatives at 5 years p=0.002. Of note patients with both IgM and IgG DSAs had greater amounts of intimal arteritis on biopsy[72]. Comparably, in this study's cohort of cAMR patients, there was greater activity in the biopsies of those with both IgG and IgM DSAs. Whilst IgM DSAs had no clear effect on allograft survival

their co-existence with IgG DSA was associated with more severe patterns of histological rejection similar to those episodes discussed by the Everly.

There was a significantly increased incidence of IgM DSA in cases with allograft rejection, particularly aAMR and cAMR. Despite this, there was no reduction in survival in any of the cohorts. The presence of IgM DSAs has been attributed to heightened immune signalling that may herald an iso-type switch to IgG and future rejection[224]. De novo IgM DSA isotype switch has been shown in both heart and kidney transplantation to correlate with graft loss and transplant coronary artery disease[224]. In this study however, despite 6/50 controls having IgM DSAs present at the time of surveillance biopsy, these did not precede a future IgG switch nor an episode of future rejection. It is possible that production of IgM DSA is a feature of continuous endothelial HLA exposure despite the absence of inflammation.

The role of IgM DSAs alone has been previously investigated; here it was explored whether or not the presence of an IgM DSA would result in a significantly poorer outcome when also in the presence of an IgG DSA. Interestingly, there appears to be no significant increase in the risk of allograft failure with the double positivity of IgM and IgG DSAs. This study demonstrates that the presence of an IgG DSA significantly reduces graft survival across all phenotypes of allograft rejection. The suggestion that IgM may have few pathological effects in non-infectious diseases is not novel. For example, patients with anti-phospholipid syndrome develop thrombocytopaenia, arterial and venous thromboses due to high levels of anti-cardiolipin (ACL) antibody. Those with only the IgM isotype ACL antibody are generally asymptomatic[225]. This paradigm has also been demonstrated in transplantation. Roelen et al determined that patients with pre-formed IgM DSA against class I HLA did not develop hyperacute rejection following transplantation. This finding was reproduced by McCalmon and colleagues[73,226]. It might seem counterintuitive given the high affinity with which IgM can

bind and fix complement, however the possible immunoregulatory effect of IgM has been known for some time. Within serum, IgM exists in a pentameric form in lower concentration than IgG making up only 10% of total Ig. Despite high affinity, specificity for non-self-antigen is low and affinity maturation does not occur. Visentin et al indicated that at high enough concentration IgM may directly compete with IgG for binding of HLA epitopes through steric hindrance[227]. Furthermore, the presence of IgM may also affect binding of IgG Fc regions with complement through steric inhibition[227]. The protective immunomodulatory effects of IgM have been considered within renal transplantation[221]. Labarrere et al reported lower mortality in recipients of cardiac allografts that demonstrated IgM deposits on vascular endothelial cells compared to those that did not[228]. In contrast, Stasney et al tested 38 cardiac transplant recipients, 18 of which went on to develop transplant coronary artery disease (TCAD). IgM DSA was shown to be strongly associated with the development of TCAD. 16 of the 18 patients were shown to have IgM anti-donor HLA prior to the diagnosis in the absence of IgG. On the other hand, only 2 of the 20 patients without TCAD had such IgM DSA. The same group also selected 34 renal transplant recipients with negative pre-transplant sera for IgG antibodies against donor HLA. 8 of the 34 developed AMR of which a significantly greater proportion tested positive for IgM DSA, 62% compared to only 15% of 26 patients who maintained normal function, $p<0.02$[224].

With the addition of IgM DSA detection, the number of patients with a de novo alloimmune response increases significantly. Given the transient nature of IgM in serum, one might postulate that a much higher proportion of patients actually mount an antibody response against their donor, than when considering IgG DSA alone. As might be expected, predicting isotype switch of IgM to IgG is similar in terms of variables to those anticipated for IgG DSA development. HLA class II locus mismatch and prior rejection were significant on univariate

analysis. Lynch et al suggested that the majority of recipients will develop a B-cell response towards their allograft. Using a novel ELISA in 9 subjects they identified that all recipients had donor-specific antibody-secreting cells eight weeks after transplant in the absence of rejection and in spite of adequate immunosuppression[229].

There are limitations to this study. It is retrospective in its design. Also, single sample testing at the time of diagnostic biopsy limits us from commenting on the timeline of IgM DSA production. Whether an IgM DSA was formed prior to testing and has a role to play is beyond the scope of this study. This study did not demonstrate a protective role for IgM on survival, however this does not prove that the presence of IgM attenuates the effect of IgG. Modification of the assay using DTT to reduce IgM interference may have resulted in the detection of supressed IgG DSA or higher MFIs of those IgG DSAs already identified.

It has been suggested that IgM DSA surveillance would be potentially beneficial to transplant recipients however, this would be difficult in clinical practice. Most IgM anti-HLA is generated in a thymus-dependent manner from antigen. Production is fluctuant and transient with a significantly shorter half-life than IgG of approximately 10 days. Although the development of IgM anti-HLA suggests a de novo alloimmune response and may be the first sign of under immunosuppression heralding impending class switch[223,224], the practical implications of increased serum monitoring and cost of single antigen do not merit increased surveillance.

In conclusion, this study demonstrates that the presence of an IgM DSA alone or in association with IgG DSA does not result in inferior allograft outcomes. Instead, the presence of an IgG DSA in allograft rejection appears to be the main driver which significantly reduces graft survival.

Chapter 6: Anti-HLA IgE: good, bad or the indifferent?

6.1 Introduction

Anti-HLA antibodies have been demonstrated in IgG, IgM and IgA isotypes. More recently, Farkas et al demonstrated the existence of IgE DSA in mouse models and humans[79]. The group showed the production of IgE against donor MHC class I and class II three weeks after acute rejection of both skin and heart allografts in mouse models. The target receptor of IgE, the FcεRI is present on cells present throughout the medulla and cortex of human kidneys giving functional activity to donor specific IgE both in vivo and in vitro. Binding resulted in mast cell and basophil degranulation leading to increased vascular permeability. Secondly, IgE DSA were confirmed in broadly sensitised human kidney transplant recipients. Finally, an abundance of Th2 cells were seen in the spleens and lymph nodes, which were confirmed to be reactive with donor cells. These data raise the question of the role of IgE in alloimmunity.

Lefaucheur et al demonstrated an association between subclinical chronic rejection and IgG4[13]. Production and regulation of IgG4 and IgE have significant similarities. Both require help from Th2 cells for production as well as many months of chronic exposure to antigen before a significant response becomes prominent[230,231]. The effector cells of IgE, mast cells and basophils have also been identified in lung, kidney, heart and skin allografts and reportedly increase during certain pathogenic circumstances, including fibrosis[232-235]. Reports have also demonstrated an association between graft injury and exposure to Th2 associated cytokines, linking allograft rejection to mast cell and eosinophilic infiltrates in solid organ transplants. Taken together, these finding are of interest and lead to the consideration of a pathogenic role for IgE DSA in cAMR.

It must also be considered that whilst IgG DSA detection is a valuable diagnostic tool, it is often absent, despite the presence of histological features of rejection in allograft biopsies.

Non-HLA antibodies may indeed play an important role in mediating alloimmune injury in these patients however a further question arises as to whether IgE DSA may exist independently explaining seemingly DSA negative tissue damage[153].

The aims of this study are:

1) To investigate if IgE anti-HLA antibodies can be detected using highly sensitised patients.
2) To demonstrate whether anti-HLA IgE DSA was detectable in patients with a known IgG4 DSA following the diagnosis of cAMR.
3) To determine if IgE DSA could be detected in patients with histological features of cAMR but without a detectable IgG DSA.

6.2 Methods

6.2.1 Patient Selection

Three cohorts of patients were identified from prior studies. These patients had all undergone IgG subclass phenotyping. From these, all patients with a positive IgG4 DSA were selected. The first included 7 sensitised patients 24 months after allograft nephrectomy with a broad number of IgG DSAs. The second included 7 patients with a biopsy diagnosis of chronic active antibody mediated rejection and a confirmed anti-HLA IgG DSA in which the subclass profile included IgG4. The final cohort included 10 patients without an identifiable anti-HLA IgG DSA but clear histological features of chronic active AMR including a combined microvascular inflammatory score of >2. Patients in the second and third groups are from the same cohort used in chapters 3 and 4.

6.2.2 Detection of anti-HLA IgE

Identification of HLA specific IgE antibodies was performed by means of a modified protocol for anti-HLA IgG detection using One Lambda SAB kits (OneLambda Inc, Canoga Park, CA.). Briefly, samples were thawed, mixed and spun in a centrifuge at 13000 rpm for 5 minutes. A 6% dilution of EDTA was made up using 25μL of EDTA and 475μL of wash buffer. 10μL of EDTA was added to each well followed by 10μL serum. 5μL of single antigen beads (LABScreen Single Antigen HLA class I or HLA class II beads) were then added to each well and then incubated at room temperature on a shaker protected from light for thirty minutes. Beads were then washed with 200μL of wash buffer and the plate vacuumed five times. Given the abundance of IgE in serum is considerably lower than other immunoglobulin the standard IgG protocol was adjusted to amplify IgE detection. Washed beads were incubated with 100μL of biotin-conjugated anti-human IgE (dilution 1:20, clone MHE-18; BioLegend, San Diego,

California) and incubated in the absence of light for a further thirty minutes. A final thirty-minute incubation with 100μL of PE labelled streptavidin (dilution 1:100; ThermoFisher, Waltham, Massachusetts) was performed prior to five final washes and re-suspension of beads in PBS. Analysis was carried out on the Luminex machine.

Four non-sensitised males were tested to determine baseline reactivity patterns for each bead. Given different beads had varying reactivity, each bead was evaluated separately. Negative cut-off values for each bead were determined based on the mean raw MFIs of each control plus 5 standard deviations.

6.3 Results

The demographics for each group of transplant recipients tested for IgE anti-HLA antibodies are shown in table 6.1

Table 6.1: Demographics

	Tx Nephrectomy (n=7)	**cAMR IgG4 DSA pos (n=7)**	**cAMR IgG DSA neg (n=10)**
Female n (%)	3 (42.9)	1 (14.3)	5 (50.0)
Age at Tx, years	43±16	43±15	52±10
Ethnicity, n (%)			
- Caucasian	3 (42.9)	5 (71.4)	5 (50.0)
- Asian	2 (28.6)	0	4 (40.0)
- Afro-Caribbean	2 (28.6)	2 (28.6)	1 (10.0)
- Other	0 (0.0)	0 (0.0)	0 (0.0)
Live donor, n (%)	2 (28.6)	2 (28.6)	2 (20.0)
Pre-emptive, n (%)	2 (28.6)	0 (0.0)	0 (0.0)
Induction, n (%)			
- Anti-CD52	5 (71.4)	7 (100.0)	6 (60.0)
- Anti-IL-2R	2 (28.6)	0 (0.0)	4 (40.0)
HLA-A/B MM	3 (2-3)	2 (2-3)	2 (1-3)
HLA-DR MM	2 (2-3)	2 (1-2)	1 (0-2)
HLA-DQ MM	1 (1-1)	1 (1-1)	1 (0-1)
Total MM	3 (3-4)	4 (3-4)	4 (2-5)

Of note, there were small numbers of females in cAMR IgG4 positive group, however this cannot be commented on for significance as the groups are quite small.

