Antibodies in

Kidney Transplantation

Andrew John Bentall

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Department of Nephrology

School of Immunity and Infection

College of Medical and Dental Sciences

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Abstract

The aim of this thesis is to examine the effect of anti-donor antibodies in the clinical management and outcomes of antibody incompatible kidney transplantation. Initial studies were conducted to improve measurement of anti-ABO specific blood group antibodies. The specificity of antibody binding to blood group antigens depended upon the assay platform and the nature of the core structure to which the blood group antigen was bound. A standardised haemagglutination assay was shown to produce excellent reproducibility, which was then applied to the analysis of samples derived from the ABOUT-K study. In this study of ABO incompatible kidney transplantation (ABOiKTx) in the UK, good clinical outcomes were achieved but there was wide variation in the method and result reported in local assays quantifying blood group antigen specific antibodies. This did not seem to alter graft survival within the limitations of a study of 100 patients however may have altered the exposure to treatment.

In a highly sensitised HLA incompatible kidney transplant recipients (HLAiKTx), I demonstrated long term outcomes were poor compared to a compatible cohort. In particular outcomes were worse with pre-formed donor specific anti-HLA Class II antibodies than with HLA class I alone. The histological injury of antibody damage occurred significantly earlier than with Class I antibodies. I then demonstrated that despite the activation of complement, anti-donor ABO specific antibodies did not display the same inflammatory phenotype in allograft biopsies as anti-HLA antibodies. Finally, the inhibition of terminal complement activation, whilst reducing early antibody-mediated rejection did not abrogate all inflammation. This resistance to inhibition of terminal complement activation may be contributed to by IgM DSA which may play a

role in cellular recruitment into the allograft but can be removed easily through plasma exchange.

Reproducible and standardised assays are needed for antibody assessment in order to make good clinical decisions to improve patient outcomes. Further studies are needed to stop production or block mechanisms of ongoing cellular infiltrate to improve patient outcomes.

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1. **A Bentall,** LD Cornell, JM Gloor, WD Park, MJ Gandhi, JL Winters, MF Chedid, PG Dean and MD Stegall. Five-Year Outcomes in Living Donor Kidney Transplants With a Positive Crossmatch. Am J Transplant. 2013 13(1) p76-85
2. **A. Bentall,** L.P. Herrera, L.D. Cornell, P.G.Dean, W. D. Park, M.J. Gandhi, J.L.Winters and M.D. Stegall. Differences in Chronic Intragraft Inflammation between Positive Crossmatch and ABO Incompatible Kidney Transplants. Transplantation. 2014 Jun 6. [Epub ahead of print].
3. **A Bentall,** DB Tyan, F Sequeira, M Everly, MJ Gandhi, LD Cornell, H Li, , NA Henderson, S Raghavaiah, JF Winters, PG Dean, MD Stegall. Antibody-Mediated Rejection Despite Inhibition of Terminal Complement. Transpl International. 2014 Jul 2. [Epub ahead of print].
4. ND Evans, D Lowe, D Briggs, R Higgins, **A Bentall,** S Ball, D Mitchell, D Zehnder and MJ Chappell. . Estimation of Antibody Binding Affinities with Surface Plasmon Resonance Experiments in Incompatible Blood Type Renal Transplants. 8th IFAC Symposium on Biological and Medical Systems, Budapest, Hungary, 29-31 August 2012

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1. **A Bentall**, M Braitch, N Mamode, D Briggs, N Kessaris & S Ball, Anti-ABO specific Assay **Variabilit**y Affects Antibody Removal in ABO incompatible Kidney Transplantation (ABOiKTx) – UK multicentre study, World Transplant Congress 2014. POSTER
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Abbreviations

ABOcKTx	ABO compatible Kidney Transplant
ABOi	ABO incompatible
ABOiKTx	ABO incompatible Kidney Transplant
AECA	Anti-endothelial cell antibodies
AHG	anti-human globulin
AMR	antibody-mediated rejection
BFXM	B cell flow cytometric crossmatch
CB	Super ChemiBlock
CDC	complement dependent cytotoxicity
CRF	calculated reaction frequency
DBD	Donation after Brain Death
DCD	Donation after Cardiac Death
DCGS	Death Censored Graft Survival
dDGF	dialysis dependent delayed graft function
DRT	Direct agglutination at Room Temperature
DSA	donor specific antibody
DTT	dithiothreitol
eAMR	early antibody-mediated rejection
EART	Extracorporeal Antibody Removal Treatment
EDC	N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
ESRF	End Stage Renal Failure
FBC	Full Blood Count
fDGF	functional delayed graft function
FSGS	focal segmental glomerulosclerosis

Fuc	L-fucose
FXM+	flow cytometric crossmatch positive
Gal	D-galactose
GBM	glomerular basement membrane
GDP	Guanosine diphosphate
Glc	D-glucose
GlcNAc	N-acetyl-D-glucosamine
HG	Haemagglutination
HLA	human leukocyte antigen
HLAiKTx	HLA antibody incompatible Kidney Transplantation
HSA	Human Serum Albumin
IAT	Indirect Anti-globin Test
IRB	Institutional Review Board
IVIG	intravenous immunoglobulin
KTx	Kidney Transplant
MAC	membrane attack complex
MBL	mannose binding lectin
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MICA	MHC-1 related chain-A
NEQAS	National External Quality Assessment Service
NHSBT	National Health Service Blood and Transplant
PE	plasma exchange
PRA	panel reactive antibodies
PTC	peritubular capillary
PTCitis	peritubular capillaritis
PTFE	polytetrafluoroethylene
PVA	Polyvinyl alcohol

PVP	Polyvinylpyrrolidone
PVX	Mixture of PVA and PVP at 1:1 ratio
RBC	Red Blood Cell
RPM	revolutions per minute
SAB	single antigen bead
S-NHS	N-Hydroxysulfosuccinimide sodium
SPR	surface plasmon resonance
TG	transplant glomerulopathy
UDP	Uridine diphosphate
-XMKTx	Negative Crossmatch Kidney Transplant
+XMKTx	Positive Crossmatch Kidney Transplant

Chapter 1 Introduction

1.1 End Stage Renal Failure in the UK

Chronic kidney disease significantly increases the mortality and morbidity in patients [1, 2]. The incidence of end stage renal failure (ESRF) has doubled in the UK since 1995 and is a growing international health problem [3]. This high mortality in patients on dialysis is significantly reduced following kidney transplantation [4]. Kidney transplantation has been shown to improve quality as well as length of life for recipients and this has driven increasing numbers of transplants being performed each year for an expanding recipient population [5-7]. The time from initiating dialysis to transplantation reduces the longevity of the kidney allograft and reduces the benefit with respect to mortality [8, 9]. The ability to pre-emptively transplant patients before commencing dialysis increases patient and allograft survival [10].

1.2 Live Donor Transplantation as a treatment option for ESRF

The first successful kidney transplantation took place in 1954, in Boston, USA [11, 12]. It was allowed due to the genetic identity of both recipient and donor, because they were identical twins. Previous attempts at renal transplantation, although technically surgically successful, had failed within the first fortnight due to graft rejection.

The current major barrier to organ transplantation is the lack of adequate numbers of available organs to potential recipients. The development of Donation after Cardiac Death (DCD) criteria deceased donor organs has provided an additional source of organs for transplantation. The application of DCD is growing, but there are still potential developments to improve outcomes. While Donation after Brain Death (DBD) kidney donation rate is static, an increase in the donation is beginning to bridge the gap between supply and demand [13]. Live donor kidney transplantation is beneficial in many ways, but significantly improves clinical outcomes for allograft survival, timing of

transplantation and health of the donor kidney without the consequences of brain stem death, peri-operative trauma and reduced ischaemic time. In the UK, about 30% of any potential unrelated live kidney donor and recipient pairs are ABO blood group incompatible in which cases the majority of recipients are blood group "O" and blood group "B". The median waiting time for deceased donor kidneys in the UK is higher in these blood groups compared to blood group "A" (1381 days, 1329 days and 925 days respectively, data according to NHSBT Dec 2013)[13].

1.3 Immunological barriers to transplantation

The blood group barrier to transplantation was discovered through early clinical experience in which incompatibility was associated with hyperacute rejection. This became an immediate absolute contraindication [12]. However, similar events could occur despite blood group compatibility and in 1960's Terasaki and colleagues developed a crossmatching tool to enable reactivity of donor lymphocytes and recipient serum to be assessed prior to transplant surgery [14, 15]. The combination of improved immunosuppression and increased tissue typing technology has improved the early survival of kidney transplants year on year [16]. An increasing number of patients with failed transplants are waiting for another kidney transplant, which means that although historically pregnancy and transfusion were the most common pathways to allo-sensitisation, there is increasing sensitisation through prior transplantation [17].

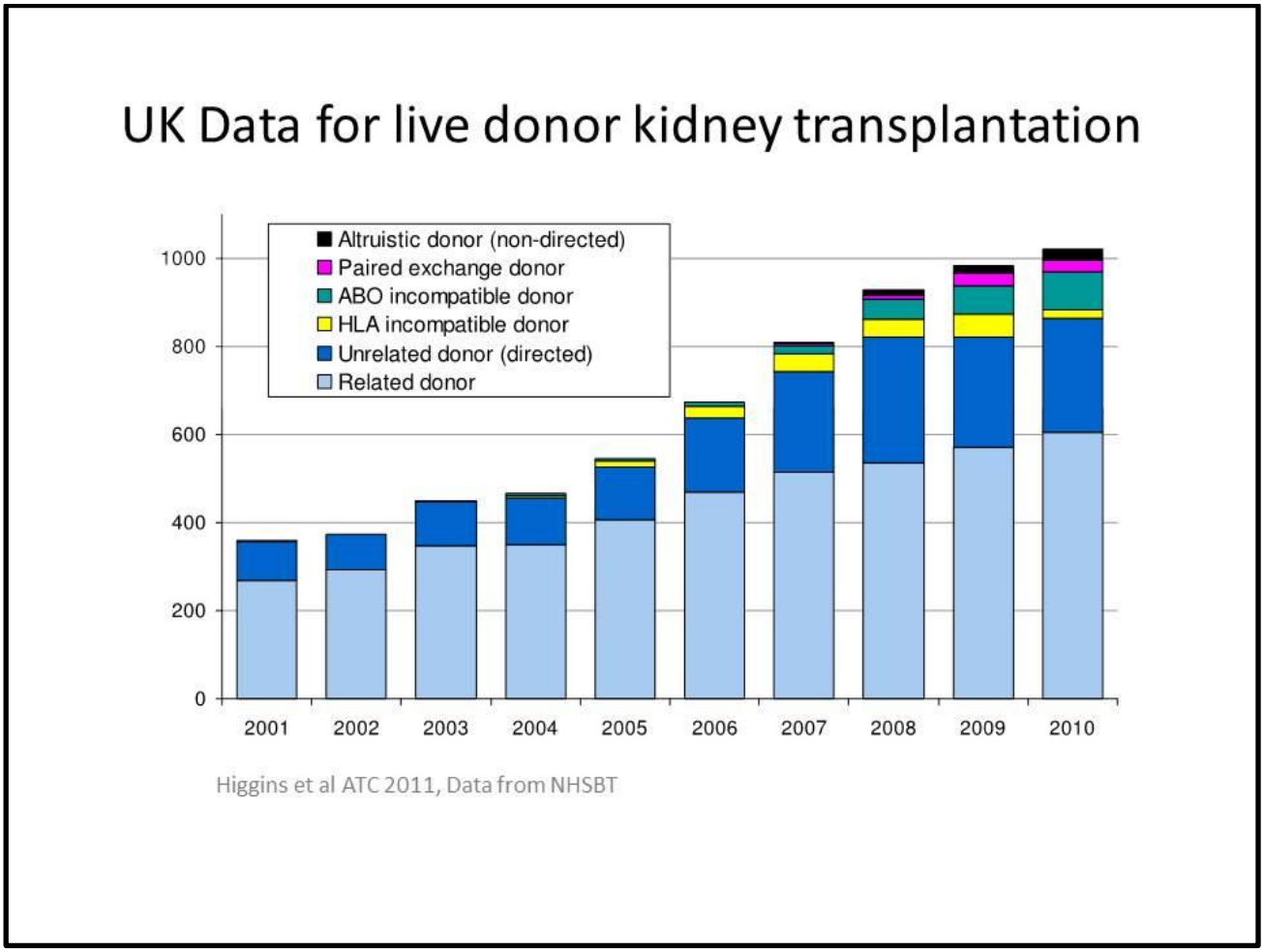
As the development of assays for detecting HLA antibodies in potential kidney recipients have become more sensitive, the task of matching donors and recipients has become increasingly complex. These immunological barriers may preclude transplantation in otherwise anatomically possible kidney transplantation.

Pooled exchange live kidney donation is an alternative for avoiding immunological risk, though this will not be the solution to all kidney transplant donor and recipient pairs [18, 19]. In the pooled exchange programmes, blood group 'O' recipients awaiting kidney transplantation will accumulate, as will the highly HLA-sensitized patients. As increased time on dialysis is associated with poorer allograft survival and increased mortality, timely transplantation is an important goal in improving patient outcomes [20].

Desensitization is the name given to the process of removing alloantibodies and preventing subsequent alloantibody production to allow successful transplantation [21, 22]. The application of different techniques for desensitization have been used to allow transplantation across these barriers, in order to achieve an antibody level at which the solid organ can be implanted without hyperacute rejection [23]. While techniques for desensitization vary between centres and between ABO incompatible or HLA incompatible transplantation, the practice in UK transplantation is growing (Figure 1.1). Published data have been mainly from single centres using differing techniques. The survival benefit of desensitization was shown in a large single centre cohort by Montgomery, who demonstrated that despite higher immunological risk, sensitized patients transplanted across immunological barriers had better patient survival than those who had a successful wait for a compatible transplant, or those who remained on dialysis [24].

Figure 1.1. UK Registry Data on Live Donor Kidney Transplantation.

The considerable increase in antibody incompatible kidney transplantation activity in the UK over the last 5 years is demonstrated. From 2004 to 2010 there is an increase from <1% to ~10% of total live donor kidney transplantation [25].



1.3.1 ABO Blood Group

1.3.1.1 Chemistry of A/B antigen formation

Chromosome 9 encodes for a glycosyl transferase which conjugates a terminal oligosaccharide onto the H antigen on cell surfaces (Figure 1.2A), following prior fucosyltransferase activity encoded on chromosome 19 [26]. Individuals of blood group 'A' have a terminal α-N-acetylgalactosamine molecule, whilst in blood group 'B' the terminal molecule is D-galactose (Figure 1.2B).

1.3.1.2 Development of anti-ABO specific antibodies

Anti-ABO specific antibodies are not produced at birth and it is postulated that anti-ABO specific antibodies develop secondary to exposure to exogenous carbohydrate antigens. The presence of anti-ABO specific antibodies is explained according to Landsteiner's rule. They develop against the A/B antigen that is absent in that individual [27]. The quantity of antibody present or titre increases with early childhood immune development [28]. The blood group "O" population has higher titres than blood group "A" or "B" populations although there is significant overlap, and the amount of antibody so quantified may be affected by age, gender and dietary intake [29, 30].

The delayed development of anti-ABO specific antibodies has been used for clinical benefit in ABO incompatible infant heart transplantation by West and colleagues who demonstrated that cardiac transplantation in very young infants had good clinical outcomes, despite them later developing anti-ABO specific antibodies [31]. Interestingly, blood group "O" recipient infants develop anti-ABO specific antibodies only to the antigen not present on the allograft, suggesting an immunologically tolerant phenotype.

Figure 1.2. Biochemical pathway and structure of A/B conjugation and antigen.

Diagrammatic process of terminal oligosaccharide conjugation and structure of the terminal sugar. [32]. Abbreviations: Fuc, L-fucose; Gal, D-galactose; GDP, Guanosine diphosphate; GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; UDP, Uridine diphosphate; N-acetylgalactosamine (GalNAc)

Figure 1.2A. Activity of H-transferase and A and B transferase for antigen production.

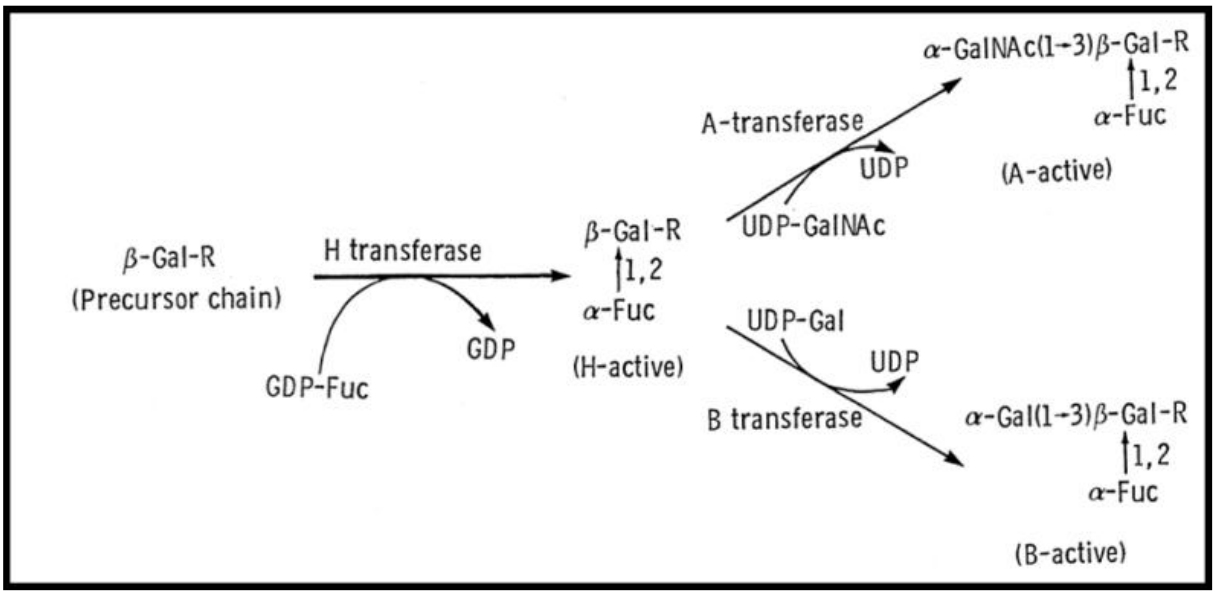
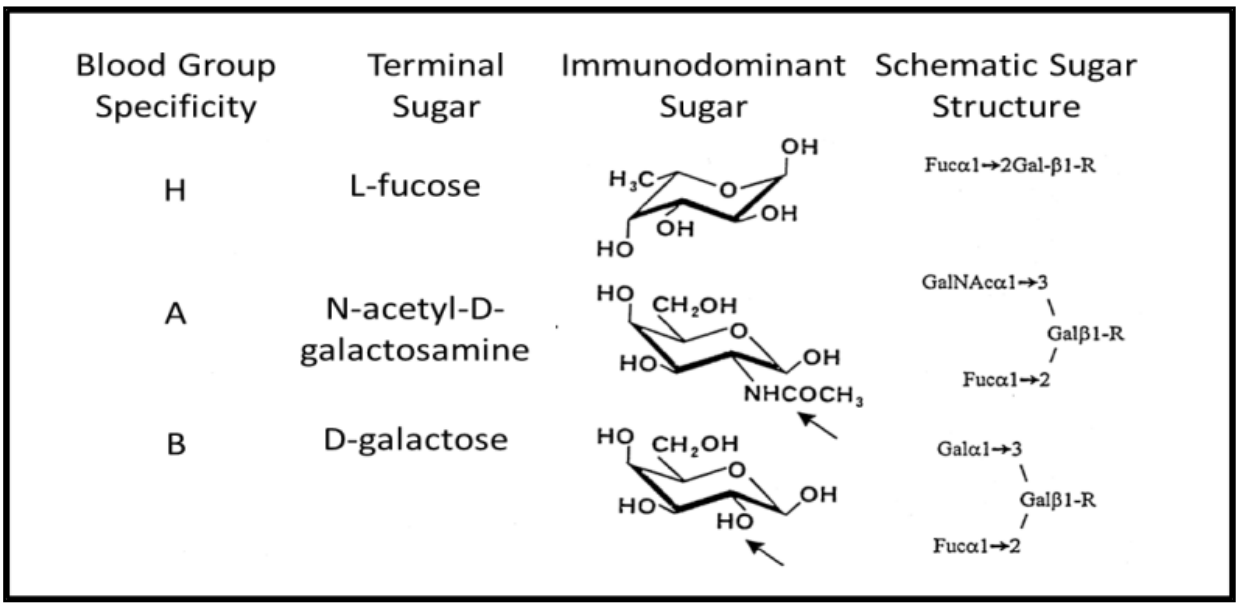


Figure 1.2B – ABO blood group antigen and structure with terminal sugar structure. Arrows demonstrate difference between A and B structure with N-acetylgalactosamine.



1.3.1.3 Subtyping of ABO Blood Group Individuals

ABO blood groups are divided into 4 main groups (O, A, B and AB), but these can be further sub-divided according to antigen density and structure. For blood group “A”, these are often identified as “A1” antigen or “non-A1”. The “A1” cohort can be identified directly by Helix pomatia lectin binding in haemagglutination assay, which is specific for “A1” antigen molecule N-acetyl-d-galactosamine. About 80% of blood group “A” individuals are “A1” which is differentiated by the concentration of the pre-dominant antigen which is of clinical importance. “Non-A1” individuals have fewer “A” epitope molecules on red cells and in the kidney [33]. The lower "A" antigen density is due to reduced levels of A transferase activity in “non-A1” blood groups (only 10%), compared to “A1”. Also this glycosyl transferase in “non-A1” individuals does not conjugate H antigen on with type 3 and type 4 structures. Blood group “AB” individuals express both A and B antigens, but since the action of these ‘A’ and ‘B’ transferases is competitive, with a fixed amount of cell surface H antigen, there is a reduction in A or B antigen density, relative to an individual in those who are homozygous or indeed compound heterozygoteswith a null allele [34].

1.3.1.4 ABO antigen and core structures

While anti-ABO specific antibody measurement will be explored quantitively and qualitatively in the next section, it is important to note that the red cell assay on which haemagglutination is based depends on the expression of A/B antigen on red cells. This is predominantly a type 2 chain structure. The core structures of A and B antigens in the kidney are not only different from the red cell, but different for each antigen. Breimer’s laboratory demonstrated that A antigen is predominantly a type 4 structure with small amounts of type 2 and minimal type 1 and type 3 involvement. However, B antigen is a

type 2 structure with type 4 being a relatively minor structure [35]. The distribution of core saccharide type structures in different tissues is shown in Table 1.1.

In ABOiKTx, clinical management is directed by the ability to monitor patients with serological tests following transplantation, which often demonstrate low levels of anti-ABO specific antibodies. However, follow up in the Japanese ABOiKTx cohort has demonstrated that over a longer period there is reduction in A/B antigen expression on biopsies of the donor kidneys and a profile similar to the recipient blood group is seen [36]. There is also a reduction in circulating A/B glycosyl transferase [37].

Table 1.1. Blood group core chain expression in different tissues

The core oligosaccharide chain expression varies significantly between red blood cells, kidneys and other visceral organs, adapted from Rydberg [38].

	Core Saccharide Chain Type			
Organ	Type 1	Type 2	Type 3	Type 4
Red Blood cell	+	+++	++	+
Vascular Tissue	++	+	++	-
Liver	++	-	-	-
Kidney	+	+	(+)	+++
Pancreas	+++	+	(+)	
Stomach	+++		-	-
Intestinal Epithelial Cells	+++	(+)	-	-

1.3.1.5 Clinical effect ABO Blood Groups

The majority of kidney transplant recipients are adults who have already developed anti-ABO specific antibodies. There are racial differences in ABO blood group distribution which poses additional barriers within ethnic communities where there is a more limited donor and recipient pairing or availability of deceased donor organs. Asian ethnic groups have a significantly higher blood group "B" population than European Caucasians and are over-represented in kidney transplant waiting lists [39]. In a small UK study, there was no major difference in anti-A titre in blood group "B" Asian patients waiting for a transplant compared to Caucasians [40]. Analysis of the UNOS database for "A2" donor kidneys showed that a significantly higher non-white proportion of recipients benefitted when "A2" kidneys were used across A to B blood group barriers, with similar clinical outcomes for patient or allograft survival [41]. Implementation of ABOiKTx in deceased donor transplantation with high anti-ABO specific titres against the donor is difficult due to timings after organ retrieval and the need for antibody removal. However, there is the possibility of low titre ABOiKTx being an effective tool in transplanting blood group "B" waiting recipients on a deceased donor waiting list. This has been modelled in paediatric kidney transplantation for potential A2-to-B transplantation in low titre recipients. It has also been successfully practiced in order to increase transplantation rate with quarterly ABO titre measurement and similar outcomes to ABO compatible kidney transplants in an adult population in the USA with an increase of 25% more "B" recipients receiving transplants [42-44]. This would allow an increase in the overall availability of group "B" kidneys in a national scheme, if local "B" recipients with low anti-A titres have all been successfully transplanted, and thus have increased benefit to other non-white ethnic groups in different regions.

1.3.2 HLA sensitization

MHC class I and II proteins are cell surface glycoproteins. Class I expression is present on most cells, whilst Class II is restricted to predominately B lymphocytes; dendritic cells and some endothelial cells, but can be up-regulated in inflammatory responses. It is the specific role of MHC class I and class II molecules in presenting antigenic peptide to the T Cell receptor (TCR) that underlies their significance as barriers to transplantation. The consequences of TCR engagement and co-stimulation are proliferation and differentiation of the T cells into effector phenotypes and concomitant production of cytokines. The role of immunosuppression is to target signalling pathways responsible for T cell activation, including TCR/CD3 engagement by the MHC, co-stimulatory pathways such as CD28 binding by CD80/CD86 and cytokine signalling pathways such as IL-2 binding to the IL-2 receptor including CD25.
Exposure to HLA alloantigen leads to the development of anti-HLA antibodies through B cell activation which at least for these protein antigens requires T cell help. Anti-HLA antibodies are produced against both Class I and Class II antigens. The specific nature of the sensitizing event on antibody production has been described to affect the IgG subclass and isotype [45].

1.4 Antibodies and the measurement of antibodies

1.4.1 Isotype production

Naïve B lymphocytes are present in the germinal centres, where activated CD4+ T lymphocytes recognise MHC class molecule and peptide presentation and the TCR binds to the MHC class 2 molecule. CD40/CD40L interaction triggers cytokine release from the effector T lymphocyte, causing proliferation and class switching of the B lymphocytes.

Antibody class switching takes place in the DNA sequence with switch regions marking all the constant regions for each class. The human sequence of class switching after IgM and IgD is: IgG3; IgG1; IgA1; IgG2; IgG4; IgE and IgA2. Different isotypes can occur at the same time in the B cell's progeny, during maturation and proliferation [46]. The structure of the constant region is important as it affects the immunological function of the isotype. The Fc region of the antibody is recognised by the Fc Receptors which initiate a response in specific effector cells (such as macrophage and neutrophils), bind complement and aid antibody transportation. Once the B lymphocyte has matured, antibody production is either short lived (plasmablasts) or long lived (plasma cells). Plasma cells find their niche in the bone marrow and survive through anti-apoptotic mechanisms, which include stimulation through CXCR4, APRIL and IL-6[47]. The determinant of pathways is important as long lived plasma cells continue to produce antibody, whilst short lived plasmablasts will have a finite life span and if there is no further stimulation of B cells, antibody production will be halted. Understanding of the longevity of these cells plays a crucial role in understanding persistent alloantibody production and guides appropriate therapeutic interventions [48, 49]. IgG is the predominant form of affinity matured antibody population, and its presence has been associated with acute antibody-mediated rejection. However, the presence of non-IgG HLA antibodies has been detected and the role of these antibodies are debated.

1.4.2. Significance of Isotype

The isotype or subclass of anti-HLA antibody has potential to affect the clinical consequences of these antibodies in the circulation and on the allograft. The FcR and consequent complement activation varies with each class (Table 1.2). The persistence of alloantibody after transplantation is associated with poor outcomes, whilst the

elimination from the circulation of alloantibody is associated with improved outcomes following a positive crossmatch [50]. These data suggest that an immunological response to the allograft may be followed by cessation of alloantibody production, without specific immune-modulatory therapies.

Recently, DSA IgG has been eluted from transplant biopsies, and correlates well with the specificity of the circulating DSA IgG, C4d deposition and features of antibody-mediated injury [51]. In a small series of highly sensitized kidney transplant recipients with a negative CDC crossmatch but with solid phase DSA, there was no difference in allograft survival up to 8 years, compared with controls with non-donor specific HLA antibodies. Patients with DSA had more episodes of rejection which all took place within the first 45 days [52]. The presence of anti-HLA IgG has also been shown to be deleterious to the graft regardless of whether it was donor specific or non-donor specific [53].

1.4.3. Anti-HLA IgM

The role of IgM has previously been thought of as not clinically relevant, without prognostic significance and in many cases simply auto-reactive. The presence of anti-donor IgM has been described by a positive result in CDC or FCX which became negative on dithiothreitol (DTT) treatment or heat treatment. Thus, positive crossmatches become negative in the assay with the reducing reaction. In these cases a transplant can proceed without immediate immunological complication [54]. Arnold et al showed that there was a low prevalence of IgM (17%) anti-HLA antibodies in patients waiting for transplantation and the distribution of IgM antibodies was more against class I than class II HLA specificities [55]. This has also been demonstrated by Diaz where only 3% of patients were described as having only an IgM isotype. However, 30% of patients had an antibody population which was changed by DTT treatment, suggesting the presence

of IgM contributing to antibody binding [56]. The detection of IgM in acute rejection could indicate that these antibodies have an active role in allograft dysfunction. This may be non-specific binding and only IgG is clinically useful according to McCalmon who demonstrated no clinical effect of IgM [57, 58]. However, there was strong staining for IgM in kidneys with hyperacute rejection that demonstrated no IgG staining, suggesting there may be a pathogenic role for IgM alloantibody [59]. In a different study, despite early IgM development, there was no longer term detrimental [60]. No conclusive data has been published, which in part reflects the difficulty in standardising the methodology for assessment of IgM anti-HLA antibodies across centres.

Table 1.2. Description of proportions and properties of different isotypes and subclasses of immunoglobulins.

Adapted from Schroeder. [61]

	Serum (%)	Structure	Complement Fixation	Opsonizing	Other function	Fc Receptor
IgG	75	Monomer	+	+++	For all IgG subclasses:	FcγR
IgG1	67% IgG	Monomer	Yes	Yes	Secondary response	I, II, III
IgG2	22% IgG	Monomer	Yes	Yes	Neutralize toxins and virus	II
IgG3	7% IgG	Monomer	Yes	Yes		I, II, III
IgG4	4% IgG	Monomer	No	No		I, II
IgM	10	Pentamer	++++	-	Primary response	
IgA	15	Monomer, dimer	-	-	Mucosal response	FcαR (CD89)
IgA1		Monomer, dimer	-	-		
IgA2		Monomer, dimer	-	-		
IgD	<0.5	Monomer	-	-	Homeostasis	FcδR
IgE	<0.01	Monomer	-	-	Allergy	FcεR I, II

1.4.4. IgG Subclasses

The development of anti-HLA antibody and its subclass may be dependent on the context in which sensitisation had taken place. Lowe at al showed in all patients with DSA, there were highest levels of IgG1detected. If sensitization to HLA occurred through transfusion or transplantation, IgG2 was the second most frequently detected subclass. This is in contrast to sensitization secondary to pregnancy where IgG3 subclass is the second most frequently detected subclass [45]. Arnold el at demonstrated, patients waiting for a kidney transplant had 25% prevalence of IgG2 and/or IgG4, of which 88% had had a previous transplant as their sensitizing event [55]. The entire IgG4 antibody population was directed against HLA class II antigens. In a different study by Karuppan et al, poor-complement fixing anti-HLA antibodies (IgG2 and IgG4) were found in patients who had graft loss, although the subclass distribution was heterogeneous. The presence of only IgG2 and IgG4 in two separate patients who experienced early graft loss was noted [62]. Monteiro reported that IgG1 against class I HLA mirrors the total IgG response to the same class I antigens. The significance of higher levels of PRA against class I was correlated with poorer graft outcome in this patient group [63]. The effect of complement binding of IgG subclasses with anti-HLA antibodies is described by Kushihata et al. They showed the predominant subclass was IgG1, with other subclasses detected infrequently [64]. While there is consensus that detection of anti-HLA antibody IgG1 is the most common subclass, there is no consensus about the significance of the presence of other subclasses and associated clinical outcomes. With the development of complement binding assays (C1q), functional assessment of antibody subclasses may be tested and associated with clinical events.

In ABOiKTx, the IgG isotype is clinically significant in predicting outcome and risk of rejection, however no IgG was found in allografts that experienced humoral rejection,

only IgM [65]. Stussi described that anti-ABO specific antibody subclass distribution of healthy donors was dominated by IgG2 subclass, with IgG1 and IgG3 being detected, but no IgG4 by flow cytometry bound to RBCs [66]. The range of subclass detected in an early study by Kay, using haemagglutination with subclass specific AHG, demonstrated a blood group distribution variability. Blood Group "A" and "B" donors produced all 4 IgG subclass antibodies in less than 14% of donors, whereas 48% of blood group "O" donors produced all 4 IgG subclasses. A large proportion of blood group "O" donors also produced complement binding subclasses IgG1 and IgG3, >87% and >74% respectively [67]. The assessment of IgG subclass antibodies has not been extensively studied in ABOiKTx.

1.4.4. HLA antibody measurement

The development of technology for measuring anti-HLA antibodies has led to an increase in sensitivity with newer assays. Complement Dependent Cytotoxicity (CDC) testing was the gold standard test for donor-recipient matching and significantly reduced the cases of hyperacute rejection. A positive CDC crossmatch vetoed a potential transplant. Assessing recipients before transplantation, to avoid lengthy CDC testing, led to the development of panels of lymphocytes with known common HLA phenotypes against which patients waiting transplantation would be tested. The degree of reactivity against these lymphocyte panels would then profile patients into an antibody risk profile: panel reactive antibody (PRA). Flow cytometry can differentiate between B and T lymphocytes and calculations can be made for donor-recipient reactivity based on T lymphocyte, predominantly Class I expressing and B lymphocytes with both Class I and II expression.

Recombinant HLA proteins were developed for solid phase assays and initially used in ELISAs and then flow cytometry machines. This allowed identification of potential recipient serum reactivity against known HLA protein profiles, without donor cells required. This is now used with microspheres in Luminex® technology. The detection of antibodies against HLA molecules allows more specific identification of anti-HLA antibodies. Defining these specificities for a patient leads to a calculated reaction frequency (CRF) which has replaced PRA in predicting the degree of sensitization of the patient against the potential donor pool.

1.4.3.1 Complement Dependent Cytotoxicity (CDC)

Complement dependent Cytotoxicity was developed in the 1960s and became a clinical tool following the development of the Terasaki plate [15]. Figure 1.3A demonstrates the assay. The incubation of recipient serum with potential donor lymphocytes, with the addition of complement, followed by staining to determine apoptosis of donor lymphocytes became the gold standard for donor-recipient compatibility.

1.4.3.2. Flow Cytometric Crossmatch (FXM)

The development of FXM technique has been proven to increase the sensitivity of detecting anti-donor antibodies. Whilst the early experience was developed in different single centre laboratories, a comparative study demonstrated agreement in 80% of cases [68]. This study, however, demonstrated a discrepancy in 20% of cases which may have had a positive result in one centre, and a negative in a different centre. Figure 1.3B demonstrates FXM which involves incubating donor lymphocytes with recipient serum. Following washing, secondary staining was performed with antibodies for cell phenotype (CD19/20 for B cells; CD3 for T cells) and for IgG. Thus, separation of cell types and binding of IgG antibody can be assessed. The histogram median value is the

channel shift, which is either subtracted or divided by the negative control crossmatch, thus providing an internal control for each crossmatch. Different laboratories use different defined points of reactivity to determine a positive result.

1.4.3.3. Single Antigen Bead Detection (SAB)

Luminex® technology allows multiple microspheres to be assessed simultaneously in the same well for the same sample (Figure 1.3C). This technology for identifying anti-HLA antibodies is ideally suited to screening and characterising antigen specificity, as reactivity against different antigens can be simultaneously tested. The machine automatically gates the microspheres into the appropriate region, based on the fluorescence of the microspheres (containing different ratios of two fluorochromes). The binding of antibody to the antigen on the microspheres is determined by secondary labelling of the antibody by Phycoerythrin (PE).

1.4.3.4. Combination of techniques

A component of this thesis is based on the immunological assessment of antibodies in transplanted patients at Mayo Clinic, Rochester, MN, USA. Gloor used all these antibody assessment techniques in a cohort of patients, which showed the relationship between antibodies quantified by CDC, FCX and Luminex. These are evidently progressively more sensitive techniques with however significant inter-patient variation in their correspondence to one another (Figure 1.4A) [69]. Prior to this, Burns had compared different antibody measure methods with histological injury and the degree of injury with the different antibody levels seen in Figure 1.4B [70].

Figure 1.3. A comparison of different techniques in clinical use for analysing anti-HLA antibodies in transplantation

There are different techniques using cellular and solid phase assays to assess anti-HLA antibodies and each are demonstrated below (complement dependent cytotoxicity (CDC) in panel A; flow cytometric crossmatch (FXM) in panel B and Luminex®(Bead) in panel C) [71].

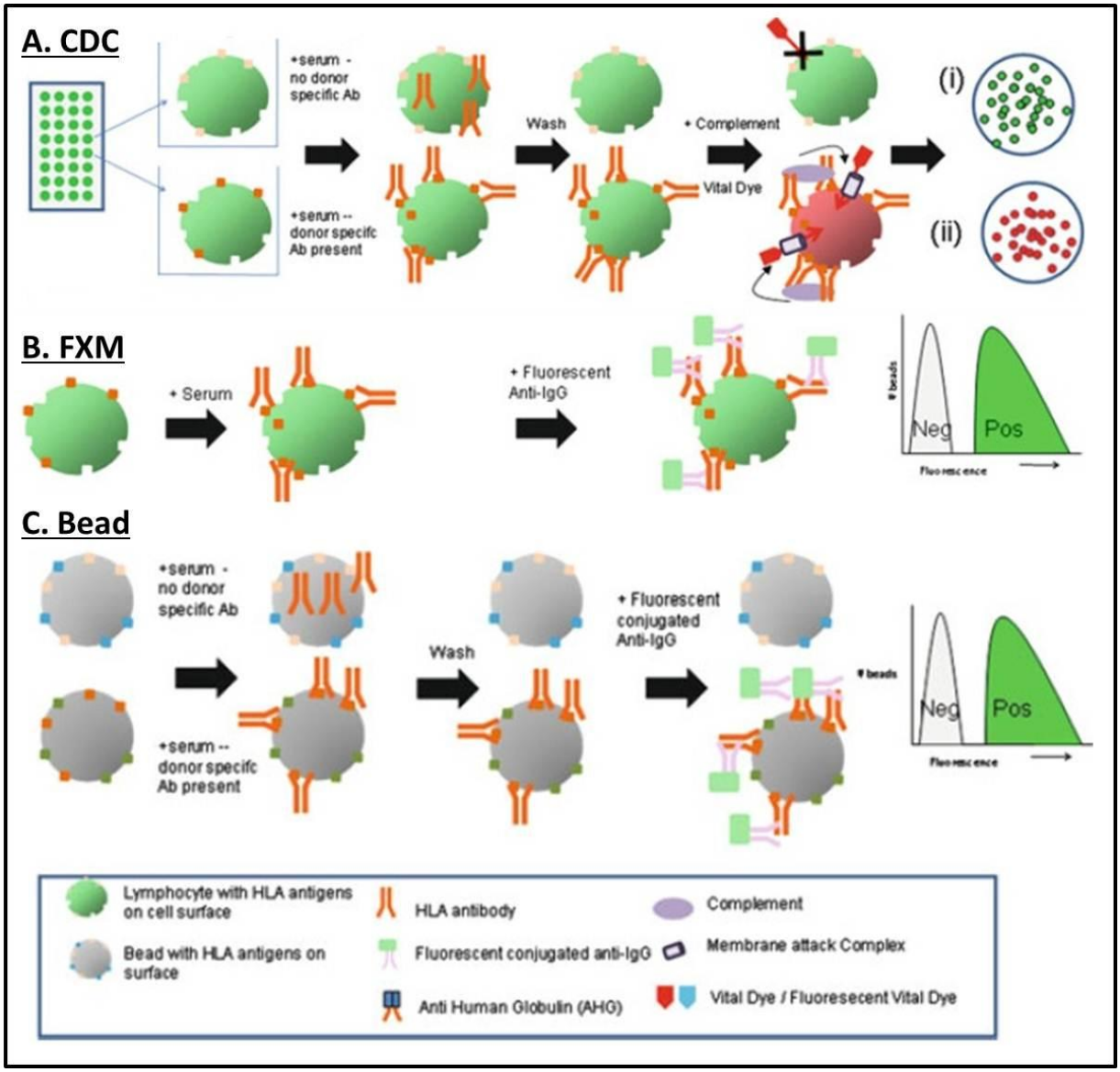


Figure 1.4 Comparison of results of different anti-donor testing in +XMKTx at Mayo Clinic, USA.