In order to establish whether anti-HLA IgE was detectable in renal transplant recipients with an anti-HLA IgG DSA, 7 patients who were broadly sensitised following allograft nephrectomy

were investigated. These patients were selected following recently published work by Lucisano et al, demonstrating that allograft nephrectomy significantly impacts the degree of DSA development particularly in the long term[236]. All 7 patients were sensitised against both class I and class II HLA. 7/7 (100%) of patients had IgE HLA against both class I and class II. In all 7, there was at least one reactivity that was specific for donor HLA antigens (see table 6.2).

Table 6.2: Pan IgG DSA specificities for each patient alongside the corresponding IgE DSA detected.

Patient	IgG Class I Specificities	IgE Class I Specificities	IgG Class II Specificities	IgE Class II Specificities
1	A3, B8, B57, Cw7	A3, B8, B57, Cw7	DQ2	DQ2
2	A11, A24, B39 Cw7	A11, A24, B39	DR11, DR52, DQ7	DQ7
3	A1, A2, B44, Cw5, Cw7	A1, A2, B44, Cw5	DR4, DR8, DR53, DQ4, DQ7	DR4, DR8
4	A1, B7, Cw7	A1, B7	DR15, DR51, DQ6	DR15, DQ6
5	A29, B44, Cw16	A29, B44	DQ2	DQ2
6	A32, B8, B44, Cw5, Cw7	A32, B8, B44, Cw5, Cw7	DQ7	DQ7
7	A11, B18, Cw14	A11, B18, Cw14	DR52, DQ5	DQ5

A greater proportion of class I IgG DSAs, 23/26 (88.5%), were also present in the IgE isotype. Of the 16 class II IgG DSA specificities 9 (56.3%) were also IgE DSAs. None of the IgE DSAs identified were present in the absence of an IgG DSA of the same specificity. These results demonstrate that anti-HLA IgE DSAs are present in the serum of sensitised renal transplant recipients with a corresponding IgG DSA.

Considering the physiological similarities between IgG4 and IgE, 7 further cases were tested. These patients were selected from prior studies investigating IgG subclass phenotypes in cAMR. All patients had an IgG DSA at the time of cAMR diagnosis. The IgG subclass composition of each case was separated for immunodominant (ID) class I and II DSA. Every patient had at least one IgG4 present either in isolation or as part of a mixture of subclasses (see table 6.3).

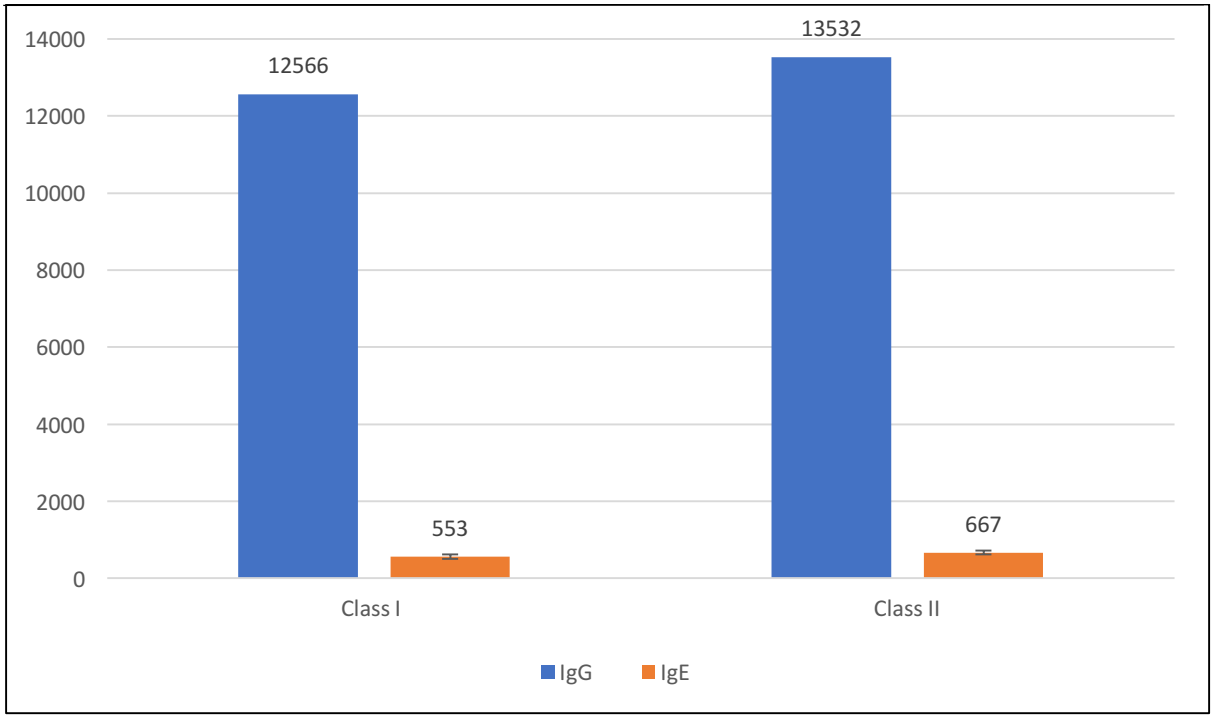
Table 6.3: Subclass (SC) composition of the class I and class II DSAs along with the IgE positive DSAs.

Patient	Pan IgG DSAs	ID Class I DSA (IgG SC)	Class I IgE DSA	ID Class II DSA (IgG SC)	Class II IgE DSA
1	Cw4, Cw5, DQ5	Cw4 (1,2)	Neg	DQ5 (1,4)	DQ5
2	DQ7, DR53	Neg	Neg	DQ7 (1,2,3,4)	DQ7
3	B44, Cw5, DQ6	B44 (1)	Neg	DQ6 (1,2,3,4)	DQ6
4	Cw10, DQ2	Cw10 (4)	Cw10	DQ2 (1,2,4)	DQ2
5	A11, B44, Cw5, DQ8	B44 (1)	Neg	DQ8 (1,2,4)	DQ8
6	DR53, DQ8	Neg	Neg	DQ53 (1,4)	Neg
7	A24, B64, DR53, DQ6	B64 (1)	Neg	DQ6 (1,2,4)	DQ6

Interestingly, only 1/10 (10%) immunodominant DSAs with an IgG4 did not have a corresponding IgE DSA. Conversely, all immunodominant DSAs that lacked IgG4 (4/4) did not have an IgE DSA present. This might suggest that the development of an IgE DSA is more likely once the humoral response has expanded to include IgG4. This would fit with the concept of sequential class switch from IgG to IgE rather than directly from IgM. The MFIs of IgE DSAs

was much lower than those of corresponding IgG DSAs. This is unsurprising given the significantly lower levels of IgE in serum compared with IgG (see figure 6.1)

Figure 6.1: Comparison of Mean MFI of IgG and IgE DSAs according to HLA class



When comparing the degree of active inflammation, those patients without IgE DSA appeared to have lower total MI scores compared to those with IgE DSA, though the numbers were too small to test significance (See table 6.4).

Table 6.4: Composite g+ptc scores of all patients alongside their IgE DSA status

Patient	IgE Class I	IgE Class II	MI score	g-score	ptc-score
1	Neg	DQ5	5	3	2
2	Neg	DQ7	4	1	3
3	Neg	DQ6	4	1	3
4	Cw10	DQ2	4	2	2
5	Neg	DQ8	3	1	2
6	Neg	Neg	2	0	2
7	Neg	Neg	1	0	1

Finally, to test whether or not IgE anti-HLA might be present even in the absence of IgG, 10 patients with histological features of chronic active AMR but without an identifiable IgG DSA were tested. Each case had significant microvascular inflammation with MI scores of 3 or more. None of the 10 patients tested were positive for IgE DSA. Although, 3 patients had significant IgE HLA. On further testing all 3 were shown to be sensitised with IgG HLA of the same specificities as the IgE HLA. These data demonstrate that anti-HLA IgE does not occur in isolation in the absence of an IgG anti-HLA antibody.

6.4 Discussion

This is the largest series investigating IgE anti-HLA antibodies in renal transplant recipients to date. The study has validated the work of Farkas et al demonstrating that IgE DSA is present in the serum of highly sensitised transplant recipients. Building on this, it has also shown a correlation between IgE DSA and IgG DSA in cAMR, particularly in those with IgG4. Lastly, that IgE likely requires IgG as a precursor for class switch in rejection as no patient with histological features of cAMR had IgE anti-HLA in the absence of an IgG HLA antibody.

IgE has the lowest concentration in serum compared to all other isotypes, existing mostly in tissue, with levels up to 10,000 times less than IgG[237]. With a half-life of approximately 2 days it is one of the shortest-lived of the immunoglobulins[238]. Effects are mediated through mast cells and basophils via the high affinity FcεR1 receptor, after the crosslinking of IgE bound to specific antigen [239]. Degranulation releases potent proinflammatory cytotoxins including, eicosanoids, TNF-α and IL-6.

IgE is strongly associated with Th2 related immunopathology including allergic pathogenesis and helminth immunity but also autoimmune diseases such as atopic dermatitis and systemic lupus erythematosus (SLE)[240,241].

Although there is little current evidence with regards to the role of IgE in alloimmunity, studies have revealed features of Th2 driven immunity in cellular rejection, raising the question as to whether there may be a function for IgE in humoral rejection[242,243].

Class switching to IgE was until recently thought to only occur directly from IgM, resulting in the production of relatively short lived IgE plasma cells and low affinity antibody[244,245]. This does not explain how IgE responses can be long lived and can often require chronic antigen stimulation to appear. Recent studies in murine models have identified a sequential class switch pathway in which IgM undergoes an intermediary switch to IgG1 followed by IgE[246].

This occurs mostly in germinal centres resulting in the production of longer lived plasma and memory cells capable of producing high affinity antibody[247]. Importantly, it must be noted that mouse IgG1 is more similar to human IgG4 than human IgG1. The absence of IgE anti-HLA antibodies in patients without an IgG, suggests that the majority of IgE produced following sensitisation is likely derived from this sequential class switching pathway. Whilst not performed in this study, it would be interesting to examine for the presence of IgM DSA in these patients, which would help to rule out direct class switch in the production of IgE DSA.

IgG4 is similar to IgE in many ways; firstly, production of both immunoglobulins depends on Th2 related cytokines (including IL-4, IL-5 and IL-13); secondly, as previously mentioned, IgG4 is often the upstream Ig in sequential class switch from IgM to IgE; finally, their ability to interact with other Igs has led to debate as to whether they occupy more of a regulatory role[248-250]. Considering IgE and IgG4 both require Th2 cell help it is unsurprising that most antigens that induce one will also efficiently induce the other. This might provide further evidence to explain why a greater number of IgG DSAs in the cAMR group had an equivalent IgE if the subclass mixture included IgG4[237].

It remains unclear as to what role IgE might play in transplant rejection. The recent study by Farkas et al established that functional IgE against both class I and class II donor MHC develops in murine and human models of solid organ transplantation during rejection and persists long term[79]. A systemic Th2 response was demonstrated in murine models by the detection of CD4+ cells positive for IL-4 in the spleens and lymph nodes of mice with skin grafts. Both humans and mice had similarly functional IgE which potently stimulated effector cell (mast cells and basophils) degranulation. Coupled with the fact that effector cells expressing FcεR1

receptors are present in human kidneys, these results present a compelling model for IgE possessing injurious alloreactive qualities.

Although some studies have shown evidence of tolerance in the setting of Th2 immunity, there is increasing evidence indicating that sustained Th2 responses can lead to allograft rejection[251]. Mast cells can mediate responses in both the innate and adaptive immune system. Indirect recruitment of neutrophils and macrophages occurs through cytokine release, as well as activation of alloreactive T cells through antigen presentation via the class II MHC. Yousem et al, first demonstrated a role for mast cells in both acute and chronic forms of rejection in lung transplantation. Increasing numbers of mast cells correlated with recruitment of effector T cells, B cells, macrophages and NK cells in transbronchial biopsies of 29 patients with allograft rejection[252]. This phenomenon was later corroborated in studies of other solid organ transplants[253-256].

Mast cell effects on acute rejection remain somewhat controversial, however mast cell activity in chronic rejection is better established. The release of histamine, TGF-β, chymase and basic fibroblast growth factor contribute to fibrosis by activating fibroblasts that induce collagen synthesis. In renal transplants, mast cell numbers have been correlated with the severity of interstitial fibrosis at the time of transplantation[257,258]. Immunohistochemical investigation by Ishida et al revealed mast cell chymase to be a potent profibrotic mediator in chronic allograft fibrosis[258]. The hypothesis that chronic allograft rejection may be a Th2 mediated pathology is further underpinned by the indirect role of eosinophils. Nolan et al examined 24 allograft nephrectomy specimens, 15 of which had histological evidence of chronic rejection. Eosinophils were identified in 14 of 15 cases. A dose dependent effect on DNA synthesis in smooth muscle cells was noted in eosinophil conditioned media[259]. Degranulation of eosinophils releases major basic protein (MBP) leading to mast cell tryptase

and chymase production[260]. This pathway is of potential importance given the majority of allograft failures are due to chronic rejection.

Recently, Henault et al have shown that IgE can cause tissue damage independently of mast cells and basophils. IgE, specific for double stranded DNA was able to enhance the inflammatory response of IgG by activating plasmacytoid dendritic cells and increasing the secretion of IFN-α and thus tissue damage in both murine and human models of SLE[261]. With the importance of allo-antibody characterisation becoming more evident, it would be interesting to investigate whether the presence of IgE causes different phenotypes of rejection although, this is beyond the scope of the present study.

While significant evidence exists providing conceivable functions for the pathogenic role of IgE, studies have also found a role for IgE and mast cells promoting immune tolerance. Lu et al investigated a Treg dependent tolerance model, in which allografts transplanted into mast cell deficient mice were rejected. Wild type mice accepted the skin grafts and upon transfer of their bone marrow derived mast cells into mast cell deficient animals, there was prolonged graft survival[262].

This study has demonstrated that IgE DSA is present in the serum of transplant recipients with chronic antibody mediated rejection provided an IgG anti-HLA is also present. This was a proof of concept study, the numbers being too small to infer statistical significance. Further work is required on a larger series of cases to investigate whether IgE specific for HLA is able to cause allograft injury as well as produce specific phenotypes of allograft rejection.

Chapter 7: Summary of Findings

Work from this thesis has highlighted the importance of antibody characterisation in identifying future risk specific to each individual receiving a renal allograft. Phenotyping isotype, subclass and complement fixing ability of DSAs can help identify clinical subgroups of patients that may require increased surveillance in the clinical setting, as well as consideration for more tailored therapies.

Work by Lefaucheur et al in 2016 demonstrated the importance of antibody characteristic phenotyping in acute antibody mediated rejection. However, chronic antibody mediated rejection is the most common cause of allograft failure in the long term. In chapter 3, there are three important take home messages. Firstly, patients with de novo IgG DSAs in the setting of histological features of cAMR have poorer graft survival than those without, particularly if there is an identifiable subclass pattern. These patients tend to have a more severe presentation with lower eGFRs and increased degrees of proteinuria. Secondly, the most significant hazards to graft survival are the presence of class II IgG2 and IgG3 DSAs, with class II IgG3 leading to the worst mean allograft survival of 12.5 months from diagnosis. Subclass profiles also correlated with histological characteristics. Finally, class II IgG2 and IgG3 associated strongly with increased levels of microcirculatory inflammation. Class II IgG2 also correlated with the chronic lesion of transplant glomerulopathy.

Development of de novo class II DSAs are of particular concern in recipients of solid organ transplants. The preponderance of anti-DQ antibodies underpins the need for change in matching algorithms. The classic three locus match is outdated and in need of reform. Prospective trials are required, where matching at the DQ locus is taken into consideration.

Phenotyping of IgG subclass demonstrates greater stratification of risk for those with cAMR.

Consideration should be taken towards subclass phenotyping of all de novo DSAs in order to help stratify patients into appropriate surveillance and treatment cohorts. Incorporating subclass phenotyping into clinical practice will at present provide significant cost concerns given the cost of single antigen beads. The assay needs to be run eight times, four times for both classes. Thinking practically, this could be reduced to twice. Subclass analysis of class I DSAs did not provide any convincing evidence of superiority over panIgG testing. Equally, although abundant, IgG1 was not shown to correlate with reduced allograft survival. The role of IgG4 remains unclear with regards to immunomodulation. IgG4 was always present with other subclasses making its identification of questionable use. Early evidence by the Paris transplant group suggests that IgG4 may correlate with chronic rejection lesions but this does not provide any further prognostic benefit that is not provided by allograft biopsy. Therefore, restricting the assay to class II IgG2 and IgG3 detection makes the most sense until the cost of solid phase assays reduces.

Testing of C1q binding of an IgG DSA in cAMR is of questionable value. In chapter 4, only class II DSAs that fixed complement were associated with reduced allograft survival on a univariate analysis. There was no correlation between C1q positivity and graft loss on multivariable Cox regression. This raised the question as to whether patients with cAMR have complement independent mechanisms of allo-immune injury. This would fit well with the results of therapeutic trials into terminal complement inhibition, finding no benefit in cAMR. Critics of the C1q bead assay claim that MFI is a reasonable surrogate of complement binding. To some extent this is an oversimplification. MFI is not a linear scale; assumptions of complement fixing by DSAs with MFIs in the mid-range cannot be made. Some antibodies at low MFI can still fix complement, similarly others at high MFI cannot. Once again taking a pragmatic cost-effective approach, there is little role for C1q testing in the setting of cAMR. A combination

of MFI, specificity of HLA and class II IgG2/IgG3 testing would therefore be adequate in risk stratifying these patients. The role of complement binding in acute AMR has not been addressed in this thesis.

Not all DSAs have a negative impact on graft survival. De novo IgM allo-immune responses are common across transplantation whether or not in the presence of rejection. In particular, patients with cAMR demonstrated higher numbers of de novo IgM DSAs compared to cases of TCMR and controls. These patients were often more broadly sensitised as a result of prior transplantation. The possible explanation for the presence of IgM DSA in controls may come from continuous endothelial HLA antigen exposure despite the absence of inflammation.

Previous studies of IgM DSA provided conflicting data of both protective and damaging roles. Work in chapter 5 demonstrated that in renal transplantation, across all phenotypes of rejection, IgM DSAs do not add any cumulative risk with regards to allograft survival, even in the presence of an IgG DSA. Although IgM antibodies can activate complement there have been no cases of hyper acute rejection secondary to pre-formed IgM DSAs published in the literature reinforcing a lack of injurious potential to allografts.

Of concern is the fact that the development of an IgM DSAs may be the first signs of under immunosuppression and herald impending class switch to IgG[223]. In studies associating IgM DSAs with poor long-term graft survival, the likely cause is future class switch.

In chapter 5, the development of a class II IgM DSA was a significant risk for class switch and could provide an argument for IgM DSA screening. However, given the transient and fluctuant production of IgM as well as the short half-life of ten days, this would be impractical, both with regards to the frequency of sampling required and the cost. From this work, it is clear that testing for IgM DSA in the post-transplant period is not helpful in predicting graft loss following development of rejection and should not be undertaken.

The presence of IgE DSA in IgG DSA positive patients is a new finding and has been shown in highly sensitised patients with an IgG DSA. In chapter 6, IgE DSA was not detected in patients in the absence of IgG DSA, illustrating that IgG is required for class switch to IgE. In the absence of IgG, despite features of alloimmune tissue injury on biopsy, no patients had an IgE response. It was very tempting to consider IgE as a candidate responsible for IgG DSA negative AMR.

Of particular interest was the suggestion that IgE was more likely to develop in patients with an IgG4 DSA. Though the numbers were too small for statistical comparison, this is a plausible theory, as both IgE and IgG4 are strongly associated with Th2 related immunopathology. This raises the question with regard to the role of IgE DSA in transplantation. Arguments for both tolerance and injurious models can be made. Hypothesising that cAMR across solid organs may in part be a Th2 related phenomenon is exciting and opens up new avenues for potential research and treatments. Clearly, further work is required to assess whether the presence of IgE DSAs have more than a bystander role in both acute and chronic forms of rejection.

In conclusion, the findings from this thesis are:

- Patients with de novo IgG DSAs in the setting of histological features of cAMR have poorer graft survival than those without. In particular, patients with class II IgG2 and IgG3 DSAs present with more severe clinical phenotypes of cAMR, with a dramatically increased risk of allograft loss.
- C1q binding class II DSAs are associated with reduced allograft survival, however, graft loss in cAMR is unlikely to be a complement dependent process.
- Whether alone or in the presence of an IgG DSA, IgM DSAs do not add any cumulative risk with regards to allograft loss.

- Patients who have developed a de novo IgG allo-immune response often have a co-existing IgE DSA. This antibody may indeed be functional and have pathologic implications. The presence of IgG4 DSA correlates with the development of IgE DSA.

Chapter 8: Future Work

The work in this thesis has shown has highlighted the significance of DSA characteristic phenotyping to better predict outcomes of patients with renal transplants with rejection. Future work will be aimed at expanding this theme.