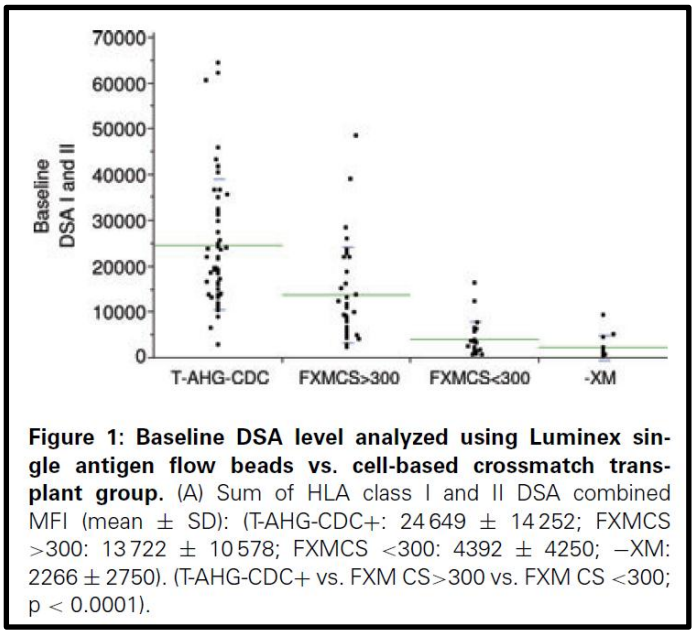


Figure 1: Baseline DSA level analyzed using Luminex single antigen flow beads vs. cell-based crossmatch transplant group. (A) Sum of HLA class I and II DSA combined MFI (mean ± SD): (T-AHG-CDC+: 24 649 ± 14 252; FXMCS >300: 13 722 ± 10 578; FXMCS <300: 4392 ± 4250; −XM: 2266 ± 2750). (T-AHG-CDC+ vs. FXM CS>300 vs. FXM CS <300; $p < 0.0001$).

Figure 1.4A - Baseline comparison of crossmatch techniques and cumulative MFI in Luminex® assessment of DSA at Mayo Clinic. This demonstrates the significant differences in sensitivity between crossmatch techniques, and highlights that even negative crossmatch recipients are not without significant levels of DSA [69]. T-AHG = T cell anti-human globulin CDC; FXMCS = flow cytometric crossmatch; -XM = negative crossmatch

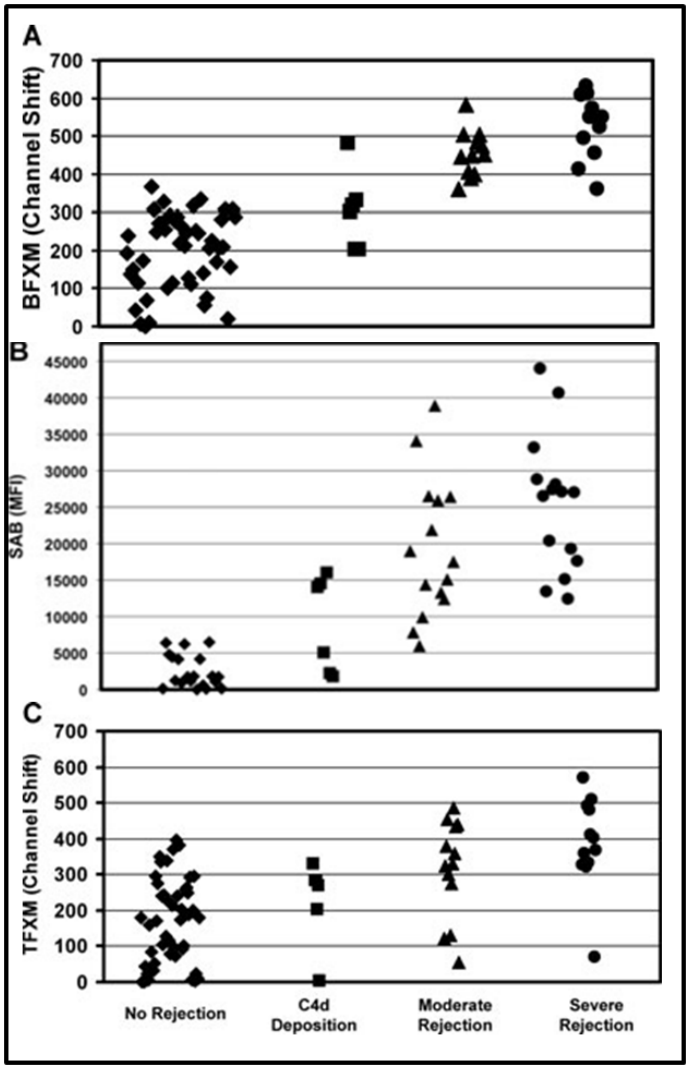


Figure 1.4B – Comparison of different antibody detection techniques with histological assessment with BFXM (A); Luminex ® SAB (B) and TFXM (C) levels in patients. Higher levels of antibody were associated with the development of more severe antibody-mediated tissue injury [70].

1.4.5. Anti-ABO specific antibody measurement

While anti-HLA measurement has progressed in the last 50 years, the measurement for anti-ABO specific antibodies is still largely based on techniques from Landsteiner's report from over 100 years ago. This haemagglutination technique is a serial doubling dilutions of patient plasma until no reactivity is found. Antibody reduction in ABOiKTx is seen as the key to improving outcomes, in particular the high risk of hyperacute rejection. Target titre levels of equal or less than 1:8 on the day of transplant have been shown to have improved outcomes [65]. The importance of lowering antibody titres to less than 1:8 to prevent negative outcomes with higher anti-graft antibodies is logical as early clinical outcomes in Japan showed that higher pre-operative IgG levels were associated with worse clinical outcomes both in graft survival and humoral rejection [65].

Despite good outcome data, the need to improve the delivery of care with accurate titre levels continues to be a goal that has not yet been achieved. The number of antibody removal therapies are based on accurate measurement, and this has an impact on patients; staffing and ultimately the cost of the process of transplantation [72, 73].

Early rejection of transplanted kidneys is most likely to be humoral rejection, involving antibody binding to renal vascular endothelium. This antibody-mediated process may not initially be accompanied by elevated antibody levels as adsorption of antibodies in the kidney may keep levels constant, until saturation of the binding sites leads to antibody titre levels rising [74]. Antibody titres of greater than 1:128 have been successfully transplanted with post-transplantation titres of <1:16 being maintained, without antibody-mediated rejection occurring [75].

In assessing transplantation across blood groups, analysis of antibody titres is crucially important. The assay used defines the titre of antibody present with a margin of error

usually of one titre either side of the median. IgM and IgG are often both individually measured. Chapter 2 outlines in more detail assays to describe anti-ABO specific antibody.

1.4.6. Other anti-donor antibodies

Major-histocompatibility-complex (MHC) class I–related chain A (MICA) antibodies were first shown to have a detrimental effect on kidney allograft by Zou et al [76]. A subsequent study showed that the effect of MICA positive antibody recipients did not have inferior outcomes to MICA negative recipients which supported data from Terasaki et al [77, 78].

Anti-endothelial cell antibodies (AECA) found in one study were predominantly IgM rather than IgG (66% vs 14%). Other non-HLA antibodies measured include vimentin, glomerular basement membrane protein agrin [79]. The presence of anti-idiotypic antibodies, directed against the idiotype of an HLA antibody, has been associated with a reduction of chronic rejection, despite the presence of donor allo-antibody although its role is still controversial [80]. More recently, angiotensin II type-1 receptor antibodies have been measured and associated with worse outcomes. Larger studies are needed to determine the clinical effectiveness for monitoring and the longer term outcomes [81, 82].

Anti-galili antibodies, the immunological barrier to xenotransplantation, are produced by natural antibody producing B cells. Anti-galili antibodies are about 1% of circulating IgG and 4% of IgM. Large amounts of IgM are deposited in xenografts in rejection. The presence of anti- galili IgM and IgG can increase with exposure to antigen, though there is a class switch at day 21 with a decline in IgM and replaced by IgG in circulation [83]. In the context of allotransplantation, not xenotransplantation, anti-gal antibody does not

affect allograft outcomes. There is a suggestion that anti-Gal binding may overlap with that of other blood group antibodies (A or B), as the epitopes are similar structures, though the clinical relevance of this has not been demonstrated [84].

1.5 Preformed antibodies as a barrier to transplantation.

1.5.1 ABO incompatible kidney transplantation

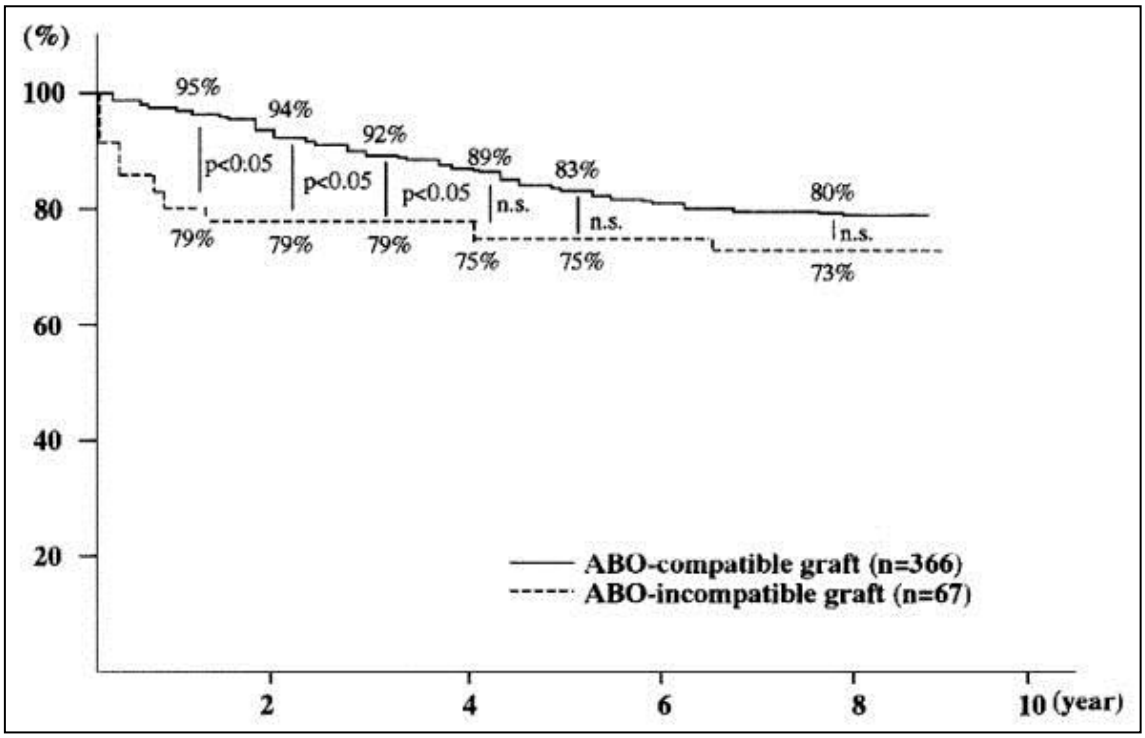
Despite Starzl and colleagues publishing a series of ABO incompatible and ABO compatible donor-recipient pairs with comparable results, early experience led to blood group incompatibility being considered an absolute barrier to transplantation. USA Transplant registry data from 1967 demonstrated a very significant poor outcome of ABOiKTx, with 45.8% (11/24) never functioning and only 7.7% (1/13) functioning more than 1 year post-transplant [85-87]. However, an “inadvertent” breach of the blood group compatibility in 1980 in Portsmouth, UK demonstrated successful reversal of antibody-mediated damage with anti-A antibodies with 65% reduction in antibody levels measured, and this led to more centres developing treatment regimens to overcome this barrier [88]. Alexandre et al in Belgium expanded practice in ABOiKTx, initially with “A2” donor kidneys with reasonable clinical outcomes [89] . As previously described, “A2” donors have fewer "A" antigens present on cell surface and thus pose less of an immunological risk and were initially used, before progression to “A1” donor kidneys. Breimer and colleagues demonstrated this by comparing “A antigen” expression on kidney transplant biopsies, demonstrating less expression in “A2” kidneys compared to “A1” kidneys [90].

There has been greater use of ABOiKTx in Japan where the largest cohorts have been carried out due to cultural reasons preventing a large deceased donor pool and so live donation has been the main source of organs for kidney transplantation [91]. This was

initially treated with regime that included heavy burden of immunosuppression, which has become less intensive. The first regime published had five pharmacological agents and splenectomy and this was comprised of methylprednisolone, cyclosporine, azathioprine, antilymphocyte globulin and deoxyspergualin [92]. The earliest protocols also included total irradiation of the allograft prior to implantation. The patient survival of these ABOiKTx compared to the ABOcKTx cohort in historic controls was not significantly different. There was significant early graft loss up to 3 years in the ABOiKTx, but no significant difference after this time period (Figure 1.5, [93]). The emphasis of therapies since then has been on the reduction in burden of immunosuppression.

Figure 1.5. Early graft loss is significantly higher in ABOiKTx compared to ABOcKTx cohorts.

Despite increased early allograft losses in the ABOiKTx cohort, medium to longer term graft survival outcomes were not significantly different in Japanese data [93].



1.5.2 HLA incompatible kidney transplantation

The presence of anti-HLA antibodies is associated with poor allograft outcome and this is worse with anti-HLA antibodies against donor HLA [53]. However, re-transplantation is increasing, leading to an increased number of patients with HLA sensitization at the time of transplant. The amount and specificity of the antibody are defined by different techniques. The methods of anti-HLA antibody testing have been described in this chapter. The result of these methods has an impact on both the treatment of these patients and on the transplant outcomes. The level of antibody against donor HLA (often defined by the test used for detection) and its specificity will significantly impact the allograft survival [94].

Early experience used pre-transplantation conditioning similar to that used for treatment of vasculitis in patients with high PRA antibody levels. The use of cyclophosphamide and plasma exchange demonstrated satisfactory antibody reduction [21, 95]. After treatment of a CDC positive crossmatch could be changed to negative, a cohort study demonstrated early allograft survival benefit using immunoadsorption rather than plasma exchange for antibody removal, although this conclusion remains controversial [96].

Experience with T-Cell CDC negative transplants, but B-cell positive crossmatches demonstrated some degree of transplantation success at 1 year post transplant, however there were still allografts lost due to CDC negative but flow cytometric lymphocyte positive reactivity against donor T-cells [97, 98]. Seven patients (7/10) in whom anti-B lymphocyte antibodies were detected had chronic rejection or early severe rejection. This resulted in recommendations to avoid transplanting patients with anti-donor positive flow crossmatches, despite negative T-cell CDC crossmatches due to the high rates of antibody-mediated vasculitis [99]. Kidney transplantation with historic

positive crossmatches, but negative at the time of transplant, also demonstrated early humoral rejection and poor clinical outcomes.

Many series of antibody incompatible kidney transplantation have now been reported from different centres across the world; however comparison is difficult due to heterogeneous techniques, both in desensitization and in particular, measuring and defining anti-HLA antibodies.

1.5.3 Limitations of pooled exchange programme

The clinical implementation of paired exchange, multiple exchanges and domino chains benefit patients who have an incompatible live donor in order to overcome antibody and other barriers. However, the application of pooled exchange will not be successful in every donor/recipient pair and there remains a limitation in transplanting the highly sensitised (>95% PRA) and in accumulating blood group "O" recipients. A combination of exchange transplantation with desensitisation is another option [100]. Further to this, exchange to avoid DSA but with acceptable ABOi combinations has been demonstrated to have good outcomes [101].

1.6 Desensitization

Desensitization can be can be categorised as reducing antibody production, removing antibody and preventing the action of antibody on the allograft after transplantation. There are no large scale randomised controlled studies demonstrating comparative efficacy in the different techniques used in current practice. The numbers from single centre experience are small and there is much heterogeneity within these cohorts.

1.6.1 Antibody Production

Cyclophosphamide was initially used in early protocols to halt antibody production. Intravenous immunoglobulin (IVIg) can modulate the levels of HLA antibodies prior to

transplantation. The use of regular high dose IVIg (2g/kg) reduced the levels of antibody in potential live donor recipients to a negative crossmatch (CDC) in 92.3% (24/26), and allowed successful deceased donor transplantation in 94.1% (16/17) of patients with high PRAs (>50%) [102]. Low doses of IVIg have been used after antibody removal. Its mechanistic role is not understood.

Splenectomy has also been used as a therapy to reduce antibody production, but has now largely been replaced by the use of rituximab therapy, a monoclonal antibody directed against CD20 expressing B lymphocytes, which has demonstrated equivalent outcomes in ABOiKTx [103-106]. The current role of splenectomy in order to salvage allografts is in accelerated early antibody-mediated rejection which has been demonstrated in case series [107, 108].

Using rituximab can have an effect on reducing anti-HLA antibodies after transplantation, but the beneficial effect on anti-HLA antibody production has not been clearly demonstrated pre-transplantation [109]. Its efficacy on depleting circulating B cells and in removing B cells from sensitised patients' lymphoid tissue is well described in the ABOiKTx cohort by Genberg [110-112]. However, in some centres current protocols have abandoned the routine use of anti-CD20, rituximab [104].

In HLAiKTx, there is less agreement on regimes for reducing antibody levels. The role of bortezomib, a proteasome inhibitor, to reduce plasma cell production has yet to demonstrate a significant reduction in circulating antibodies in large cohort studies, despite encouraging in vitro results demonstrating reduced plasma cells producing antibodies [113-115]. Newer proteasome inhibitors are under development which may have improved efficacy.

1.6.2 Extracorporeal Antibody Removal Treatment

The use of protein-A columns to remove antibody has been replaced with plasma exchange which is widely available in most nephrology and transplant centres. The use of plasma exchange to remove antibodies to a level acceptable to the transplantation team is widely published and is often used routinely post transplantation [88, 116-118]. Plasma exchange removes a volume of circulating plasma and replaces it with the same volume of fluids, which usually consist of a combination of crystalloid, human albumin and plasma [119, 120]. The protocol developed by Tyden and colleagues, introduced A/B antigen specific antibody removal, Glycosorb® ABO (Glycorex, Sweden) with rituximab therapy [121, 122]. This technology conjugates A/B trisaccharides to Sepharose beads, and plasma is filtered through these antigens, thus avoiding removal of important clotting proteins, complement, other immunoglobulins and albumin [123]. While Glycosorb® columns reduce the use of infusion products and reduce removal of clotting agents, there is still a reduction in anti-carbohydrate antibodies, namely pneumococcal and haemophilus, while protein-based immunity is unaffected [124]. Early data comparing ABOi and ABO compatible (ABOcKTx) using this technique demonstrated equivalent clinical outcomes between the groups [125]. More recently, a pan-immunoglobulin technique has been introduced into this expanding area of transplantation and demonstrated similarly good clinical outcomes in transplantation and removal of anti-ABO specific antibodies and anti-HLA antibodies [126-129]. This uses sheep anti-human antibodies to remove human immunoglobulin, and can be eluted and re-used unlike Glycosorb® columns.

Whichever technique is used, an intervention to reduce antibody before transplantation is currently considered a pre-requisite in order to achieve successful transplantation. In ABOiKTx, an antibody haemagglutination titre of 1:8 dilution is a target that is used

widely, however there is a range that different centres find acceptable up to 1:16 and 1:32 dilution of titre [130, 131]. It is difficult to compare centre to centre final ABO titre cut-off values, as different assays are used. The maximum titre pre-treatment is yet to be defined, as this is dependent on the titre assay used for measuring anti-ABO specific antibody. Reproducible and thus comparative titre results will direct successful antibody reduction and ultimately a good allograft and patient outcomes at transplantation. In the study by Lawrence et al, a titre of 1:256 was considered the maximum titre that could be successfully reduced to enable transplant surgery [132]. In HLAiKTx, Extracorporeal Antibody Removal Treatment (EART) is often targeted at making a crossmatch negative before transplantation; this can either be CDC or FXM depending on the transplant unit's protocol.

Post-transplant surgery, antibody removal is based either on an individual unit's set protocol, a rise in titre to pre-determined level (often 1:16-1:32) or only in response to antibody-mediated rejection [121, 133-135]. Antibody-mediated rejection is treated with EART, along with pharmacological intervention, after transplantation until resolution of the acute clinical deterioration.

1.6.3 Prevention of allograft damage

Post-operative therapies to prevent allograft damage have been based on a combination of techniques above. In addition, C5 inhibition with eculizumab has been used for trials and for 'rescue therapy' in cases of aggressive AMR successfully [136, 137]. The drug works by blocking terminal complement activation and thus membrane attack complex formation associated with cell apoptosis. In addition, splenectomy has been effective in reducing early AMR in transplanted patients and in response to aggressive early AMR, without significant side-effect profile [138, 139].

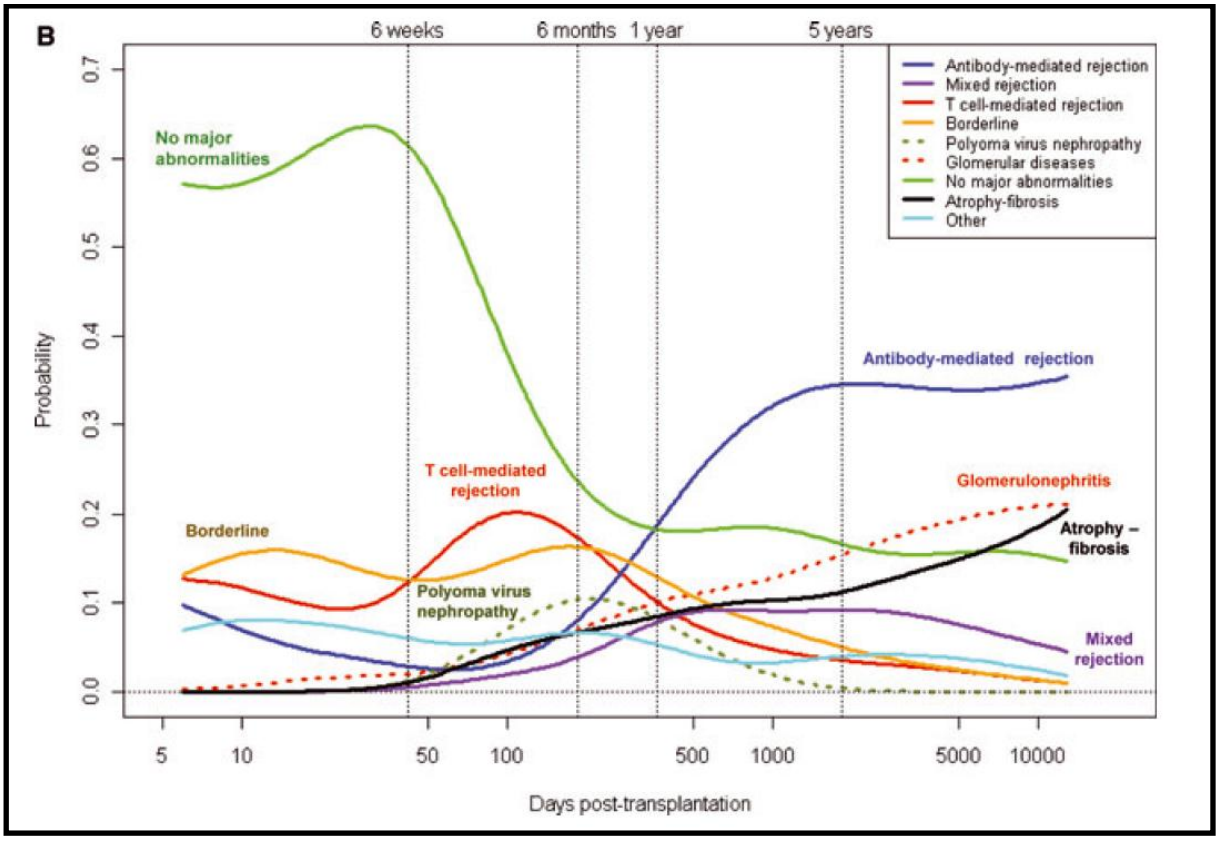
Early experience in Japan for ABOiKTx used anti-coagulation to prevent early allograft thrombosis which demonstrated an increased allograft survival benefit of 13% at 5 years, though this has not become standard practice in the majority of centres performing ABOiKTx at present [91].

1.7 Rejection

The presence of anti-donor antibody is strongly correlated with early antibody-mediated rejection, which in turn is associated with reduced long term allograft survival. In addition to early antibody-mediated rejection, Figure 1.6 details the processes occurring in the allograft loss over time and demonstrates antibody-mediated injury as the predominant process involved after 1 year in a cohort of indication transplant biopsies [140]. Thus, after surviving the early transplant period, antibody-mediated injury becomes the major contributor; indeed 64% of allografts that failed after biopsy were AMR related. On further analysis of the cohort, Sellares et al describe the same phenotype in allografts regardless of C4d presence or absence, with the same molecular phenotype of antibody-mediated rejection, endothelial and NK transcript expression [141]. The Banff criteria for transplant histology have evolved over the past 8 years to reflect the role of antibody on histological samples and correlate with clinical outcomes [142, 143]. More recent studies demonstrate that whilst allograft function can be within normal or acceptable range, there can be histological injury associated with alloantibody which is only detected on surveillance or protocol biopsies [144]. Thus understanding the processes involved in humoral damage to the allograft may guide future therapies.

Figure 1.6. Distribution of histopathology diagnoses in biopsies expressed as probability plots conditional on the time of biopsy post-transplantation.

Sellares' diagram demonstrates the profile of mechanisms impacting allograft injury over time [140]. The probability of antibody-mediated injury increases after 6 months at a faster rate than other pathological processes.



1.7.1. Defining Antibody-Mediated Rejection

The diagnostic criteria of early antibody-mediated rejection are the presence of donor specific alloantibody, tissue injury in keeping with AMR and the presence of C4d [145]. Each of these elements will be discussed in the following sections. Histological findings consistent with antibody-mediated injury are shown in Table 1.3. Risk factors for humoral rejection include a previous transplant, female gender, pregnancy, historical positive cross-match, and increased PRA [146].

1.7.2 Peritubular capillaritis (PTCitis)

A study by Liptak et al of ultrastructural changes in early AMR describes peritubular capillary infiltration comprised of 59% monocytes; 14% neutrophils and 12% lymphocytes, with the rest as unspecified mononuclear cells. In the acute biopsies, there was evidence of lytic injury with excessive swelling and disorganization of endothelial cells with fibrin fibre attachments and denudation of basement membrane; pericapillary endothelial cell apoptosis was increased in frequency with no direct contact with inflammatory cells and lastly fragmentation of the endothelial layer and formation of apical blebs with detachment of fragments from the basement membrane. The follow up biopsies for 2 patients (2 of 25) showed signs of transplant glomerulopathy at 26 and 27 days respectively, but ultimately all allografts failed. There was evidence of subendothelial space widening and multiplication of the basement membrane. The rapid progression to TG within 1 month following allograft implantation shows that this process is a spectrum following antibody-mediated injury [147]. Infiltration of the allograft with scoring for peritubular capillaritis was recommended by the Banff 2007 meeting [142]. The scoring system is based on presence of inflammation and the extent of inflammatory cells in the lumen. The type of cell and the minority/majority of population of these cells should be noted as well as the association of C4d deposition with PTCitis. The technique for C4d staining should be noted, as immunofluorescence is more sensitive and correlated with clinical data better than immunohistochemistry.

1.7.3 Glomerulitis

Glomerulitis (Table 1.3) is defined by the percentage of glomeruli with inflammation and the extent of involvement of each glomerulus. The inflammation of the glomeruli is defined by the degree of mononuclear cell infiltration and endothelial cell enlargement

[148]. Glomerulitis can be present in both cellular and antibody-mediated rejection. In the context of AMR, glomerulitis is associated with staining for CD68, macrophage lineage cells, but these are absent in the context of cellular rejection [149]. The impact of the presence of glomerulitis and its severity is associated with worse allograft outcomes and other lesions associated with chronic antibody-mediated rejection [150].

1.7.4 Microcirculation injury scores

In order to identify allografts with increased risk of failure, combined scores using both glomerulitis and peritubular capillaritis have been used to assess risk of allograft failure and prediction of outcome [151]. The presence of late (>1 year) microcirculation inflammation is highly predictive of the presence of donor specific antibody and allograft failure, whilst C4d positivity was not predictive ($p=0.581$). Furthermore, receiver operating characteristic analysis demonstrates that in patients with de novo analysis, the severity of the microcirculatory score is more sensitive for allograft failure than the presence of C4d deposition alone in the allograft biopsy, which supported earlier work demonstrating equally poor allograft survival in patients with microcirculation injury as with acute antibody-mediated rejection [152, 153].

Table 1.3. Banff criteria for antibody-mediated rejection and the histological scoring of microcirculation inflammation.

Antibody-Mediated Rejection is defined by having histological injury (Criteria 1) with evidence of C4d deposition (Criteria 2) and evidence of anti-donor antibodies (Criteria 3). The Banff classification for inflammation associated with antibody-mediated rejection are shown below.

<table>
<tr><td colspan="4">Antibody-Mediated Rejection[142]</td></tr>
<tr><td>Grading</td><td colspan="2">Criteria 1</td><td>Criteria 2</td><td>Criteria 3</td></tr>
<tr><td>Grade 1</td><td colspan="2">ATN-like minimal inflammation</td><td rowspan="3">C4d deposition</td><td rowspan="3">Circulating anti-donor antibodies</td></tr>
<tr><td>Grade 2</td><td colspan="2">Capillary and or glomerular inflammation (g/ptc>0) and/or thomboses</td></tr>
<tr><td>Grade 3</td><td colspan="2">Arterial involvement - v=3</td></tr>
<tr><td colspan="5">Histological Scoring [148] [142]</td></tr>
<tr><td rowspan="4">Glomerulitis</td><td>0</td><td colspan="3">No glomerulitis</td></tr>
<tr><td>1</td><td colspan="3">Glomerulitis in less than 25% of glomeruli</td></tr>
<tr><td>2</td><td colspan="3">Segmental or global glomerulitis in 25-75% of glomeruli</td></tr>
<tr><td>3</td><td colspan="3">Glomerulitis (mostly global) in more than 75% of glomeruli</td></tr>
<tr><td rowspan="4">Peritubular capillaritis</td><td>0</td><td colspan="3">No significant cortical ptc, or <10% of PTCs with inflammation</td></tr>
<tr><td>1</td><td colspan="3">≥10% of cortical peritubular capillaries with capillaritis, with max 3 to 4 luminal inflammatory cells</td></tr>
<tr><td>2</td><td colspan="3">≥10% of cortical peritubular capillaries with capillaritis, with max 5 to 10 luminal inflammatory cells</td></tr>
<tr><td>3</td><td colspan="3">≥10% of cortical peritubular capillaries with capillaritis, with >10 luminal inflammatory cells</td></tr>
</table>

1.7.5 Complement and Antibodies

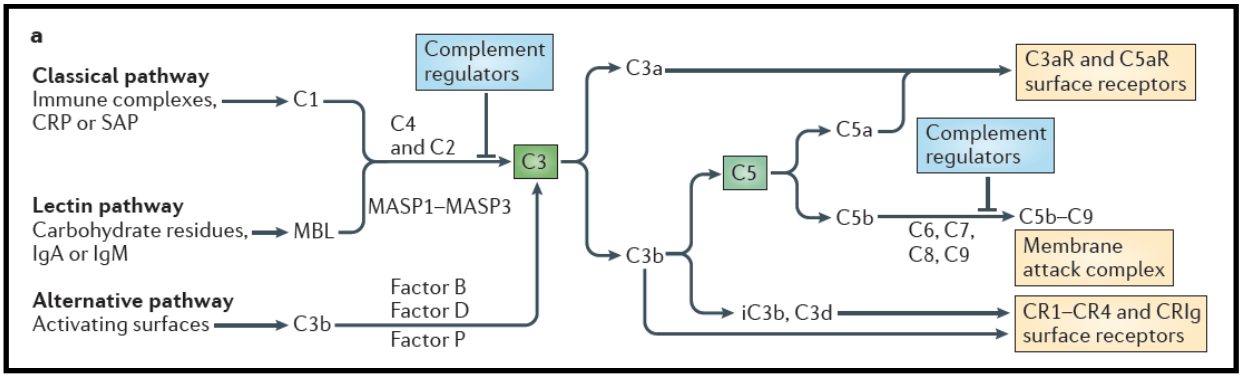
1.7.5.1 Complement Activation

The deposition of C4d in the allograft reflects the biological process of antibody binding to antigen and activating the complement cascade causing tissue injury via the classical complement pathway (Figure 1.7). C4d is a breakdown product of activated C4, when C4b is cleaved. This occurs in the classical pathway of complement activation after C1q binding to antibody-antigen complexes. C4d deposition in the allograft is due the covalent binding to tissue, whilst other C4 and C3 breakdown products do not remain in the allograft on biopsies. C4d (thought to be inactive) is bound and thus detectable in biopsies and correlates well with antibody-mediated damage [154]. C4 can also bind to mannose binding protein (lectin pathway) and follow the same activation pathway.

Figure 1.7. Activation of complement pathways.

There are 3 different pathways for complement activation which are shown in the diagram below. (MBL, mannose binding lectin; CRP, C-reactive protein; MASP, MBL‑associated serine protease; SAP, serum amyloid P;CR, complement receptor; C3aR, C3a receptor; C5aR, C5a receptor and CRIg, Complement Receptor of the Immunoglobulin superfamily)

Taken from Sacks and Zhou [155].



1.7.5.2 Complement Activation and Chronic Injury

The further evidence for rejection with C1q-mediated classical pathway activation with presence of DSA IgG was shown by C1q binding to DSA IgG on single antigen beads (SAB) correlating with the development of transplant glomerulopathy and graft loss. C1q positive DSA IgG was associated with the presence of C4d in graft histology, but also with a higher incidence of acute rejection [156, 157]. Anti-HLA antibodies that fix C4d in vitro assay are also strongly associated with early AMR, and if C4d was not activated on the SAB, there was no early AMR [158]. Furthermore, increased deposition of C4d in the allograft is associated with significantly worse allograft outcomes [159]. Whilst the presence of C4d in the biopsy is associated with the detection of circulating antibody, the absence of C4d does not signify the absence of anti-donor antibodies. C4d negative biopsies with circulating DSA still display microcirculation injury and the new Banff criteria of AMR reflect this change in viewpoint [151, 160-164]. This also reflects the temporal nature of biopsies, sampling and intensity of binding. Loupy and colleagues have recently demonstrated in a large cohort that despite significant DSA levels, if C1q binding was negative, the allografts survival was not inferior to patients without DSA, in contrast to the poor outcomes in the combined DSA/C1q+ patients [165].

C4d staining has been correlated with multi-layering of the peritubular capillary basement membrane by Regele. The presence of C4d in the graft at biopsy without transplant glomerulopathy was associated with progression to transplant glomerulopathy at follow up biopsy greater than 9 months in 82% allografts [166]. Acute antibody-mediated rejection is a risk factor for the development of transplant glomerulopathy as is the presence of anti-HLA antibodies, particularly with anti-class II antibodies [167-169]. The deposition of C4d with the presence of anti-HLA antibodies is associated with microcirculation injury, an indicator of antibody-mediated injury.

1.7.5.3 Antibody damage without Complement

Early humoral rejection in allografts that have a positive B lymphocyte flow crossmatch with DSA can be prevented by C5 inhibition (eculizumab). C5 inhibition blocks the final complement pathway step prior to terminal membrane attack complex (MAC) formation. The presence of C4d in the allograft biopsy demonstrated complement activation was initiated, but no endothelial or tubular damage occurred early which is associated with antibody associated mechanisms. There was significant reduction in the incidence of early AMR, which in the control cohort was 41.2%, and only 7.7% in treatment group in high titre HLA incompatible kidney allografts [136]. In atypical HUS (aHUS), the use of eculizumab post-transplant has shown a dramatic reduction in activated terminal complement complex. However, in the presence of anti-donor antibody without AMR, transplant glomerulopathy develops despite C5 inhibition, suggesting that the classical complement pathway for what is called chronic antibody-mediated damage may not be the only process involved in allograft damage [170-172].

Acute rejection with anti-donor antibodies does not always need the antibody-C1q complex. A murine model demonstrated that if C1q donor neg/recipient neg transplantation does not protect from early acute rejection, with presence of high anti-donor IgG and low IgM [173]. This was associated with higher levels of graft infiltration of macrophages and neutrophils, the hallmark of antibody-mediated rejection. The roles of non-classical complement activation pathways in the presence of antibody are postulated in this paper. In a different paper, the absence of circulating C4 in murine models does not improve allograft outcome when donor or recipient was depleted of C4 [174]. Furthermore, non-classical pathways of complement activation are a different mode for graft injury and complement deposition. The development of features consistent with chronic AMR can take place in the presence of anti-donor antibodies but

in the absence of C3 component. This was true with different immunoglobulin isotypes producing tissue injury with similar features of neointimal thickening and cellular infiltrates as expected in antibody-mediated rejection [175].

1.7.6 Chronic Antibody-Mediated Rejection

Transplant glomerulopathy is defined as the characteristic duplication of the glomerular basement membrane (GBM) observed by light microscopy with degree of severity determined by the percentage of glomeruli capillary loops affected (Table 1.4) [148].

Chronic damage associated with allograft loss was broadly classified as chronic allograft nephropathy without attempting to define the pathological processes that cause allograft loss. More recently, an attempt to increase the specificity by which the mechanism of injury is defined in these cases has been developed. This however represents a consensus of expert opinion using often limited data. Consequently these classifications have changed with each iteration of Banff consensus definitions. Increasingly, microarray molecular phenotyping is used in research to define immunological processes in allograft biopsies [176]. Chronic glomerulopathy describes a process that has significant impact on allograft survival and is associated with alloantibody, in the absence of other diseases that may cause the same phenotype of endothelial injury and is irreversible. With antibody-mediated injury occurring before allograft failure, it can be used as an endpoint which will allow the evaluation of both: types of anti-donor antibody and effective interventions to prevent chronic antibody-mediated damage.

1.7.7 Accommodation

The presence of alloantibody is associated with tissue injury but not all allografts display a phenotype of allograft injury, despite the presence of alloantibody. This has

been called “accommodation”. The phenomenon of accommodation was described in animal models of xenotransplantation when there was no xenograft loss despite the presence of anti-donor antibody [177]. More recently it is commonly seen in ABOiKTx, but the term accommodation has not been widely used in HLAiKTx [178-180]. The precise definition of accommodation is important because initially it was defined as the presence of alloantibody without allograft dysfunction [181]. However, histological assessment allows a more detailed analysis of allografts, prior to deterioration in renal function. Further to this, molecular markers of immunological injury/activation are now being used, which demonstrate activation of the immune system, despite good allograft function and no chronic histological injury in light microscopy. Thus accommodation may simply be “slow” rejection, as true accommodation should reflect no allograft injury in the presence of alloantibody [182].

Table 1.4. Criteria for Chronic Antibody-Mediated Renal Allograft Rejection.

Adapted from Colvin, R.B. [142, 183].

Diagnostic criteria for chronic antibody-mediated rejection	
Histological evidence of chronic injury	1. Arterial intimal fibrosis without elastosis 2. Duplication of glomerular basement membrane 3. Multilaminated peritubular capillary basement membrane 4. Interstitial fibrosis and tubular atrophy
Evidence of antibody action/deposition in tissue	1. C4d deposition
Serological evidence of DSA	1. Anti-HLA antibodies 2. Other anti-donor antibody

1.8 What happens after an antibody incompatible transplant

1.8.1 Clinical Outcomes in HLAiKTx

The outcomes of HLAiKTx have been described in single-centre case series. There are few studies which report longer term outcomes (more than 2 years follow up, Table 1.5). As previously described, the level of the antibody is key to defining the degree of HLAiKTx. The current three main methods of stratifying HLAi are by complement dependent cytotoxicity (CDC) assay; lymphocyte flow crossmatch (FXM) (either B or T lymphocytes) and using solid phase assay beads (SAB) coated with HLA proteins. A comparison of data between these methods in a single centre demonstrates the relationship between each of these testing methods and clinical outcomes; namely that across a population there is an association between levels of anti-HLA antibody and tissue injury [70].

Allograft outcomes are difficult to compare directly between centres due to lack of standardisation of the anti-HLA antibody assays, different antibody targets and the different therapies used. The results suggest higher antibody levels associated with increased rates of AMR and worse allograft outcomes [184]. The outcomes of centres report allograft survival and creatinine, however the histological analysis of HLAiKTx are not widely published [185]. More recently, the development of C1q binding assays, detecting the activation of complement for a particular DSA will further impact the interpretation of these results, as patients with high DSA levels, but without C1q binding have significantly better clinical outcomes [165].

Table 1.5. A comparison of outcomes in early period HLAiKTx for patient and allograft survival in different centres and with registry data.

The rate of AMR across different centres is wide, with centres using more sensitive methods of DSA detection having lower rates of AMR, suggesting lower antibody levels. Similarly, the 1 year allograft survival rate ranges from 78% to 100%.