8.1 DSA Phenotype Kinetics

This thesis identified a subgroup of patients with cAMR that had particularly poor outcomes based on the phenotype of their DSA. I have established a database of patients with acute AMR and divided them according to their treatment. These include augmented immunosuppression with MMF and steroids, increased FK dosing as well as plasma exchange with IVIg. Given that IgG3 and C1q binding have been shown to be predictors of poor allograft survival in acute AMR. I plan to investigate whether IgG subclasses and C1q profiles change following different treatment algorithms. It would be important to understand whether DSA characteristics change as patients progress from acute to chronic AMR. Considering C1q activity was not found to have as significant an effect on patients with cAMR as initially expected, does the complement fixing ability of their DSAs change as patients progress from into chronic forms of rejection? It would also be interesting to assess whether particular subclass switches may indicate efficacy of novel treatments.

8.2 Correlating Molecular Histological Phenotypes with DSA

The use of transcript analysis has been added to the Banff 2015 criteria to aid in the diagnosis of AMR. NanoString technologies now allow us to assess biopsy specimens stored in paraffin for pathogenic-gene-transcripts. Sets of transcripts have been associated with AMR. I hope to correlate particular transcripts with DSA profiles in our cohorts of cAMR and acute AMR. This

will add to future risk prediction models allowing more accurate diagnoses of rejection phenotypes and help individualise potential future therapies.

8.3 The relevance of non-HLA DSA

Many of the patients with histologic changes of rejection tested in this thesis have been found to be negative for anti-HLA antibodies. It is still unclear as to how many simply have an HLA DSA which is either sequestered in the kidney, whether the sensitivity of solid phase assays remains too low or if the causative antibody is in fact a non-HLA DSA. Immunisation against non-HLA antigens has been shown to result in worse long-term outcomes[85]. Non-HLA antibodies are not tested routinely and can occur against a broad range of antigens, making assays impractical to perform as part of standard screening. Yet many patients may in fact be missing out on standard of care treatments should they have an undetected antibody in the context of histological allo-immune injury. Biotech companies are now producing solid phase assays that can test against an array of non-HLA antigens broadening the potential scope to identify and start to understand the clinical relevance of these antibodies. This has the potential to significantly change practice as a broader array of antigens are likely to mediate expanded rejection phenotypes. For example, angiotensin II type I receptor antibodies (AT1R-Ab) have been associated with varying forms of vascular rejection[263]. Recently, Dr Michelle Willicombe demonstrated that class II anti-HLA DSAs are associated with transplant renal artery stenosis (TRAS)[264]. We postulate that AT1R-Abs may be associated with development of TRAS. We plan to test the serum of patients who have developed TRAS for AT1R-Abs. This may suggest a role for augmented immunosuppression alongside traditional stenting.

8.4 IgE Anti-HLA Antibodies

The presence of IgE DSA in IgG DSA positive patients is a new finding. In order to better understand the circumstances for class switch to IgE patients with different IgG subclass mixtures will be tested. Initially we have postulated that IgE will develop after chronic antigen stimulation often in the presence of IgG4. In order to prove this, a larger cohort of patients without IgG4 will be compared. It would also be of benefit to assess DSA positive patients without histologic features of AMR (ATI, TCMR and significant IFTA) to see if IgE might play a role. I have already discussed how Th2 pathways are significant in cellular rejection. IgE would therefore be of interest if present in the absence of AMR.

8.5 IgA Anti-HLA Antibodies

Finally, I have investigated the role of IgM, IgG and IgE DSAs in rejection. Anti-HLA IgA can also occur in immunised patients. Recent evidence suggests that anti-HLA IgA may cumulatively reduce allograft survival in re-transplant recipients if in the presence of IgG[77,265]. This may be mediated via MBL complement pathway. It is not yet clear what role, if any, anti-HLA IgA may play alone in patients who have not had an episode of rejection. A small cohort of patients with multiple phenotypes of rejection, with and without IgG DSA will be tested and compared to see if there are variations in rejection phenotype and survival outcomes.

Financial Acknowledgements

This research is supported by the Imperial Charitable Fund and the National Institute for Health Research (NIHR) Biomedical Research Centre base at Imperial College Healthcare NHS Trust and Imperial College London.

References

1. Garcia GG, Harden P, Chapman J, World Kidney Day Steering C. The global role of kidney transplantation. *Lancet.* 2012;379(9820):e36-38.
2. Evans RW, Manninen DL, Garrison LP, Jr., et al. The quality of life of patients with end-stage renal disease. *N Engl J Med.* 1985;312(9):553-559.
3. Port FK, Wolfe RA, Mauger EA, Berling DP, Jiang K. Comparison of survival probabilities for dialysis patients vs cadaveric renal transplant recipients. *JAMA.* 1993;270(11):1339-1343.
4. Morris PJ, Williams GM, Hume DM, Mickey MR, Terasaki PI. Serotyping for homotransplantation. XII. Occurrence of cytotoxic antibodies following kidney transplantation in man. *Transplantation.* 1968;6(3):392-399.
5. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am J Transplant.* 2009;9(11):2520-2531.
6. Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation.* 2008;86(3):377-383.
7. Lefaucheur C, Loupy A, Hill GS, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *J Am Soc Nephrol.* 2010;21(8):1398-1406.
8. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *Am J Transplant.* 2012;12(5):1157-1167.
9. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. *Am J Transplant.* 2011;11(3):450-462.
10. Montgomery RA, Cozzi E, West LJ, Warren DS. Humoral immunity and antibody-mediated rejection in solid organ transplantation. *Semin Immunol.* 2011;23(4):224-234.
11. Aubert O, Loupy A, Hidalgo L, et al. Antibody-Mediated Rejection Due to Preexisting versus De Novo Donor-Specific Antibodies in Kidney Allograft Recipients. *J Am Soc Nephrol.* 2017;28(6):1912-1923.
12. Orandi BJ, Chow EH, Hsu A, et al. Quantifying renal allograft loss following early antibody-mediated rejection. *Am J Transplant.* 2015;15(2):489-498.
13. Lefaucheur C, Viglietti D, Bentlejewski C, et al. IgG Donor-Specific Anti-Human HLA Antibody Subclasses and Kidney Allograft Antibody-Mediated Injury. *J Am Soc Nephrol.* 2016;27(1):293-304.
14. Viglietti D, Loupy A, Vernerey D, et al. Value of Donor-Specific Anti-HLA Antibody Monitoring and Characterization for Risk Stratification of Kidney Allograft Loss. *J Am Soc Nephrol.* 2017;28(2):702-715.
15. Montgomery RA, Loupy A, Segev DL. Antibody-mediated rejection: New approaches in prevention and management. *Am J Transplant.* 2018;18 Suppl 3:3-17.
16. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature.* 1999;401(6756):921-923.
17. Gruen JR, Weissman SM. Evolving views of the major histocompatibility complex. *Blood.* 1997;90(11):4252-4265.
18. Murphy K, Weaver C. *Janeway's immunobiology.* 9th edition. ed. New York, NY: Garland Science/Taylor & Francis Group, LLC; 2016.

19. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol.* 1977;29(1):1-5.
20. Ingulli E. Mechanism of cellular rejection in transplantation. *Pediatr Nephrol.* 2010;25(1):61-74.
21. Brown K, Sacks SH, Wong W. Coexpression of donor peptide/recipient MHC complex and intact donor MHC: evidence for a link between the direct and indirect pathways. *Am J Transplant.* 2011;11(4):826-831.
22. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol.* 2004;4(5):336-347.
23. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science.* 1995;270(5234):286-290.
24. Halloran PF, Homik J, Goes N, et al. The "injury response": a concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. *Transplant Proc.* 1997;29(1-2):79-81.
25. Hidalgo LG, Halloran PF. Role of IFN-gamma in allograft rejection. *Crit Rev Immunol.* 2002;22(4):317-349.
26. Forbes RD, Zheng SX, Gomersall M, al-Saffar M, Guttmann RD. Evidence that recipient CD8+ T cell depletion does not alter development of chronic vascular rejection in a rat heart allograft model. *Transplantation.* 1994;57(8):1238-1246.
27. Glick B, Chang TS, Jaap GR. The Bursa of Fabricius and Antibody Production. *Poult Sci.* 1956;35(1):224-225.
28. Henderson A, Calame K. Transcriptional regulation during B cell development. *Annu Rev Immunol.* 1998;16:163-200.
29. Martensson IL, Almqvist N, Grimsholm O, Bernardi AI. The pre-B cell receptor checkpoint. *FEBS Lett.* 2010;584(12):2572-2579.
30. Almqvist N, Martensson IL. The pre-B cell receptor; selecting for or against autoreactivity. *Scand J Immunol.* 2012;76(3):256-262.
31. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. *Blood.* 2008;112(5):1570-1580.
32. Clatworthy MR. Targeting B cells and antibody in transplantation. *Am J Transplant.* 2011;11(7):1359-1367.
33. Noorchashm H, Reed AJ, Rostami SY, et al. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. *J Immunol.* 2006;177(11):7715-7722.
34. Halloran PF, de Freitas DG, Einecke G, et al. The molecular phenotype of kidney transplants. *Am J Transplant.* 2010;10(10):2215-2222.
35. Tsai EW, Rianthavorn P, Gjertson DW, Wallace WD, Reed EF, Ettenger RB. CD20+ lymphocytes in renal allografts are associated with poor graft survival in pediatric patients. *Transplantation.* 2006;82(12):1769-1773.
36. Ding Q, Yeung M, Camirand G, et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest.* 2011;121(9):3645-3656.
37. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity.* 2008;28(5):639-650.