Centre	Year Published	Patient Number	DSA technique	Patient Survival			Allograft Survival			AMR rate
				1yr	3yr	5yr	1yr	3yr	5yr	
Hopkins [22]	2000	4	CDC/ELISA	100%	n/a	n/a	100%	n/a	n/a	100%
Mayo Clinic [186]	2003	14	CDC/ELISA	86%	n/a	n/ a	78%	n/a	n/ a	43%
UCLA [102]	2003	42	CDC	98%	n/a	n/a	90%	n/a	n/a	31%
Paris [187]	2007	32	CDC	n/a	n/a	n/a	84%	78%	78%	41%
Maryland [185]	2009	41	CDC/ELISA/Luminex	n/a	n/a	78%	90%	74%	70%	12%
Chicago [188]	2005	12	Flow	100%	n/a	n/a	100%	n/a	n/a	25%

1.8.2 Clinical Outcomes in ABOiKTx

Whilst single centres report good outcomes, equivalent to ABO compatible cohorts within centre, there is data suggesting a significant difference between ABOi and ABOc across registry data [25, 189]. This is in contrast to Futagawa's study with earlier data from UNOS which showed equivalent outcomes in ABOi to ABOc between 1995 and 2003 [190]. This study was smaller than Montgomery et al (n=392 vs. n=738) and compared both deceased donor and live donor together, but this was in the early experience of ABOiKTx. There are many factors that could explain the differences in outcomes in these cohorts. Table 1.6A shows data from some centres performing ABOiKTx, reflecting the differences in outcomes.

There is a higher rate of antibody-mediated rejection in the ABOiKTx group than in ABOcKTx (Mayo Clinic data, 46% vs. 2%)[191]; however, this was not reported in Tyden's study, in which the cellular rejection rate was very low and not significantly different between the ABOiKTx and ABOcKTx (Table 1.6B) [125, 192].

The risk factor associated with allograft loss through hyperacute antibody-mediated rejection and early antibody-mediated rejection is the level of the anti-donor ABO titre, in particular the IgG isotype [193]. It is difficult to separate the analysis of IgG and IgM data, as often they are both present at similar levels – insofar as high titre patients generally have high levels of both isotypes.

Table 1.6. Clinical outcomes of ABOiKTx with survival and AMR as endpoints.

Table 1.6A - A comparison of outcomes in ABOiKTx for patient and allograft survival in different centres and with registry data.

Centre	Patient Number	Patient Survival			Allograft Survival		
		1yr	3yr	5yr	1yr	3yr	5yr
Hopkins [194]	60	96.3%	96.3%	89.4%	98.3%	92.9%	88.7%
Mayo Clinic [73]	40	95%	89%	n/a	96%	80%	n/a
Guys Hospital [195]	62	94.5%	91.9%	n/a	98.4%	98.4%	n/a
Karolinska, Sweden [196]	45	100%	n/a	n/a	96%	91.1%	n/a
US Transplant Registry [189]	738	96.8%	93.7%	88.3%	94.1%	89.6%	82.6%
Japanese Transplant Registry – early era [197]	451	92%	89%	86%	82%	76%	70%
Japanese Transplant Registry – modern era [197]	1427	98%	97%	96%	96%	93%	91%

Table 1.6B. A comparison of rejection rates for ABOiKTx in different centres demonstrating wide range of clinical outcomes in each data set.

Centre	Number	Rejection Rates	
		AMR	Cellular
Montgomery [194]	60	16.7% (n=10)	18.3% (n=11)
Mayo Clinic [191]	24	45.8%(n=11)	16.7% (n=4)
Japan [198]	494	Combined 58% (n=256)	
Karolinska, Sweden [125]	60	0%	6.7% (n=1)

1.9 Summary and Scope of this thesis

The benefit of kidney transplantation is proven, yet antibody-mediated rejection significantly reduces the allograft survival. For patients with antibody incompatible donors, it is very important to provide accurate information in order to make clinical decisions, both for the clinician and for the patient.

The aim of this thesis is to:

1) Develop an assay for reproducible measurement of anti-ABO specific antibodies
2) Compare the anti-ABO specific antibodies between UK transplant centres
3) Examine the clinical outcomes in ABO incompatible kidney transplantation in the ABOUT-K study and the effect of differences in anti-ABO specific antibody measurement on these outcomes.
4) Define the long term clinical and histological outcomes of positive crossmatch kidney transplants with regards to their HLA class of antibody
5) Assess the cellular infiltrate in antibody incompatible kidney transplant recipients and the clinical outcomes
6) Investigate the possible cause for early antibody-mediated rejection in patients treated with C5 inhibition.

Chapter 2 Novel Assay Development for anti-ABO blood group antigen specific antibodies

2.1 Introduction

2.1.1 ABO Titration

The measurement of anti-ABO specific antibodies has not substantially changed in the last 100 years since their identification by Landsteiner. Identification of an individual's blood group has been done, by testing patient red blood cells (RBC) against known reagents that agglutinate known red cell phenotypes or more recently genetically. Alternatively the blood group may be deduced from the specificity of binding from the individual's plasma, referred to as reverse blood group typing (Landsteiner's rule) [199]. Red cells of known blood group are incubated with plasma of the patient and if there is agglutination, then a positive result is recorded. The antibody reaction is determined by serial doubling dilution of plasma against red cells, known as ABO titration and quantification of anti-ABO specific antibody is determined by the number of dilutions necessary to abrogate the reaction. There have been different variations on this technique's protocol, but all are based on the same principle of serial doubling dilutions until the lowest concentration of plasma that agglutinates is recorded as the titration value. This application has been used for assessing risk in haemolytic disease of the newborn and for determining transfusion from donors with high or low anti-ABO specific antibody titres [200].

Measuring the amount of antibody is considered a pre-requisite for ABOiKTx. The reduction of anti-ABO specific antibody to acceptable levels and monitoring for early complications is routinely performed in clinical practice. The method of quantification of anti-ABO specific antibody was transferred from transfusion practice into transplantation practice.

2.1.2 Immunoglobulin Isotypes

ABO titration has been adapted for isotypes; in that measurements can be taken for total isohaemagglutination; treated with DTT for measurement of non-IgM isotype (presumed IgG) and total agglutination using different ionic strength diluents to measure predominantly IgM [201-203]. The addition of anti-human globulin (AHG) to increase agglutination and measurements at different temperatures have also been used to differentiate plasma antibody reactions attributable to IgG and IgM [204, 205]. The significance of isotypes in transfusion reactions is important. Greenbury and colleagues inferred that only 25 anti-A1 IgM molecules per RBC are required for direct agglutination of A1 red blood cells, but 20,000 IgG molecules were necessary [206]. In order to initiate complement binding, it has been estimated that 7000 molecules of anti-A IgG per RBC are required to bind complement using an anti-C3c reagent, although data for IgM is not available [207]. The proportion of anti-A IgG of total IgG was found by Buchs to be $1/10^4$-$1/10^6$ [208]. Furthermore, the temperature at which agglutination takes place helps to identify isotype, for example at 4ºC IgM anti-ABO specific antibodies agglutinate better while IgG have increased agglutination at 37ºC.

The agglutination of RBC by different IgG subclasses demonstrates high levels of IgG2 binding – but interestingly a quarter of patients had all 4 subclasses found, although these were not quantified [209]. While IgG2 binding is high, maternal anti-ABO specific antibodies causing haemolytic disease of the new-born are predominantly IgG1 and IgG3 in distribution, because IgG2 does not cross the placenta.

2.1.3 Poor reproducibility of ABO titration

Data from Japan and Sweden was the first to demonstrate significant variation in results from different centres and users performing the ABO titration [210, 211]. There was up

to an 8 $\log_2$ dilution difference between the lowest and highest readings in Ishida's study. Kumlien and colleagues demonstrated that using a defined protocol significantly reduced the variation in assay; however this did not eliminate variation within an intra-laboratory standard of ±1 dilution from the median titre. The American Pathology Society coordinated an international study in laboratory variation and if a set protocol was followed using a gel card technique at a set temperatures using a defined endpoint, this produced more reproducible results [205]. This study demonstrated that at 37ºC there was less variation than at 4ºC. The use of pooled red cells or donor cells is still debated; however this study also demonstrated that using the same cells reduced variation between laboratories. However, pooled RBCs have the possibility of true standardisation of antigenicity and may be "normalised" through an adequate quality assurance process. National pooled RBCs will reflect the genetic distribution with each region, but will not be applicable to different international regions.

The assumption that donor A/B antigen RBC expression reflects the kidney A/B antigen expression remains unproven. Indeed, A/B antigen expression will also vary, as transferase activity will also depend on the expression of core chains, the variation of which remains ill described. Thus, ultimately testing anti-ABO specific antibodies against RBCs may not be testing the appropriate antigen-antibody interactions in transplantation [212]. The primary function of haemagglutination is to assess the agglutination function of the antibody, rather than the quantity of antibody. There are also qualitative differences in antibody binding that cannot be assessed by haemagglutination.

2.1.4 Alternative techniques for anti-ABO antibody assessment

Flow cytometry is a different technique for analysing antibody binding to RBCs demonstrated by Stussi et al to be sensitive and specific for both isotype and subclass of anti-ABO specific antibodies [66]. Krishnan and colleagues assessed flow cytometric analysis with correlation to haemagglutination titre in clinical cases and demonstrated the ability to correlate anti-ABO specific antibody changes over time with both haemagglutination and flow cytometry and in response to clinical therapies [213].

Further analysis of clinical assessment of antibodies using ELISA assays demonstrated that different isotypes and subclasses of anti-ABO specific antibodies may play an important role. Ishida et al demonstrated a difference between rejecters and non-rejecters during the first month post ABOiKTx. Whilst at 1 week both groups had an initial rise in IgM, IgG1 and IgG2, at 1 month there was a further rise of IgM; IgG1 and IgG2 in rejecters but falling levels of IgM and IgG2 in non-rejecters [214]. This paper does acknowledge that at 1 month all rejecters had returned to dialysis. This data has yet to be reproduced by other groups. Importantly, the assessment of antibody after a clinical event has very little benefit to clinical management. To be able to predict risk and to guide subsequent management, an assay to determine higher risk patients is required [212, 215]. Clinical data shows that despite the return of anti-ABO specific antibodies, there is seldom rejection or allograft dysfunction and while there is an increased risk of having antibody-mediated rejection if the starting titres are higher; there are still a large proportion of patients who have very good clinical courses who had initial high titres [216].

Anti-ABO specific antibody binding to A/B antigens was shown using surface plasmon resonance [217, 218]. Surface plasmon resonance is a platform that allows molecular interaction to be assessed, as association and dissociation of molecules are displayed

and data can be extrapolated for the binding affinities of binding molecules [219]. Different binding properties of anti-ABO specific antibodies may explain the differences in outcome between the same agglutination titre of antibody.

The aim of these studies was to explore the development of reliable and reproducible assays to measure anti-ABO specific antibodies. Improved anti-ABO specific antibody quantification, specifically improved precision and reproducibility is an important step towards:

1) Prospective multicentre studies of intervention, including EART and immunosuppression

2) Risk stratification based upon antibody quantification, with a view to identifying patients likely to develop early allograft loss or antibody-mediated rejection who may be offered alternative treatment strategies such as paired exchange.

It may also be possible to differentiate antibodies on the basis of antigen specificity, isotype or other characteristics that specifically identify those more or less likely to contribute to organ damage (rather than just haemagglutination)

2.2 Anti-ABO specific antibody assay development

2.2.1 Haemagglutination

2.2.1.1 Introduction

The measurement of anti-ABO specific antibody by agglutination is the current best practice in red cell immunohaematology. I aimed to produce a robust, reproducible assay for haemagglutination and differentiate IgG binding from IgM. In order to differentiate isotypes, DTT was used in the methodology to determine isotype distribution and thereby the effects of different isotypes on the allograft outcomes, specifically rejection and allograft survival [220].

2.2.1.2 Methods

Using protocols based on NHSBT Red Cell Immunohaematology laboratory practice and UK NEQAS (National External Quality Assessment Service), day to day variability; different red cell batches and with freeze/thaw samples were investigated. Plasma was obtained by centrifuging human blood in EDTA for 10 minutes at 5000rpm. A DiaMed gelcard was used according to manufacturer's protocol and both direct and indirect agglutination were measured.

2.2.1.2.1. Preparation of Red Blood Cell Test Blood Group Sample.

This is same for both Haemagglutination (HG) techniques. Red Blood Cells (RBC) were obtained from NHSBT Supplies (NHSBT Liverpool); A1rr (product code PR014); Brr (product code PR035) and OR1r (product code PR041), in a Cell Stat suspension, 0.8 ±0.2% concentration/ml used for all assays requiring red cells.

A 0.8% red cell suspension was prepared in ID-Diluent 2 using red blood cell blood group according to specificity of binding required. The ID-Diluent-2 solution was allowed to reach room temperature before use and 1ml of ID-Diluent-2 was dispensed into a clean tube. Ten microL of packed red cells was added and gently mixed. The cell suspension could be used immediately, or stored at 2-8°C for 48hours.

One aliquot of plasma was treated with 0.1M DTT (Sigma, Poole Dorset, UK). Three hundred microL of plasma was diluted with 300 microL of 0.1M DTT for 30minutes at 37ºC. ID Diluent 2 was used for serial dilutions of plasma using a doubling dilution method.

Direct agglutination at room temperature (DRT) was performed using neutral DiaMed cards (005015, NaCl, Enzyme Test and cold Agglutinins, BioRad, UK) with 50 microL of RBCs suspended in ID Diluent 2 to each microtube. Fifty microL of each

plasma dilution was added to the corresponding microtube and incubated at room temperature for 15 minutes. The microtubes gel cards were centrifuged (85g) for 10 minutes in DiaMed (I-Centrifuge 12S-II, BioRad, UK). The gel cards were analysed over a bench lamp and the last well giving a positive result of agglutination was recorded as the titre result for that sample. The indirect agglutination using antiglobulin technique (IAT) used 50microL of red cells suspended in ID Diluent 2 to each microtube and then 25microL of the DTT treated plasma serially diluted from 1:1 with ID Diluent 2. Gelcards already had anti-IgG in the columns (004025, Coombs Anti-IgG, BioRad, UK). This was incubated at 37ºC for 15 minutes and then centrifuged (85g) and read as per previous assay. A single user performed multiple haemagglutination assays for historic controls to attain laboratory competencies.

Different batches of RBCs were used to assess variability between HG results. Two different batches were obtained and HG was performed on the same sample by the same operator. The same sample was tested on 2 sequential days with the same batch of RBC to determine day to day variation. The effect of freeze/thaw was determined by testing samples on arrival to the laboratory. Samples were frozen at -40°C for a minimum of 1 week and then thawing took place in a water bath at 25°C for 30minutes.

2.2.1.3 Assay variability

Pooled red blood cells were obtained from NHSBT processing laboratory in Liverpool for ABO blood groups. Haemagglutination was tested with different RBC batches and intra-sample variability assessed. The results were analysed using a weighted kappa quadric analysis, which tests the agreement between two different tests and gave a result of 0.867 (0.799 to 0.936, n=16) demonstrating very good agreement of the two tests. The calculation is based on the difference between how much agreement is

actually present ("observed" agreement) compared to how much agreement would be expected to be present by chance alone[221]. The effect of freeze/thaw on samples was tested using stored samples which had been tested on day of arrival for ABO titration and then re-tested after thawing and the results of both titrations were within 1 dilution of each other.

Finally, HG assays were performed on different days and there was good reproducibility (Figure 2.1). Bland-Altman plots show the difference between test 1 and test 2 for reproducibility. The weighted kappa quadric assessment for different day agreement of IgG was 0.945 (CI 95% 0.901-0.989) and IgM 0.979 (CI 95% 0.971-0.988) demonstrating excellent agreement between the two tests (Figure 2.2). The statistical methods are explained in more detail in section 2.2.5 on page 125.

Figure 2.1. Reproducibility of HG assay on different days by same operator.

This figure demonstrates the reproducibility of the HG assay on inter day variability testing for anti-A1 IgG (A) and IgM (B) reproducibility. HG data is $\log_{10}$ transformed from $\log_2$ dilution titre result. There is good reproducibility of the assay on the same sample tests on different days by the same operator. The reproducibility for anti-B IgG (C) and IgM (D) is also very good. The Bivariate Plot with Linear Fit is plotted with r^2 regression coefficient. n=number of samples

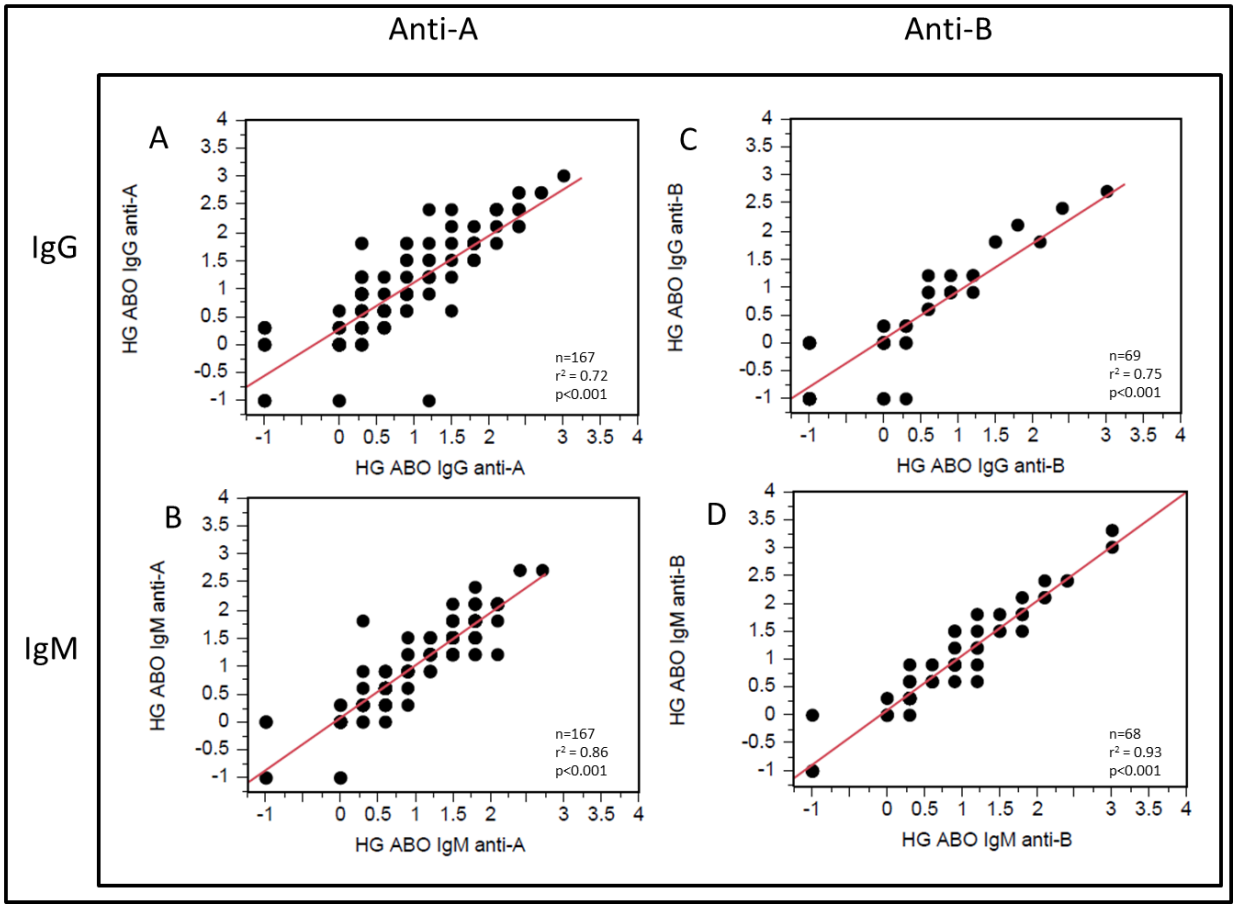
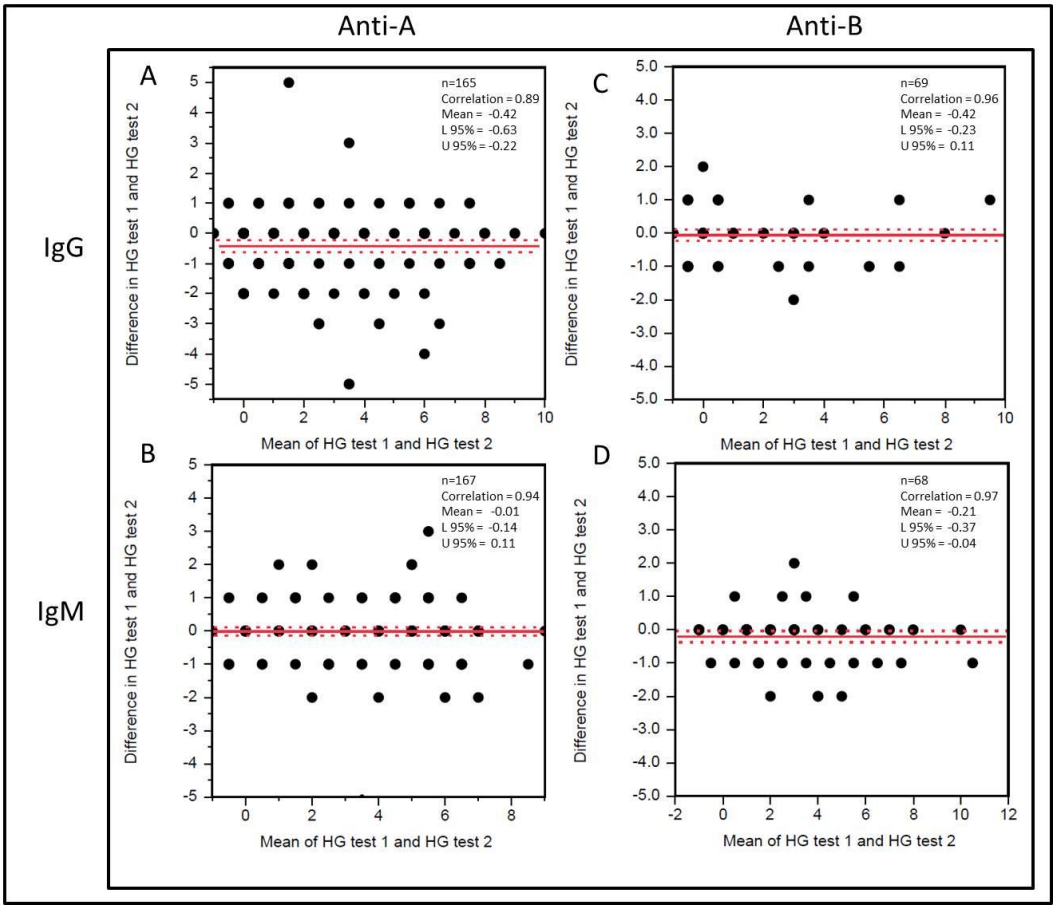


Figure 2.2. Intra-test agreement between HG assays for IgG and IgM.

These Bland-Altman plots demonstrate agreement between test 1 and test 2 for each isotype by HG, with a single user for both IgG and IgM against both blood group A (graphs A and B respectively) and both IgG and IgM against blood group B RBCs (graphs C and D respectively). The data is plotted as a continuous variable, however is categorical in nature and thus there is overlap of data points. The plots demonstrate very good correlation and mean difference between the 2 haemagglutination (HG) tests as low in all 4 tests.



2.2.1.4. Conclusions

A single user with a single methodology for ABO titration can produce results that are within the limits of the units of measurement of the test itself. Intra-laboratory quality control accepts a difference of 1 titre dilution. The use of doubling dilution in these assays reflects the limits of the assay performance. These reproducibility experiments demonstrate a mean difference between tests of one titre step (± half titre step), which is the limit of assay performance as defined by the size of the (minimum) increment.

A reliable and reproducible assay is important in the comparison of clinical data and to assess clinical variables between different centres performing ABOiKTx. In order to compare antibody levels during transplant assessment and treatment, a reproducible method is required. Freeze/thaw had no significant effect on these results. Different red cell pooled batches validation has been performed so that there was very high level of agreement over time.

This assay will be used to the clinical study in Chapter 3 describing the clinical outcomes in a multicentre observational study in ABOiKTx.

2.2.2 Flow Cytometry Assay

An alternative approach to measuring anti-ABO specific antibodies uses flow cytometry and I adapted this technique from a previously published method for identifying IgG and IgM [213]. Flow cytometry has the potential advantage of allowing identification and quantification of antigen specific isotype and subclass. Furthermore, the identification of complement binding IgG subclasses would allow risk stratification in the assessment of anti-ABO specific antibody. The protocol for running samples in both isotype and subclass experiment was similar with different secondary labelled antibodies.

2.2.2.1 Methods

A suspension of RBCs from NHSBT (as used in haemagglutination assays) was measured using a haemocytometer to a required concentration of 10^7 cells/ml in flow cytometry diluent. This concentration was determined by Krishnan et al to reduce the effect of agglutination on the assay when setting flow cytometry gating [213]. The RBC suspension for both blood group "A1" and blood group "B" were vortexed for 10 seconds to ensure adequate suspension. Twenty five microL of RBC suspension was added to the appropriate tube, and a further 20microL of FACS diluent was added. Five microL of the plasma to be tested was added to the appropriate tube. Each tube was vortexed for 10 seconds and incubated at room temperature for 30minutes. The cells were washed with PBS (pH 7.4) 3 times using a Cellwash centrifuge (5 minutes at 1000g, DiaCent-CW, BioRad, UK) and the supernatant discarded, to leave a RBC pellet. One hundred microL of secondary antibody (according to isotype/subclass to be identified) was added according to dilution of each secondary antibody, and then incubated at 4°C in the dark for 15 minutes.

Following incubation, 300microL of flow cytometry diluent was added to each tube and vortexed for 10 seconds. The tube was then put on the flow cytometer (BD FACSCanto™ II, BD Biosciences). Gating of the flow cytometer was based on the red cell population size using forward and size scatter. Counts of 5,000 cells were acquired per tube in the gated area. Data was analysed using the BD FACSCanto™ software (BD Biosciences, California, USA). In order to report the binding of each antibody, the relative median fluorescence intensity (RMFI) was calculated from the sample median fluorescence intensity (MFI) of sample binding divided by the MFI in the presence of "AB" serum.

2.2.2.1.1 Isotypes

The concentration of each secondary antibody for each isotype was determined using standard curves for each of the following antibodies: 1:800 Goat Anti-Human IgG (γ-chain specific), F(ab′)2 fragment–FITC antibody (F1641-2ML, Sigma, UK); 1:200 mouse anti-human IgM (μ chain specific) R-phycoerythrin (R-PE) conjugate (9022-09. Southern Biotech, Birmingham, USA) and 1:100 anti-human IgA APC (130-093-113, Miltenyi Biotec, UK).

2.2.2.1.2 Subclasses

In order to perform multicolour flow cytometry on the IgG subclasses, it was necessary to conjugate a fluorochrome to an isotype. This comprised of incubating the mouse anti-human IgG4 purified (9190-01, Southern Biotech, Birmingham USA) with Lynx Rapid PerCP conjugation kit (LNK073PERCP, AbD Serotec, UK). Conjugation was achieved by adding 1 microL of the Modifier reagent for every 10 microL of antibody and mixing gently. This sample was added directly to the LYNX lyophilized mix and mixed by gently pipetting in order to re-suspend. This mixture was incubated in the dark for 3 hours at room temperature. One microL of Quencher reagent was added for every 10 microL of antibody used, following incubation and left for a further 30 minutes before use. The other antibodies used were mouse anti-human IgG2 FITC (9080-02, Southern Biotech, Birmingham, USA); Mouse anti-human IgG3 R-PE (9210-09, Southern Biotech, Birmingham, USA) and anti-human IgG1 APC (130-093-189, Miltenyi Biotec, UK).

2.2.2.1.3 Purification of antibody

For positive controls in both flow cytometry and surface plasmon resonance, purification of both murine monoclonal and human polyclonal antibody against both A and B antigens was carried out. A 50ml syringe was connected to a Glycorex™ sephadex

column which was connected in series with a Pharmaxia LKB P1 pump and Pharmacia Single Path Monitor. The circuit was washed with 150ml of PBS (pH 7.4) using rate of 8ml/min. The solution of protein for purification was added to the 50ml syringe and pump speed of 2ml/min. Monitoring of the protein concentration determined the protein adsorption to the column. Glycine elution buffer (40ml, pH 2.8 100mM) was added to the syringe and the pump speed reduced to 1ml/min. Eluate was collected in 15ml Falcon Tube containing 0.5ml Tris solution(1M, pH 8.3) for neutralisation of the acid. Continue with elution until the protein peak returns to baseline. The eluate was concentrated using Vivaspin 20 columns. The columns were initially blocked with 20ml of 0.1% BSA (Sigma, UK) by incubating for 30minutes at room temperature on a roller. The column was washed with de-ionised water and shaken in order to dry. Using 20ml of eluate in column, this was centrifuged at 4000g until concentrated. The volume was checked volume at 30minute intervals and repeat centrifuge process was performed if necessary to reduce volume to 5ml. The flow through was discarded.

This protocol was used for both murine monoclonal and human polyclonal antibodies. The blood group typing agents against A or B antigen were used as murine monoclonal antibodies (clone Birma-1 or clone LB-2, Millipore, UK). The human polyclonal antibody was eluted from waste Glycosorb™ columns after clinical discard.

2.2.2.2 Results

2.2.2.2.1 Isotypes

The assessment of blood group antibodies represented a normal distribution for both IgG and IgM isotypes relative to the background binding against AB serum (RMFI) which is shown in Figure 2.3. The relationship of the $\log_2$ transformed MFI strongly correlated with the HG titre data plotted on a transformed scale ($\log_2$) shown in Figure 2.4A. The

relationship of IgM to both IgA and IgG as measured by flow cytometry also correlate with each other in Figure 2.4B

Figure 2.3. Histograms demonstrating the distribution of anti-A specific antibodies in flow cytometry and HG.

Figure 2.3A. The distribution of the log_2 transformed central haemagglutinin titre (anti-A IgG) measured prior to antibody removal is normally distributed with a mean of 4.5 and standard deviation of 2.7. (In this and subsequent figures a HG titre of 0 is neat and -1 no reactivity)

Figure 2.3B. The distribution of the log2 transformed RMFI (anti-A IgG) measured prior to antibody removal is normally distributed with a mean of 2.3 and standard deviation of 1.4.

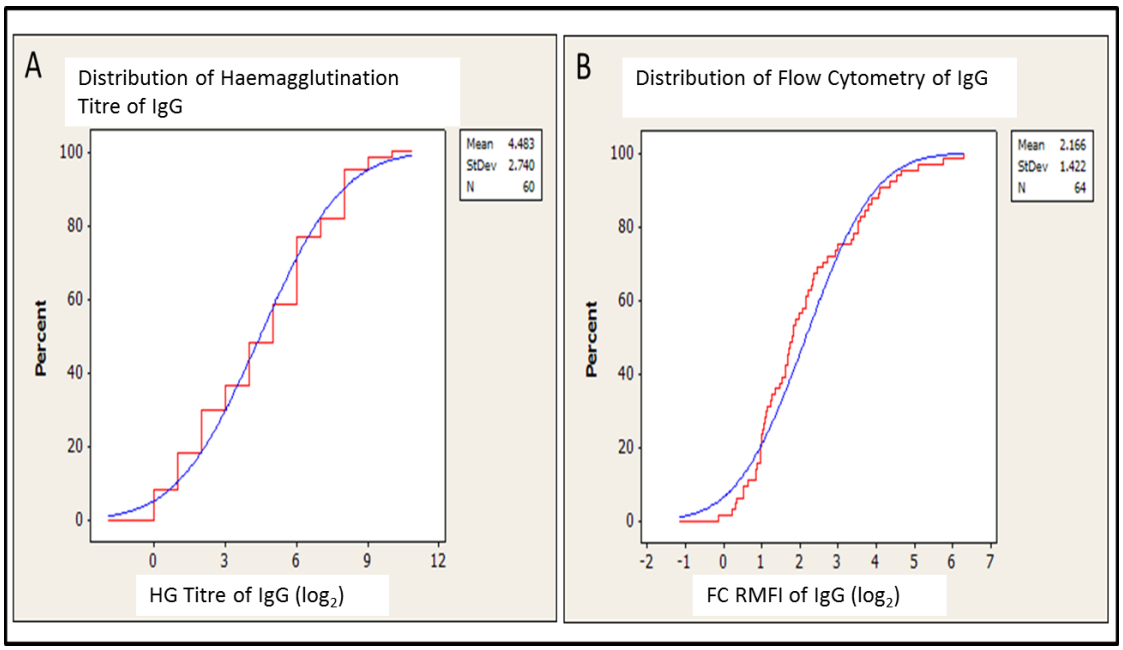


Figure 2.4. The relationship of HG titre and flow cytometric analysis of anti ABO specific antibodies.

Figure 2.4A. The log_{10} haemagglutination titre (log HG - x axis) and flow cytometry data represented by the log_2 relative median fluorescence intensity (Flow RMFI log_2- y axis) for both IgG and IgM isotypes against both A and B blood group antigens. The Bivariate Plots with Linear Fit (red lines) demonstrate the strongest correlation for IgG binding to A antigen (graph A) as demonstrated by the regression coefficient (r^2). n= number tested.

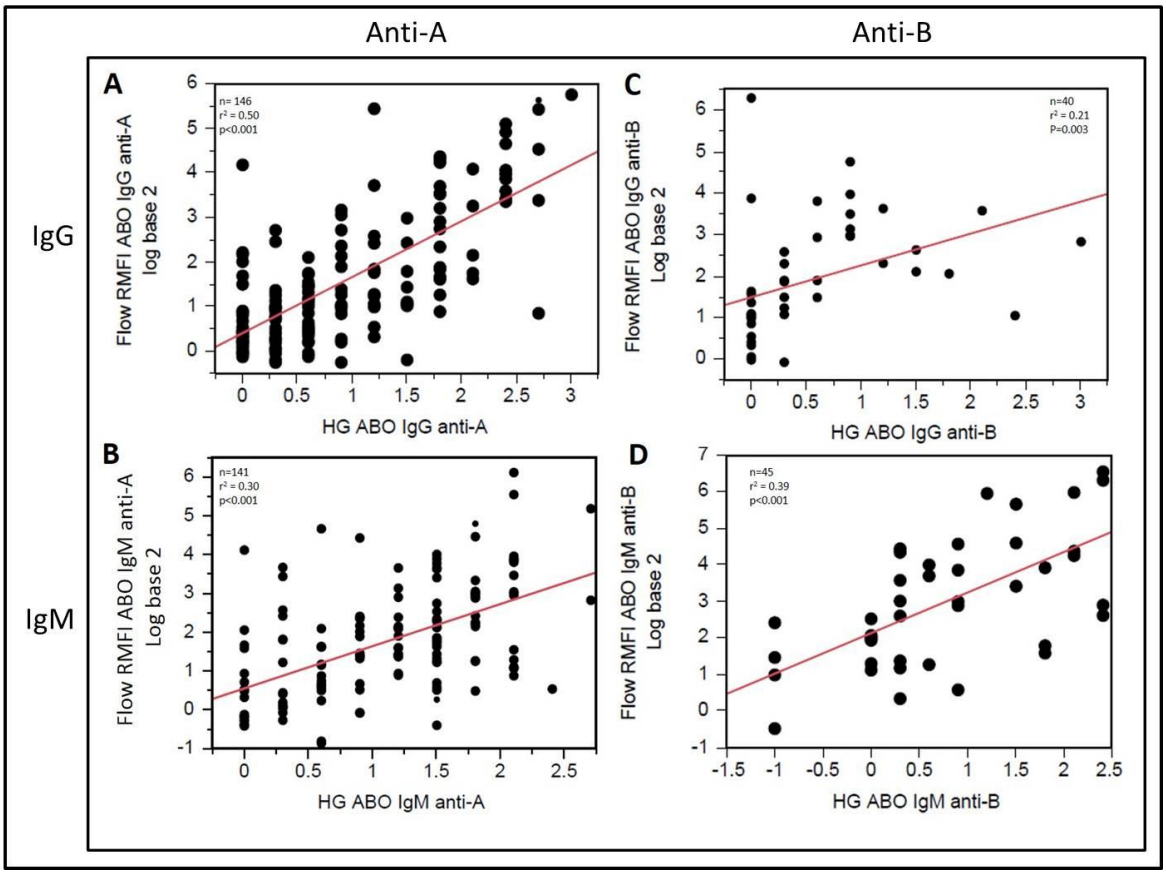
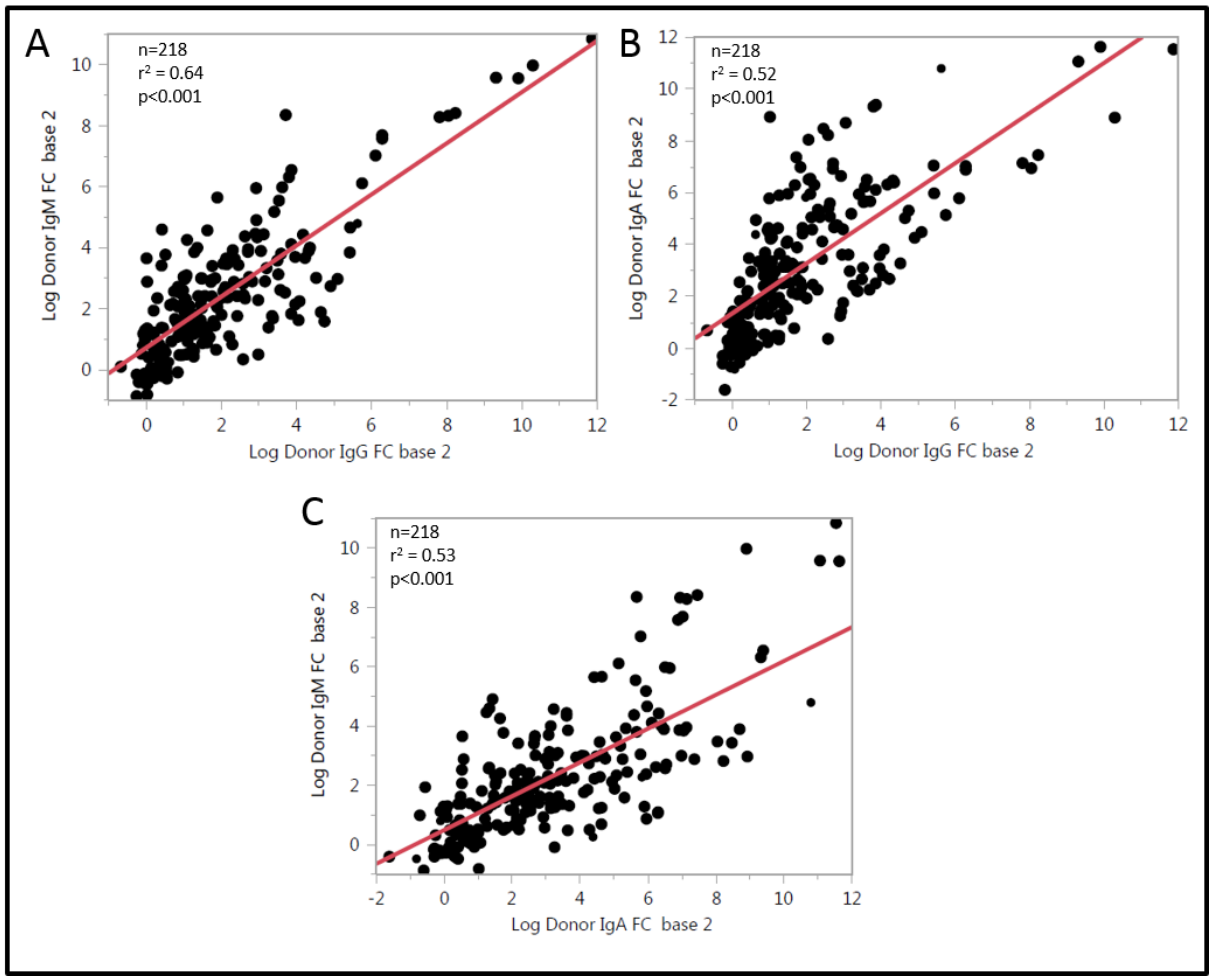


Figure 2.4B. The assessment of anti blood group antibody isotypes by flow cytometry (FC) for IgG, IgM and IgA.

The relationships of the log base$_2$ transformed relative median fluorescence intensity of each isotype to the other isotypes are shown below. There is the greatest correlation with IgG and IgM (r^2=0.64) relative to the other isotypes. The data are plotted as a Bivariate Plot with Linear Fit (red lines) with a regression coefficient. n= number tested; r^2=coefficient.

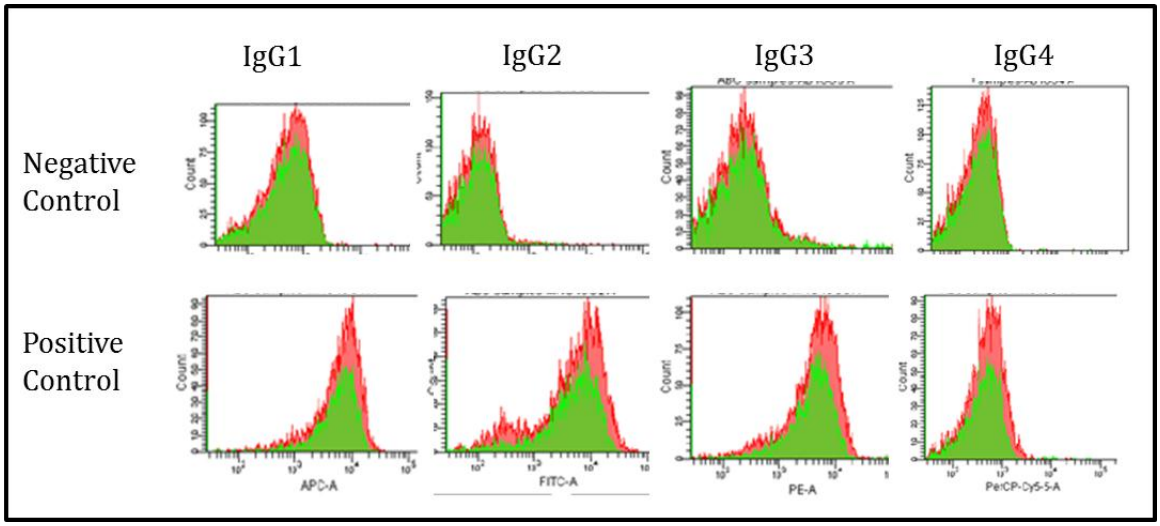


2.2.2.2.2 Subclasses

Optimisation of the concentration of the individual subclass secondary antibodies and then combined in performing 4-colour flow cytometry demonstrated strong binding to the positive control with minimal background binding (Figure 2.5). In a small cohort of patients (n=4) with antibody-mediated rejection collected from clinical practice, there was binding for the different subclasses which demonstrated different distributions of IgG subclasses over early post-transplant time points (Figure 2.6). These experiments require further exploration and refinement.

Figure 2.5. Flow cytometry histograms for subclass analysis of IgG

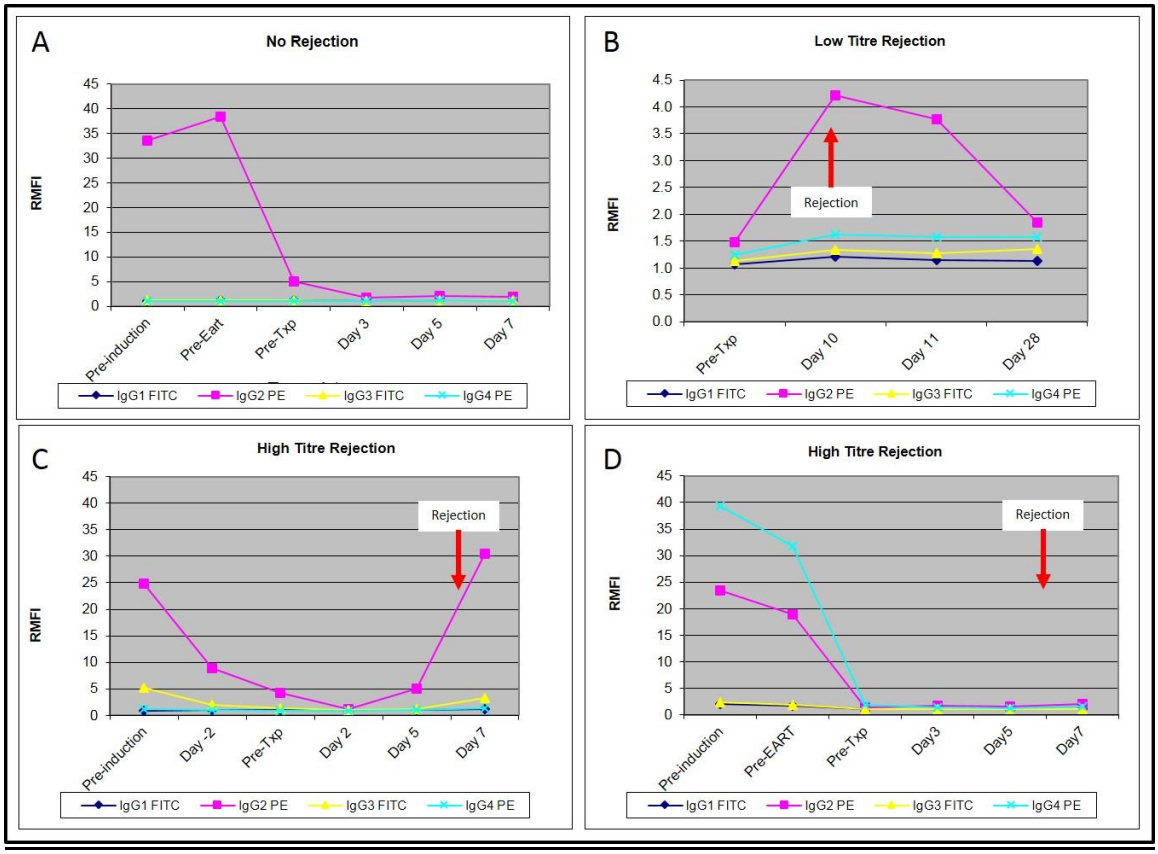
These flow cytometry subclass assay histograms demonstrating the human positive control and negative control (human AB serum). The median fluorescence shift for each subclass against blood group A red cells in the positive control is shown (a concentrated eluted human polyclonal antibody from Glycosorb™ columns, method described in 2.2.2.1.3). The red and green histograms represent different gated areas on the flow cytometer. The table shows the median fluorescence intensity (MFI) binding and the relative MFI (RMFI, calculated by divided the test MFI by the negative control MFI) for each subclass.



N=1	IgG1 MFI	IgG1 RMFI	IgG2 MFI	IgG2 RMFI	IgG3 MFI	IgG3 RMFI	IgG4 MFI	IgG4 RMFI
Negative Control	174	-	49	-	31	-	137	-
Positive Control	5381	**30.9**	9905	**202.1**	5577	**179.9**	608	**4.4**

Figure 2.6. Preliminary Data for IgG subclass analysis of patient with ABOiKTx

These data demonstrate changes in the IgG subclasses in patients with antibody-mediated rejection compared to no rejection in the patient represented in graph A. IgG2 is the most prevalent subclass across all patients, however no rejection was found in the patient represented in graph A despite high IgG2 levels. High levels of IgG4 above baseline were found in 1 patient (D) prior to transplantation and IgG3 was present at higher levels than baseline in (C), which had a high (relative to background) response at the time of rejection. Time of rejection is annotated in the graphs for the patients, which occurred at day 10 (Graph B), Day 6 (Graph C) and Day 6(Graph D). Two of these patients (represented in graph A and D) were collected as part of the ABOUT-K study (Chapter 3), and 2 further patients not enrolled in the ABOUT-K study with AMR were analysed from another centre, thus these sample times (shown in Graph B and C) differed from the ABOUT-K study protocol defined in Chapter 3.



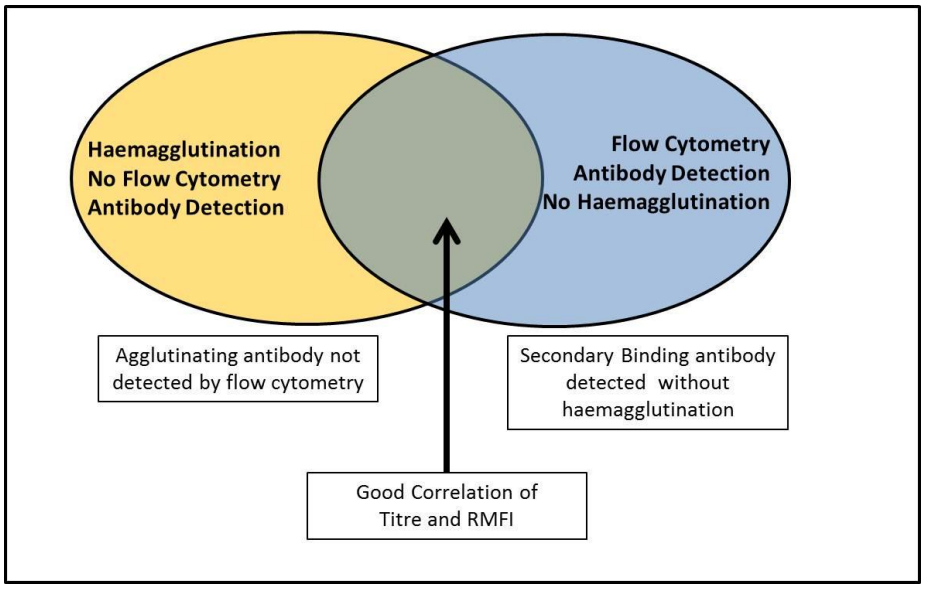
2.2.2.3 Conclusions

Binding to RBC with antibodies was able to be quantitatively assessed using flow cytometry and there was a significant correlation of the flow cytometry assay and the HG assay. While the two assays correlated, there was significant scatter across different samples (different individuals). This degree of variation was greater than expected based solely on the performance of the assays: primarily inter-assay variation in HG. For example: in Figure 2.4A which documents HG and flow cytometric binding to rbc attributed to IgG, in one sample the Log2 RMFI was greater than 4 but HG was only detected when the sample was used neat (log10 HG = 0). Conversely, a sample with a HG titre of 1/512 (log10 HG = 2.75) was associated with a much lower RMFI (Log2 RMFI = 1).1(Graph A). Importantly this may be reflect differences in the nature of antibody detected by the two techniques, which correlate at a population level but are not necessarily wholly congruent in an individual as illustrated schematically in Fig 2.7. These comments might equally apply to blood group antigen specific antibody binding to vascular endothelium, given the different pattern of core saccharide chains from which blood group antigens arise. This is however beyond the scope of these studies.

IgG subclass assessment was performed in a cohort of 4 patients, but demonstrated in most patients an IgG2 predominance in keeping with published literature [66]. The association with IgG subclass and clinical outcomes is planned in future work developing from these results. One patient who had antibody-mediated rejection had increasing levels of blood group antigen specific antibody measurement tested highly positive for anti-A specific IgG4, however this was not seen in the others tested (n=4).

Figure 2.7. Venn diagram to represent the potential different antibody populations in the two different assays.

Schematic illustration of potential relationship of antibody populations and assay measurements. (RMFI, relative median fluorescence intensity)



2.2.3 Solid Phase Assay

2.2.3.1 Microsphere Assay

2.2.3.1.1 Introduction

The development of assays to measure anti-A/anti-B binding, instead of using haemagglutination has been a goal over decades. The use of biological antigen in enzyme-linked immunosorbent assay (ELISA) has previously been investigated and whilst there was an appropriate sensitivity of antibody detection, there was background non-specific binding. [208]. Use of a synthetic assay will reduce the biological variability of different batches of red cells and allow a standardised assay. The development of synthetic ELISAs for measuring anti-ABO specific antibodies has demonstrated a correlation between the optical density measurement and the haemagglutination titre

for IgG and IgM isotypes. However, the correlation is variable for different isotypes with each blood group antigen [222].

The use of microspheres with synthetic blood group A antigen was reported by Holgersson. This report demonstrated a good correlation of anti-A antibody binding to the microspheres with reference to the haemagglutination titre. This paper did not however report the specificity of binding of anti-A antibody to A antigen alone, or crossreactivity of anti-B antibody binding to A-antigen beads [223]. In general these solid phase techniques have been developed using purified anti-ABO specific antibodies as test samples. I set out to repeat these investigations with a view to further development and study of the specificity of binding of anti-ABO specific antibodies in plasma.

2.2.3.2 Methods and Results

2.2.3.2.1 Coupling carbohydrate antigen to microsphere beads

Carbohydrate antigens were coupled to microspheres using a carboxyl substitution reaction. Carboxyl coated microspheres (BioRad; Seromap; Qiagen) were selected for different bead regions, according to their ratio of internal fluorescence using Luminex® technology. Oligosaccharides for A trisaccharide (L305, Dextra, Reading, UK) and B trisaccharide (G32, Dextra, Reading, UK) with terminal amine groups were a kind donation from Dextra Laboratories (Reading, UK).

The microspheres were brought to room temperature and vortexed for 30 seconds and then sonicaided for a further 60 seconds. A suspension of 500µL microspheres were pipetted into a microcentrifuge tube. This tube was centrifuged at 13,400g for 4 minutes to achieve a microsphere pellet. The supernatant was removed and 100microL of bead wash buffer (BioRad, UK) was added. This was vortexed for 10 seconds and sonicaided

for a further 10 seconds and centrifuged again with the same settings. The supernatant was removed and 80microL of bead activation buffer (BioRad, UK) was added. This was vortexed for 30 seconds and sonicaided for a further 90 seconds and centrifuged again with the same settings. Fresh *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma, UK) and N-Hydroxysulfosuccinimide sodium (S-NHS, Sigma, UK) were both diluted in bead activation buffer at 50mg/ml in separate microcentrifuge tubes. Ten microL of EDC solution and then 10microL of S-NHS solution were added to the bead suspension. The reaction of EDC created an unstable reactive ester replacing the carboxyl group, which was replace by a semi-stable amine-reactive NHS-ester after the addition of S-NHS. This solution was vortexed for 30 seconds and agitated at room temperature in the dark for 20 minutes.

One hundred and fifty microL of PBS (pH 7.4) was added to the solution at the end of incubation and centrifuged at 13,400g for 4 minutes to achieve a microsphere pellet. The supernatant was removed and 100microL of PBS (pH 7.4) added. This was vortexed for 30 seconds and sonicaided for a further 15 seconds and centrifuged again with the same settings.

Five hundred microL of PBS (pH 7.4) containing the following masses (concentration) of oligosaccharide used in conjugation: 200microg (400microg/ml); 400 microg(800microg/ml); 600 microg(1200microg/ml); 800 microg(1600microg/ml); 1000microg(2000microg/ml) and 1200 microg(2400microg/ml) respectively were added to the activated bead pellet. The mass of oligosaccharide in the conjugation process will be used to describe the different concentrations. The oligosaccharides all had amine terminal conjugations, but varied depending on the conjugation structure (simple amine or 8 carbon linker) for each experiment and the blood group antigen required. Terminal A-antigen, B-antigen and H-antigen were used, as well as

unconjugated beads as controls. The trisaccharides were either with a terminal amine group directly conjugated onto the trisaccharides, or had an 8 carbon chain structure (Linker). These were donated by Dextra Ltd, Reading, UK. This solution was agitated at room temperature in the dark for 2 hours. This tube was centrifuged at 13,400g for 4 minutes to achieve a microsphere pellet, following 2 hour incubation. Supernatant was removed and 500microL of PBS (pH 7.4) was used to wash the beads and this was centrifuged at 13,400*G for 4 minutes. The microspheres were resuspended in 250microL blocking buffer (BioRad, UK) by vortexing for 15 seconds. The microcentrifuge tube was agitated for 30 minutes in the dark and then centrifuged at 13,400g for 4 minutes and the supernatant removed. The microspheres were washed by 500microL storage buffer (BioRad, UK) and centrifuged at 13,400*G for 6 minutes. The supernatant was removed and the beads re-suspended in 200microL of storage buffer (Figure 2.8). Centrifuge tubes with conjugated beads are stable for 1 year stored at 4ºC in the dark according to manufacturing instructions.

Figure 2.8. Coupling of carbohydrate antigens to microspheres was achieved using a carboxyl substitution reaction.

Adapted from Thermoscientific.com

(http://www.piercenet.com/instructions/2160650.pdf)

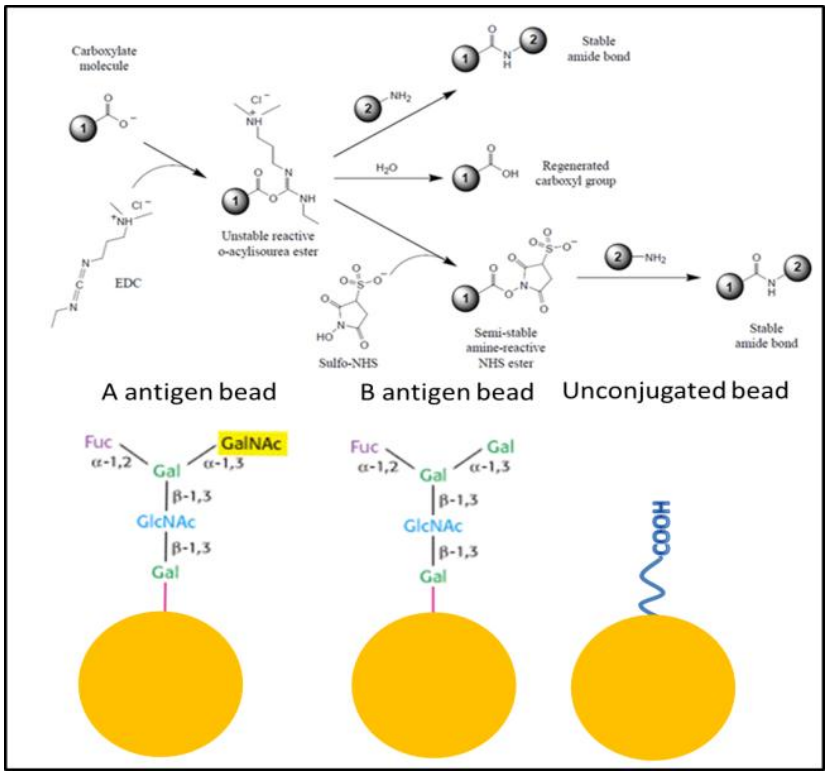
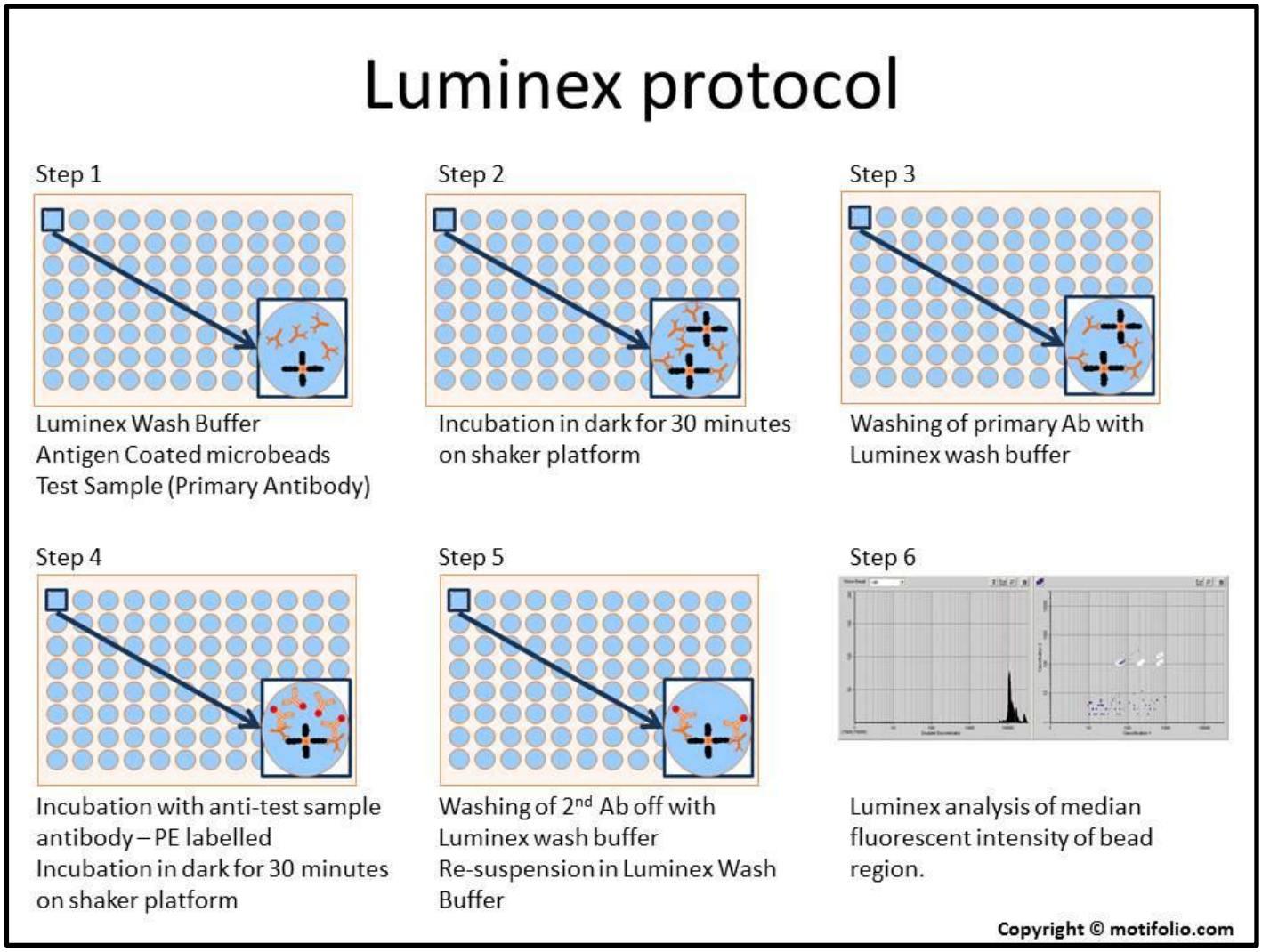


Figure 2.9. Diagram of Luminex protocol for analysis of antibody binding



2.2.3.2.2 Microsphere assay protocol

A 96 well filter bottom plate (Millipore, UK) was covered with well cover, and the required number of wells exposed. Each required well was washed twice with 200 microL Luminex Wash Buffer (LWB, One Lambda, CA, USA) and buffer removed using a Millipore Vacuum suction apparatus (Millipore, UK). Following 10 seconds of vortexing, the required microsphere suspensions were sonicaided for 5 minutes, to resuspend the microspheres.

Into each well 20µl of LWB was added, then 1.5µl of microsphere suspension. 8µl of sample was added to each well, as per experiment protocol, and then incubated with agitation for 30 minutes at room temperature in the dark. Two wash cycles were performed following incubation and 80µl of PE-conjugated secondary antibody was added to each well, according to protocol. For positive controls for A/B carbohydrates, murine monoclonals against A or B antigen were used (clone Birma-1 or clone LB-2, Millipore, UK), and a rat anti-mouse IgM (µ chain specific) PE conjugate (Clone 1B4B1, Southern Biotech, Birmingham, USA) was used as a secondary antibody. When human samples were used, a mouse anti-human IgG (γ chain specific, Clone H2) R-phycoerythrin (R-PE) conjugate and mouse anti-human IgM (µ chain specific, Clone UHB) R-phycoerythrin (R-PE) conjugate were used at 1:100 dilution (Southern Biotech, Birmingham, USA). Following a further incubation with agitation for 30 minutes at room temperature in the dark, two more wash cycles were performed, but re-suspending the beads in 80µl of LWB (Figure 2.9).

The plate was then inserted into the Luminex machine and the fluorescence of each microsphere determined the region, and the median fluorescence intensity of PE for each region determined the amount of binding of the tagged primary antibody.

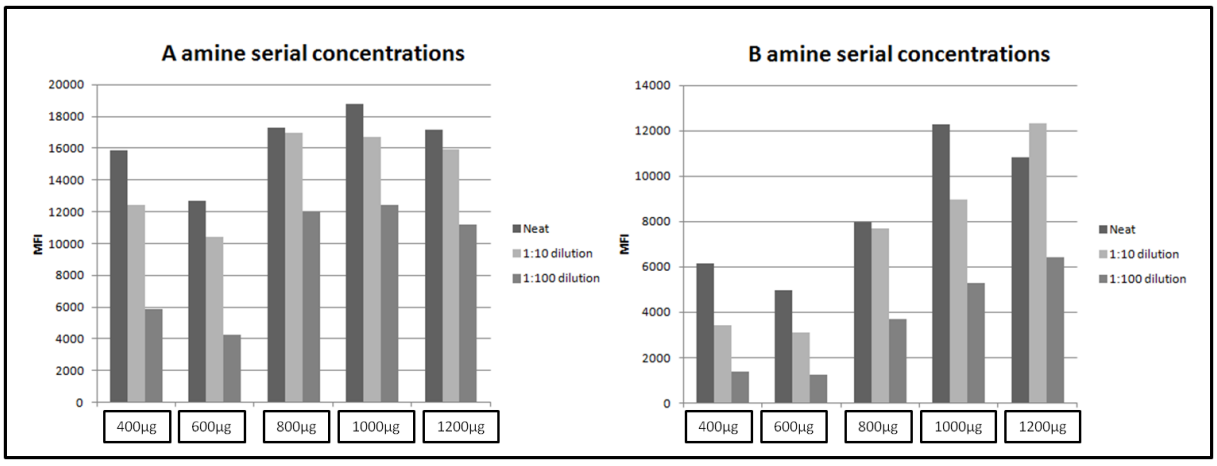
2.2.3.2.3 Microsphere assay development strategies

The presence of conjugated A/B antigen on microspheres was determined by murine monoclonal typing agents as described above and the MFI response to different concentrations of antigen was used to determine the amount of antigen bound to microspheres.

In order to optimise the concentration of trisaccharides for conjugation to the microspheres, different concentrations of antigen were incubated with microspheres to enable saturation of binding. The concentrations of A and B amine trisaccharides were titrated up from 400microg (800microg/ml) in conjugation solution to 1200microg (2400microg/ml) in solution for each trisaccharides. The effect of increase in conjugation trisaccharides was measured by the binding of the murine monoclonal MFI. There was a continued increase in B trisaccharides MFI with increasing concentrations, which did not reach saturation (Figure 2.10).

Figure 2.10. Increasing concentrations of trisaccharide incubation increase binding fluorescence.

These graphs demonstrate the binding of blood group specific murine monoclonal IgM against each antigen dependant on the different concentrations of trisaccharide used in conjugation. There was a rise in antibody binding between 400 and 1200micrograms microspheres and a dose response to dilution of antibody at neat; 1:10 and 1:100. The B amine, on the other hand, did not reach a saturation plateau and the MFI binding of the murine monoclonal antibody was lower than that of the A amine binding MFI data.



2.2.3.2.4 Protocols used for Reducing Non-specific Binding

Throughout all the experiments, binding specificities of the plasma samples and the microbeads did not distinguish between blood group A samples (anti-B antibody alone) or blood group B samples (anti-A antibody alone) due to binding to self-antigen. That is to say, antibodies in blood group A samples bound to A trisaccharide and antibodies in blood group B samples bound to B trisaccharide. This section describes processes that were undertaken unsuccessfully to develop specificity and reduce non-specific binding from the microbead assay. The binding of antibody to beads represented a hook effect representative of serial dilutions of true antibody binding (Figure 2.11)

2.2.3.2.4.1 Beads from Different Manufacturers

Different manufacturers of microsphere beads were used to demonstrate if there was a reduction in background binding between manufacturers. Qiagen and BioRad microspheres were used. A further bead product, SeroMap has been produced specifically to reduce non-specific binding [224]. While all of these products had conjugation of oligosaccharides, there was no improvement in non-specific binding. Protocols for conjugation and for testing were the same between these different manufacturers' microspheres.

2.2.3.2.4.1 Conjugation Testing

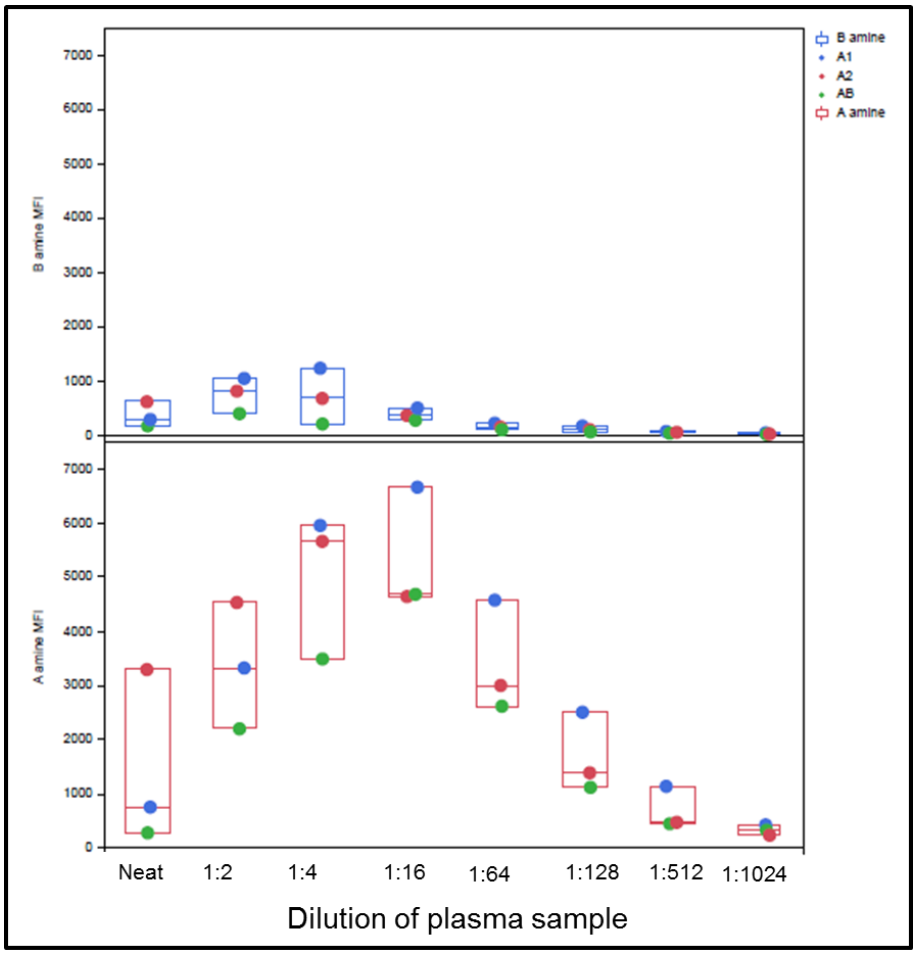
The non-specific binding of antibodies in plasma could be due to structures either present, or formed during the conjugation process. To determine if the conjugation process was increasing non-specific binding, a sample of 20microL of beads were removed after each process in the conjugation protocol. These were stored and then tested against LWB, blood group "A" plasma and blood group "B" plasma. No

oligosaccharides were conjugated in this protocol. These time points were pre-treatment, after initial washing, after carboxyl activation, after EDC-NHS reaction, after blocking and after final wash (Figure 2.12). There was an increase in binding fluorescence during the conjugation process with anti-A binding higher than anti-B binding.

Using trisaccharides with terminal amine or terminal 8 carbon-linker trisaccharides, microbeads were conjugated using the same conjugation protocol. The effect of binding between these two differently linked trisaccharides is shown in Figure 2.13. The reactivity to the trisaccharides linker did not improve specificity of binding. Similarly, a hook effect is seen in dilutions of AB plasma.

Figure 2.11. Non-specific binding of human antibodies to conjugated blood group trisaccharides.

These graphs demonstrate the non-specific binding of IgM antibodies of blood group "A" plasma (samples A1 and A2) and IgM antibodies of blood group "AB" plasma against the A and B amine (both 1200mg conjugated) microspheres with serial dilution of each plasma sample. The hook effect is demonstrated for each of these samples for both amine antigens. Binding against both A and B antigens occurred for both "A" plasma samples and "AB" plasma and MFI readings were greater against A trisaccharides than B trisaccharides. (n=3)



2.2.3.2.4.3 Pre-incubation protocols

Twenty microL 1:10 dilution in LWB of Rabbit Serum (DAKO X0902) was used in the initial incubation with plasma and microspheres and incubated for 30minutes. The plates were washed twice with LWB and then the 20microL of LWB was added to resuspend the microspheres before the addition of the 8microL of plasma sample to test. The rest of Luminex protocol was then followed (Figure 2.14A). Super Chemiblock (10.5mg/ml, Millipore) was used to block non-specific binding after Waterboer et al using 2.5% concentration. Following the initial double washing of the wells, the Luminex protocol was adapted to include an additional first incubation period of 20microL of LWB, 1.5microL of beads and 10microL of Super Chemiblock in each well. This was incubated and agitated at room temperature for 60minutes. I used 10microL of serial dilutions of Super Chemiblock, starting with neat (10.5%); 1:2 (5.25%), 1:4 (2.625%), 1:8 (1.313%) and 1:16 (0.656%) at the incubation stage. Eight microL of plasma was added to the well before completing the rest of the Luminex protocol (Figure 2.14B).

0.5% Polyvinyl alcohol (PVA) and 0.8% Polyvinylpyrrolidone (PVP) can be used to reduce non-specific binding. Neither of these agents had a significant effect on increasing specificity when used in the protocol. They were added to microspheres just before adding plasma to the Luminex protocol. Waterboer et al also used a combination of PVA and PVP (called PVX). A 1:1 mixture of solutions was made. PVX at 1:10 ratio of PVX to neat plasma and incubated at 4°C for 1hour with agitation. The PVX plasma was then centrifuged 4 minutes at 13,400g. The supernatant was diluted to 1:10 dilution with LWB and added to the microbeads according to Luminex protocol and the rest of the protocol was carried out unchanged. This did not demonstrate a reduction in non-specific binding (Figure 2.14A).

Marvel was prepared at 5% using distilled water. Twenty microL of serial dilutions of 5%, 2.5% and 1.25% Marvel were used in the initial incubation with the microbeads and incubated for 60minutes with agitation at room temperature before addition of plasma and following the routine Luminex protocol. These concentrations of Marvel did not reduce non-specific binding (Figure 2.14C). Human Serum Albumin (HSA) was used at 0.5% (Sigma, UK) for microbead blocking without a beneficial effect to reduction in non-specific binding. Twenty microL of 0.5% HAS was added to 20microL of LWB and 1.5microL of microbeads and incubated for 30minutes in an agitator at room temperature. The wells were washed with 200microL of LWB as per standard protocol and then 20microL of 0.5% LWB resuspended the microbeads and 8microL of plasma was added to each well. The remaining part of the experiment followed the standard Luminex protocol (Figure 2.14D).

Figure 2.12. Background binding to microspheres increased during the conjugation protocol.

There was non-specific binding to microspheres regardless of the manufacturer of the microspheres and the non-specific binding increased through the conjugation process. These used BioRad beads (left) and Qiagen (right) beads with no reduction in non-specific binding. There was greater reactivity to blood group "B" plasma, than blood group "A" plasma.

EDC-NHS is chemical used to activate the carboxyl group on the microsphere. Final – represents the unconjugated bead, following buffer washing to remove the blocking agent

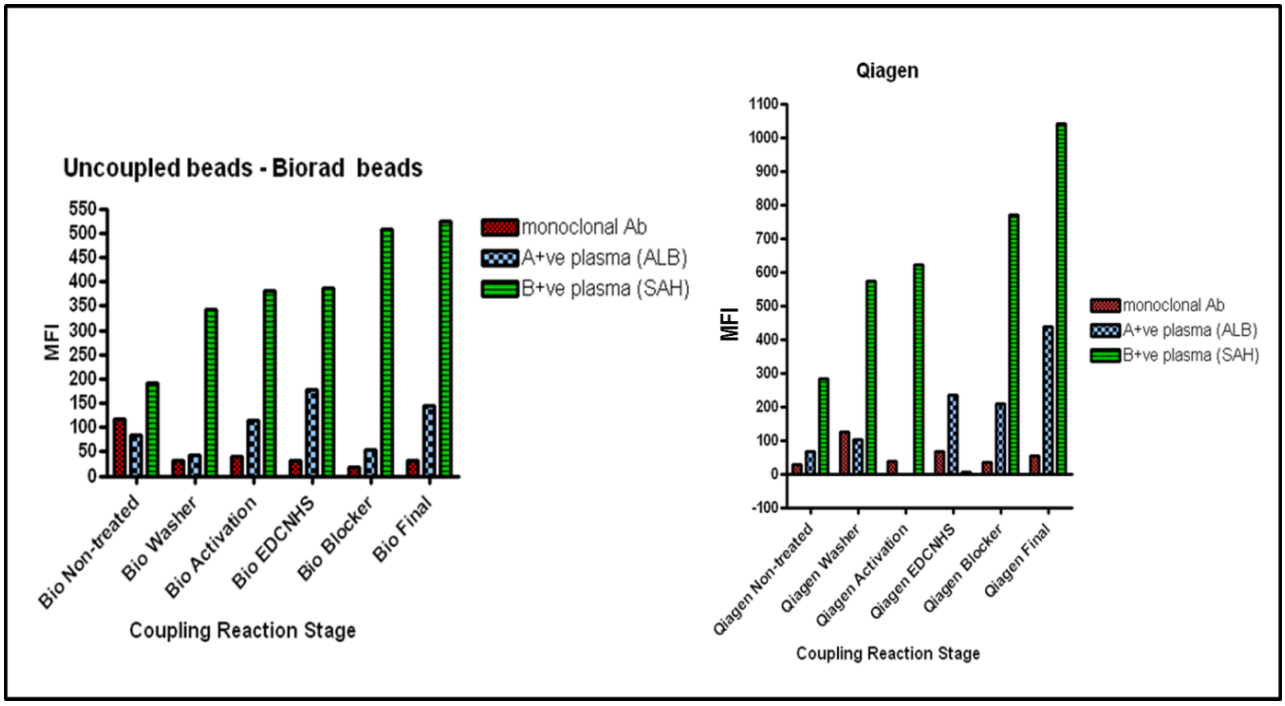


Figure 2.13. Comparison of non-specific binding between Amine and Linker trisaccharides

For serial dilutions AB serum, the hook effect was demonstrated for anti-trisaccharide IgM binding but there was no reduction in non-specific binding between the amine-derived or the carbon-linker derived trisaccharides.

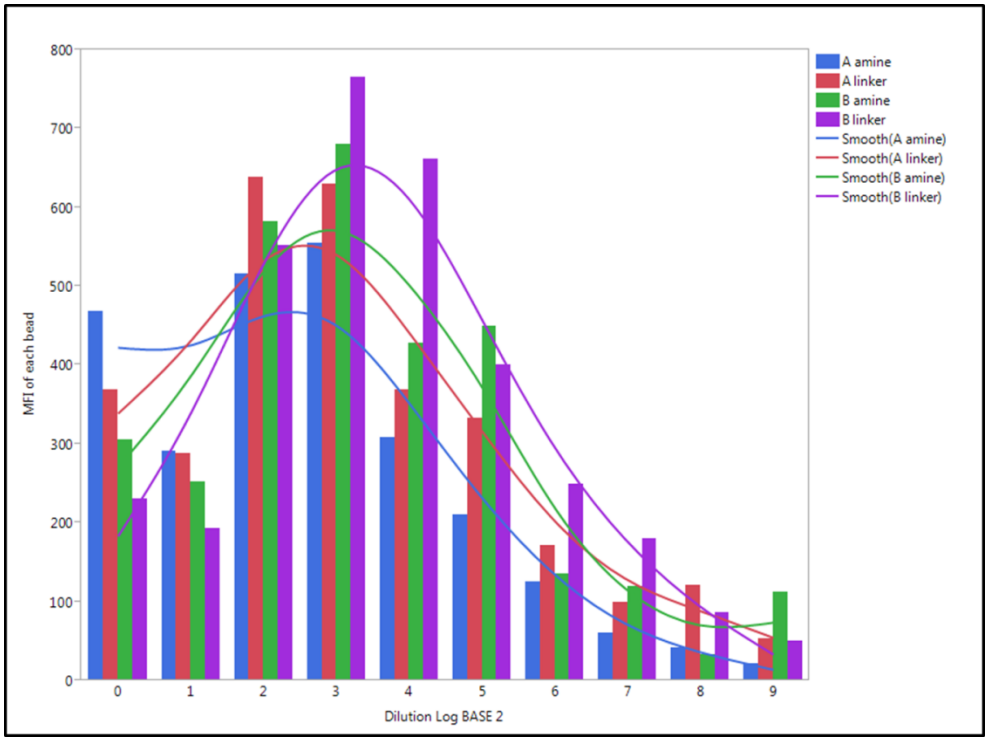


Figure 2.14. Incubation with blocking antibodies.

Figure 2.14A .The use of Rabbit serum to reduce non-specific binding did not affect the MFI between samples tested. There was no different if PVX was used in this assay with or without Rabbit serum.