38. Moreau A, Blair PA, Chai JG, et al. Transitional-2 B cells acquire regulatory function during tolerance induction and contribute to allograft survival. *Eur J Immunol.* 2015;45(3):843-853.
39. Dunnick W, Hertz GZ, Scappino L, Gritzmacher C. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res.* 1993;21(3):365-372.
40. Smith TF. IgG subclasses. *Adv Pediatr.* 1992;39:101-126.
41. Jefferis R, Kumararatne DS. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin Exp Immunol.* 1990;81(3):357-367.
42. Valenzuela NM, Schaub S. The Biology of IgG Subclasses and Their Clinical Relevance to Transplantation. *Transplantation.* 2018;102(1S Suppl 1):S7-S13.
43. Allhorn M, Briceno JG, Baudino L, et al. The IgG-specific endoglycosidase EndoS inhibits both cellular and complement-mediated autoimmune hemolysis. *Blood.* 2010;115(24):5080-5088.
44. Woof JM, Burton DR. Human antibody-Fc receptor interactions illuminated by crystal structures. *Nat Rev Immunol.* 2004;4(2):89-99.
45. Heinemann FM, Roth I, Rebmann V, et al. Immunoglobulin isotype-specific characterization of anti-human leukocyte antigen antibodies eluted from explanted renal allografts. *Hum Immunol.* 2007;68(6):500-506.
46. Stites E, Le Quintrec M, Thurman JM. The Complement System and Antibody-Mediated Transplant Rejection. *J Immunol.* 2015;195(12):5525-5531.
47. Perugino CA, Mattoo H, Mahajan VS, et al. Emerging Treatment Models in Rheumatology: IgG4-Related Disease: Insights Into Human Immunology and Targeted Therapies. *Arthritis Rheumatol.* 2017;69(9):1722-1732.
48. Murata K, Fox-Talbot K, Qian Z, et al. Synergistic deposition of C4d by complement-activating and non-activating antibodies in cardiac transplants. *Am J Transplant.* 2007;7(11):2605-2614.
49. Zhang X, Reed EF. Effect of antibodies on endothelium. *Am J Transplant.* 2009;9(11):2459-2465.
50. J B. Les serums hemolytiques, leurs antitoxines et lestheories des serums cytolytique. *Annales de L'Institut Pasteur.* 1900(14):257-296.
51. Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. *Nat Rev Immunol.* 2008;8(1):48-58.
52. Stegall MD, Chedid MF, Cornell LD. The role of complement in antibody-mediated rejection in kidney transplantation. *Nat Rev Nephrol.* 2012;8(11):670-678.
53. Peng Q, Li K, Sacks SH, Zhou W. The role of anaphylatoxins C3a and C5a in regulating innate and adaptive immune responses. *Inflamm Allergy Drug Targets.* 2009;8(3):236-246.
54. Feucht HE, Felber E, Gokel MJ, et al. Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol.* 1991;86(3):464-470.
55. Racusen LC, Colvin RB, Solez K, et al. Antibody-mediated rejection criteria - an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant.* 2003;3(6):708-714.
56. Jeannet M, Pinn VW, Flax MH, Winn HJ, Russell PS. Humoral antibodies in renal allotransplantation in man. *N Engl J Med.* 1970;282(3):111-117.
57. Gould DS, Auchincloss H, Jr. Direct and indirect recognition: the role of MHC antigens in graft rejection. *Immunol Today.* 1999;20(2):77-82.

58. Murphey CL, Forsthuber TG. Trends in HLA antibody screening and identification and their role in transplantation. *Expert Rev Clin Immunol.* 2008;4(3):391-399.
59. Soosay A, O'Neill D, Counihan A, Hickey D, Keogan M. Causes of sensitisation in patients awaiting renal transplantation in Ireland. *Ir Med J.* 2003;96(4):109-112.
60. Sargent IL, Arenas J, Redman CW. Maternal cell-mediated sensitisation to paternal HLA may occur, but is not a regular event in normal human pregnancy. *J Reprod Immunol.* 1987;10(2):111-120.
61. Honma K, Parker KC, Becker KG, McFarland HF, Coligan JE, Biddison WE. Identification of an epitope derived from human proteolipid protein that can induce autoreactive CD8+ cytotoxic T lymphocytes restricted by HLA-A3: evidence for cross-reactivity with an environmental microorganism. *J Neuroimmunol.* 1997;73(1-2):7-14.
62. Ramon DS, Huang Y, Zhao L, et al. Use of complement binding assays to assess the efficacy of antibody mediated rejection therapy and prediction of graft survival in kidney transplantation. *Hum Immunol.* 2017;78(2):57-63.
63. Malheiro J, Tafulo S, Dias L, et al. Determining donor-specific antibody C1q-binding ability improves the prediction of antibody-mediated rejection in human leucocyte antigen-incompatible kidney transplantation. *Transpl Int.* 2017;30(4):347-359.
64. Guidicelli G, Guerville F, Lepreux S, et al. Non-Complement-Binding De Novo Donor-Specific Anti-HLA Antibodies and Kidney Allograft Survival. *J Am Soc Nephrol.* 2016;27(2):615-625.
65. Ntokou IS, Boletis JN, Apostolaki M, et al. Long-term post transplant alloantibody monitoring: a single center experience. *Clin Transpl.* 2011:341-350.
66. Chong AS, Alegre ML. The impact of infection and tissue damage in solid-organ transplantation. *Nat Rev Immunol.* 2012;12(6):459-471.
67. Filippone EJ, Farber JL. Humoral Immune Response and Allograft Function in Kidney Transplantation. *Am J Kidney Dis.* 2015;66(2):337-347.
68. Valenzuela NM, Reed EF. Antibodies in transplantation: the effects of HLA and non-HLA antibody binding and mechanisms of injury. *Methods Mol Biol.* 2013;1034:41-70.
69. Anthony RM, Nimmerjahn F. The role of differential IgG glycosylation in the interaction of antibodies with FcgammaRs in vivo. *Curr Opin Organ Transplant.* 2011;16(1):7-14.
70. Hidalgo LG, Sis B, Sellares J, et al. NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection. *Am J Transplant.* 2010;10(8):1812-1822.
71. Bryan CF, Martinez J, Muruve N, et al. IgM antibodies identified by a DTT-ameliorated positive crossmatch do not influence renal graft outcome but the strength of the IgM lymphocytotoxicity is associated with DR phenotype. *Clin Transplant.* 2001;15 Suppl 6:28-35.
72. Everly MJ, Rebellato LM, Haisch CE, et al. Impact of IgM and IgG3 anti-HLA alloantibodies in primary renal allograft recipients. *Transplantation.* 2014;97(5):494-501.
73. McCalmon RT, Jr., Tardif GN, Sheehan MA, Fitting K, Kortz W, Kam I. IgM antibodies in renal transplantation. *Clin Transplant.* 1997;11(6):558-564.

74. Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol.* 2001;167(5):2861-2868.
75. Susal C, Kropelin M, Wiesel M, et al. Pretransplant IgA-anti-hinge and IgA-anti-Fab autoantibody activity is associated with good kidney graft survival. *Transplant Proc.* 1995;27(5):2663-2665.
76. Susal C, Wiesel M, Staehler G, Groth J, May G, Opelz G. Excellent kidney graft survival in patients with high pretransplant serum IgA concentrations and IgA-anti-Fab autoantibody activity. *Transplant Proc.* 1995;27(1):1072-1074.
77. Arnold ML, Bach C, Heinemann FM, et al. Anti-HLA alloantibodies of the IgA isotype in re-transplant candidates part II: Correlation with graft survival. *Int J Immunogenet.* 2018;45(3):95-101.
78. Farges O, Buffello D, Shi YM, Berth A, Bismuth H. Anti-donor antibody class switching after liver transplantation. *Transplantation.* 1995;60(3):296-300.
79. Farkas AM, Baranyi U, Bohmig GA, et al. Allograft rejection is associated with development of functional IgE specific for donor MHC antigens. *J Allergy Clin Immunol.* 2019;143(1):335-345 e312.
80. Tambur AR, Herrera ND, Haarberg KM, et al. Assessing Antibody Strength: Comparison of MFI, C1q, and Titer Information. *Am J Transplant.* 2015;15(9):2421-2430.
81. Yell M, Muth BL, Kaufman DB, Djamali A, Ellis TM. C1q Binding Activity of De Novo Donor-specific HLA Antibodies in Renal Transplant Recipients With and Without Antibody-mediated Rejection. *Transplantation.* 2015;99(6):1151-1155.
82. Loupy A, Lefaucheur C, Vernerey D, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. *N Engl J Med.* 2013;369(13):1215-1226.
83. El-Awar N, Terasaki PI, Nguyen A, et al. Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood. *Hum Immunol.* 2009;70(10):844-853.
84. Lefaucheur C, Viglietti D, Mangiola M, Loupy A, Zeevi A. From Humoral Theory to Performant Risk Stratification in Kidney Transplantation. *J Immunol Res.* 2017;2017:5201098.
85. Opelz G, Collaborative Transplant S. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. *Lancet.* 2005;365(9470):1570-1576.
86. Zhang Q, Reed EF. Non-MHC antigenic targets of the humoral immune response in transplantation. *Curr Opin Immunol.* 2010;22(5):682-688.
87. Reindl-Schwaighofer R, Heinzel A, Kainz A, et al. Contribution of non-HLA incompatibility between donor and recipient to kidney allograft survival: genome-wide analysis in a prospective cohort. *Lancet.* 2019;393(10174):910-917.
88. Delville M, Lamarthee B, Pagie S, et al. Early Acute Microvascular Kidney Transplant Rejection in the Absence of Anti-HLA Antibodies Is Associated with Preformed IgG Antibodies against Diverse Glomerular Endothelial Cell Antigens. *J Am Soc Nephrol.* 2019;30(4):692-709.
89. Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A.* 1994;91(14):6259-6263.

90. Zwirner NW, Marcos CY, Mirbaha F, Zou Y, Stastny P. Identification of MICA as a new polymorphic alloantigen recognized by antibodies in sera of organ transplant recipients. *Hum Immunol.* 2000;61(9):917-924.
91. Mizutani K, Terasaki P, Rosen A, et al. Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure. *Am J Transplant.* 2005;5(9):2265-2272.
92. Terasaki PI, Ozawa M, Castro R. Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival. *Am J Transplant.* 2007;7(2):408-415.
93. Zou Y, Stastny P, Susal C, Dohler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. *N Engl J Med.* 2007;357(13):1293-1300.
94. Paul LC, Baldwin WM, 3rd, van Es LA. Vascular endothelial alloantigens in renal transplantation. *Transplantation.* 1985;40(2):117-123.
95. Sun Q, Liu Z, Yin G, Chen H, Chen J, Li L. Detectable circulating antiendothelial cell antibodies in renal allograft recipients with C4d-positive acute rejection: a report of three cases. *Transplantation.* 2005;79(12):1759-1762.
96. Sun Q, Liu Z, Chen J, et al. Circulating anti-endothelial cell antibodies are associated with poor outcome in renal allograft recipients with acute rejection. *Clin J Am Soc Nephrol.* 2008;3(5):1479-1486.
97. Glotz D, Lucchiari N, Pegaz-Fiornet B, Suberbielle-Boissel C. Endothelial cells as targets of allograft rejection. *Transplantation.* 2006;82(1 Suppl):S19-21.
98. Ferry BL, Welsh KI, Dunn MJ, et al. Anti-cell surface endothelial antibodies in sera from cardiac and kidney transplant recipients: association with chronic rejection. *Transpl Immunol.* 1997;5(1):17-24.
99. Grandtnerova B, Mackova N, Hovoricova B, Jahnova E. Hyperacute rejection of living related kidney grafts caused by endothelial cell-specific antibodies: case reports. *Transplant Proc.* 2008;40(7):2422-2424.
100. Castillo S, Revelen R, Bordron A, Renaudineau Y, Dueymes M, Youinou P. Anti-endothelial cell reactivity, the unresolved enigma. *Int J Immunopathol Pharmacol.* 2001;14(2):109-118.
101. Jackson AM, Lucas DP, Melancon JK, Desai NM. Clinical relevance and IgG subclass determination of non-HLA antibodies identified using endothelial cell precursors isolated from donor blood. *Transplantation.* 2011;92(1):54-60.
102. Jackson AM, Kuperman MB, Montgomery RA. Multiple hyperacute rejections in the absence of detectable complement activation in a patient with endothelial cell reactive antibody. *Am J Transplant.* 2012;12(6):1643-1649.
103. Dragun D, Muller DN, Brasen JH, et al. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *N Engl J Med.* 2005;352(6):558-569.
104. Hiemann NE, Meyer R, Wellnhofer E, et al. Non-HLA antibodies targeting vascular receptors enhance alloimmune response and microvasculopathy after heart transplantation. *Transplantation.* 2012;94(9):919-924.
105. Lefaucheur CV, D; Aubert, O; Philippe, A; Halloran, P; Loupy A; Dragun, D. Anti-angiotensin II type1-receptor antibodies (AT1R-Ab) Induce a Specific Phenotype of Rejection Disinct from HLA antibody-Mediated Rejection. Transplantion; 2018.
106. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. *N Engl J Med.* 1969;280(14):735-739.
107. Pena JR, Fitzpatrick D, Saidman SL. Complement-dependent cytotoxicity crossmatch. *Methods Mol Biol.* 2013;1034:257-283.