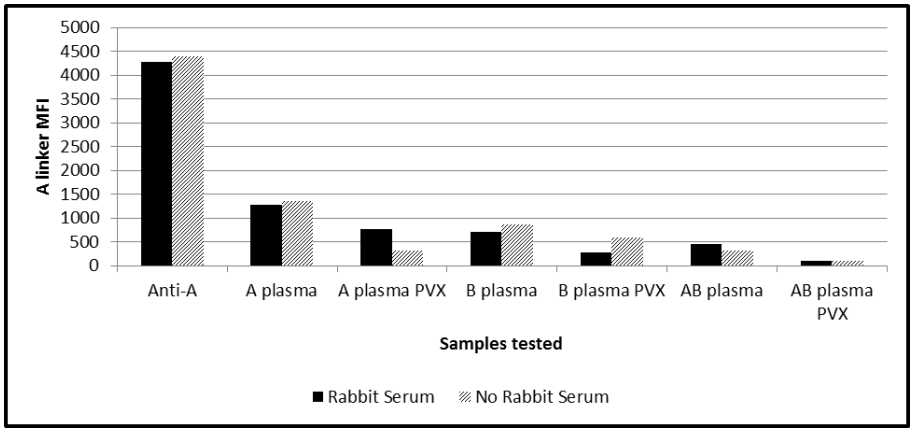
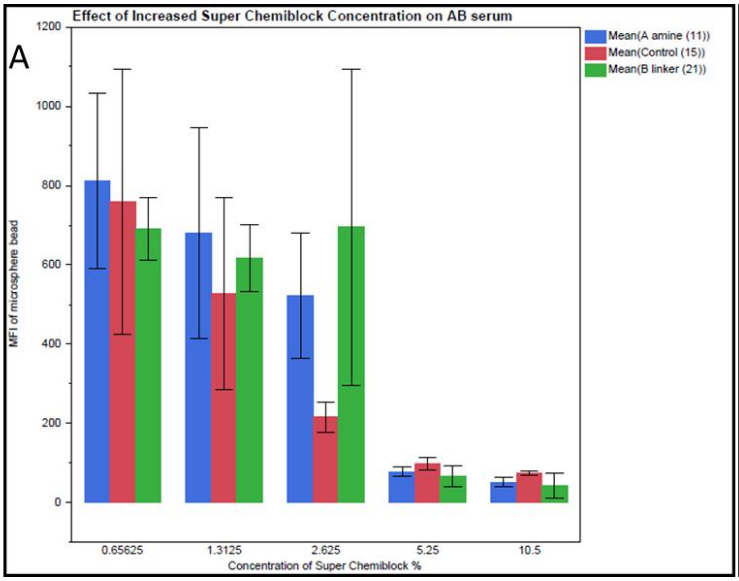


Figure 2.14B. The increase in Super Chemiblock reduces the non-specific binding of AB serum to both A (blue) and B (green) conjugated microspheres as well as unconjugated control beads (red) shown in graph A. However, the effect of Super Chemiblock at higher concentrations significantly reduces the binding of the anti-A murine monoclonal antibody, shown in graph B.



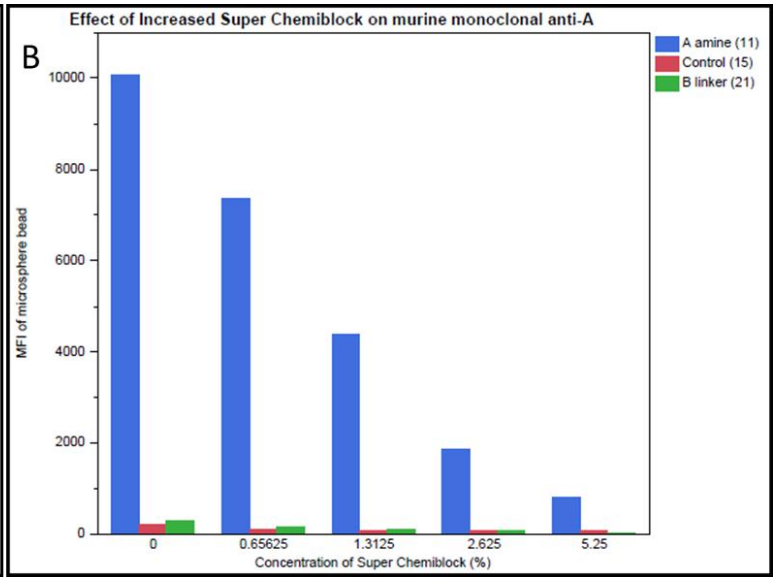


Figure 2.14C. The addition of Marvel at different concentrations (5%, 2.5% and 1.25%) did not reduce non-specific binding of human AB serum to both A (grey) and B (dark) conjugated microspheres as well as unconjugated control beads (white spotted). This was true for both IgM and IgG isotypes (graph A and B respectively)

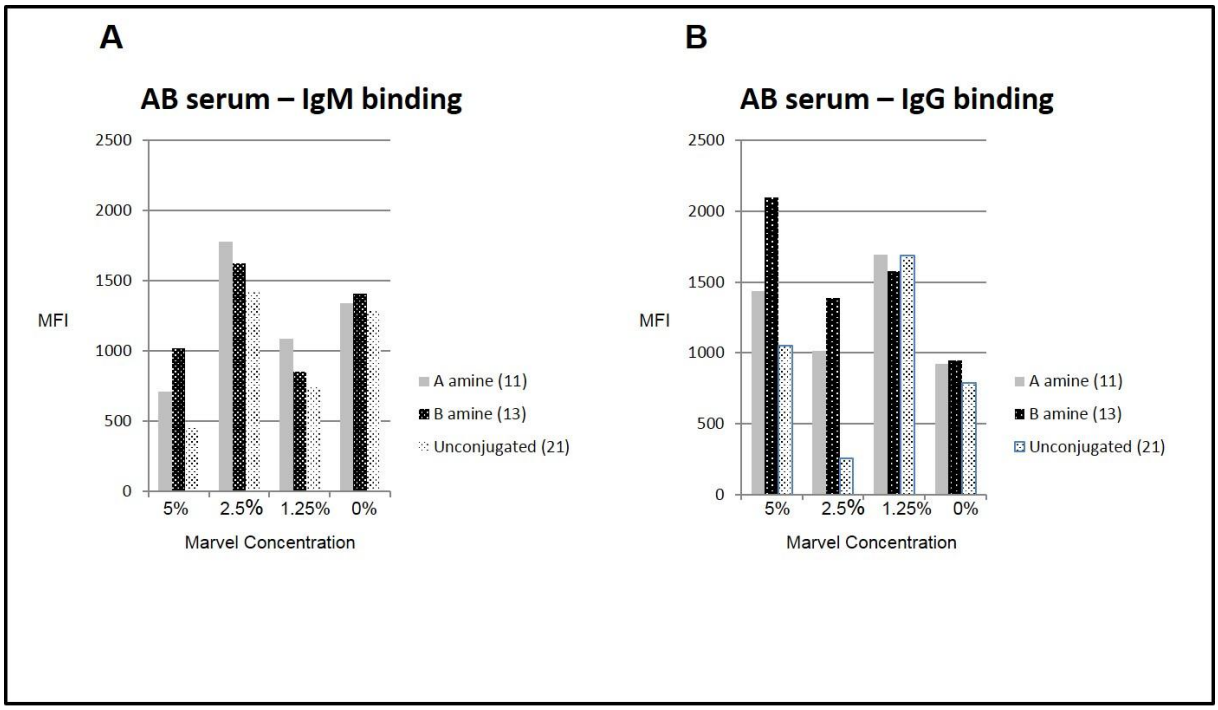
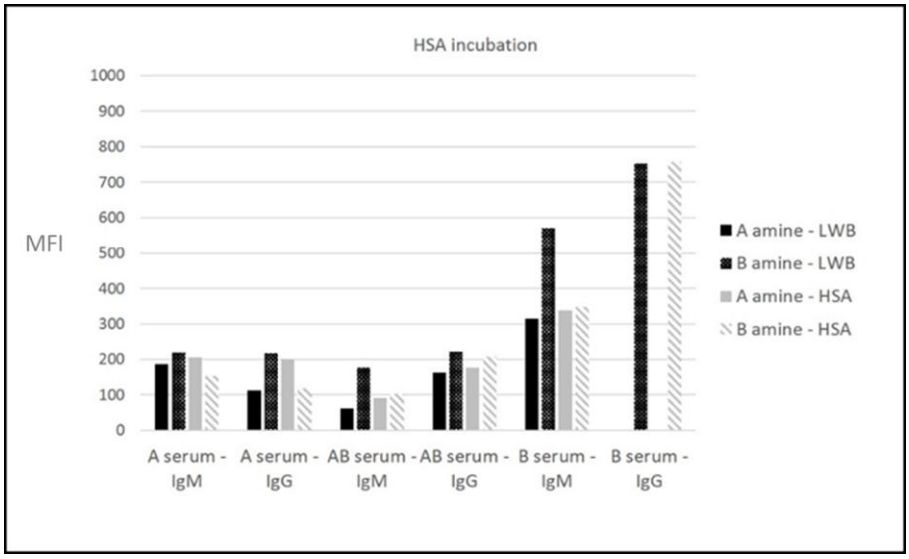


Figure 2.14D. Addition of Human Serum Albumin (HSA) to block microbeads and as a suspension fluid for the microbeads did not change the non-specific binding compared to Luminex Wash Buffer (LWB). This was true for both IgM and IgG isotypes.



2.2.3.2.4.4 Different Wash Regimes

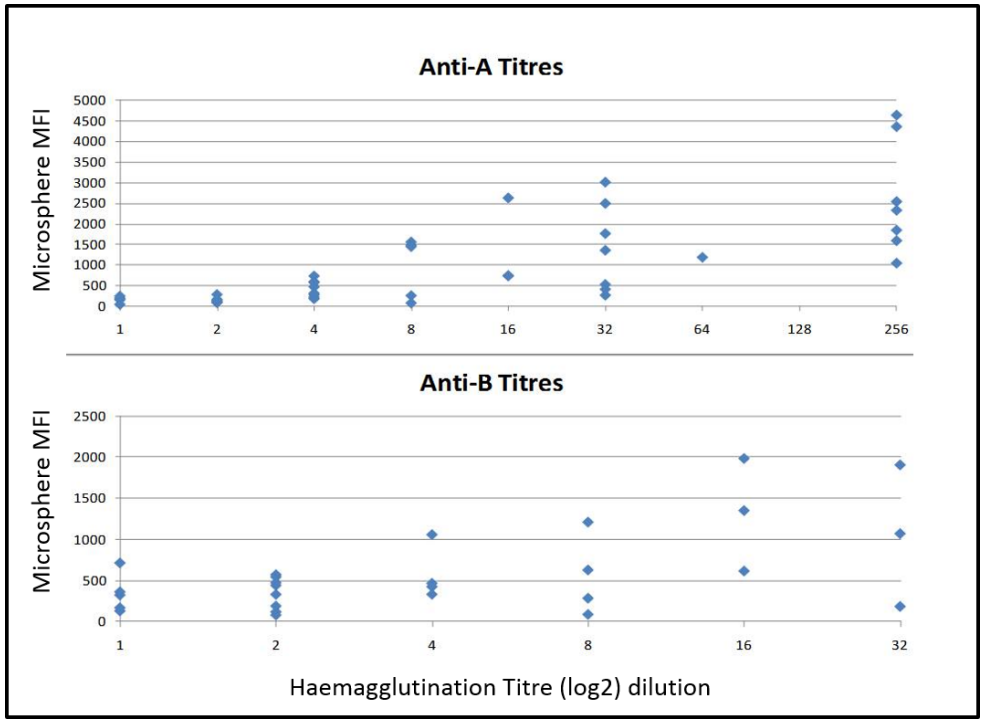
The number of washes performed at each time point was varied after incubation was changed from 2, 4, 6 or 8 without any reduction in non-specific binding.

2.2.3.2.4.5 Plasma testing

Using samples from haemagglutination assays, the Luminex beads were tested for correlation of antibody titre and MFI of bead reading. There was an increase in MFI with higher titres, and the titres produced higher MFIs in the anti-A blood group antibodies than in anti-B antibodies (Figure 2.15).

Figure 2.15. The relationship of HG titre and MFI of conjugated trisaccharide to microspheres.

Using the microspheres conjugated with **(**1600microg/ml beads); there was high background binding, and lack of specificity. There was however, a quantitative increase in binding (MFI, y-axis) relative to the haemagglutination titre (x-axis) for anti-A titres and for anti-B titres (n=80). The HG titres are higher against A antigen and this reflected in MFI in the Luminex assay.



2.2.3.3 Conclusions

In these experiments for anti-ABO specific antibody binding, I demonstrated successful conjugation of A and B trisaccharides to microspheres, with an increase in monoclonal binding suggesting that there is increased antigen conjugation with increased concentrations of A/B trisaccharides used.

Although the concentration of the trisaccharides used in conjugation was the same for both A and B antigens, B trisaccharide conjugation did not yield the same MFI as with A trisaccharide when tested using the murine monoclonal typing antibody and using the same 2nd PE-labelled anti-murine antibody. The explanation for this could be that B trisaccharide does not as effectively conjugate to the microspheres or possibly that the anti-B murine monoclonal or second stage antibody binding kinetics differ in this setting, such that there are lower amounts of second stage antibody bound even in the event of identical bead surface density of blood group trisaccharide

Despite using different reagents, I was not able to reduce background MFI binding to beads. Whilst binding to beads was consistent to increased haemagglutination titres, there was no specificity. Whilst this assay would not allow specificity of binding for blood group identification, it may allow a more sensitive assay to demonstrate changes in quantity in anti-ABO specific antibody during desensitization protocols. This, however, would need further validation.

2.2.4 Surface Plasmon Resonance

2.2.4.1 Introduction

The binding affinity of antibody is determined by the relationship between association and dissociation of the antibody/antigen complex. The effect of affinity of antibodies is represented by the binding avidity. Thus IgM is usually a low affinity antibody, however,

its pentameric structure significantly increases binding avidity. IgG, through germinal centre selection, is a higher affinity antibody.

Surface plasmon resonance (SPR), as detailed in the Figure 2.16A, is a tool that can measure the association and dissociation of molecular interaction in real time (Figure 2.16B). In brief, the gold layer fixes a molecule (ligand); we used an A/B trisaccharide, via a carboxylation reaction. Buffer is then used to normalise the chip and equilibrate the machine. The effect of mass, caused by running the samples (analyte) over the gold chip causes a change in diffraction of light through the prism to be detected by the light detector.

Previous work using this technique for antigen specific purified anti-ABO specific antibody, describe the antibody binding related to this purified antibody [217, 218]. Given my interest in these studies in assessing the individual variation of anti-ABO specific antibody and correlating this with the clinical outcome requiring a functional test deemed that it was necessary to assess whole plasma. The protocol from Kimura et al was adapted using a different machine Proteon XPR36 (BioRad, UK) [217]. The aim was to demonstrate antibody-antigen binding to determine specificity to antigen and to determine the binding characteristics. This work was done in collaboration with biomathematical engineers at University of Warwick.

The change in mass of the gold chip due to antibody binding alters the refractive index and the sensogram demonstrates this in schematic format. Stopping the analyte, and allowing buffer to flow, demonstrates the dissociation curve. Finally, a regeneration fluid is required to remove all bound analyte and then the reading is back to baseline.

SPR, in particular the Proteon XPR36, is a tool used to analyse high numbers of analytes and their interaction with determined ligands. This machine can demonstrate the

binding of 6 analytes with 6 different ligands in one experiment, thus 36 interactions can take place in each experiment.

Figure 2.16. Schematic diagrams of SPR technology.

Figure 2.16A

This diagram demonstrates the interaction between immobilised ligand on the gold layer and the analyte in fluidics passing the ligand. The light is refracted at a different angle and detected and a refractive unit (RU) is calculated. The plotting of the RU against time demonstrates the association and dissociation rate in the second diagram and then removal of the analyte during regeneration to return to ligand without analyte before next experiment [225].

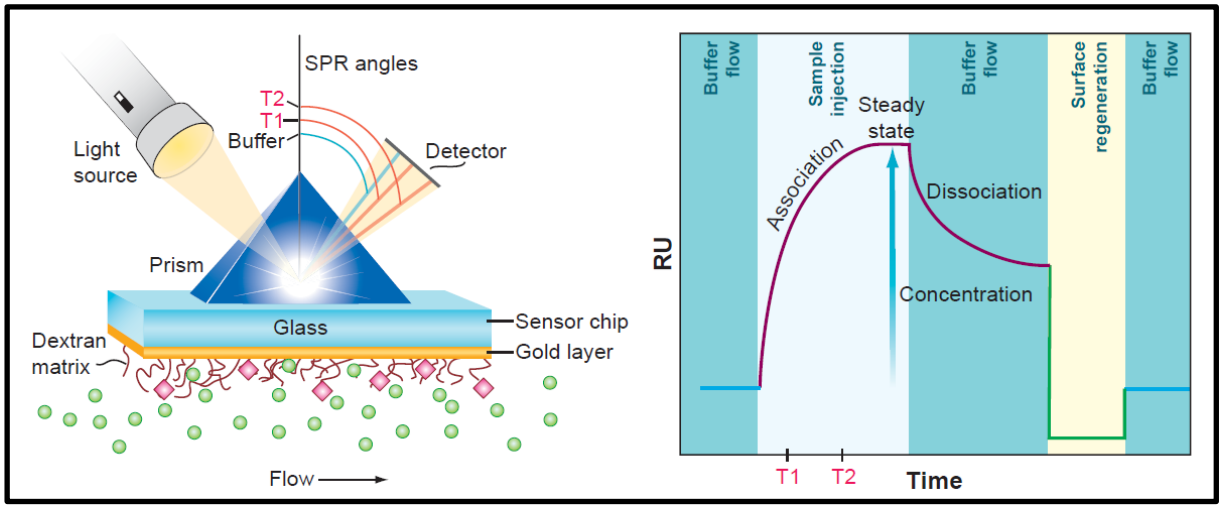
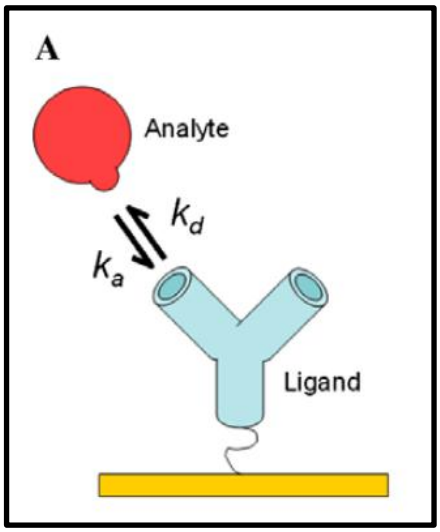


Figure 2.16B

Although the association stage (K_a) continues to increase, there is ongoing dissociation (K_d) throughout, until a steady state is reached [226].



2.2.4.2 Methods

2.2.4.2.1 Antigen coupling

Antigen coupling to the SPR chip was performed according to the manufacturer's recommendations. A maintenance chip was inserted and running buffer used to calibrate the machine. A cleaning chip was inserted to ensure a clean system, and then a new GLH (amine coupling) chip was inserted and labelled on machine specific software. Normalisation of the new chip took place using software manager. Activation of carboxyl groups on chip was achieved by combined mixture of *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma, UK) and N-Hydroxysulfosuccinimide sodium (S-NHS, Sigma, UK) were both diluted in borate (pH 8.5) buffer at 50mg/ml. The volume of each solution was 125microL to give a combined volume of 250microL. The SPR machine was then run on an activation setting to activate the chip. The injection of ligand (250microL at 1mg/ml concentration of oligosaccharides) in each channel was automated by the protocol and then deactivation of chip using 1M ethanolamine (100microL) was performed, using 250microL per channel. Running buffer then stabilised the chip before experiments were performed. Regeneration after each experiment was performed with Glycine 0.1mM (pH 2.5) with two regeneration steps.

2.1.4.2.2 Buffering dilution

The mass effect of buffer solution interfered with the reading for this assay. Thus adjusting the buffer solution to be as physiological as possible for plasma would reduce the difference between plasma sample and running buffer mass effect. We performed a series of experiments with different concentrations of Tween 20 (Sigma, UK) to

minimise the delta mass effect between sample and buffer and optimised the Tween 20 concentration at 0.5%.

2.2.4.2.3 Control testing

Antigen attachment through EDC and S-NHS carboxylation was demonstrated by the mass effect on the Proteon chip as demonstrated by the increase in response units (RU). The mass effect increased with increased concentration of A/B antigen, and the increased mass of the A antigen had a greater RU value than the same concentration of B antigen.

Murine IgM monoclonal anti-A and anti-B with high affinity antibodies were used as positive controls for assessing the binding specificity in this platform. The results of these correlated with the microspheres assays with high RU values, which demonstrated very good association and the dissociation was slow, demonstrating very high avidity. The response of the murine monoclonals was dependent on the concentration of antibody and ligand (Figure 2.17). Anti-A1 Lectin (100405, BioRad, UK) was used for A1 specificity, to demonstrate that the antigen reflected the A1 antigen that I wanted to study. There was excellent binding association and dissociation with this and again very good specificity (Figure 2.18).

2.2.4.2.4 Binding

Having optimised the buffer for running human plasma, it was important to demonstrate that the binding would be specific for anti-A and anti-B and plasma from "A", "B" and "AB" donors was used. There was non-specific binding to the trisaccharide with amine terminal chain; however the 8 carbon chain linker demonstrated antigen specific antibody binding - in keeping with Landsteiner's rule (Figure 2.19A and Figure 2.19B and Figure 2.19C).

The murine monoclonal IgM was purified using a Glycosorb® column as described above and dialysed into running buffer in order to quantify a monoclonal antibody. Quantification was performed using a Rad Diffusion assay as described below.

The Rad Diffusion (The Binding Site, Birmingham, UK) plate was left open for 10minutes at room temperature to allow condensation to evaporate. The supplied lyophilised calibrators were reconstituted with distilled water. The calibrator were made for final concentrations of 100%, 60% and 10% using distilled water. The calibrator and samples were micro-pipetted into the wells on the plate (20microL) and the lids were replaced and incubated at room temperature (20-24°C). The plates were left for 120 hours to allow complete diffusion to occur. The ring diameters were then measured to the nearest 0.1mm using backlighting. The 3 calibrators were plots to produce a linear calibration curve with protein concentration on horizontal axis and ring diameter on vertical axis. The test concentration was read off the calibration curve.

Using a constant concentration of both anti-A and anti-B monoclonal, inhibition assays were performed with incubation of respective trisaccharides in antibody solution prior to injection on the SPR machine. This demonstrated serial binding inhibition with increasing concentration of trisaccharides for both anti-A and anti-B (Figure 2.20A). A similar experiment was performed using purified human antibody, eluted from a Glycosorb® column (method in 2.2.2.1.3). At a known quantity of antibody in solution, there was reduction in binding when diluted. At a defined concentration of purified human anti-blood group antibody, there was inhibition of anti-A antibody binding with increased concentrations of A trisaccharides in solution (Figure 2.20B).

Figure 2.17. Concentration dependant binding of murine monoclonal antibody.

With different concentration of ligand (antigen) applied to each lane of the chip, there were different response units for antibody binding. With serial dilutions of a pre-determined concentration of murine monoclonal IgM, there was a reduction in association response units; however the dissociation rate of the antibody was similar across each concentration. Using either anti-A monoclonal (left graphs) or anti-B monoclonal (right graphs) there was better differentiation of binding characteristics at lower ligand concentration for the A-trisaccharide, but no difference for the B-trisaccharide. Antibody (analyte) concentrations are shown in the legend on the right of each pair of graphs.

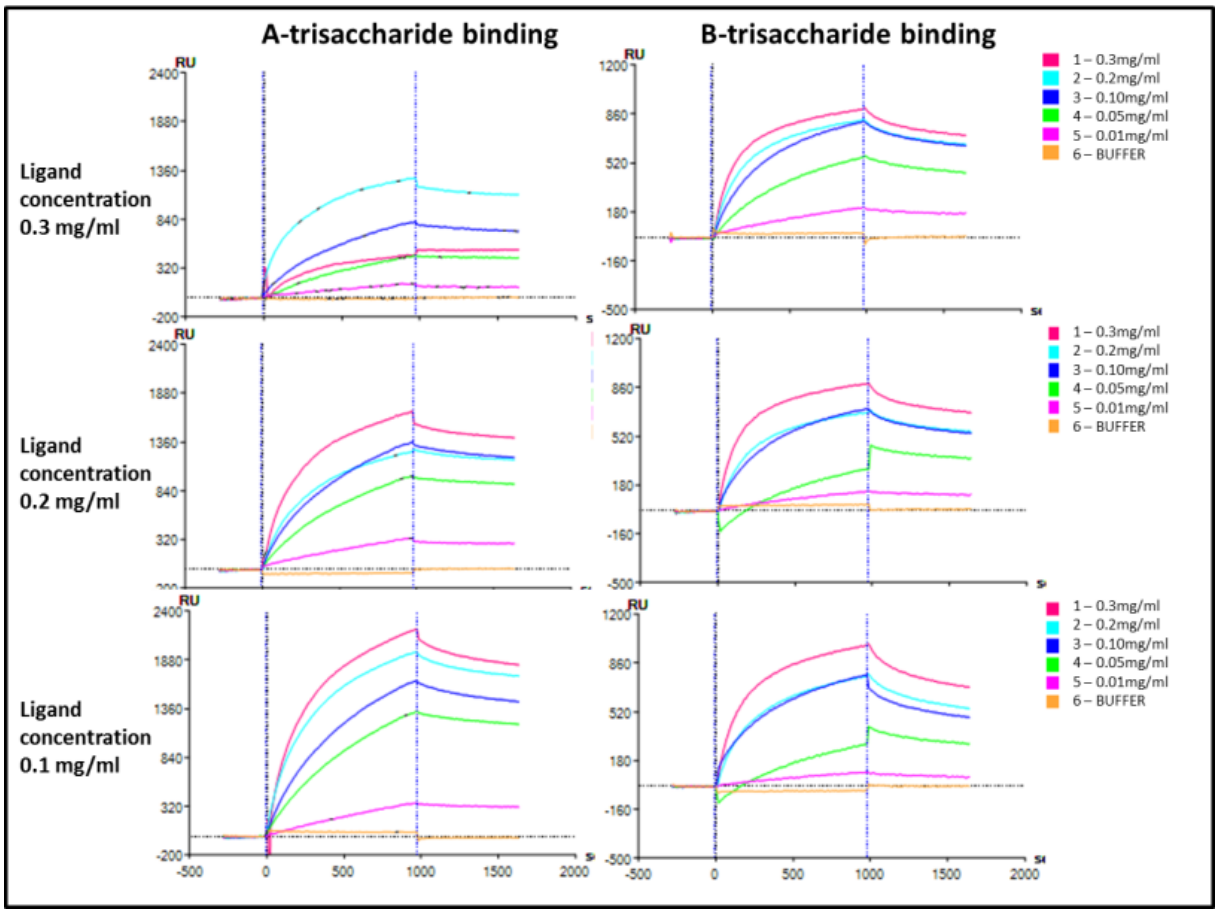


Figure 2.18. Binding specificity of lectin to A-linker antigen on SPR.

Anti-A1 lectin (pink) binding to A-trisaccharide ligand(antigen) in lanes 1-3 (left) demonstrated good association, but rapid dissociation and there was no binding to B-trisaccharide in lanes 4-6, compared to running buffer (orange) as control.

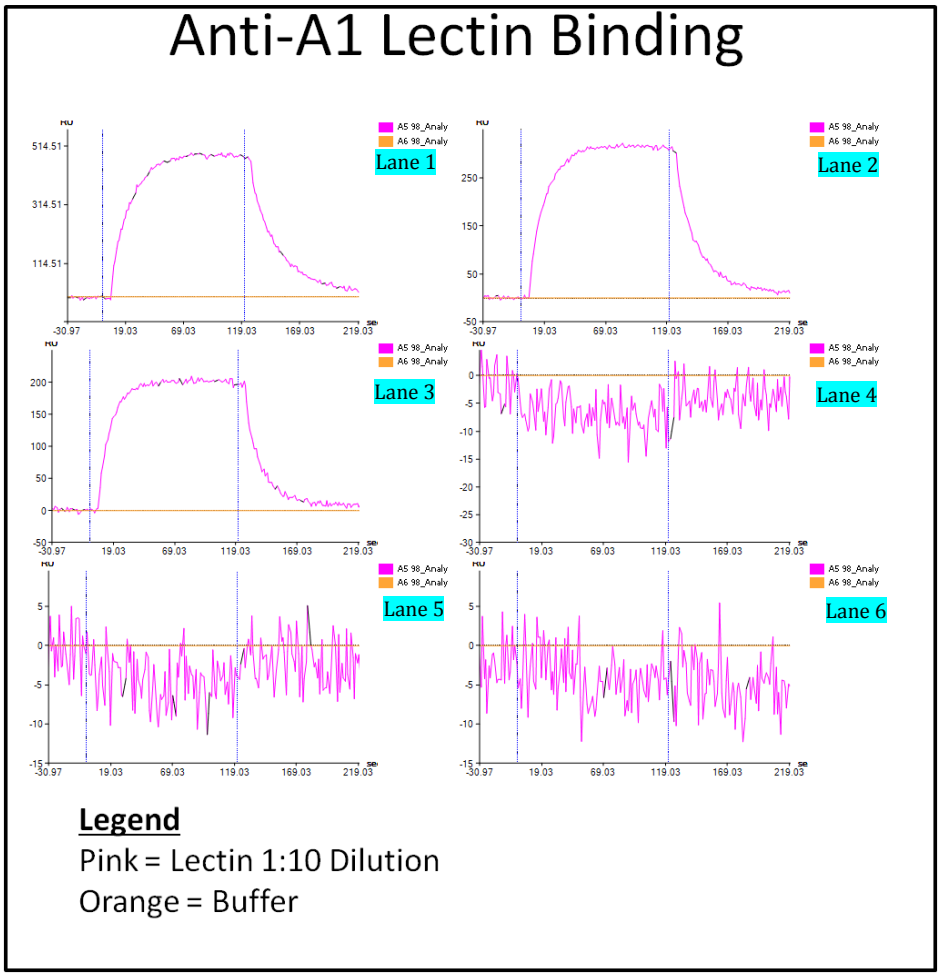


Figure 2.19 Binding specificity of plasma to Linker Blood group Antigens, compared to amine derived antigens.

Figure 2.19A (Binding of A Plasma)

Binding specificity between the amine and 8 carbon linker trisaccharides is demonstrated in the following diagrams for blood group "A" plasma. In the upper two graphs, bloods group "A" plasma binds to both A and B trisaccharide amines (A and B respectively). However, there is no binding of blood group "A" plasma to A trisaccharide with 8 carbon linker, but still good binding to B trisaccharide, in the lower two graphs (C and D).

Each differently coloured line demonstrates different concentrations of plasma used (1:5; 1:10; 1:20; 1:50 and 1:100). There is a reduction in response units as the dilution of the plasma increases.

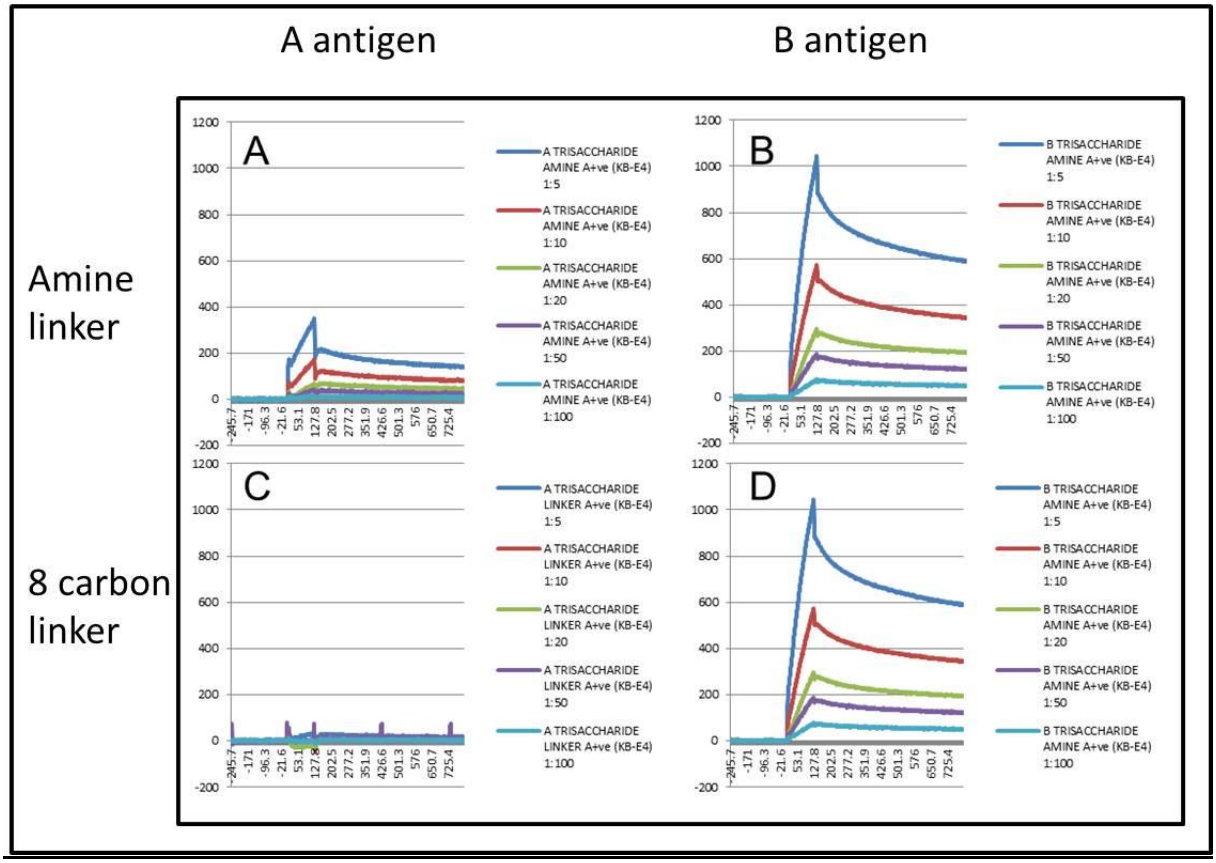


Figure 2.19B (Binding of B plasma)

As demonstrated with blood group "A" plasma in figure 2.19A, a similar pattern is displayed with blood group "B" plasma. Binding specificity between the amine and 8 carbon linker trisaccharides is demonstrated with non-specific binding to both A and B trisaccharide amines in the upper two graphs (A and B respectively). While there was still binding to A trisaccharide 8 carbon linker, there was no binding to B trisaccharide in the lower two graphs (C and D respectively).

Each graph demonstrates different concentrations of plasma used (1:5; 1:10; 1:20; 1:50 and 1:100). There is a reduction in response units as the dilution of the plasma increases.

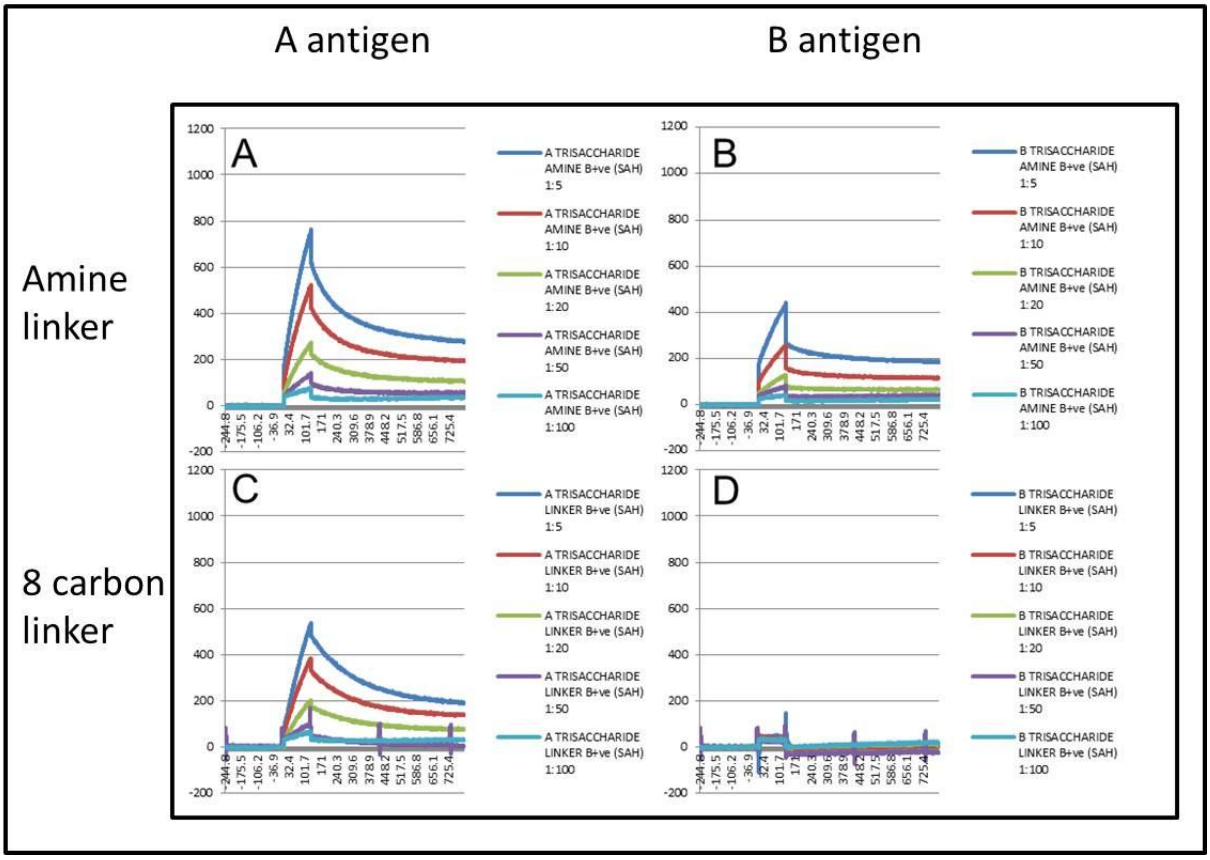


Figure 2.19C – This figure demonstrates control AB serum binding to the A and B trisaccharides amine structures in both upper graphs (A and B respectively) but no binding to the A and B 8 carbon liner trisaccharides in the lower graphs (C and D respectively).

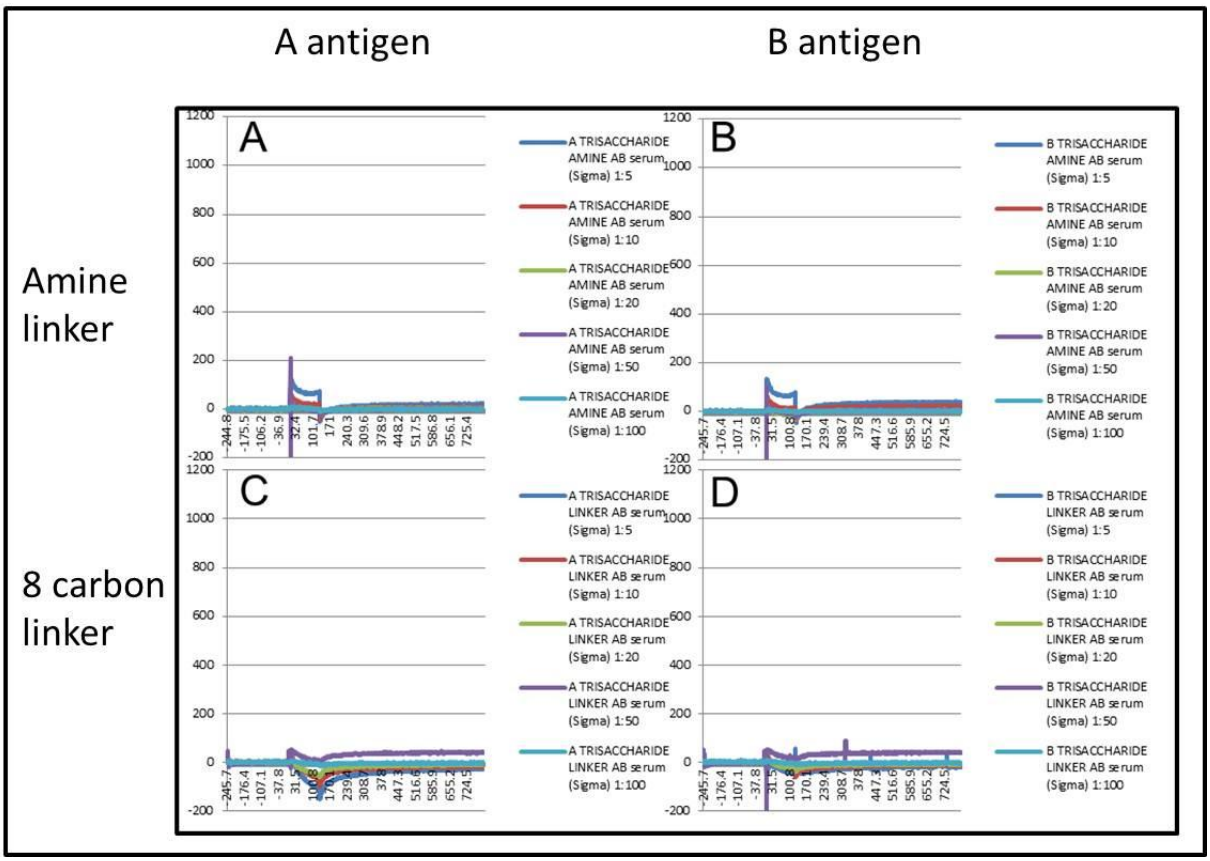


Figure 2.20. Inhibition studies of murine monoclonal antibodies and purified human antibody

Figure 2.20A - I performed inhibition studies with increasing concentrations of A or B trisaccharides with each purified murine IgM monoclonal typing agent. There was a serial reduction in RU values with the increased concentration of A or B trisaccharides.

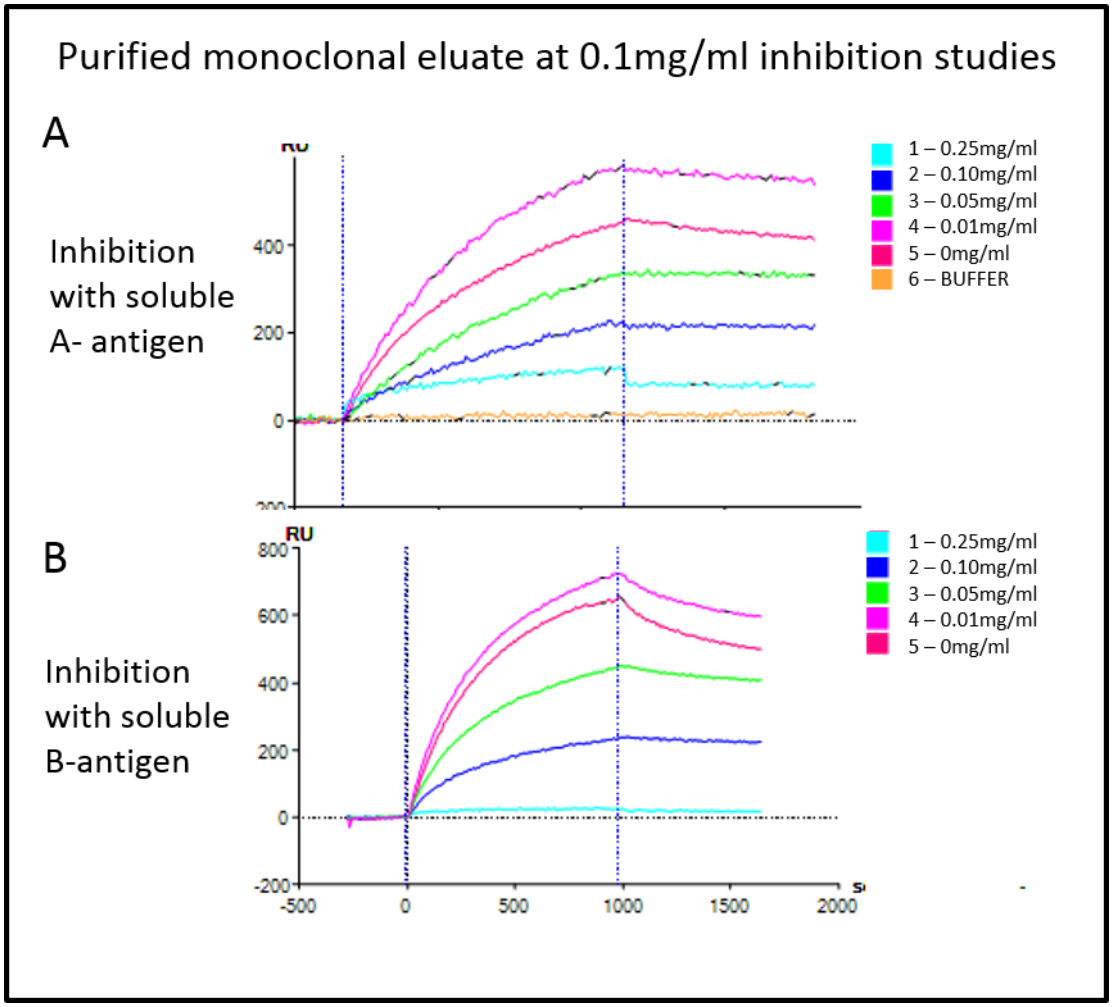
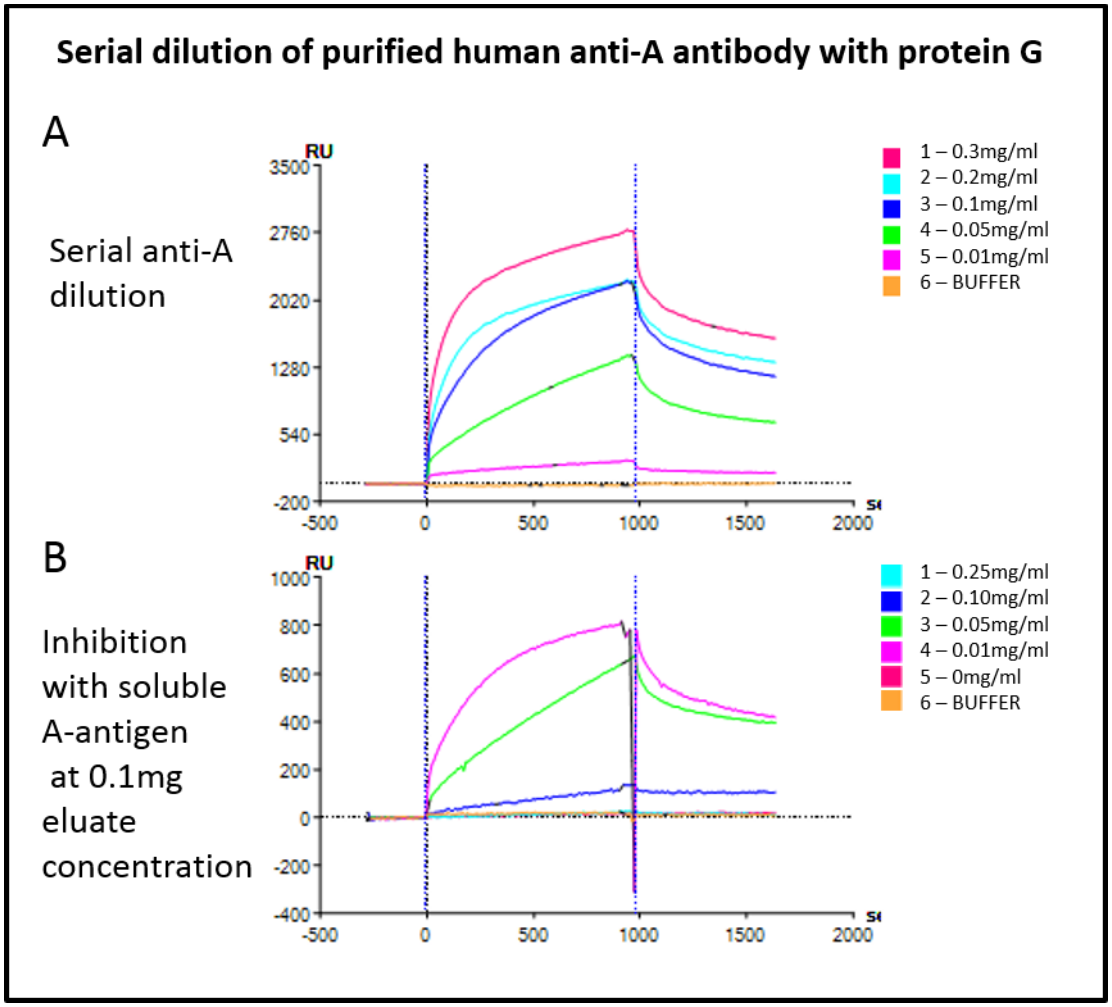


Figure 2.20B. Purified human anti-A IgG was quantified and serially diluted, and inhibited with soluble A-trisaccharide, both demonstrating a dose dependent reduction in binding.



2.2.4.2.5 Modelling

Work undertaken in collaboration by N. Evans and M.J. Chappell, University of Warwick, demonstrated that simple models for prediction of antibody binding did not predict the pattern of binding (Figure 2.21A), however, this was refined and was very predictive of the binding (Figure 2.21B). The paper is attached at the end of this thesis.

Figure 2.21. Modelling of antibody binding characteristics.

Figure 2.21A – These graphs demonstrate the true SPR output data in light grey and the heavier black line of the Langmuir Model. Initial association and dissociation rates are similar, however the final on/off rates around the time of analyte stopping are not well modelled. Work undertaken by N. Evans

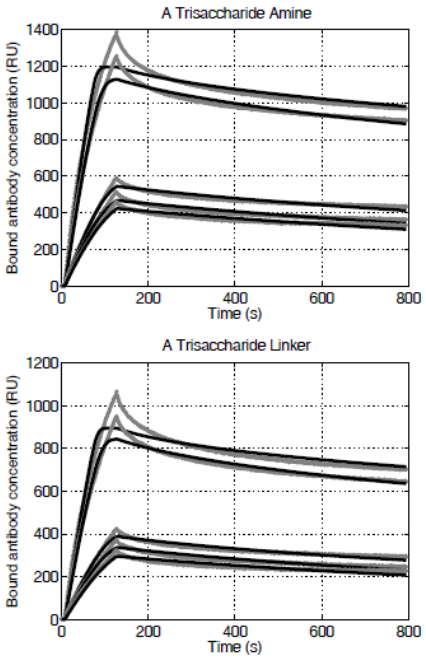


Fig. 1. Full model output (black lines) plotted with corresponding experimental data (grey lines).

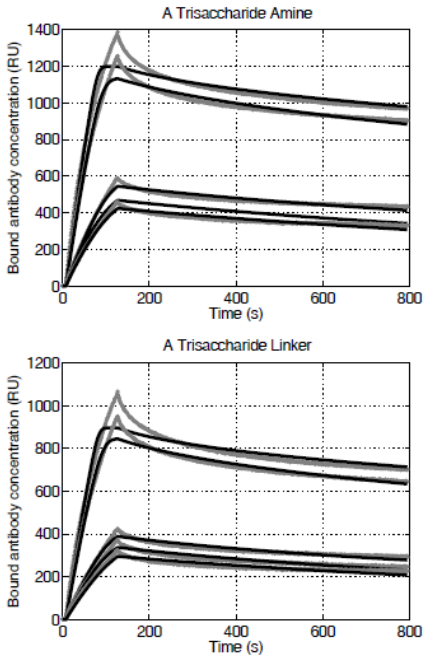
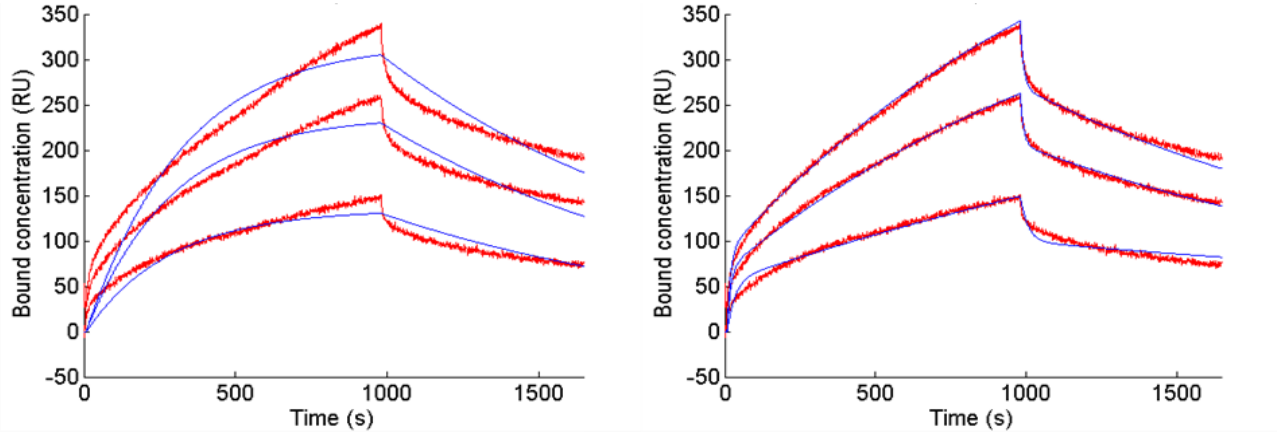


Fig. 2. QSS model output (black lines) plotted with corresponding experimental data (grey lines).

Figure 2.21B – These graphs demonstrate the true SPR output data in blue line and the new modelling in red demonstrating that the more variables included in the model, whether real data or assumed data increased the predictability of the model of anti-ABO specific binding.



2.2.4.5 Conclusions

Binding of anti-A and anti-B specific human polyclonal antibodies were demonstrated to be specific for A/B antigen with an 8 carbon linker structure, but not with amine alone terminal group. These data support the observation by Bovin and colleagues suggesting that the presentation of the terminal A/B antigen determines the specificity of binding [227]. It may be that a cryptic epitope is "hidden" by the carbon linker and this may be revealed on the microsphere platform, but not on SPR platform with the 8 carbon linker, or that the proximity of antigen conjugation on the microspheres allows binding to different antigens to occur. There may also be other antibodies which are binding to these structures, as there was no cross reactivity with commercial monoclonal antibodies to suggest that A/B antigen conformation is changed but that a different epitope is revealed in the conjugation process to allow binding to take place.

In these experiments, the SPR platform demonstrated that in fluidics system binding of human plasma samples enabled specific binding. SPR showed that using an 8 carbon linker it was possible to achieve binding specificity; functional assessment of antibody binding; application of plasma samples to this assay. The application of techniques from laboratory to clinical to practice using clinical samples is a technical challenge which was not achieved in these sets of experiments. Further work to correlate the binding characteristics of plasma samples on SPR with the effective removal of antibody in EART and episodes of rejection needs to be performed.

2.2.5. Statistical Analysis

Descriptive statistics were used to assess the data from this study. Titres are reported as doubling dilutions and the $\log_2$ transformation is used for titres in order to normalise the data. Data are presented as mean and standard deviation (Mean± StDev) if found to be normally distributed by the Shapiro-Wilk test, and data are represented as median and first and third quartiles (Median, Q1-Q3) if there is a non-parametric distribution, unless otherwise specified. The Wilcoxon rank-sum test was use for comparison of non-parametric data, which is equivalent to a Mann-Whitney U test. Correlation between test data was performed using bivariate plots with linear fit, assessed with regression coefficient.

Weighted kappa quadric assessment was used for agreement of data between two tests (treating haemagglutination titre data as continuous variables) following discussion with a statistician. The value of Kappa signifies the strength of the agreement between 2 tests, with excellent agreement value being 1.

Comparison of reproducibility of haemagglutination titres between tests was performed using weighted kappa inter agreement analysis. For the Bland-Altman plots,

the data was treated as continuous data to assess the differences of each method from the mean of both tests and is plotted as the difference between test 1 and 2 on the y-axis against the average of test 1 and 2 on the x-axis.

Probability of values (p) of less than 0.05 was considered significant for these studies. All statistics were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA), except the haemagglutination analysis performed using MedCalc (Ostend, Belgium).

2.3 Anti-ABO specific Antibody Conclusions

In this chapter I have demonstrated that a single user, using a standard technique can produce haemagglutination assay results that approach the theoretical limits of a dilution assay with respect to reproducibility, that is to say a mean inter-assay variation equivalent to a single titre step (bearing in mind that the choice of doubling dilution in HG is reflects the underlying performance of the assay). The application of these results is important for Chapter 3, the ABOUT-K study, examining the differences in local and central titres in assessing risk of ABOiKTx.

The development of ABO synthetic assays remains a question unanswered. While Luminex® technology has been used for the development of different antibody measurements in other disease process, such as pneumococcal antibodies and HLA antibodies, the specificity in ABO antibodies was not achieved [228, 229]. This was due to a constant lack of specific binding to A or B blood group antigens.

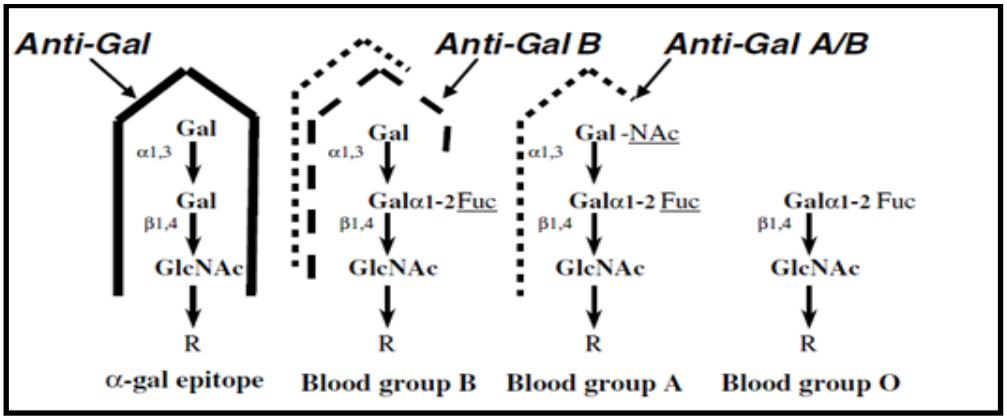
The absorption of anti-A or anti-B antibody in O serum has been demonstrated which reduces the titre against both A and B antigens and is labelled anti-A,B antibody. The proportion that is pure anti-A, pure anti-B and combined anti-A,B has not been quantified [230].

The lack of specificity demonstrated using amines in the Luminex® assay and in SPR suggests cross reactivity of antibody. This could be explained by the anti-ABO specific antibodies reacting with a core structure with the two glycolipid structures. Holgersson's group have demonstrated this phenomenon in ELISA and microsphere assays. They have extended the structure from a trisaccharide to a tetrasaccharide and show better specific binding with the tetrasaccharide structure [231]. This work extended their previous data from 2007 which demonstrated good binding to synthetic antigen, however via a personal communication, similar difficulties were found with cross reactivity [223] (Holgersson - personal communication). These data suggest that some anti-ABO specific antibodies are type chain specific, and thus the configuration of the antigen changes binding; data support by Huflejt and colleagues who demonstrated that antibody binding differed between trisaccharide and tetrasaccharide structures between individuals and between A and B antigens [227]. The clinical significance of this is yet to be determined, with regards to antigen specific antibody removal. The data in my experiments on SPR support different antigen structures affecting antibody binding characteristics. . Cross reactivity was demonstrated by Galili, explaining the common epitopes as targets for anti-Gal antibodies and anti-A/B antibodies see Figure 2.22, binding to the terminal structure of the 1-3α gal terminal oligosaccharide [84].

The assessment of anti-ABO specific antibody using different assay platforms demonstrates different characteristics of antibody-antigen interaction. As Buchs et al conclude in their paper "it is difficult to compare agglutination…..with binding to A or B substance since the agglutination assay depends not only on antigen binding but also on agglutinating capacity of the antibodies" [208].

Figure 2.22. Similarities in antibody binding epitopes between anti-carbohydrate antibodies.

This schematically shows that antibodies binding to the Gal antigen can also bind to the terminal molecule on A or B antigen, but not H-antigen, due to the α1,3 galactose molecule [84].



Synthetic trisaccharides B antigen conjugated to either microspheres or the SPR platform had lower measurements than the corresponding A antigen, but antigen was present as evidenced by the high affinity murine monoclonal binding against both antigens. Thus higher concentrations of B antigen on beads were required to increase MFI in the Luminex® platform and the binding RU values of anti-B antibodies, whether murine monoclonal IgM or human plasma samples did not generate high RU values. This may reflect many different variables. The density of the trisaccharides or the conjugation process may be less efficient in the B-trisaccharide compared to the A-trisaccharide. The underlying chemistry for this is not clear, as the terminal chain of the two structures should be similar. The joining of the either the amine or the carbon linker may have bound to a different structure (part of the N-acetyl-glucosamine structure) or the configuration of this terminal structure with the amine prevents effective carboxyl substitution.

Binding of antibody to microsphere synthetic trisaccharides was shown by Pochechueva to give higher MFI in trisaccharides, rather than tetrasaccharides for A/B antigen [232]. This is in contrast to work done by Lindberg who demonstrated that tetrasaccharides removed anti-ABO specific antibodies better than trisaccharides [233].

Binding to synthetic antigens is described as being variable by Lindberg et al, and the use of IgG subclass analysis demonstrates that there are higher levels of complement fixing IgG1 and IgG3 subclasses in blood group "O" serum, whereas the blood group "A" and "B" had higher levels of IgG2, although blood group "O" serum still had high levels of this IgG subclass [231].

In summary, the conformation of the synthetic antigen, both in the assay platform and the structure of the linker to the platform seem to be important to developing improved specificity. While synthetic assays are yet to deliver reproducible results, HG is still key to assessing antibody. The use of flow cytometry, however, would allow identification of isotypes of binding which may give more information for clinical decisions regarding antibody profile risk. This needs to be evaluated in a larger cohort.

Chapter 3 The ABOUT-K study

A prospective, multi-centre, observational study evaluating an antigen-specific antibody removal protocol for ABO-incompatible renal transplantation in the UK

3.1 Introduction

At the time of study design, there were 6 centres undertaking planned ABOiKTx in the UK, however this number has increased significantly over the last few years. This increase has been based on the outcomes from single centres performing ABOiKTx who reported similar clinical outcomes to their ABO compatible cohort (ABOcKTx) as described in Chapter 1. No multi-centre national data had been collected on ABOiKTx that examined clinical outcomes, complications, antibody removal and antibody titres. Furthermore, no assessment had been undertaken centrally to compare local and central titres measurements by haemagglutination. This is a pre-requisite to compare clinical outcomes between centres with a standardised assay. A prospective observational study of practice and outcome would describe clinical outcomes and unify the measurement of anti-ABO blood group specific antibody titres.

The ABOUT-K study aims to describe the prevalence of acute rejection in the first 12 months post-transplantation, patient survival, graft survival and renal function at 1 year. In addition, the local result of anti-A and/or anti-B titres was collated and compared to a centralised assay which was undertaken using a standardised technique in order to assess the impact of differences in titration technique on clinical practice.

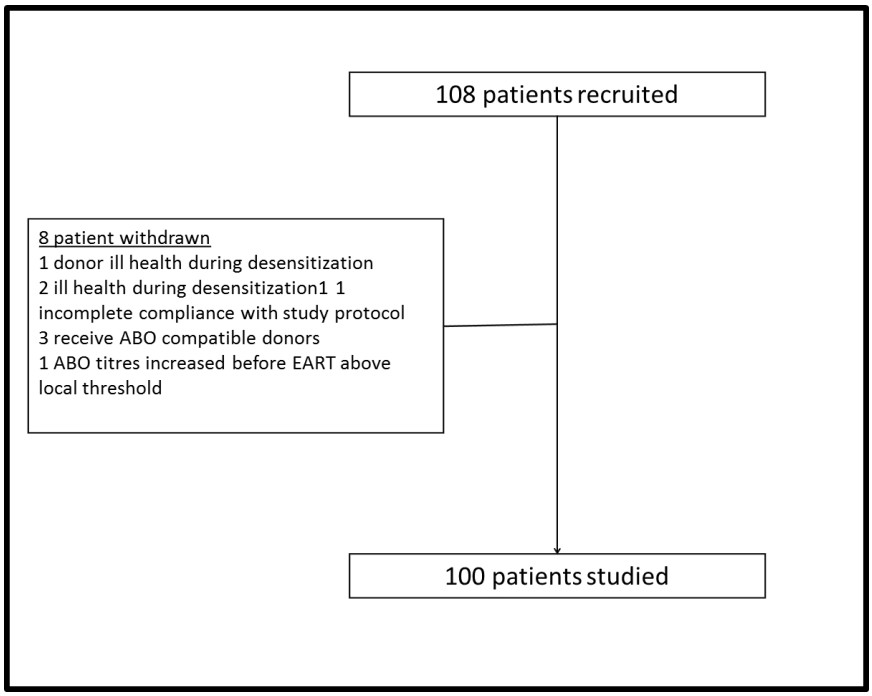
3.2 Methods

Fourteen centres were recruited to participate in the ABOUT-K study group. Six other centres were approached but did not participate in the study. One hundred and eight patients were recruited to the ABOUT-K study from 9 different transplant centres between 13th January 2009 and 4th July 2012. Eight patients were withdrawn for reasons described in Figure 3.1.

This study protocol was approved by the South Birmingham Research and Ethics committee (08 H1207 293) and study sponsor University Hospitals Birmingham NHS Foundation Trust (RXK3468) and was a NIHR portfolio study (6878). The study design was an observational, prospective, cohort study of patients accepted into an ABO incompatible kidney transplant programme and due desensitization prior to transplantation.

Figure 3.1. Total Patients recruited and exclusion with reasons

This diagram demonstrates all patients that were recruited into the study, but withdrawn with reasons for these exclusions.



3.2.1 Study Aims

The design of this study was based upon the hypothesis that differences in clinical practice, specifically in the quantification of blood group-antigen specific antibody, in the method of EART and in immunosuppressive regime affect the outcomes and

complications in ABOi kidney transplantation. The degree of variation in practice observed and the number of centres recruiting 100 patients means that the study effectively stands as a pilot;, identifying the need for greater uniformity in practice if any definitive conclusions are to be made with respect to the optimisation of ABOiKTx.

The primary endpoint was acute rejection in the first year following ABOiKTx. This was determined by the local transplant team and the centres were required to classify rejection as antibody-mediated rejection; biopsy-proven or non-biopsy proven. Treatment regimens were collected for each rejection episode. The secondary endpoints were patient survival and allograft survival; renal function in the first 12 months post-transplantation; safety and efficacy of ABOiKTx including the EART pre and post-transplant; anti-ABO antibody titres pre- and post-transplantation; the presence of non-ABO blood group antibodies post-ABOiKTx; severity of rejection in patients; the occurrence of opportunistic infections and bleeding complications.

3.2.2 Treatment Protocols

Patients were treated according to local protocol in each transplant centre. This comprised of induction therapy as part of desensitization or at time of transplantation. Induction regime consisted of rituximab in most patients with basiliximab, with a few patients receiving alemtuzumab. Maintenance therapy for immunosuppression at initiation was tacrolimus; mycophenolate and prednisolone at intention treat analysis. This therapy was adjusted according to local practice and clinical indications during follow up.

Extracorporeal antibody removal therapy (EART) was used to reduce anti-donor ABO titres to acceptable levels in keeping with the local transplant centre policy on acceptable titres using local laboratory titres. The level of acceptable titre against donor

ABO blood group was 1:8 dilution or less in the majority of centres, with others using 1:4 dilution as a target titre according to local titration assessment (n=2). EART was performed using a variety of techniques, plasma exchange; double filtration plasma exchange and immunoadsorption. Immunoadsorption was performed using either Glycosorb™(Glycorex, Sweden) columns which are blood group A/B antigen specific columns or Therasorb™ (Miltenyi Biotec Ltd, UK) [123]. Both immunoadsorption techniques require plasma separation before immunoadsorption from plasma filtrate can occur.

3.2.3 Study Selection and Clinical Assessment

The patients were recruited into the study according to local clinical practice and within the framework of the Antibody Incompatible British Transplantation Society Guidelines [234]. Participants identified by local centres for ABOiKTx were approached by the local investigator and invited to participate in the study. Once recruited, the co-ordinating centre registered the patient and entered the participant's data on the NIHR portfolio.

Patients were included in this study who were able to given written consent and who met the guidelines for live donor kidney transplantation as set out in the British Transplantation Society – Renal Association guidelines [235]. Inclusion criteria for this study were:

- Patients who were 18 years of age or over
- Undergoing a planned ABO incompatible kidney transplantation
- Able to give valid consent

Exclusion criteria for the study were:

- Patients who were less than 18 years of age
- Pregnancy confirmed by positive β-HCG pregnancy test

- The need for removal of anti-HLA antibodies pre-transplantation

Subjects could withdraw at any time if they wished, without requirement for explanation, and with the explicit understanding that their subsequent clinical care was not affected by their decision to discontinue with the study. If patients withdrew from the study, they continued to undergo routine clinical follow up with appropriate monitoring and were included in any analysis on intent to treat basis. Patients were assigned a study number in the order which they are recruited for this study, labelled according to the local transplant centre. Patient data were stored at the local transplant centre and patient assigned number. The study number was recorded at study co-ordinating centre. Data was entered onto an electronic web-based database, run by Eclinso, an electronic database company. Serious adverse event were treated according to standard clinical care and recorded in the electronic database.

The central laboratory processed samples following central registration, and stored samples under the study number. This was not an interventional study and the management of the patient was entirely dictated by the local transplant centre. There was prospective collection of relevant demographic, laboratory and outcome data. Parameters relating to antibody removal; induction and antibody levels prior to transplant were used as comparative factors between patients in this cohort. The aim of this study was to recruit a minimum of 100 patients across the UK from study start date and to follow them directly for 1 year after transplantation, with consent for 5 year follow up via NHSBT datasets for patient and allograft survival and allograft function.

The primary outcomes were defined at 1 year post-transplantation, however secondary outcomes beyond one year have been collated using data reported to NHSBT. The date

of censoring for this study when the latest data was retrieved from NHSBT for this thesis was 9th Dec 2013.

3.2.3.1 Informed consent

The notes of patients who met inclusion criteria were reviewed by one of the local investigators who arranged to see the patient in order to specifically address inclusion and exclusion criteria. Patients were given the information sheet and had the opportunity to return for a second consultation to give informed consent.

3.2.4 Data Collection

3.2.4.1 Baseline

All patients had a full medical history taken and a clinical examination. The following were recorded at consent:

a) Demographic information including date of birth, gender and race
b) Medical history including aetiology of renal disease
c) Dialysis history – pre-dialysis, current dialysis modality and length of time on dialysis
d) Transplantation history – number of previous transplants
e) HLA sensitization using peak and current calculated reaction frequency (CRF)
f) Donor/recipient HLA types and HLA matching (A,B,DR)
g) Blood group of donor/recipient (ABO)
h) Physical examination, including weight; height and blood pressure
i) Haematology: full blood count (FBC) including differential count and platelets
j) Blood chemistry: urea and creatinine, albumin, lipid profile and glucose
k) CMV status – donor/recipient
l) Anti- A/Anti-B antibody titres – locally (and the method used for)

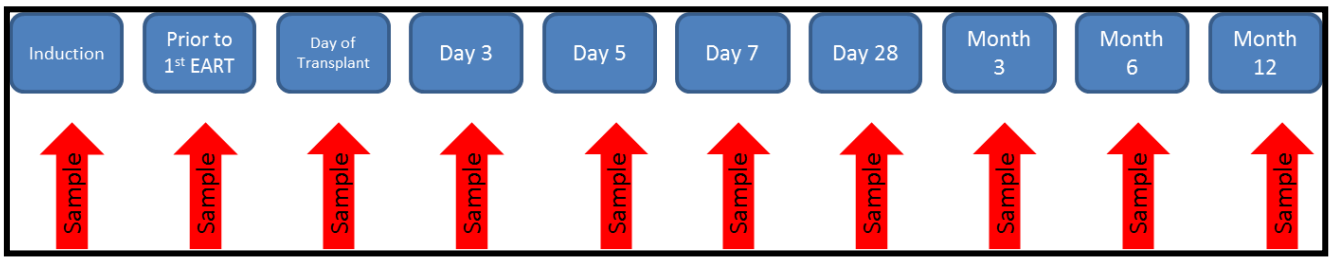
Donor patient information was collected for:

a) Demographic information including age, height, weight, gender and race
b) Donor GFR uncorrected for body surface area (BSA) if performed according to local protocol
c) Donor split renal function (right compared with left) if performed according to local protocol

3.2.4.2 Study Assessments

Participants were assessed at time points shown in Figure 3.2. Clinical assessment was made according to local protocol and managed according to local policy. Study assessment was at routine clinical appointments that correlated with the clinical study time points. Haematological, biochemical and immunological monitoring took place routinely and data was entered into the electronic database. ABO titres were measured prior to immunosuppressive drug therapy and prior to EART which was guided by the titre as well as the subsequent time points shown in Figure 3.2. The patient proceeded to transplantation according to local policy. Any complications from surgery were recorded. The number of EARTs undertaken prior to and following transplantation were recorded. The incidence of delayed graft function was recorded (defined as the requirement for dialysis in the first week post-transplantation). Following discharge from hospital, routine out-patient assessment continued for patients with assessment of graft function, immuno-suppressive medication and intercurrent complications were made at each visit and these were entered into the study database at the pre-specified time-points.

Figure 3.2. Time points for clinical; serological and immunological assessment over 1 year.



At each time point patient and allograft survival and allograft rejection was recorded. Weight and blood pressure, immunosuppressive drug regime, haematology and biochemistry results and drug levels were also recorded. Evidence of infections, malignancy, opportunistic infections and other complications were recorded at each assessment. Proteinuria was measured according to local protocol. ABO titres were measured at each time point locally and a sample sent to the central laboratory.

3.2.5 Anti-ABO specific antibody assessment

All treatment was directed by using antibody titres measured in the local laboratory to the transplant centre. Anti-ABO specific antibodies were measured at 3 timepoints pre-transplantation and 7 time points following transplantation (Figure 3.2) to assess antibody changes over time post-transplant and to correlate these with clinical events. The local technique for ABO titration measurement was recorded.

For the purposes of comparing technique performance the samples from Guys Hospital were excluded from analysis since this centre used a technique in which there was no attempted distinction between IgG and IgM haemagglutinin. In the analysis of EART and its relationship to locally reported assays (compared to the central quantification of haemagglutinin), the results of the HG assay used by Guys were incorporated into the analysis of IgG, since this was the measurement on which decisions to treat with EART

and to proceed to transplantation was made in the other centres. This is also referred to as the decision point assay below.

The method for measurement of central anti-ABO specific antibody is described in Chapter 2.2.1

3.2.6 Statistical Analysis

Descriptive statistics were used to assess the data from this study. Within the study cohort, analysis was made between different EART treatment groups; local and central titre methods and titre groups according to median titre of each method. Data are presented as mean and standard deviation (Mean± StDev) if tested as normally distributed by the Shapiro-Wilk test, and data are represented as median and first and third quartiles (Median, Q1-Q3) if there is a non-parametric distribution, unless otherwise specified.

Comparison of reproducibility of haemagglutination titres between tests was performed using weighted kappa inter agreement analysis. For the Bland-Altman plots, the data was treated as continuous data to assess the differences of each method from the mean of both tests.

Kaplan-Meier survival plots for survival analysis outcomes with the log-rank test were used to test differences. Comparison of data between cohorts was done using t-test and Wilcoxon rank-sum test depending on the distribution of the data in each cohort. Chi squared tests were performed on categorical data. Probability of values (p) of less than 0.05 was considered significant for this study. All statistics were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA), except the haemagglutination analysis performed using MedCalc (Ostend, Belgium).

3.3 Results

3.3.1 Patient and Donor characteristics

One hundred patients were recruited and received an ABOiKTx in this study. Seventy patients were blood group 'O' of which 51 received an 'A' donor; 3 'AB' donor and 16 received a 'B' donor. Nine 'B' recipients received an 'A' donor and 14 'A' recipients were donated 'B' kidneys. The remaining 7 recipients were 'AB' donors into 'A' (n= 4) or 'B' (n=3) recipients. The mean age of the recipients was 46.8 ± 13.2 years. There were more males (n=59) and the majority of recipients were Caucasian (n=86). Overall demographics are shown in Table 3.1. The main causes of ESRF were glomerulonephritis (n=26); hypertension/ischaemic (n=14); reflux/pyelonephritis (n=14) and cystic kidney disease (n=14), shown in Figure 3.3. Eighty six patients received a first transplant. Twelve patients received their second transplant, with the other two patients having had 2 and 3 previous transplants. The median dialysis time was 32 (13-46) months, with about two thirds receiving haemodialysis as mode of renal replacement therapy (41/63). The mean recipient body mass index (BMI) was 26.6 ± 4.6kg/m^2 and the median weight loss in the 12 months following transplantation was 2.2kg (-4.8 to 2.4). The overall cohort were not sensitized to HLA antigen with a median calculated reaction frequency (CRF) of 0 with 3rd quartile CRF still being 0, despite 14 patients with failed allograft being re-transplanted with an ABOiKTx. Only 2 patients had a CRF >50%. The donor characteristics reflected the ethnicity and age of the recipients. The majority (n=70) had hand assisted laparoscopy as the technique for nephrectomy (Table 3.2).

3.3.2 CMV status and prophylaxis

There were a similar number of patients in all groups of CMV status (Table 3.3). Prophylaxis was used in most D+/R- and 25.8% of D+/R+ had prophylaxis. CMV prophylaxis was valganciclovir adjusted to renal function. This was started according to local clinician if treated for rejection episodes in the other CMV status cohorts.

Table 3.1. Demographics and baseline clinical data of ABOi kidney transplant recipients in the ABOUT-K study

DEMOGRAPHICS TABLE	
Age (Mean ± StDev) (years)	46.8 ± 13.2
Gender (Males) (n)	59
Ethnicity (Caucasian) (n)	86
Pre-emptive transplantation (n)	**36**
First Transplants (n)	**86**
Renal Replacement Time (months)	32 (13-52)
Dialysis time (months)	32 (13-46)
Haemodialysis (n)	41
Peritoneal Dialysis (n)	15
Failing Transplant (n)	8
Recipient Blood group(n)	
O	**70**
A	18
B	12
Donor Blood group(n)	
A1	**59**
A2	1
B	30
AB	10
HLA mismatch [Median(Q1-Q3)]	
A	1 (1-1)
B	1 (1-2)
DR	1 (1-1)
ABDR	3 (2-4)
HLA sensitization	
Current CRF	Median 0 (range 0-59)
Peak CRF	Median 0 (range 0-82)

CRF - calculated reaction frequency

Figure 3.3. The underlying cause of end stage kidney disease in patients in the ABOUT-K study

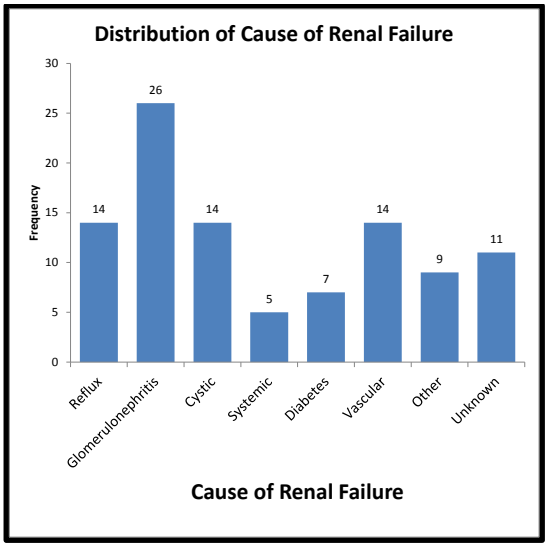


Table 3.2. Demographics and baseline clinical data of ABOiKTx donors in the ABOUT-K study

Donor details	
Age (Mean ± StDev) (years)	47.7 ± 12.1
Gender (Male) (n)	41
Ethnicity (Caucasian) (n)	77
BMI (Mean ± StDev) (kg/m^2)	26.5 ± 4.8
GFR (Mean ± StDev) (ml/min)	97.9 ± 20.7
Surgical technique for nephrectomy	
Hand-assisted laparoscopic (n)	70
Laparoscopic (n)	18
Other (n)	12

Table 3.3. Table of Donor/Recipient CMV status and the use of CMV prophylaxis in ABOUT-K patients determined by the donor/recipient

		CMV prophylaxis		
CMV status (n)		Day 7	Day 28	Month 3
D+/R+	31	25.8% (8/31)	22.6% (7/31)	16.1% (5/31)
D+/R-	21	85.7% (18/21)	81.0%(17/21)	61.9% (13/21)
D-/R+	31	12.9% (4/31)	9.4% (3/31)	12.9% (4/31)
D-/R-	17	11.8% (2/17)	29.4% (5/17)	0.0% (0/17)

3.3.3 Blood Pressure Control

Blood pressure was well controlled before and after transplant with mean blood pressure control in target limits, except for a transient rise at day 28 (Table 3.4). Patients were on median 1 (IQR 1-2) anti-hypertensive agents prior to transplantation, which did not change within the study period at either 3 months or 12 months.

Table 3.4. Blood pressure measurement in study cohort

Blood pressure compared at recruitment and through study timepoints during 12 month follow up demonstrates no improvement in blood pressure control over the 1 year follow up.

		Consent	Day28	Month3	Month12
BP systolic (SBP)	Mean ± StDev	137 ± 21	143 ± 14	139 ± 24	137 ± 26
BP diastolic (DBP)	Mean ± StDev	81 ± 11	83 ± 10	81 ± 11	79 ± 14
BP medications	Median (Q1-Q3)	1 (1-2)	1 (1-2)	1 (1-2)	1 (1-2)

3.3.4 Induction and Antibody removal techniques

Eighty patients had rituximab as an induction agent. Median time of rituximab administration was 27 days prior to transplant (Q1-Q3= 12-30days). Four patients had alemtuzumab as a single induction agent. Basiliximab was used on the day of transplantation in 56 patients, of which 38 patients received rituximab as well. Intravenous Immunoglobulins (IVIg) were administered in 4 patients prior to transplantation. The majority of patients had immunoadsorption (n=55), and 14

patients did not have any EART. A summary of immunological characteristics and desensitisation therapies are shown in Table 3.5.

Discussion of titre differences are further described in a later section; however analysis of titre reduction in EARTs was performed on local titres, as these directed the number of therapies. Median reduction of local IgG log_2 dilution titre per EART therapy was similar in IA than PEx (0.75, Q1-Q3 0.25-1 vs. 0.54, Q1-Q3 0.17-0.75, p=0.099). The number of sessions of EART was significantly higher in the PEx group than IA group (median 6[3-8] vs. 4[2-5], p=0.006). Importantly the IgG titres (or other decision point assay) in the local assay were also significantly higher in PEx group (Median log_2 dilution 6[5-7] vs. 4[3-7], p=0.007, Figure 3.4A and 3.4B). This may explain the higher number of EART episodes. There was, however, no difference in median IgG log_2 dilution titre between the PEx and IA groups when analysed using the central assay (Median log_2 dilution 4.5[2-6] vs. 3[2-6], p=0.487, Figure 3.9C). Differences in assay technique may affect the result reported, which may account for the higher number of treatments given in the plasma exchange cohort, where the local titre was reported as significantly higher than the central titre. It is not however possible to definitively attribute causation in a study of this design.