108. Vaidya S, Ruth J. Contributions and clinical significance of IgM and autoantibodies in highly sensitized renal allograft recipients. *Transplantation.* 1989;47(6):956-958.
109. Bray RA, Lebeck LK, Gebel HM. The flow cytometric crossmatch. Dual-color analysis of T cell and B cell reactivities. *Transplantation.* 1989;48(5):834-840.
110. El-Awar N, Lee J, Terasaki PI. HLA antibody identification with single antigen beads compared to conventional methods. *Hum Immunol.* 2005;66(9):989-997.
111. Martins TB. Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clin Diagn Lab Immunol.* 2002;9(1):41-45.
112. Leffell MS. Real benefits from virtual crossmatches. *Transplantation.* 2010;89(2):138-139.
113. Tuttle-Newhall JE, Krishnan SM, Levy MF, McBride V, Orlowski JP, Sung RS. Organ donation and utilization in the United States: 1998-2007. *Am J Transplant.* 2009;9(4 Pt 2):879-893.
114. Feucht HE, Schneeberger H, Hillebrand G, et al. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int.* 1993;43(6):1333-1338.
115. Mauiyyedi S, Crespo M, Collins AB, et al. Acute humoral rejection in kidney transplantation: II. Morphology, immunopathology, and pathologic classification. *J Am Soc Nephrol.* 2002;13(3):779-787.
116. Mauiyyedi S, Pelle PD, Saidman S, et al. Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J Am Soc Nephrol.* 2001;12(3):574-582.
117. Kayler LK, Kiss L, Sharma V, et al. Acute renal allograft rejection: diagnostic significance of focal peritubular capillary C4d. *Transplantation.* 2008;85(6):813-820.
118. Haririan A, Kiangkitiwan B, Kukuruga D, et al. The impact of c4d pattern and donor-specific antibody on graft survival in recipients requiring indication renal allograft biopsy. *Am J Transplant.* 2009;9(12):2758-2767.
119. Loupy A, Hill GS, Suberbielle C, et al. Significance of C4d Banff scores in early protocol biopsies of kidney transplant recipients with preformed donor-specific antibodies (DSA). *Am J Transplant.* 2011;11(1):56-65.
120. Haas M, Mirocha J. Early ultrastructural changes in renal allografts: correlation with antibody-mediated rejection and transplant glomerulopathy. *Am J Transplant.* 2011;11(10):2123-2131.
121. Sis B, Jhangri GS, Riopel J, et al. A new diagnostic algorithm for antibody-mediated microcirculation inflammation in kidney transplants. *Am J Transplant.* 2012;12(5):1168-1179.
122. Trpkov K, Campbell P, Pazderka F, Cockfield S, Solez K, Halloran PF. Pathologic features of acute renal allograft rejection associated with donor-specific antibody, Analysis using the Banff grading schema. *Transplantation.* 1996;61(11):1586-1592.
123. Halloran PF, Schlaut J, Solez K, Srinivasa NS. The significance of the anti-class I response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. *Transplantation.* 1992;53(3):550-555.
124. Halloran PF, Wadgymar A, Ritchie S, Falk J, Solez K, Srinivasa NS. The significance of the anti-class I antibody response. I. Clinical and pathologic features of anti-class I-mediated rejection. *Transplantation.* 1990;49(1):85-91.
125. Haas M. An updated Banff schema for diagnosis of antibody-mediated rejection in renal allografts. *Curr Opin Organ Transplant.* 2014;19(3):315-322.

126. Gupta A, Broin PO, Bao Y, et al. Clinical and molecular significance of microvascular inflammation in transplant kidney biopsies. *Kidney Int.* 2016;89(1):217-225.
127. Sellares J, Reeve J, Loupy A, et al. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. *Am J Transplant.* 2013;13(4):971-983.
128. Sellares J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. *Am J Transplant.* 2012;12(2):388-399.
129. Solez K, Colvin RB, Racusen LC, et al. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant.* 2008;8(4):753-760.
130. Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant.* 2007;7(3):518-526.
131. Aubert O, Higgins S, Bouatou Y, et al. Archetype Analysis Identifies Distinct Profiles in Renal Transplant Recipients with Transplant Glomerulopathy Associated with Allograft Survival. *J Am Soc Nephrol.* 2019;30(4):625-639.
132. Banfi G, Villa M, Cresseri D, Ponticelli C. The clinical impact of chronic transplant glomerulopathy in cyclosporine era. *Transplantation.* 2005;80(10):1392-1397.
133. Gloor JM, Sethi S, Stegall MD, et al. Transplant glomerulopathy: subclinical incidence and association with alloantibody. *Am J Transplant.* 2007;7(9):2124-2132.
134. Bentall A, Cornell LD, Gloor JM, et al. Five-year outcomes in living donor kidney transplants with a positive crossmatch. *Am J Transplant.* 2013;13(1):76-85.
135. Redfield RR, Ellis TM, Zhong W, et al. Current outcomes of chronic active antibody mediated rejection - A large single center retrospective review using the updated BANFF 2013 criteria. *Hum Immunol.* 2016;77(4):346-352.
136. Choi J, Aubert O, Vo A, et al. Assessment of Tocilizumab (Anti-Interleukin-6 Receptor Monoclonal) as a Potential Treatment for Chronic Antibody-Mediated Rejection and Transplant Glomerulopathy in HLA-Sensitized Renal Allograft Recipients. *Am J Transplant.* 2017;17(9):2381-2389.
137. Roufosse C, Simmonds N, Clahsen-van Groningen M, et al. A 2018 Reference Guide to the Banff Classification of Renal Allograft Pathology. *Transplantation.* 2018;102(11):1795-1814.
138. Monga G, Mazzucco G, Novara R, Reale L. Intertubular capillary changes in kidney allografts: an ultrastructural study in patients with transplant glomerulopathy. *Ultrastruct Pathol.* 1990;14(3):201-209.
139. Wavamunno MD, O'Connell PJ, Vitalone M, et al. Transplant glomerulopathy: ultrastructural abnormalities occur early in longitudinal analysis of protocol biopsies. *Am J Transplant.* 2007;7(12):2757-2768.
140. de Kort H, Willicombe M, Brookes P, et al. Peritubular Capillary Basement Membrane Multilayering in Renal Allograft Biopsies of Patients With De Novo Donor-Specific Antibodies. *Transplantation.* 2016;100(4):889-897.
141. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised Diagnostic Criteria for Chronic Active T Cell-Mediated Rejection, Antibody-Mediated Rejection, and Prospects for Integrative Endpoints for Next-Generation Clinical Trials. *Am J Transplant.* 2017.
142. Loupy A, Vernerey D, Tinel C, et al. Subclinical Rejection Phenotypes at 1 Year Post-Transplant and Outcome of Kidney Allografts. *J Am Soc Nephrol.* 2015;26(7):1721-1731.

143. Gosset C, Viglietti D, Rabant M, et al. Circulating donor-specific anti-HLA antibodies are a major factor in premature and accelerated allograft fibrosis. *Kidney Int.* 2017;92(3):729-742.
144. Senev A, Coemans M, Lerut E, et al. Histological picture of antibody-mediated rejection without donor-specific anti-HLA antibodies: Clinical presentation and implications for outcome. *Am J Transplant.* 2018.
145. Martin L, Guignier F, Mousson C, Rageot D, Justrabo E, Rifle G. Detection of donor-specific anti-HLA antibodies with flow cytometry in eluates and sera from renal transplant recipients with chronic allograft nephropathy. *Transplantation.* 2003;76(2):395-400.
146. Snanoudj R, Claas FH, Heidt S, Legendre C, Chatenoud L, Candon S. Restricted specificity of peripheral alloreactive memory B cells in HLA-sensitized patients awaiting a kidney transplant. *Kidney Int.* 2015;87(6):1230-1240.
147. Lucia M, Luque S, Crespo E, et al. Preformed circulating HLA-specific memory B cells predict high risk of humoral rejection in kidney transplantation. *Kidney Int.* 2015;88(4):874-887.
148. Duquesnoy RJ. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. I. Description of the algorithm. *Hum Immunol.* 2002;63(5):339-352.
149. El-Awar N, Terasaki PI, Cai J, et al. Epitopes of HLA-A, B, C, DR, DQ, DP and MICA antigens. *Clin Transpl.* 2009:295-321.
150. Schellekens J, Vanderlocht J, Groeneweg M, et al. The Maastricht Transplant Center: clinical setting and epitope searches in HLA class II molecules: does the structural localization of a polymorphic site contribute to its immunogenicity? *Transpl Immunol.* 2014;31(4):213-218.
151. Milongo D, Kamar N, Del Bello A, et al. Allelic and Epitopic Characterization of Intra-Kidney Allograft Anti-HLA Antibodies at Allograft Nephrectomy. *Am J Transplant.* 2017;17(2):420-431.
152. Suciu-Foca N, Ho E, King DW, et al. Soluble HLA and anti-idiotypic antibodies in transplantation: modulation of anti-HLA antibodies by soluble HLA antigens from the graft and anti-idiotypic antibodies in renal and cardiac allograft recipients. *Transplant Proc.* 1991;23(1 Pt 1):295-296.
153. Zhang Q, Reed EF. The importance of non-HLA antibodies in transplantation. *Nat Rev Nephrol.* 2016;12(8):484-495.
154. Hayde N, Bao Y, Pullman J, et al. The clinical and genomic significance of donor-specific antibody-positive/C4d-negative and donor-specific antibody-negative/C4d-negative transplant glomerulopathy. *Clin J Am Soc Nephrol.* 2013;8(12):2141-2148.
155. Sis B, Jhangri GS, Bunnag S, Allanach K, Kaplan B, Halloran PF. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. *Am J Transplant.* 2009;9(10):2312-2323.
156. Adam B, Afzali B, Dominy KM, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. *Clin Transplant.* 2016;30(3):295-305.
157. Halloran PF. T cell-mediated rejection of kidney transplants: a personal viewpoint. *Am J Transplant.* 2010;10(5):1126-1134.