Median (Q1-Q3) local IgG log_2 titre reduction was 3 (1-4) vs. central IgG log_2 titre 2 (1-4) (Wilcoxon rank-sum test, p=0.676) and the median (Q1-Q3) local IgM log_2 titre reduction was 3(2-4) vs. central IgM 3(1-4) (Wilcoxon rank-sum test, p=0.878). When the reductions were analysed by EART, there was no difference at either local or central IgG assay [median (Q1-Q3) IgG log_2 titre reduction (local IgG titre IA 3(1-5) vs. PEx 3 (2-5) titre reduction, p=0.721; central IgG log_2 titre IA 2(1-4) vs. PEx 3(2-5) reduction, p=0.223)]. There was a higher median (Q1-Q3) IgM log_2 titre reduction in the PEx cohort than the IA cohort in the central IgM log_2 titre IA 3(2-4) vs. PEx 4 (3-5) titre reduction,

p=0.006, but this was not significantly different with local IgM (local IgM log_2 titre IA 3(2-3) vs. PEx 3 (2-5) titre reduction, p=0.346).

3.3.4.1 Albumin concentrations in the recipients

There was no significant change in serum albumin in those who received IA, PEx or no EART at each timepoints before transplantation or in the early follow up period (less than 28 days). After EART, there was no difference between the groups with mean albumin on the day of transplant (IA 36.2±5.5; PEx 33.5±7.3 and No EART 36.4±5.2, p=0.404)

3.3.5 Acute Rejection post ABOiKTx

There were 29 patients who experienced episodes of acute rejection during follow up. Only four of these were diagnosed with antibody-mediated rejection (Figure 3.5). All AMR took place in blood group "O" recipients with kidneys with blood group "A" as donor antigen (either as A [n=3] alone or as AB [n=1]), which was not statistically significant compared to the other non-"O" recipients (5.9% vs 0%, p=0.17). There was an increased risk of rejection in blood group "B" recipients compared to other blood groups (58% [n=7] p=0.017) with an odds ratio (OR 4.2, 1.22-15.5). Three (16%) blood group "A" recipients (16%, n=3) and 19 (27%) blood group "O" recipients had rejection.

Renal biopsies were performed in 44 patients over the 1 year follow up period. Seventeen patients had biopsies in the first week after transplant. Twenty two patients had biopsies after 1 week before 1 month. Twenty one patients had biopsies between month 1 and month 3. There were 17 biopsies performed between 3 months and 1 year. In total, 77 biopsies were performed for all patients in 1 year follow up.

Table 3.5. Antibody data and desensitisation techniques for the ABOUT-K study

The predominant method for local ABO titration technique was by test tube. The titres measured locally and centrally had similar distribution; however, the central IgG titre was 1 dilution lower than local assays overall. Guy's titre values are presented separately as the isotype was not distinguished in this assay.

Immunological Details TABLE	
Local HG method	
Test Tube	63
Gel Card	37
Starting Titre*#	
Local measurement IgG	5 (3-7)
Local measurement IgM	5 (3-7)
Central measurement IgG	4 (2-6)
Central measurement IgM	5 (4-6)
Guy's local titre measurement	5 (4-7)
Guy's central measurement IgG	1 (1-5)
Guy's central measurement IgM	4 (2-6)
Induction Agent	
Rituximab	80
Alemtuzumab	4
Basiliximab	56
EART – pre transplantation	
Immunoadsorption	55
Plasma Exchange	31
No EART	14

* Titres are pre-induction, or pre-EART if this data was not available and expressed as $\log_2$ dilution. # Titres are expressed as Median (1^{st} Quartile -3^{rd} Quartile)

Figure 3.4 Comparison of titres between local and central assays.

Figure 3.4A. Comparison of anti-ABO specific donor titre IgG (log_2) before antibody removal using HG assay by local and central assay assessment reveals non standardized assays given clinically significant different titre results, but not on the central assay.

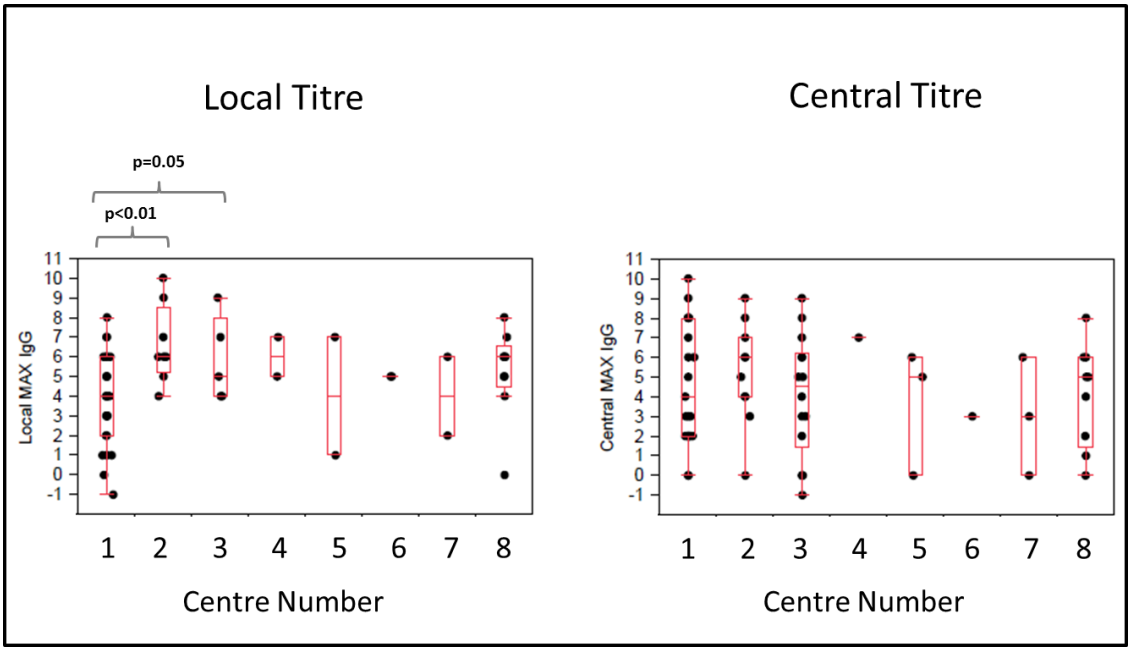


Figure 3.4B. Comparison of anti-ABO specific donor titre IgG (log_2) by EART therapy group demonstrates local titres were higher in the PEx group and this is reflected in significantly higher number of EART therapies (p<0.01).

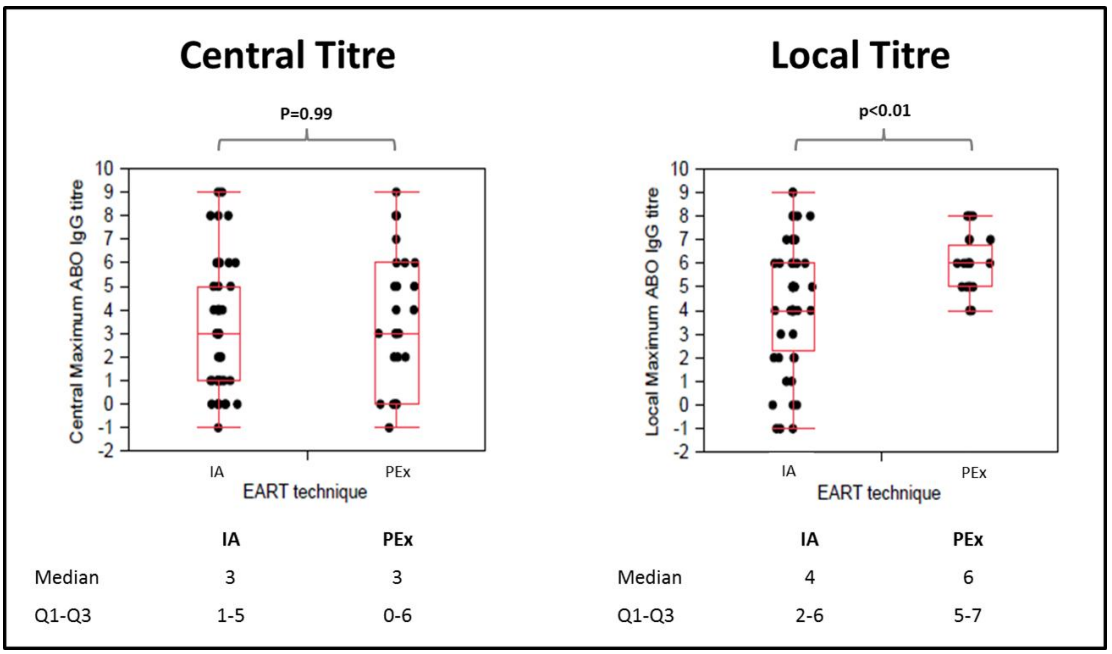
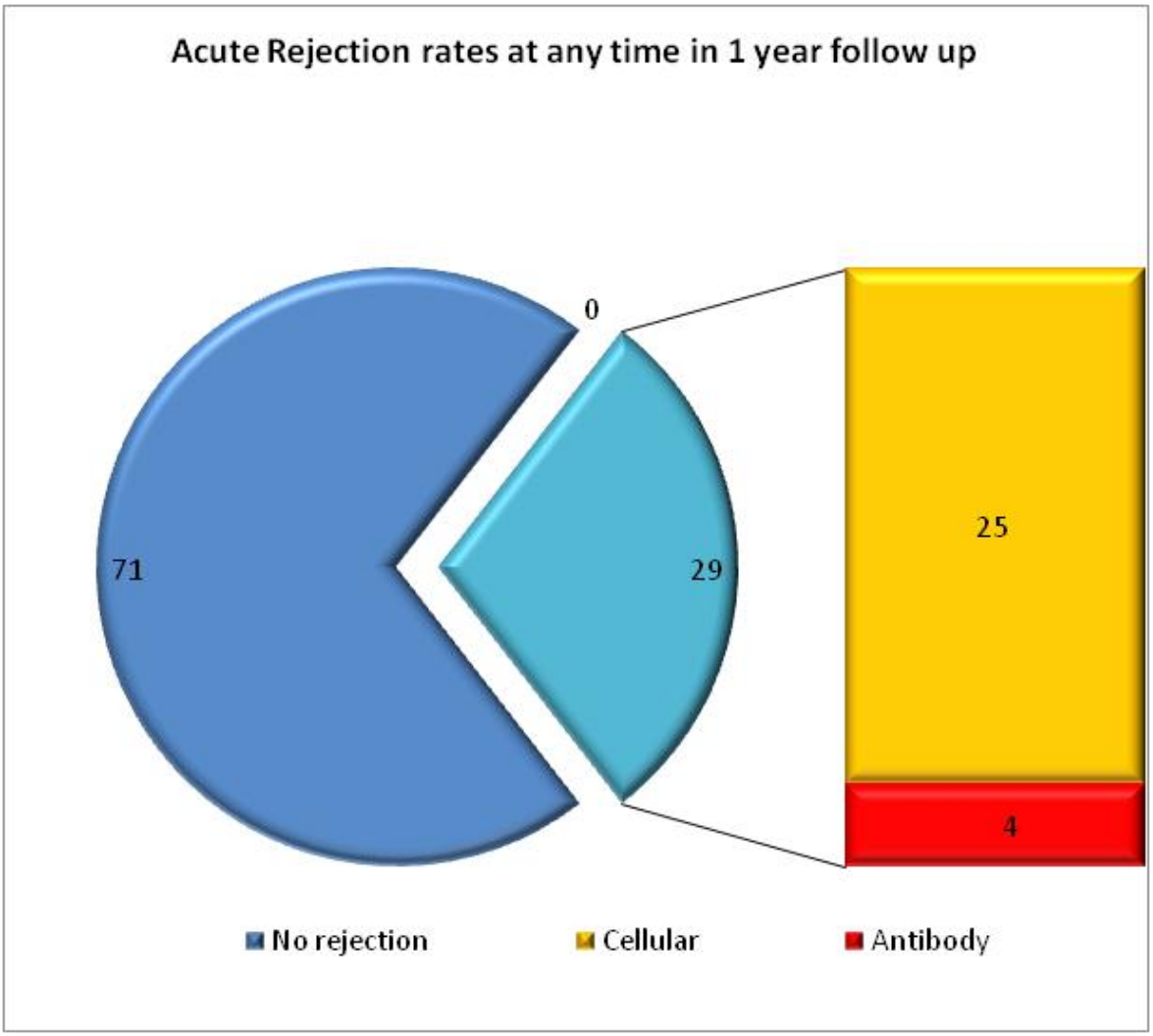


Figure 3.5. The rejection rates of patients after ABOiKTx.

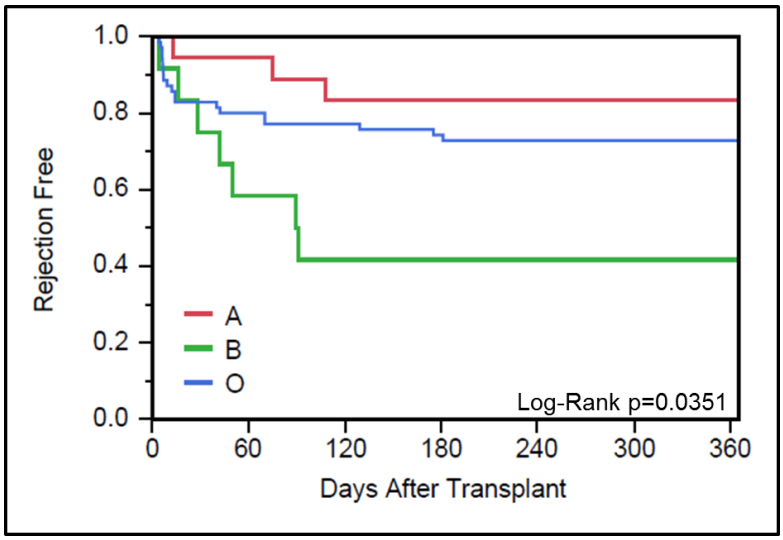
Antibody-mediated rejection occurred in 4 (13.8%) of the patients who had rejection.



Analysing rejection by antibody titre, all rejection episodes were grouped together into a rejection group as the number of antibody-mediated rejection episodes was too low for statistical analysis in this cohort. Acute rejection episodes occurred in the early post-transplant period and this was at a significantly higher rate in blood groups "B" and "O" recipients compared to "A" (p=0.035, Figure 3.6). The majority of rejection occurred within 30 days. After 120 days post-transplant there were few episodes of rejection (n=3).

Figure 3.6. Rejection free survival in allograft following ABOiKTx

Blood group "A" recipients (red) has significantly less rejection than blood group B, not O in follow up in the first year following transplantation ("A" vs. "B" 17.6% [3/17] vs 53.8% [7/13], p=0.035, "A" vs. "O" 17.6% [3/17] vs. 27.1% [19/70], p=0.419, Pearson Chi squared). Blood group "B" recipients (green) had more rejection than other blood group recipients ("B" 53.8% [7/13] vs. "non-B" vs. 25.3% [22/87], p=0.034, Pearson Chi squared).



3.3.6 Patient and Allograft Survival

One patient died secondary to sepsis at 97 days after transplantation, giving a 1 year survival of 99%. Allograft failure occurred in 6 patients by 1 year. Two grafts were lost early secondary to antibody mediated rejection refractory to intervention by antibody removal and immunosuppressive agents. One graft was lost on day 1 from acute renal artery thrombosis, attributed to surgical complication in the absence of a rise in ABO titre and the kidney was pale and ischaemic. Histological examination demonstrated severe acute tubular injury with no inflammation or tubulitis; the C4d stain was negative and there was no peritubular capillaritis, which would support an acute AMR episode. One graft was lost on day 79 due to recurrence of FSGS. One graft loss was attributed to BK nephropathy at 146 days post-transplant and another was attributed to mixed cellular humoral rejection at 177 days. A latter graft loss at 483 days was attributed to non-compliance. There was no allograft survival difference correlated with blood group of recipients. There was no statistical difference in 2 year allograft survival between different antibody removal techniques (IA 94.6% vs. 81.6%, p=0.406). The Kaplan-Meier plots in Figures 3.6A, B and C demonstrate allograft loss over time in relation to overall allograft survival; blood group and EART.

Figure 3.7 Allograft survival divided into overall survival; survival by recipient blood group and by antibody removal technique

Figure 3.7A. All allograft Kaplan-Meier death censored survival (median follow up 700 day (IQR 355-1057)

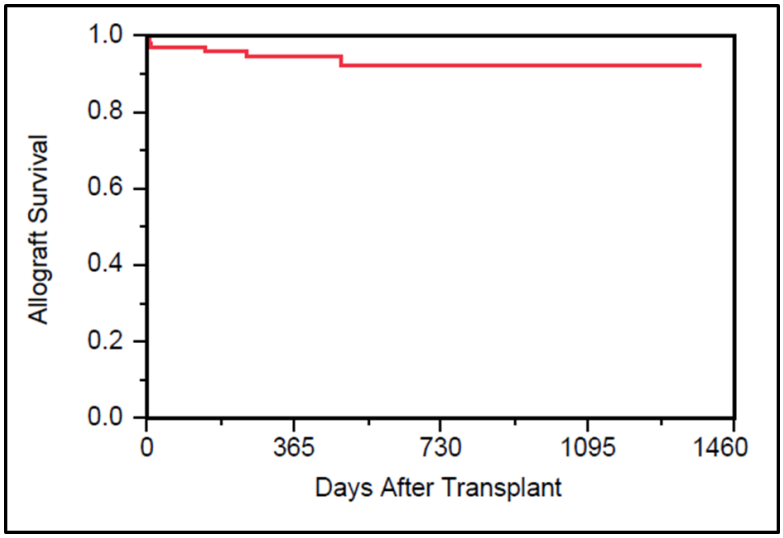


Figure 3.7B. Allograft Kaplan-Meier death censored allograft survival by recipient blood group. Death censored allograft survival by blood group type. Both "A" and "B" recipient cohorts did not lose any allografts and the lines overlap. All transplants that failed were in blood group "O" recipients; this did not reach statistical significance.

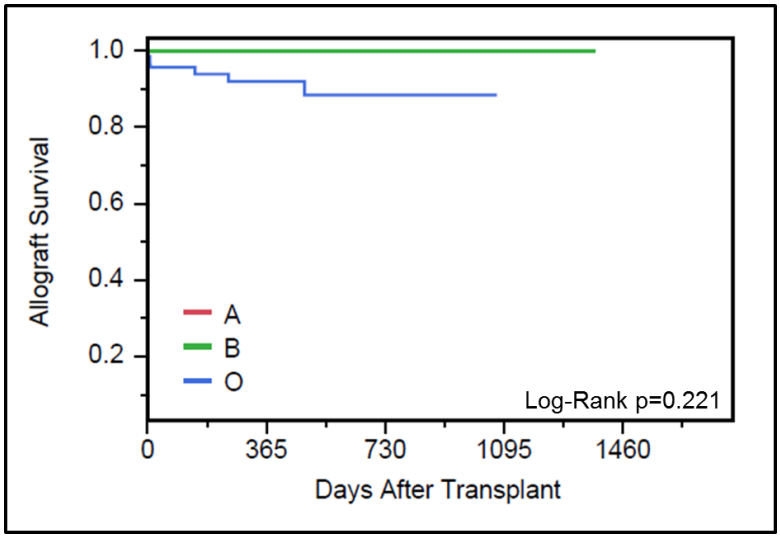
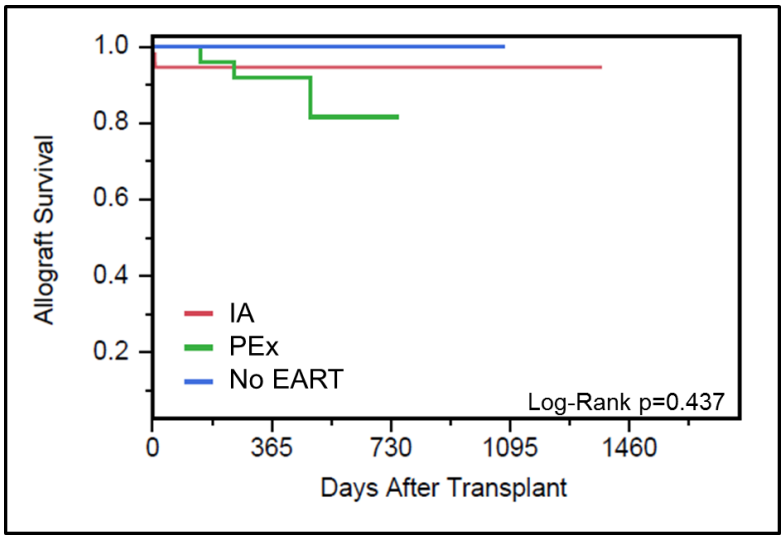


Figure 3.7C. Death censored allograft survival by EART technique.

Patients without EART (i.e. Low titre patients) had 100% graft survival. There was no statistical significant difference in allograft survival between IA, PEx or No EART techniques.



3.3.7 Renal function following transplantation

At last follow up, serum creatinine for this cohort was median 121 micromol/L (Q1-Q3 95.5-154.5) with median follow up 700 days (Q1-Q3 355-1057). There was no significant difference in creatinine at any time point within the first 12 months for the different EART therapies. The incidence of dialysis dependant delayed graft function (dDGF) was 7. Our transplant centre has published on the importance of functional delayed graft function (fDGF) and 16/100 patients met the criteria of less than 10% reduction in daily creatinine [236]. This occurred at day 3 in 11 patients and at day 5 in 3 patients and 2 patients met criteria at day 7.

The pre-treatment titre of patients did not distinguish between patients with delayed graft function needing dialysis regardless of isotype measurement or local/central assessment. Higher pre-desensitisation titres were associated with no fDGF regardless of isotype or local/central measurement (Figure 3.8). There was no association with allograft failure with either dDGF (p=0.40) or fDGF (p=0.96). The function of the allograft was no different at most recent follow up for dDGF (Median creatinine (Q1-Q3) 147 micromol/L, (87-163) vs. 124 micromol/L, (94-150), p=0.70) however, fDGF was associated with poorer function at last follow up (Median creatinine(Q1-Q3) 150 micromol/L, (120-187) vs. 121 micromol/L, (93-143), p=0.05).

Regardless of the time of rejection experienced post-transplantation, those with rejection had significantly higher creatinines even at Day 3 post-transplant prior to the rejection episode (p=0.008). There continued to be a difference after transplant at Day 5; Day 7; Day 29; Month 3; Month 6 and Month 12 (Table 3.6). This was true for cellular rejection, but there was no difference in the few with antibody-mediated rejection. There was no difference in renal function at each time point stratified by recipient or donor blood group, or by blood group donor/recipient pairs.

Figure 3.8. Prediction of delayed graft function by starting titre.

Figure 3.8A – Functional Delayed Graft Function was associated with lower starting anti-donor titres by either local or central assays. Patients with fDGF (red triangles) are displayed in both graphs, as dialysis dependent delayed graft function is a subgroup of fDGF. Black circles are all patients without DGF. (Wilcoxon rank-sum test)

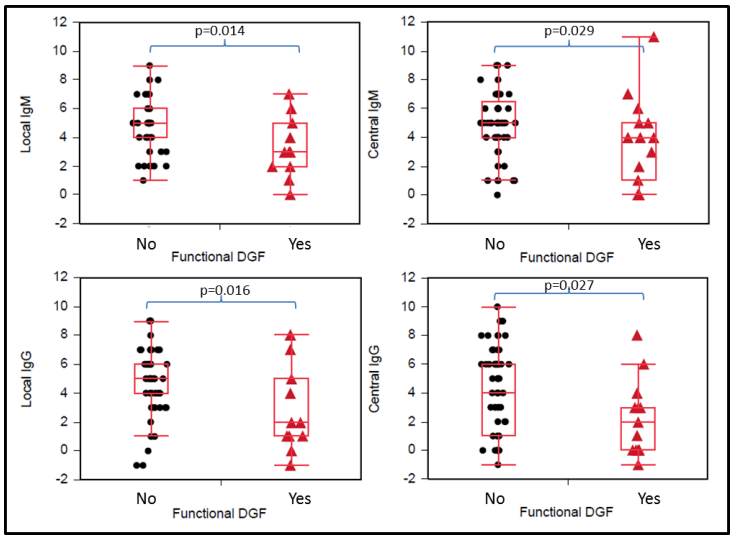


Figure 3.8B – Lower local starting anti-donor titres were associated with Delayed Graft Function by dialysis(dDGF). Higher starting titres by either local or central assays were not predictive of dDGF. fDGF (red triangles) are displayed in both graphs as dialysis dependent delayed graft function is a subgroup of fDGF. Black circles are all patients without DGF. (Differences calculated by Wilcoxon rank-sum test)

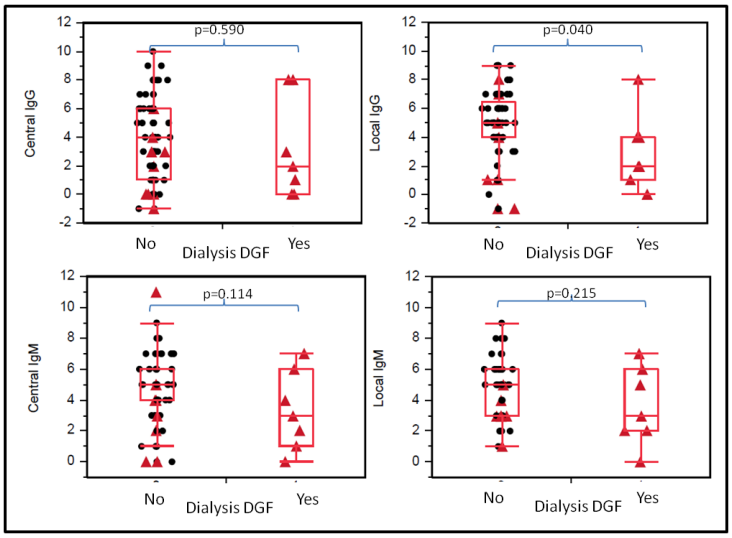


Table 3.6. Biochemical analysis in the patient cohort at each study time point

		Consent	Pre-induction	Pre-EART	Pre-Txp	Day 3	Day 5	Day 7	Day 28	Month 3	Month 6	Month 12
Creatinine (µmol/L)	Median (IQR)	596 (404-778)	560 (429-718)	596 (445-726)	502 (399-634)	138 (96-254)	124 (96-186)	122 (95-166)	120 (94-151)	123 (107-145)	132 (104-161)	124 (103-155)
Urea (mmol/L)	Median (IQR)	20.6 (16.7-25.9)	21.2 (16.8-25.0)	21.2 (15.9-24.5)	14.9 (10.3-19.1)	9.5 (5.8-15.0)	9.1 (6.2-14.2)	8.5 (6.0-14.1)	8.3 (6.9-9.8)	7.5 (6.0-10.0)	7.8 (6.1-10.3)	7.5 (6.3-9.3)
Albumin (µmol/L)	Median (IQR)	42 (38-46)	41 (37-46)	39 (35-42)	36 (32-40)	31 (27-34)	32 (29-35)	34 (30-34)	40 (36-43)	43 (40-46)	43 (40-45)	43 (40-46)
Glucose (mmol/L)	Median (IQR)	5.3 (4.3-6.2)	5.2 (4.7-6.0)	5.7 (4.7-6.7)	5.4 (4.7-6.7)	7.0 (5.4-8.4)	6.7 (5.2-8.6)	4.3 (3.9-5.5)	6.4 (5.6-7.8)	5.2 (4.3-6.5)	5.5 (4.7-6.4)	5.7 (4.9-6.6)
Cholesterol (mmol/L)	Median (IQR)		4.5 (3.9-5.4)					4.3 (3.9-5.5)	5.4 (4.7-6.3)	5.2 (3.9-5.4)	5.3 (4.5-7.8)	5.1 (4.5-6.0)

Table 3.7. Comparison of creatinine in patients with rejection at any time point

Rejection was defined as biopsy or non-biopsy proven rejection by local clinical team and analysis demonstrated regardless of time of rejection. Serum creatinine was significantly higher in those who had rejection in follow up data at all time points. Creatinine in micromol/L (median [Q1-Q3]). Wilcoxon rank-sum test.

Time point	n	Rejection group	No Rejection group	p-value
Day 3	86	183 (121-450)	128 (92-181)	0.008
Day 5	73	203 (115-356)	115 (88-158)	0.002
Day 7	95	174 (120-303)	111(84-146)	<0.001
Day 28	92	151 (97-182)	115 (89-133)	0.004
Month 3	86	155 (125-191)	116 (93.5-139)	<0.001
Month 6	73	157 (124-241)	126 (95-147)	<0.001
Month 12	88	142 (115-198)	119 (95.4-142)	0.010
Month 24	57	158 (129-182)	115 (92-144)	0.001

3.3.8 Anti-ABO specific antibody comparison from local to central analysis and effect on clinical outcomes

Inter-rater agreement was tested using kappa agreement, as there is no gold standard test against which both tests could be compared. The weighted kappa for anti-donor IgG at induction was 0.613 (0.461-0.765). This decreased to 0.547 (0.389-0.705) before EART and down to 0.339 (0.200-0.477) on the day of transplant surgery. Thus, the agreement between local and central titre measurement was divergent the closer to the time of transplant at lower titres. Figure 3.9A demonstrates the differences between the central titre and the local titre and Figure 3.9B demonstrates intra-rater agreement for the central titration assay.

Analysis was made at each corresponding time point prior to transplantation, namely pre-induction; pre-EART and at time of transplant. The antibody range before EART is displayed in Figure 3.10 for both local and central titres. In the 3 major contributing centres, there was a significant difference in IgG titres measured locally, however this was not so when measured centrally as illustrated for samples quantified prior to EART in Figure 3.4A.

Table 3.8 demonstrates the number of samples at each time point collected. There were 86 samples collected prior to induction agent; 91 prior to antibody removal and 99 at time of transplantation. There were variations in local protocols, in that no samples were measured if no induction agent or EART was administered. The median local IgG titre at assessment was 1:32 (Q1-Q3 16-64). There were a significant number that had very low IgG titres (n=15, ≤1:4) and reasonably local high IgG titres (n=9, ≥1:256).

A titre of 1:8 or below is generally used as the decision point for transplantation to proceed, all be it that this has not been related to the sensitivity of the HG assay in use

at the reporting centres [65]. The number of patients on day of transplant that had a local titre IgG which was >1:8 was 4.1% (4/98) and 4.1% (4/97) by central titre IgG. For IgM, the local titre >1:8 was 11.1% (8/72) and 6.25% (6/96) by central titre IgG. There was no significant clinical implication of these titres being above target when correlated with allograft loss or allograft rejection. There was no correlation with AMR using central titre HG assay.

In this study, the analysis of local titres is complicated as one centre performs an isohaemagglutination test and does not identify isotype. Guy's Hospital contributed a significant proportion of patients to this study (n=26), however their local laboratory assessment was the only laboratory to perform a single agglutination assay, isohaemagglutinin, not differentiating between different isotypes of anti-ABO specific antibody. These were treated as IgG for analysis purposes as titre that directed clinical therapies, as described in the methodology.

Figure 3.9.Comparison of local and central titres agreement plots

Figure 3.9A -Bland-Altman plot of local to central agreement assay titres, plotted as log_2 dilutions. Titration results are ordinal, however treated as continuous, thus significant overlap of titre results.

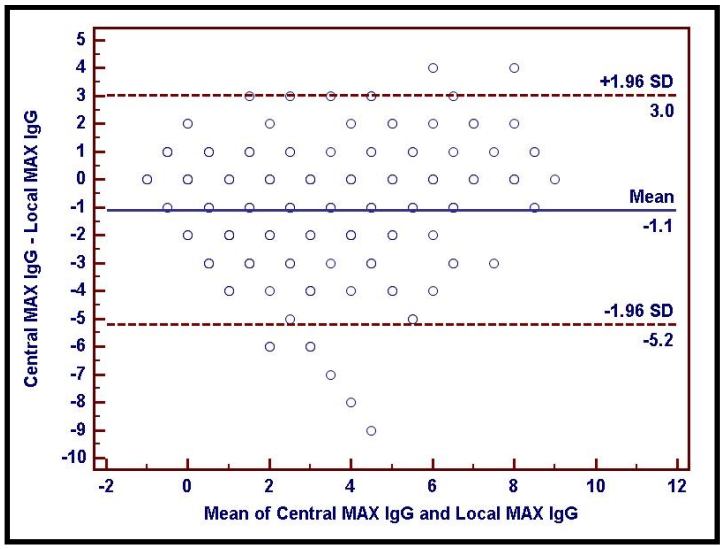


Figure 3.9B- Bland-Altman plot of intra-rater agreement, demonstrating excellent agreement on reproducibility – previously described in more detail in Chapter 2.

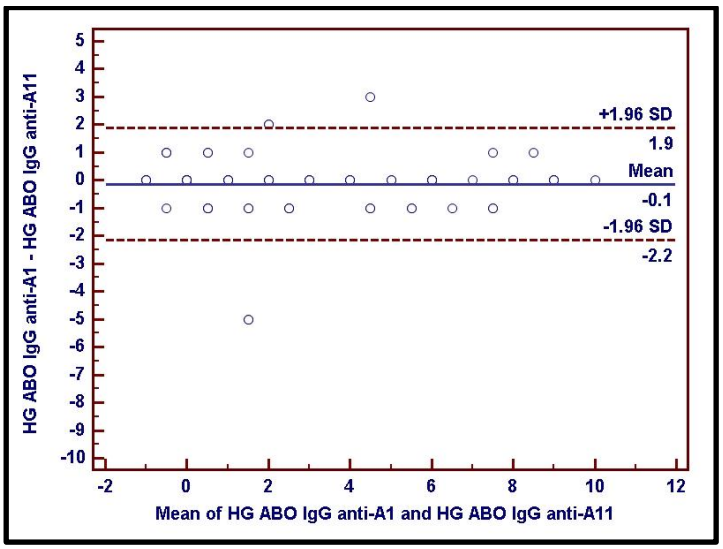


Figure 3.10. Distribution of ABO titre values at different time points and at different transplant centres and the variation in EART therapies based on these assay variations.

Histograms of local and central assay assessment of anti-ABO specific antibodies (log_2 dilution).

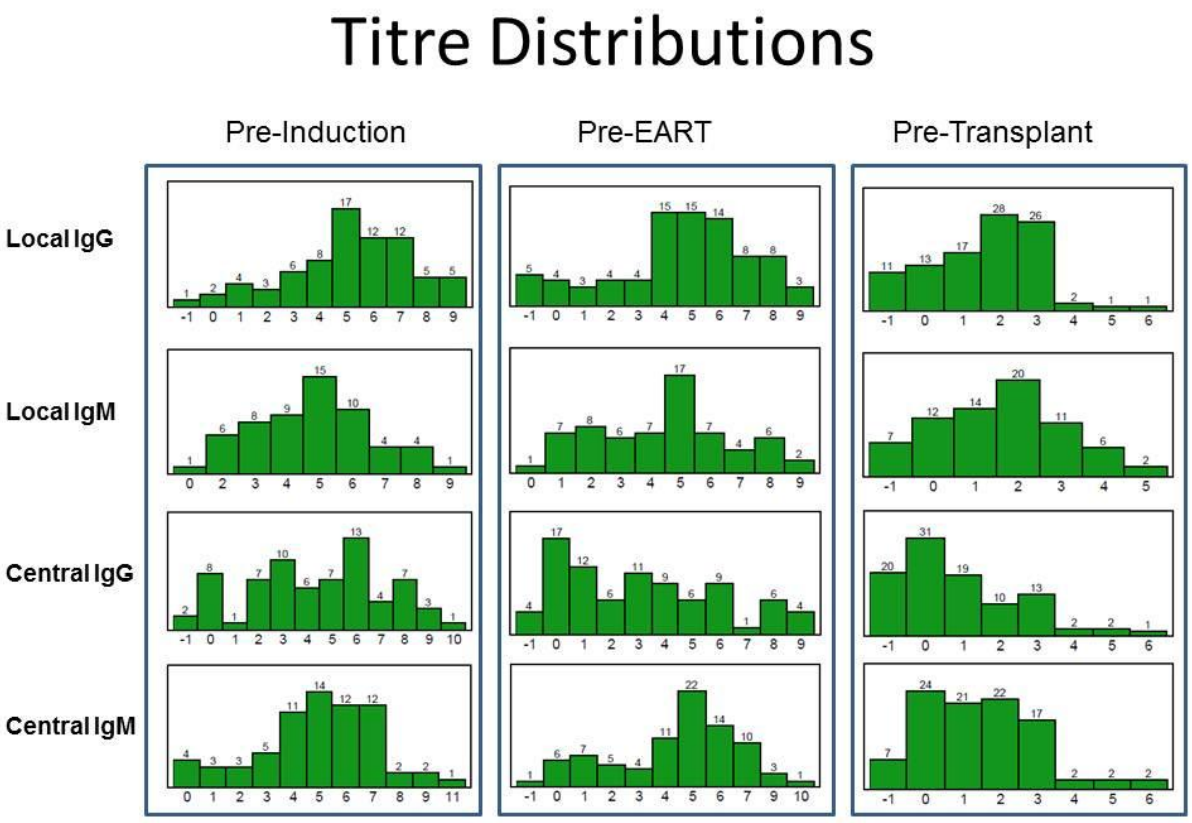


Table 3.8. Number of samples at local and central collection at pre-transplant time points.

The number of samples collected centrally at time point for the ABOUT-K study patients.

	Pre-Induction	Pre-EART	Pre-Transplant
Local Titre completed	100	70	100
Central Sample sent	86	91	99

3.3.9 Adverse Events and Infectious Complications

As previously described, 1 patient died from sepsis secondary to ischaemic bowel with a retroperitoneal haematoma. Two other patients required bowel resection in the first week post-transplant. One patient had ischaemic small bowel and the second had a perforated caecum. Both of these have had reversal bowel surgery during the study with no further complications (Table 3.9)

3.3.9.1 Vascular

There were 4 renal artery surgical complications; 2 with bleeding requiring significant resuscitation, 1 of which required cardiopulmonary resuscitation. These did not correlate with either type of antibody removal; the starting titre or degree of titre reduction. One renal artery dissected and needed a polytetrafluoroethylene (PTFE) graft. In one allograft failure, there was a primary renal artery thrombosis, which following thrombectomy did not improve function to the allograft. The allograft was pale and hypo-perfused, not necrotic nor infarcted. Biopsy revealed an ischaemic kidney with no evidence of AMR. One patient developed a post-transplant polyclonal lymphoproliferative disease 3 months post-transplant.

Three patients had ischaemic cardiac events. Two patients had non-ST elevation myocardial infarctions which occurred in the peri-operative and early post-operative period (<24hours). One patient had a myocardial infarction during DFPP and had coronary stent inserted and operation delayed. One further patient has chest pain following recruitment and had coronary stenting performed. This patient had a delay of the transplant operation by 6 months, and then proceeded to undergoing EART and transplantation. One patient who had transplant renal artery stenosis was successfully treated with angioplasty within the first 3 months post-transplant.

There were 6 peri-allograft haematomas that needed intervention and 4 lymphocoeles that required intervention. One patient required vacuum therapy to the transplant wound, but had not had any of the above collections.

3.3.9.2 Infections

Reported infections requiring admission to hospital were: 4 chest infections and 7 urine infections in the 100 patients. Of the chest infections, there were 2 cases of pneumocystis jirovecii (PCP) in one institution. This was at the same time of an apparent UK national outbreak of PCP. Both recovered well with therapy. The number of recorded viraemias was 6 CMV viraemias, 6 BK viraemias and 3 JC viraemias. This was defined as a detectable PCR without clinical symptoms, blood changes or allograft dysfunction. One patient had varicella viral re-activation. Six patients had BK nephropathy and 4 patients had CMV disease, defined as kidney dysfunction with viraemia [237].

3.3.9.3 Other Adverse Events

Other reported serious adverse events were: 2 SVC obstructions (1 secondary to line insertion and 1 spontaneous); small bowel resection for strangulated hernia; venesection for polycythaemia; incision and drainage of scrotal abscess; severe tubulo-interstitial nephritis drug reaction; severe duodenitis on endoscopy; calculi in submandibular gland; slow respiratory wean for 4 days post-bowel resection. There were 5 cases recorded of new onset diabetes after transplantation. One patient had suicidal ideation at 6 months post-transplant and continues to have regular psychiatric review.

Significant reactions to immunoadsorption were only reported in 1 patient on first therapy and responded to local therapy and then proceeded to transplant. Three

patients had their operation delayed due to complications; 1 with positive blood cultures for Staphlococcus aureus and 2 patients with acute cardiovascular events.

Two patients had recurrence of focal segmental glomerulosclerosis (FSGS) in the allograft; of which one had early rapid recurrence by Day 3 post-transplant and re-commenced haemodialysis early despite PEx therapy within the first year; the second re-occurred at 11 months post-transplant. One patient was non-compliant with his medication after the 1 year follow up and his allograft failed at 451 days after transplant with combined cellular infiltrate and chronic antibody-mediated rejection on the biopsy. Two patients had thrombotic microangiopathy on biopsy with elevated tacrolimus levels.