158. Halloran PF, Pereira AB, Chang J, et al. Potential impact of microarray diagnosis of T cell-mediated rejection in kidney transplants: The INTERCOM study. *Am J Transplant.* 2013;13(9):2352-2363.
159. Willicombe M, Roufosse C, Brookes P, et al. Acute cellular rejection: impact of donor-specific antibodies and C4d. *Transplantation.* 2014;97(4):433-439.
160. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. *N Engl J Med.* 2010;363(15):1451-1462.
161. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol.* 2002;13(1):234-241.
162. Sis B, Einecke G, Chang J, et al. Cluster analysis of lesions in nonselected kidney transplant biopsies: microcirculation changes, tubulointerstitial inflammation and scarring. *Am J Transplant.* 2010;10(2):421-430.
163. Lefaucheur C, Loupy A, Vernerey D, et al. Antibody-mediated vascular rejection of kidney allografts: a population-based study. *Lancet.* 2013;381(9863):313-319.
164. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.
165. Willicombe M, Blow M, Santos-Nunez E, Freeman C, Brookes P, Taube D. Terasaki Epitope Mismatch Burden Predicts the Development of De Novo DQ Donor-Specific Antibodies and are Associated With Adverse Allograft Outcomes. *Transplantation.* 2018;102(1):127-134.
166. de Kort H, Willicombe M, Brookes P, et al. Microcirculation inflammation associates with outcome in renal transplant patients with de novo donor-specific antibodies. *Am J Transplant.* 2013;13(2):485-492.
167. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation.* 2013;95(3):410-417.
168. Hidalgo LG, Campbell PM, Sis B, et al. De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. *Am J Transplant.* 2009;9(11):2532-2541.
169. Haas M. The relationship between pathologic lesions of active and chronic antibody-mediated rejection in renal allografts. *Am J Transplant.* 2018;18(12):2849-2856.
170. Marfo K, Lu A, Ling M, Akalin E. Desensitization protocols and their outcome. *Clin J Am Soc Nephrol.* 2011;6(4):922-936.
171. Khovanova N, Daga S, Shaikhina T, et al. Subclass analysis of donor HLA-specific IgG in antibody-incompatible renal transplantation reveals a significant association of IgG4 with rejection and graft failure. *Transpl Int.* 2015;28(12):1405-1415.
172. Sis B, Campbell PM, Mueller T, et al. Transplant glomerulopathy, late antibody-mediated rejection and the ABCD tetrad in kidney allograft biopsies for cause. *Am J Transplant.* 2007;7(7):1743-1752.
173. Issa N, Cosio FG, Gloor JM, et al. Transplant glomerulopathy: risk and prognosis related to anti-human leukocyte antigen class II antibody levels. *Transplantation.* 2008;86(5):681-685.
174. Kaczmarek I, Deutsch MA, Kauke T, et al. Donor-specific HLA alloantibodies: long-term impact on cardiac allograft vasculopathy and mortality after heart transplant. *Exp Clin Transplant.* 2008;6(3):229-235.

175. Palmer SM, Davis RD, Hadjiliadis D, et al. Development of an antibody specific to major histocompatibility antigens detectable by flow cytometry after lung transplant is associated with bronchiolitis obliterans syndrome. *Transplantation.* 2002;74(6):799-804.
176. Walsh RC, Everly JJ, Brailey P, et al. Proteasome inhibitor-based primary therapy for antibody-mediated renal allograft rejection. *Transplantation.* 2010;89(3):277-284.
177. Freitas MC, Rebellato LM, Ozawa M, et al. The role of immunoglobulin-G subclasses and C1q in de novo HLA-DQ donor-specific antibody kidney transplantation outcomes. *Transplantation.* 2013;95(9):1113-1119.
178. Kaneku H, O'Leary JG, Taniguchi M, Susskind BM, Terasaki PI, Klintmalm GB. Donor-specific human leukocyte antigen antibodies of the immunoglobulin G3 subclass are associated with chronic rejection and graft loss after liver transplantation. *Liver Transpl.* 2012;18(8):984-992.
179. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol.* 2014;5:520.
180. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. *Immunity.* 2006;24(1):19-28.
181. Yeap WH, Wong KL, Shimasaki N, et al. CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes. *Sci Rep.* 2016;6:34310.
182. Walker MR, Woof JM, Bruggemann M, Jefferis R, Burton DR. Interaction of human IgG chimeric antibodies with the human FcRI and FcRII receptors: requirements for antibody-mediated host cell-target cell interaction. *Mol Immunol.* 1989;26(4):403-411.
183. Arnold ML, Ntokou IS, Doxiadis, II, Spriewald BM, Boletis JN, Iniotaki AG. Donor-specific HLA antibodies: evaluating the risk for graft loss in renal transplant recipients with isotype switch from complement fixing IgG1/IgG3 to noncomplement fixing IgG2/IgG4 anti-HLA alloantibodies. *Transpl Int.* 2014;27(3):253-261.
184. Kushihata F, Watanabe J, Mulder A, Claas F, Scornik JC. Human leukocyte antigen antibodies and human complement activation: role of IgG subclass, specificity, and cytotoxic potential. *Transplantation.* 2004;78(7):995-1001.
185. Honger G, Amico P, Arnold ML, Spriewald BM, Schaub S. Effects of weak/non-complement-binding HLA antibodies on C1q-binding. *HLA.* 2017;90(2):88-94.
186. Schaub S, Honger G, Amico P. The complexity of the humoral immune response against HLA antigens. *Transpl Int.* 2014;27(3):249-250.
187. van der Zee JS, van Swieten P, Aalberse RC. Inhibition of complement activation by IgG4 antibodies. *Clin Exp Immunol.* 1986;64(2):415-422.
188. Ronco P, Debiec H. Molecular dissection of target antigens and nephritogenic antibodies in membranous nephropathy: towards epitope-driven therapies. *J Am Soc Nephrol.* 2006;17(7):1772-1774.
189. Griffiths EJ, Nelson RE, Dupont PJ, Warrens AN. Skewing of pretransplant anti-HLA class I antibodies of immunoglobulin G isotype solely toward immunoglobulin G1 subclass is associated with poorer renal allograft survival. *Transplantation.* 2004;77(11):1771-1773.
190. Honger G, Hopfer H, Arnold ML, Spriewald BM, Schaub S, Amico P. Pretransplant IgG subclasses of donor-specific human leukocyte antigen antibodies and development of antibody-mediated rejection. *Transplantation.* 2011;92(1):41-47.

191. Chin C, Chen G, Sequeria F, et al. Clinical usefulness of a novel C1q assay to detect immunoglobulin G antibodies capable of fixing complement in sensitized pediatric heart transplant patients. *J Heart Lung Transplant.* 2011;30(2):158-163.
192. Sutherland SM, Chen G, Sequeira FA, Lou CD, Alexander SR, Tyan DB. Complement-fixing donor-specific antibodies identified by a novel C1q assay are associated with allograft loss. *Pediatr Transplant.* 2012;16(1):12-17.
193. Calp-Inal S, Ajaimy M, Melamed ML, et al. The prevalence and clinical significance of C1q-binding donor-specific anti-HLA antibodies early and late after kidney transplantation. *Kidney Int.* 2016;89(1):209-216.
194. Yabu JM, Higgins JP, Chen G, Sequeira F, Busque S, Tyan DB. C1q-fixing human leukocyte antigen antibodies are specific for predicting transplant glomerulopathy and late graft failure after kidney transplantation. *Transplantation.* 2011;91(3):342-347.
195. Thammanichanond D, Mongkolsuk T, Rattanasiri S, et al. Significance of C1q-fixing donor-specific antibodies after kidney transplantation. *Transplant Proc.* 2014;46(2):368-371.
196. Kulkarni S, Kirkiles-Smith NC, Deng YH, et al. Eculizumab Therapy for Chronic Antibody-Mediated Injury in Kidney Transplant Recipients: A Pilot Randomized Controlled Trial. *Am J Transplant.* 2017;17(3):682-691.
197. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant.* 2014;14(2):272-283.
198. Claisse G, Absi L, Cognasse F, Alamartine E, Mariat C, Maillard N. Relationship between Mean Fluorescence Intensity and C1q/C3d-fixing capacities of anti-HLA antibodies. *Hum Immunol.* 2017;78(4):336-341.
199. Heinemann FM, Roth I, Rebmann V, Arnold ML, Spriewald BM, Grosse-Wilde H. Characterization of anti-HLA antibodies eluted from explanted renal allografts. *Clin Transpl.* 2006:371-378.
200. Takemoto SK, Zeevi A, Feng S, et al. National conference to assess antibody-mediated rejection in solid organ transplantation. *Am J Transplant.* 2004;4(7):1033-1041.
201. Cooper JE, Gralla J, Cagle L, Goldberg R, Chan L, Wiseman AC. Inferior kidney allograft outcomes in patients with de novo donor-specific antibodies are due to acute rejection episodes. *Transplantation.* 2011;91(10):1103-1109.
202. Willicombe M, Brookes P, Sergeant R, et al. De novo DQ donor-specific antibodies are associated with a significant risk of antibody-mediated rejection and transplant glomerulopathy. *Transplantation.* 2012;94(2):172-177.
203. DeVos JM, Gaber AO, Knight RJ, et al. Donor-specific HLA-DQ antibodies may contribute to poor graft outcome after renal transplantation. *Kidney Int.* 2012;82(5):598-604.
204. Arreola-Guerra JM, Morales-Buenrostro LE, Granados J, et al. Anti-HLA-DQ antibodies are highly and independently related to the C1q-binding capacity of HLA antibodies. *Transpl Immunol.* 2017;41:10-16.
205. Taylor CJ, Kosmoliaptsis V, Martin J, et al. Technical Limitations of the C1q Single-Antigen Bead Assay to Detect Complement Binding HLA-Specific Antibodies. *Transplantation.* 2017;101(6):1206-1214.

206. Zeevi A, Lunz J, Feingold B, et al. Persistent strong anti-HLA antibody at high titer is complement binding and associated with increased risk of antibody-mediated rejection in heart transplant recipients. *J Heart Lung Transplant.* 2013;32(1):98-105.
207. Diebolder CA, Beurskens FJ, de Jong RN, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science.* 2014;343(6176):1260-1263.
208. Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. HLA antibody specification using single-antigen beads--a technical solution for the prozone effect. *Transplantation.* 2011;92(5):510-515.
209. Schaub S, Honger G, Koller MT, Liwski R, Amico P. Determinants of C1q binding in the single antigen bead assay. *Transplantation.* 2014;98(4):387-393.
210. Tangye SG, Ferguson A, Avery DT, Ma CS, Hodgkin PD. Isotype switching by human B cells is division-associated and regulated by cytokines. *J Immunol.* 2002;169(8):4298-4306.
211. Germain RN, Ashwell JD, Lechler RI, et al. "Exon-shuffling" maps control of antibody- and T-cell-recognition sites to the NH2-terminal domain of the class II major histocompatibility polypeptide A beta. *Proc Natl Acad Sci U S A.* 1985;82(9):2940-2944.
212. El-Awar N, Nguyen A, Almeshari K, et al. HLA class II DQA and DQB epitopes: recognition of the likely binding sites of HLA-DQ alloantibodies eluted from recombinant HLA-DQ single antigen cell lines. *Hum Immunol.* 2013;74(9):1141-1152.
213. Imai N, Nishi S, Alchi B, et al. Immunohistochemical evidence of activated lectin pathway in kidney allografts with peritubular capillary C4d deposition. *Nephrol Dial Transplant.* 2006;21(9):2589-2595.
214. Loupy A, Suberbielle-Boissel C, Hill GS, et al. Outcome of subclinical antibody-mediated rejection in kidney transplant recipients with preformed donor-specific antibodies. *Am J Transplant.* 2009;9(11):2561-2570.
215. Haas M, Montgomery RA, Segev DL, et al. Subclinical acute antibody-mediated rejection in positive crossmatch renal allografts. *Am J Transplant.* 2007;7(3):576-585.
216. Matsuda Y, Imamura R, Takahara S. Evaluation of Antigen-Specific IgM and IgG Production during an In Vitro Peripheral Blood Mononuclear Cell Culture Assay. *Front Immunol.* 2017;8:794.
217. Olson PR, Weiblen BJ, O'Leary JJ, Moscowitz AJ, McCullough J. A simple technique for the inactivation of IgM antibodies using dithiothreitol. *Vox Sang.* 1976;30(2):149-159.
218. Barger B, Shroyer TW, Hudson SL, et al. Successful renal allografts in recipients with crossmatch-positive, dithioerythritol-treated negative sera. Race, transplant history, and HLA-DR1 phenotype. *Transplantation.* 1989;47(2):240-245.
219. Barger BO, Fotino M, Zmijewski CM, LeFor WM. Positive crossmatches against mandatorily shared kidneys. *Transplantation.* 1992;54(2):254-257.
220. Khan N, Robson AJ, Worthington JE, Martin S. The detection and definition of IgM alloantibodies in the presence of IgM autoantibodies using flowPRA beads. *Hum Immunol.* 2003;64(6):593-599.
221. Kerman RH, Susskind B, Buyse I, et al. Flow cytometry-detected IgG is not a contraindication to renal transplantation: IgM may be beneficial to outcome. *Transplantation.* 1999;68(12):1855-1858.
222. Donaldson PT, Thomson LJ, Heads A, et al. IgG donor-specific crossmatches are not associated with graft rejection or poor graft survival after liver transplantation. An

assessment by cytotoxicity and flow cytometry. *Transplantation.* 1995;60(9):1016-1023.
223. Lietz K, John R, Burke E, et al. Immunoglobulin M-to-immunoglobulin G anti-human leukocyte antigen class II antibody switching in cardiac transplant recipients is associated with an increased risk of cellular rejection and coronary artery disease. *Circulation.* 2005;112(16):2468-2476.
224. Stastny P, Ring S, Lu C, Arenas J, Han M, Lavingia B. Role of immunoglobulin (Ig)-G and IgM antibodies against donor human leukocyte antigens in organ transplant recipients. *Hum Immunol.* 2009;70(8):600-604.
225. Lopez LR, Santos ME, Espinoza LR, La Rosa FG. Clinical significance of immunoglobulin A versus immunoglobulins G and M anti-cardiolipin antibodies in patients with systemic lupus erythematosus. Correlation with thrombosis, thrombocytopenia, and recurrent abortion. *Am J Clin Pathol.* 1992;98(4):449-454.
226. Roelen DL, van Bree J, Witvliet MD, et al. IgG antibodies against an HLA antigen are associated with activated cytotoxic T cells against this antigen, IgM are not. *Transplantation.* 1994;57(9):1388-1392.
227. Visentin J, Guidicelli G, Couzi L, et al. Deciphering IgM interference in IgG anti-HLA antibody detection with flow beads assays. *Hum Immunol.* 2016;77(11):1048-1054.
228. Labarrere CA, Pitts D, Halbrook H, Faulk WP. Immunoglobulin M antibodies in transplanted human hearts. *J Heart Lung Transplant.* 1993;12(3):394-402.
229. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B cell response to renal transplantation. *Am J Transplant.* 2013;13(7):1713-1723.
230. Deo SS, Mistry KJ, Kakade AM, Niphadkar PV. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India.* 2010;27(2):66-71.
231. Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol.* 1983;130(2):722-726.
232. Itoh S, Nakae S, Velotta JB, et al. The role of recipient mast cells in acute and chronic cardiac allograft rejection in C57BL/6-KitW-sh/W-sh mice. *J Heart Lung Transplant.* 2010;29(4):401-409.
233. Jungraithmayr W. The putative role of mast cells in lung transplantation. *Am J Transplant.* 2015;15(3):594-600.
234. Papadimitriou JC, Drachenberg CB, Ramos E, Ugarte R, Haririan A. Mast cell quantitation in renal transplant biopsy specimens as a potential marker for the cumulative burden of tissue injury. *Transplant Proc.* 2013;45(4):1469-1471.
235. Roberts IS, Brenchley PE. Mast cells: the forgotten cells of renal fibrosis. *J Clin Pathol.* 2000;53(11):858-862.
236. Lucisano G, Brookes P, Santos-Nunez E, et al. Allosensitization after transplant failure: the role of graft nephrectomy and immunosuppression - a retrospective study. *Transpl Int.* 2019.
237. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. *Nat Rev Immunol.* 2014;14(4):247-259.
238. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. *J Allergy Clin Immunol.* 2012;129(3):635-645.
239. Kraft S, Kinet JP. New developments in FcepsilonRI regulation, function and inhibition. *Nat Rev Immunol.* 2007;7(5):365-378.

240. Valenta R, Mittermann I, Werfel T, Garn H, Renz H. Linking allergy to autoimmune disease. *Trends Immunol.* 2009;30(3):109-116.
241. Charles N, Hardwick D, Daugas E, Illei GG, Rivera J. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *Nat Med.* 2010;16(6):701-707.
242. Goldman M, Le Moine A, Braun M, Flamand V, Abramowicz D. A role for eosinophils in transplant rejection. *Trends Immunol.* 2001;22(5):247-251.
243. Sadeghi M, Daniel V, Weimer R, Wiesel M, Hergesell O, Opelz G. Pre-transplant Th1 and post-transplant Th2 cytokine patterns are associated with early acute rejection in renal transplant recipients. *Clin Transplant.* 2003;17(2):151-157.
244. Chaudhuri J, Alt FW. Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. *Nat Rev Immunol.* 2004;4(7):541-552.
245. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol.* 2003;3(9):721-732.
246. He JS, Meyer-Hermann M, Xiangying D, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. *J Exp Med.* 2013;210(12):2755-2771.
247. Erazo A, Kutchukhidze N, Leung M, et al. Unique maturation program of the IgE response in vivo. *Immunity.* 2007;26(2):191-203.
248. Zack DJ, Stempniak M, Wong AL, Weisbart RH. Localization of an Fc-binding reactivity to the constant region of human IgG4. Implications for the pathogenesis of rheumatoid arthritis. *J Immunol.* 1995;155(10):5057-5063.
249. Punnonen J, Aversa G, Cocks BG, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci U S A.* 1993;90(8):3730-3734.
250. Jabara HH, Loh R, Ramesh N, Vercelli D, Geha RS. Sequential switching from mu to epsilon via gamma 4 in human B cells stimulated with IL-4 and hydrocortisone. *J Immunol.* 1993;151(9):4528-4533.
251. Tay SS, Plain KM, Bishop GA. Role of IL-4 and Th2 responses in allograft rejection and tolerance. *Curr Opin Organ Transplant.* 2009;14(1):16-22.
252. Yousem SA. The potential role of mast cells in lung allograft rejection. *Hum Pathol.* 1997;28(2):179-182.
253. Li QY, Raza-Ahmad A, MacAulay MA, et al. The relationship of mast cells and their secreted products to the volume of fibrosis in posttransplant hearts. *Transplantation.* 1992;53(5):1047-1051.
254. Greenstein SM, Katz JP, Hakki A, Simonian S, Sowinski J, Katz SM. Mast cells in ureters from rejected allografts. *Transplant Proc.* 1989;21(1 Pt 1):286-288.
255. Colvin RB, Dvorak HF. Letter: Basophils and mast cells in renal allograft rejection. *Lancet.* 1974;1(7850):212-214.
256. El-Refaie AM, Burt AD. Mast cells and c-Kit expression in liver allograft rejection. *Histopathology.* 2005;47(4):375-381.
257. Pardo J, Diaz L, Errasti P, et al. Mast cells in chronic rejection of human renal allografts. *Virchows Arch.* 2000;437(2):167-172.
258. Ishida T, Hyodo Y, Ishimura T, Takeda M, Hara I, Fujisawa M. Mast cell numbers and protease expression patterns in biopsy specimens following renal transplantation from living-related donors predict long-term graft function. *Clin Transplant.* 2005;19(6):817-824.

259. Nolan CR, Saenz KP, Thomas CA, 3rd, Murphy KD. Role of the eosinophil in chronic vascular rejection of renal allografts. *Am J Kidney Dis.* 1995;26(4):634-642.
260. Marone G, Patella V, de Crescenzo G, Granata F, Calabrese C. Immunological interactions between human eosinophils and cardiac mast cells. *Chem Immunol.* 2000;76:118-133.
261. Henault J, Riggs JM, Karnell JL, et al. Self-reactive IgE exacerbates interferon responses associated with autoimmunity. *Nat Immunol.* 2016;17(2):196-203.
262. Lu LF, Lind EF, Gondek DC, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature.* 2006;442(7106):997-1002.
263. Jobert A, Rao N, Deayton S, et al. Angiotensin II type 1 receptor antibody precipitating acute vascular rejection in kidney transplantation. *Nephrology (Carlton).* 2015;20 Suppl 1:10-12.
264. Willicombe M, Sandhu B, Brookes P, et al. Postanastomotic transplant renal artery stenosis: association with de novo class II donor-specific antibodies. *Am J Transplant.* 2014;14(1):133-143.
265. Arnold ML, Heinemann FM, Horn P, et al. 16(th) IHIW: anti-HLA alloantibodies of the of IgA isotype in re-transplant candidates. *Int J Immunogenet.* 2013;40(1):17-20.