3.3.9.4 Haematology

There was an improvement over the follow up for haemoglobin and haemocrit following transplantation despite increase immunosuppressants (Table 3.10). At Day 7 the haemoglobin (8.9±3.9g/L) were statistically lower than at all other time points ($p<0.001$). There was also a transient rise in leukocytes, commonly associated with steroid therapy at Day 7 which was higher than all other time points ($12.2±4.7*10^9$/L, $p<0.001$). At the time of transplant, there was statistically lower platelet numbers than at all other time points ($183.2±64.5*10^9$/L, $p<0.001$).

Table 3.9. Comparison of complications according to EART group.

Group	Renal Artery Complications	Haematoma	Lymphocoele	Infections		PCP	Viral Disease		IHD event	Bowel Resection	dDGF	fDGF	PTLD
				Urine	*Chest*		*CMV*	*BK*					
IA (n=55)	2	1	1	2	3	2	3	5	1	2	5	11	0
PEx (n=31)	2	4	2	3	1	0	0	1	1	0	1	3	0
No EART (n=14)	0	1	1	2	0	0	1	0	2	0	1	2	1

Table 3.10. Haematological responses following ABOiKTx.

These results demonstrate stable leukocytes and an improved haemoglobin following transplantation. A rise in leukocytes and neutrophils occurred at Day 7 in keeping with steroid immunosuppression regimes.

		Consent	Pre-induction	Pre-EART	Pre-Txp	Day 7	Day 28	Month 3	Month 6	Month 12
Haemaglobin g/L	Mean ± StDev	10.1 ± 4.0	11.3 ± 2.4	10.9 ± 1.3	10.0 ± 1.7	8.9 ± 1.6	10.4 ± 2.1	11.4 ± 2.2	12.0 ± 1.8	12.0 ± 3.2
Haematocrit (%)	Mean ± StDev	29.7 ± 13.2	34.6 ± 7.5	33.2 ± 4.3	30.4 ± 5.2	26.6 ± 5.7	32.5 ± 6.5	34.9 ± 7.6	36.4 ± 8.0	35.6 ± 12.1
Leukocytes $x10^9/L$	Mean ± StDev	7.2 ± 3.0	7.3 ± 2.8	7.5 ± 3.3	7.6 ± 3.3	12.2 ± 4.7	9.8 ± 3.9	6.9 ± 3.6	7.1 ± 2.7	7.4 ± 3.5
Neutrophils $x10^9/L$	Mean ± StDev	4.8 ± 4.9	5.7 ± 5.1	5.1 ± 2.8	6.4 ± 9.8	9.4 ± 4.5	7.7 ± 3.6	4.6 ± 3.1	4.8 ± 2.3	4.9 ± 3.1
Platelets $x10^9/L$	Mean ± StDev	210.8 ± 101.6	232.5 ± 89.7	225.2 ± 63.9	183.2 ± 64.5	257.1 ± 107.5	273.4 ± 105.4	270.5 ± 101.0	257.6 ± 74.7	238.4 ± 97.4

3.4 Conclusions

ABOiKTx is an effective option for patients with end stage kidney disease in a multi-centre study allowing pre-emptive live kidney transplantation. The overall outcomes of this patient cohort are in keeping with national allograft survival rates for live donor ABOcKTx (97%), and better than the national registry data for ABOiKTx from NHSBT, UK (91%). The increased survival at 1 year in the ABOUT-K study (94% vs 91%) was higher, although not significantly different in the number of patients in this study (Figure 1.1).

The results in ABOUT-K compared to national UK registry data suggest that the centres contributing to ABOUT-K study may be performing better in the early (1 year) period than the rest of the registry data. The ABOUT-K study had recruited about a third of the total UK ABOiKTx in the registry data however, the difficulty of comparing outcomes in the national registry data using local titre results is demonstrated well in the difference in titre measurement between centres in Figure 3.4A. Thus, significantly high titres in one centre may be associated with good outcomes, but when these results are assessed using a central assay, the titres are "mid-range". In this study, it has not been possible to demonstrate a difference in clinical outcomes between centres or based on initial titres or at the time of transplant.

In this cohort, we have not shown an adverse immunological effect with different levels of titre either at local or central assay titres at either pre-desensitization or at the time of transplantation. While the 2 early AMR allograft losses were in patients with initially higher pre-EART titres (greater than 1:128), this study does not answer the question at what level is safe to transplant patients on the day of surgery, as the study did not have sufficient events to be able to analyse this. The majority of patients were transplanted

below the threshold of ≤ 1:8 according to local and central titres. The patients that were above the 1:8 titre, either locally or centrally on the day of transplant, did not have an obviously detrimental outcome in transplantation. This value of 1:8 is still an arbitrary titre value. Flint et al demonstrate using a test tube agglutination method that 1:32 is adequate for allowing transplantation to take place while the Tyden protocol aims for 1:8 or less. In the Tyden paper, however, one centre transplanted patients with a pre-transplant titre of 1:128 [130]. The authors hypothesized that this was technique dependent, rather than true antibody level, which is likely given the similar clinical outcomes between these centres [238].

The graft survival at 1 year was 3% greater than other registry data. As registry data is not currently stratified according to anti-ABO specific antibody titre risk, no further comparisons can be made. It seems likely that assessment of clinical outcomes between centres would require an assay to be standardised and reproducible between centres and within centres to allow comparisons to be made of outcomes and for collaborative studies to take place. While each centre performs its own in-house quality assurance testing, no national programme to compare ABO titres is currently in place.

This potentially has a significant impact on patients being initially accepted into the ABOiKTx programmes at local centres. As demonstrated in this cohort, when local titres were compared between different centres, there were significant differences between titre values, which at first sight suggests that different centres have local populations with different anti-ABO specific antibody levels. However, using titres measured centrally, the population in all centres was widely and normally distributed when the major contributing centres to this study are compared. Thus, it is possible that acceptance onto an ABOiKTx programme could be limited by a local assay, rather than true anti-ABO specific antibody levels. While this study only examines patients already

accepted into local ABOiKTx programmes, it does suggest that in some centres local ABO titres may be higher than acceptable levels in the local assay, but in a central assay the titre would be appropriate for antibody removal. This requires further study. This would increase the live donor transplantation options for donor/recipient pairs, especially in blood group "O" recipients. There are no published data currently in press that suggests the number of patients that might be prevented from ABOiKTx based on high local titres, only the assay variability studies [239]. It is relevant to clinical practice because patients who would otherwise do well may be excluded on the basis of a high local titre [131].

Secondly, while we have shown that plasma exchange has significantly higher number of treatments required, prior to transplantation; this was associated with a significantly higher titre levels reported in the local assay in the plasma exchange cohort compared to the immunoadsorption cohort despite similar central measurements. Clinicians were treating local titre value produced in their laboratory compared to those centres using IA. Despite there being a higher number of treatments in the PEx cohort, there was not a significant reduction in serum albumin levels. The number of complications from viraemia and the 2 patients with bowel perforations should be noted and taken into consideration when counselling patients who may be at higher risk for these events. The impact of the method and the number of EART may have an effect on complications. The 2 allograft losses, due to antibody mediated rejection, both occurred early and developed rapidly. Both patients had good functioning allografts immediately before AMR was clinically detected. While 2 out of 100 is a small number, these are the patients that need to be studied in further detail and continuation of this work will look to identify the markers of antibody that may distinguish them from the rest of the cohort. There is limited evidenced based therapy for treating these patients. The use of early

rescue splenectomy and eculizumab are mostly likely to salvage the allograft, although eculizumab as a rescue therapy does not save all allografts [240]. Both these blood group "O" patients had higher titres prior to EART (local highest titre ≥1:128; central IgG titre 1:128 and 1:256, central IgM titre 1:256 and 1:128); had B antigen donors and a greater degree of HLA mismatch (6 and 5 respectively) but within the cohort they had no other distinguishing characteristics pre-transplant. Importantly, neither had anti-HLA DSA detected. The early allograft loss associated with ABOiKTx in registry data is likely to be comprised of the accelerated early antibody-mediated rejection that was seen in the two patients, as well as other more routine cause of early allograft loss.

ABOiKTx patients with early accelerated AMR need to be studied in more depth to be able to predict the likelihood of risk prior to transplantation. There were no specific characteristics that denoted these as higher risk. One patient was Afro-Caribbean female with lupus as an underlying disease who was a 6 antigen (ABDR) mismatch and was both A and B blood group incompatible. The second patient had hypertensive nephropathy and had a B donor who was a 5 antigen (ABDR) mismatch. Both patients were treated with immunoadsorption prior to transplantation and had local titres of 1:8 at the time of transplant surgery (central titres IgG 1:2 and 1:8 respectively).

The allograft loss from BK nephropathy had rapid onset and progression of nephropathy. The rate of BK nephropathy (6%) in this cohort seems to be similar to other published literature using immunoadsorption (8%, 3/42) and lower than plasma exchange only regimes (17.5%, 11/62), however Flint et al published a series of 37 ABOiKTx without any BK nephropathy [130, 237, 241]. The role of natural immunity in the context of transplant viral infections is yet to be fully investigated. Does antigen exposure of to A or B antigen reduce immune response to similar antigen, which are known to be expressed in BK and JC sialic receptors [242]? Both BK and JC viral

receptors can mediate haemagglutination of red cells and thus immunological tolerance to A/B incompatible antigen may down regulate immune response to both these viruses. However the level of immunosuppression is likely to play a significant factor in the higher incidence, as Flint et al demonstrated that without rituximab induction the rate of BK and CMV viraemia was equivalent between ABOiKTx and ABOcKTx [130] . The timing of onset of BK nephropathy in the paper by Sharif et al demonstrated that 75% of cases occurred in the first 6 months following transplantation and our data supported this early onset (4/6 within 6 months).

These data suggest that good outcomes in ABOiKTx can be achieved but there is however a strong argument for further standardisation of the assessment of blood group antibodies. This is a prerequisite for alignment of practice and for the initiation of any meaningful study of intervention, including different EART and optimal use of paired exchange.

In conclusion, ABOiKTx is an effective tool for kidney transplantation for recipients without an ABO compatible live donor, especially in blood group "O" recipients. A standardised technique to measure ABO titres in order to ensure equality of access and comparison across centres is necessary. The survival advantage of early, pre-emptive transplantation is likely to outweigh the higher risks of undergoing this type of transplantation; however a cohort comparison study needs to be done to compare this and require longer term follow up.

3.5 Further Work

The outcomes of these patients will continue to be monitored for 5 year outcomes via NHSBT. The analysis of study samples will continue for the post-transplant period to correlate outcomes with antibody changes in the early period after

transplantation. Assays that have been developed in this thesis will continue to be developed and applied to this study cohort.

A cohort study, using NHSBT data, matched for transplant centre, year of transplant, age, gender and transplant waiting time could be completed to compare the outcomes in this cohort. A comparison of the paired exchange patients in the UK who were ABO incompatible could also be performed as a better control cohort, who are unable to undergo direct live donor kidney transplantation. This would include the time of entry into the paired exchange programme, and the number of "matching runs" that were completed before a transplant took place. The patient and allograft survival in this cohort would be useful comparative, but would require large numbers in a longer term study.

I have also established collaborations with Prof Lori West's laboratory in the University of Alberta, Edmonton, Canada who have developed an assay to measure isotype and IgG subclass binding to the different type chain structures of A/B antigens. This work is novel and may explain why certain antibody levels are high but cause no rejection, whilst others are more immunogenic. Other local collaborations with the transplant surgery department have evolved. They are investigating biomarkers in transplantation. Assays for endothelial injury would be a useful tool for monitoring the effect of anti-ABO specific antibodies on endothelium. This work is being done by Ms Mel Field as part of her MD thesis at University of Birmingham, UK.

Chapter 4 Five-Year Outcomes in Living Donor Kidney Transplants with a Positive Crossmatch

This chapter has been published in the American Journal of Transplantation [144]

4.1 Introduction

Antibodies against donor HLA, termed donor-specific alloantibodies (DSA), pose a significant barrier to successful kidney transplantation because of the increased risk of both early and late graft loss [53, 243]. However, since many candidates have antibodies which react against a broad range of HLA, finding a donor against whom they have no antibody can be difficult. Over the past decade, novel "desensitization" protocols have been developed to enable patients with high level anti-donor antibodies (termed here positive crossmatch kidney transplants, +XMKTx) to receive a transplant with acceptable short term outcomes [22, 102, 186, 244]. Recently, paired donation and "acceptable mismatch" programs have provided other means for providing sensitized patients a donor against whom they have little or no antibody [18, 245-247]. +XMKTx appears to have higher *patient* survival compared to either dialysis or waiting for an HLA compatible transplant [24]. However, with regards to *graft* survival, few studies have provided data beyond 1 or 2 years after transplantation and those that have suggest inferior graft survival in +XMKTx compared to negative crossmatch kidney transplantation (–XMKTx) [191]. In addition, the late outcomes of transplants in patients with low levels of antibody and with antibody against Class II HLA remain unclear. The goal of the current study was to determine the *actual* 5-year graft outcomes in patients with antibodies against donor HLA.

4.2 Methods

4.2.1 Antibody Detection

The presence of anti-donor alloantibody was determined using conventional crossmatch assays as previously described [186]. During the study period, the primary methods for determining the presence and level of DSA pretransplant were crossmatch assays including: 1) complement dependent cytotoxic (CDC) crossmatch using the T cell anti-human globulin enhanced technique and; 2) T and B flow cytometric crossmatch (FXM) for CDC- patients. All serum was tested against donor cells for cytotoxicity using the T-cell anti-human globulin enhanced complement dependent cytotoxicity assay. However, since the T cells only express Class I (and not Class II), the only cytotoxic donor-specific alloantibody (DSA) identified was against Class I. DSA positive CDC negative patients were those who had a positive T-cell flow cytometric crossmatch (TFXM) and/or a positive B-cell flow cytometric crossmatch (BFXM). TFXM positive result was a channel shift of >66 and a BFXM positive result was a channel shift >99 as previously reported [248]. Solid phase assays were utilized in the programme beginning in 2006. Therefore, in 2006 using pre-desensitization stored serum, confirmation of DSA and its HLA specificity generally was performed retrospectively using a solid phase, single antigen bead assay (LABScreen, One Lambda, Canoga Park, CA) with the amount reported as the mean fluorescence intensity of the highest single level against donor Class I and/or Class II. Mean fluorescence intensity 1000 or greater was considered positive.

4.2.2 Study Populations.

The study described in this chapter required Institutional Review Board (IRB) approval at Mayo Clinic, Rochester, USA. The outcomes were analyzed of 102 patients who underwent living donor +XMKTx at the Mayo Clinic in Rochester, MN, USA between January 2000 and December 2006 and who met all the following criteria: 1) positive crossmatch against their live donor prior to transplantation (prior to starting any pre-transplant therapy); 2) retrospective verification of the presence of DSA using a solid phase assay and 3) achieved a negative complement-dependent cytotoxic (CDC) crossmatch assay on the day of transplant. Data was censored on the 1st January 2012 and all transplant recipients had been transplanted a minimum of 5 years prior to this date. A comparison group included 204 –XMKTx recipients matched 2:1 for the same time era, age (+/-10 years) and sex for +XMKTx recipients. Data for analysis was censored 1st January 2012.

In solid phase assay, a cutoff of positivity of an MFI >1000 was chosen because during the 5-year follow-up there were no graft losses occurred in any +XMKTx in which both Class I and/or Class II DSA was <1000 at baseline (i.e 100% 5 year death-censored graft survival). Of 154 +XMKTx, 102 recipients met inclusion criteria while 52 were excluded. These included: 8 lost to follow-up; 16 had no DSA detected by solid phase assay at baseline despite a positive crossmatch; 5 had DSA in which the highest MFI was <1000; 11 had no sera available for testing DSA retrospectively and 12 CDC+ patients who did not achieve a negative CDC crossmatch despite desensitization.

The outcomes of 204 living donor kidney transplants from the same time period who had a negative crossmatch (-XMKTx) without antibody removal therapy against their donor were studied. This group was developed from a pool of 831 – XMKTx recipients

from the same time period by identifying 2 –XM living donors recipients of similar age (+/-10 years) and sex for + XMKTx recipient. 34 –XMKTx patients who met these criteria were lost to follow up and were excluded from matching. This group was not retrospectively tested for HLA antibodies.

4.2.3 Desensitization and Immunosuppression

The goal of "desensitization" was to achieve a negative CDC crossmatch on the day of transplant or a channel shift less than 300 in the B-cell flow cytometric assay as previously described by Gloor and colleagues [248]. Three general approaches to desensitization were utilized including: 1) plasma exchange with low-dose intravenous immunoglobulin (100mg/kg) plus splenectomy at the time of transplant (n=16), 2) plasma exchange plus immunoglobulin (2g/kg) without splenectomy (n=48), 3) high dose immunoglobulin alone (2g/kg, n=21) and finally no pre-transplant desensitization (n=17). All +XMKTx received induction with rabbit polyclonal anti-lymphocyte antibodies and received tacrolimus, mycophenolate mofetil and prednisolone as maintenance immunosuppression. –XMKTx generally also received the same oral triple therapy except for thirty-three (16.2%) -XMKTx who received other regimens including cyclosporine and sirolimus-based immunosuppression.

4.2.4 Biopsy Scoring, Renal Function and Proteinuria

Patients underwent surveillance allograft biopsy at 1-year and 5year time-points following transplantation using a percutaneous ultrasound guided 18-gauge biopsy gun (Bard, Murray Hill, NJ) as previously described. Each biopsy was deemed adequate for interpretation by a Mayo Clinic renal pathologist and scored using the Banff 97 classification [148]. In addition, the presence of C4d was assessed by

immunofluorescence in the +XMKTx by frozen sections. Patients with both a biopsy at 1-year and 5-year timepoints were included in a paired analysis. Paired biopsies were examined retrospectively for peritubular capillaritis according to Banff 2007 criteria [142].

Graft loss was defined as return to dialysis or re-transplantation. Renal function was calculated from creatinine readings using the Modification of Diet in Renal Disease (MDRD) equation [249]. Proteinuria was assessed using 24-hour urine collection.

4.2.5 Statistical Analysis

Analysis was made on JMPv9. (SAS, Cary, NC,) using Kruskal-Wallis/ Wilcoxon rank-sum test; Pearson and paired t-tests according to the variable data comparison. Normality of distribution of variables was tested using Shapiro-Wilk test. The statistical differences in survival were calculated by the log-rank test. Matching was done using the GREEDY algorithm [250]. A multivariate analysis was not performed. There are multiple variables that may affect allograft outcome described in the literature (for example ABDR mismatch, dialysis time, crossmatch status, level of antibody, anti-class I or II, age, diabetes) and these were collected in this study. In this study there were 52 failed allografts at five years, and on this basis a multivariate analysis could only be limited in scope is potential susceptible to instability caused by variable collinearity.

4.3 Results

4.3.1 Patients and Matched Control Subjects

Of the 102 +XMKTx patients, the mean age was 46.3 ±12 years, 69.6% were women and 96.1% were Caucasian (Table 4.1). Comparing this group to 204 –XMKTx matched controls, differences existed in variables known to be associated with being sensitized to HLA including: a longer duration of dialysis pre-transplant (Median[Q1-Q3] 23.1 [10.8-53.1] vs. 9.0 [5.1-24.4] months, $p<0.0001$); a lower rate of pre-emptive transplantation (18.6% vs. 51.0%, $p<0.0001$); a higher rate of patients who had a prior kidney transplant (39.2% vs. 22.1%, $p=0.002$); and a more HLA ABDR mismatches over 2(82.4% vs. 66.7%, $p=0.005$, Pearson Chi squared).

Table 4.1. Comparison of +XMKTx and –XMKTx populations according to demographics and immunology

	Non-sensitized	HLA-Sensitized	Class I only	Combined Class II/I	
	N=204	N=102	N=36	N=66	p value
Age (years)	46.9 ± 10.2	46.3 ± 12	47.6 ± 11.7	45.6 ± 12.2	0.69 *
Gender (Female) %(n)	69.6% (142)	69.6% (71)	83.3% (30)	62.1% (41)	1.0*
Ethnicity (Caucasian) %(n)	90.7% (185)	96.1% (98)	100% (36)	93.9% (62)	0.82*
Retransplant %(n)	22.1% (45)	39.2% (40)	27.8% (10)	45.5% (30)	0.002 *
ABDR mismatch Median(Q1-Q3)	3 (2-4)	3 (3-5)	3 (2.25-4.75)	3 (3-4)	0.005 *
Pre-emptive %(n)	51% (104)	18.6% (19)	16.7% (6)	19.7% (13)	<0.001 *
Dialysis time (months) Median [Q1-Q3]	9.0 (5.1-24.4)	23.1 (10.8-53.1)	22.9 (15.1-90.3)	24.1 (9.6-24.4)	<0.001 *
Living donor %(n)	100% (204)	100% (102)	100% (36)	100% (66)	1.0 *
Living Related Donor %(n)	38.7% (79)	44.1% (45)	36.1% (13)	48.5% (32)	0.37 *
Primary Disease %(n)					
Diabetes	26.5% (54)	10.8% (11)	13.9% (5)	9.1% (6)	0.002 *
Polycystic	13.7% (28)	9.8% (10)	11.1% (4)	9.1% (6)	0.33 *
CDC negative %(n)		59.8% (61)	44.4% (16)	68.2% (45)	0.02 †
CDC positive %(n)		40.2% (41)	55.6% (20)	31.8% (21)	
AHGCDC (Dilution)		4 (2-8)	4 (2-14)	4 (2-8)	0.76 †
TFXM Channel Shift		187 ± 124.4	267.6 ± 86.8	150.6 ± 122.3	0.001 †
BFXM Channel Shift		329.7 ± 92.5	310.2 ± 80.1	338.5 ± 97.2	0.239 †
Class I only % (n)		35.3% (36)	100% (36)	-	n/a
Class II only % (n)		19.6% (20)	-	30.3% (20)	n/a
Class I and II % (n)		45.1% (46)	-	69.7% (46)	n/a
Baseline Class I MFI Median(Q1-Q3)		7378 (2342-12363)	10251 (1452-16986)	5248 (323-18317)	0.002 †
Baseline Class II MFI Median(Q1-Q3)		2735 (0-10616)	n/a	7269 (1076-23293)	<0.001†
Follow up (days)	2929 ± 741	2488 ± 911	2514 ± 900	2474 ± 923	<0.001 *
Donor gender (Female)	56.4% (115)	59.8% (61)	66.7% (24)	56.1% (37)	0.33 *
Donor age (years)	42.6 ± 12	41.0 ± 11.9	39.7 ± 11.3	41.7 ± 12.1	0.26 *

Data is expressed as mean and standard deviation, unless specified. * The results are comparisons between the total HLA-sensitized and non-sensitized matched controls, not the Class of antibody groups. † p value is comparison between sensitized (+XMKTx) with antibody against donor Class I only or donor Class II/I (Class II only or Class I and II).

The date of analysis was at least 5 years post transplantation (+XMKTx mean = 2488 ± 911days vs. -XMKTx mean 2929 ± 741days). At baseline, 40% (n=41) of the +XMKTx had a crossmatch that was CDC+ (median dilution 1:4, range 1:1-1:64). In the +XMKTx group, 36 had antibody against donor Class I only, 20 had antibody against donor Class II alone and 46 had antibodies against both donor Class I and II. The level of antibody against Class I specificities was similar in the Class I only group and in Class I and II combined group with similar mean highest MFI against donor Class I specificities (MFI= 10251 (6282-13223) vs. 7578 (3888-12480), p=0.17) and a similar proportion of CDC+ recipients (55.6% vs. 45.7%, p=0.37). The median highest MFI against Class I was higher in the CDC+ patients compared to the FXM+ group (12000 (2658-18317) vs. 3404 (0-15851), p<0.0001), however, there was considerable overlap of MFI between these CDC+ and FXM+ patients. The level of DSA against Class II specificities was similar in the Class II only and both Class I and II groups (5230 [1140-23293] vs. 6658 [672-18265], p=1.0). These two groups were combined for further analysis (called Class II/I).

4.3.2 Five-Year Patient and Graft Survival

Patient survival at 5 years was lower in +XMKTx compared to –XMKTx (86.3% vs. 92.5% p=0.01, Figure 4.1A). Death-censored graft survival at 5 years was lower in the +XMKTx than -XMKTx (70.7% vs. 88.0%, p<0.01, Figure 4.1B). +XMKTx patients therefore had significantly higher risk of a composite endpoint of patient death, allograft failure or transplant glomerulopathy (Odds Ratio 4.3, 95% CI 2.5-7.4, Logistic Regression).

At 1 year, CDC+ transplants had lower death-censored graft survival than the CDC-/FXM+ recipients (82.4% vs. 96.7%, p=0.02), but the difference was not statistically

different at 5 years (64.9% vs. 72.7%, p=0.26, Figure 4.1C) demonstrating a similar rate of graft loss in CDC- grafts between 1 and 5 years.

The anti-HLA specificity of antibody detected at baseline had an important impact on 5-year death-censored graft survival. Graft survival was higher in recipients with antibody against donor Class I only compared with those with Class II antibody (either alone or with Class I) (85.3 *vs.* 62.6%, p=0.05, Figure 4.1D) and was similar to that of the –XMKTx (85.3 *vs.* 88.0%, p=0.64). In the Class I antibody only group, 60% (3/5) of graft losses occurred in the first year and the subsequent rate of graft loss averaged only 1.6% per year in the 5 year study period. This is comparable with the rate of graft loss in the –XMKTx group (2.9% per year). In the Class I only group, patients who had a CDC-crossmatch had graft survival similar to that of –XMKTx recipients (93.3% *vs.* 88.0%, p=0.51). In contrast, antibody against donor Class II was associated with a higher rate of graft loss in the 5 year follow up (7.0% per year in the Class II/I combined group).

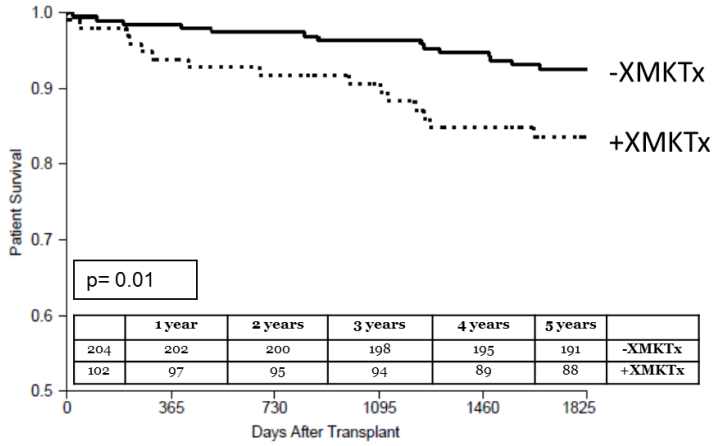
The higher 5-year graft survival of Class I alone vs Class II/I recipients did not appear to be due to differences in antibody levels, in as much as it is possible to assess antibody levels on the basis of binding to the Luminex beads (Figure 4.2). Also, there was no statistical difference in graft survival between CDC+ and CDC- patients with Class II antibodies (54.3% *vs.* 66.2%, p=0.23).

Figure 4.1 Five-year outcomes after Positive Crossmatch Live Donor Kidney Transplant (+XMKTx.)

Actual 5 year outcomes are shown for +XMKTx including patient survival (Panel A); overall death-censored graft survival (Panel B); graft survival by baseline crossmatch assay type (+XMKTx recipients who were CDC+ vs CDC-, Panel C); and graft survival by donor-specific HLA specificity (+XMKTx recipients with antibody against donor Class I only, Class II only and both Class I and II, Panel D).

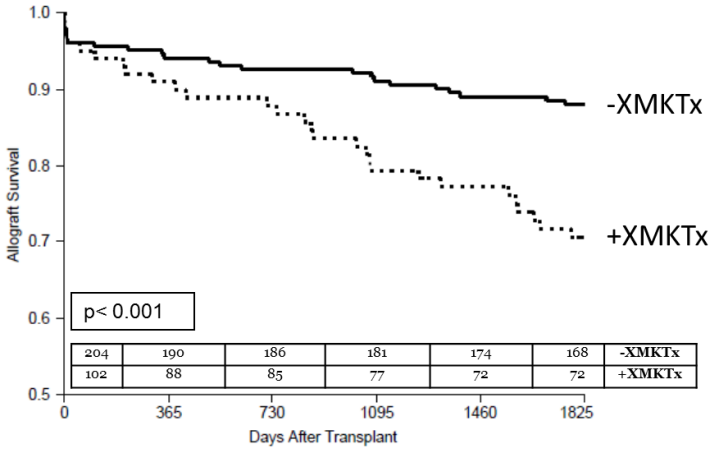
Patient numbers at each time point for survival are tabulated in each graph.

A. 5-year Patient Survival



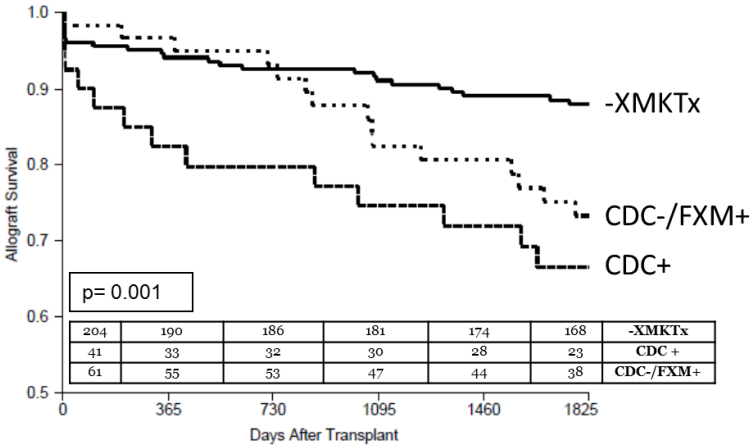
	1 year	2 years	3 years	4 years	5 years	
204	202	200	198	195	191	-XMKTx
102	97	95	94	89	88	+XMKTx

B. 5-Year Overall Graft Survival



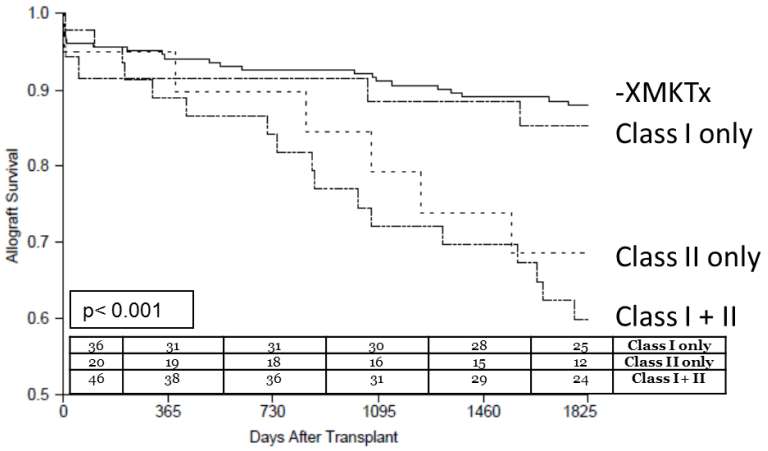
204	190	186	181	174	168	-XMKTx
102	88	85	77	72	72	+XMKTx

C. Graft Survival by Crossmatch Assay (CDC+ vs CDC-/FXM+)



204	190	186	181	174	168	-XMKTx
41	33	32	30	28	23	CDC +
61	55	53	47	44	38	CDC-/FXM+

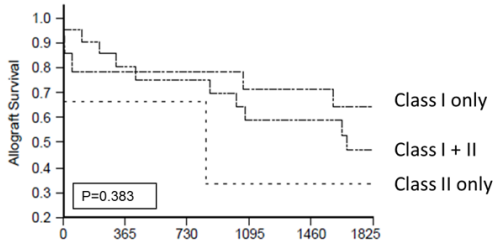
D. Graft Survival by Donor-Specific HLA Antibody Specificity



36	31	31	30	28	25	Class I only
20	19	18	16	15	12	Class II only
46	38	36	31	29	24	Class I+ II

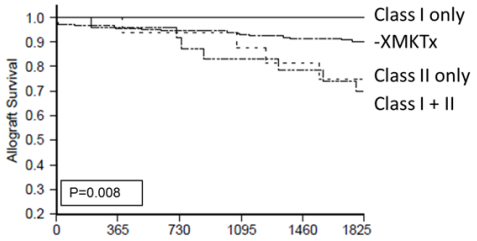
E. Graft Survival by Donor-Specific HLA Antibody Specificity and early AMR episode

A. Graft Survival with Antibody Mediated Rejection.



	Day 0	1 year	2 years	3 years	4 years	5 years
Class I only	14	11	11	10	10	9
Class II only	3	2	2	1	1	1
Class I + II	21	15	14	12	11	8

B. Graft Survival without Antibody Mediated Rejection

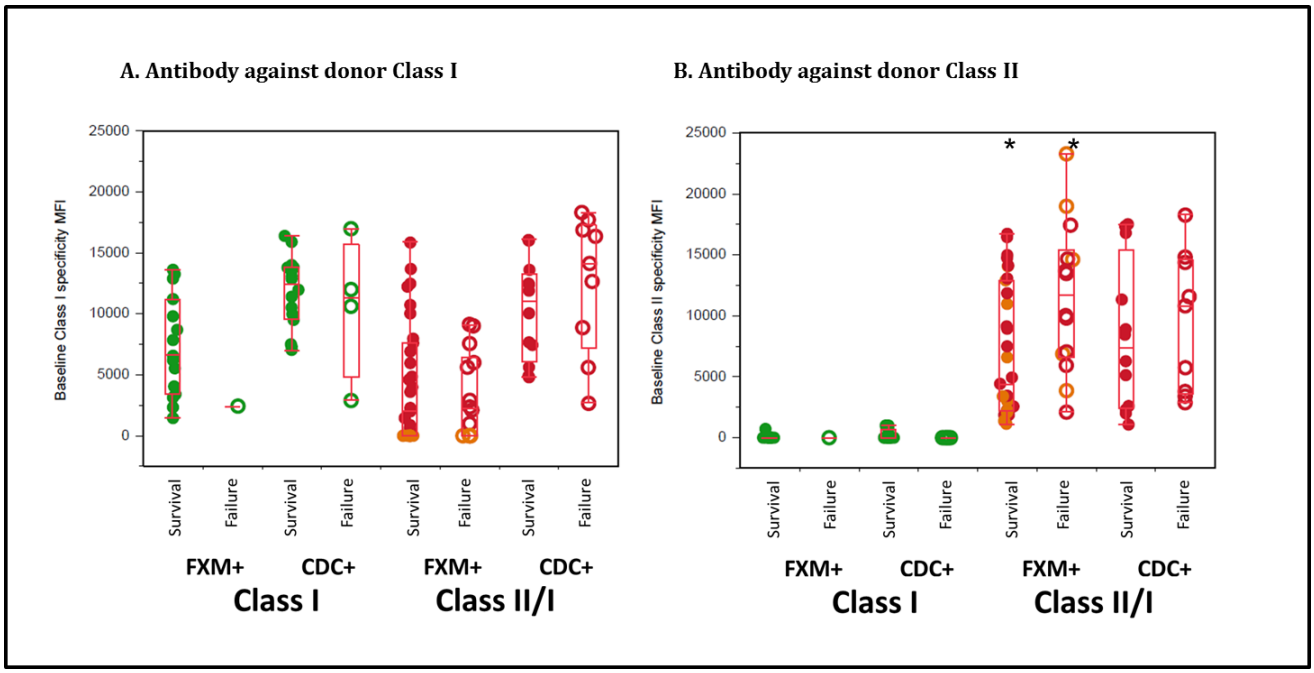


	Day 0	1 year	2 years	3 years	4 years	5 years
-XMKTx	199	187	184	179	173	165
Class I only	22	20	20	20	18	17
Class II only	17	16	15	14	13	11
Class I + II	25	23	21	19	18	16

In general, baseline antibody levels by solid phase were similar in grafts that survived compared with grafts that failed (Figure 4.2). The exception was in the presence of anti-Class II antibody, lower levels (median fluorescence intensity of <3000) had better survival compared to those with higher levels (5-year DCGS 86.7% *vs.* 54.6%, p=0.05). There was no significant difference in 5-year outcomes comparing DSA against HLA-DR or HLA-DQ. Causes of graft loss in Class I only and Class II/I groups were similar, with 60% related to transplant glomerulopathy overall (Class I 60.0% (3/5) *vs* Class II/I 60.9% (14/23), p=0.97).

Figure 4.2. Anti-Donor Antibody Levels and Outcome by Specificity for Donor HLA.

Antibody levels against donor Class I HLA were similar in the Class I only group and the Class II/I group (Panel A). For Class I, antibody levels were similar in surviving and failed allografts. Antibodies against donor Class II were absent in Class I group (Panel B). The mean antibody levels were higher in Class II/I CDC-/FXM+ grafts that failed compared to those that survived (median 11747 [2098-23293] vs. 4420 [1140-16729], p=0.018, Wilcoxon rank-sum test) denoted in the graph by *. Scatterplots of anti-donor antibody levels as determined by solid phase assay and expressed as mean fluorescence intensity demonstrate distribution across surviving and failed grafts at 5 years.



The different antibody groups are represented by different colours. Green circles are Class I only; orange circles are Class II only and Red circles are Class I + II. Data are expressed with median and interquartile ranges.

4.3.3 Antibody-Mediated Rejection

The incidence of antibody-mediated rejection (AMR) was 37.2% (n=38) in the +XMKTx group and 2.5% (n=5) in the –XMKTx group. Early AMR was less common in the Class II only group (15%) compared to either the Class I only (38.9%, p=0.06) or recipients with both Class I and II (45.7%, p=0.02). +XMKTx patients with an episode of AMR had lower graft survival compared to those without early AMR (53.2% *vs.* 81.2%, p<0.01) (Figure 4.1E).

4.3.4 Renal Function and Proteinuria at 5 Years

Renal function in the +XMKTx at 5 years spanned a wide spectrum and was not significantly different from –XMKTx (Figure 4.3A+B) [+XMKTx mean estimated glomerular filtration rate (eGFR) = 44.4±19.6 ml/min, *vs.* -XMKTx mean eGFR = 48.5±17.9, p=0.22)]. At 5 years, the percentage of surviving grafts with good function (GFR >40 ml/min) also was similar in +XMKTx *vs.* –XMKTx. (52.4% *vs.* 64.6%, p=0.09).

Proteinuria (24-hour urinary protein excretion), has been associated with chronic antibody-mediated injury [251]. At 5 years, the median 24-hour urine protein excretion was higher in the +XMKTx *vs.* –XMKTx patients (277 (range 23-10826) *vs.* 91.5 (range 14-7784), p<0.01 Wilcoxon rank-sum test; Figure 4.3C + D). In the +XMKTx, 44.4% (n=20/44) of patients had 24-hour urinary protein excretion greater than 500mg/24hrs compared to only 13.8% (n=18/130, p<0.01) in –XMKTx at 5 years. Proteinuria was significantly associated with the presence of transplant glomerulopathy at both 1-year ('CG=0' 108[range 26-7788] vs. 'CG>0' 496 [range 75-9997],p<0.01) and 5-year('CG=0' 72 [range 23-522] vs. 'CG>0' 694 [range 49-10826] ,p<0.01). The level of proteinuria at 1-year was significantly higher in patients with no CG at 1 year biopsy

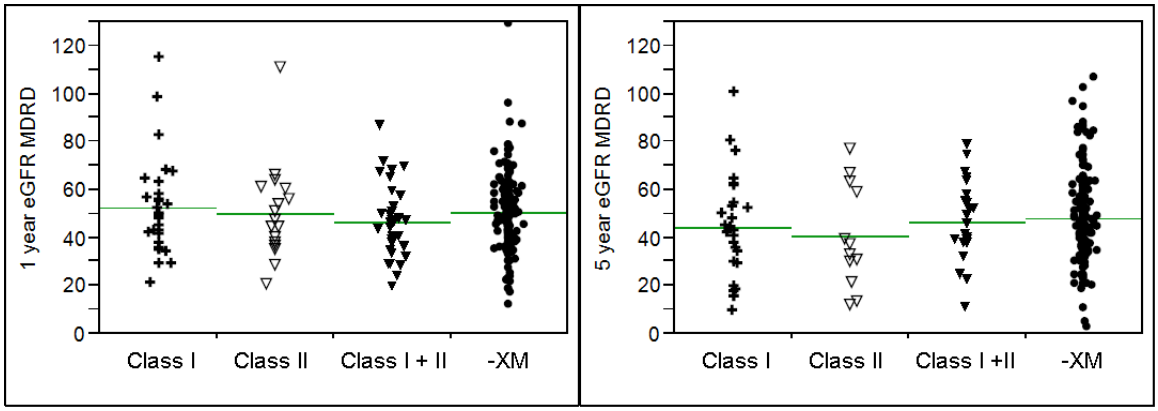
who went on to develop CG by the 5 year biopsy ('5-year CG=0' 76 [range 26-229] vs. '5-year CG >0' 134 [range 26-2405], p<0.01) by light microscopy.

Figure 4.3. Renal Function and Proteinuria after +XMKTx.

Estimated glomerular filtration rate (eGFR) by MDRD at 1 (Panel A) and 5 years (Panel B) after transplantation with mean eGFR shown by green line. Twenty-four hour urine protein excretion (mg/day) at 1 (Panel C) and 5 years (Panel D) after transplantation with box and whisker plot. Comparisons of data made using Student's t-test for eGFR and Wilcoxon rank-sum test for proteinuria.

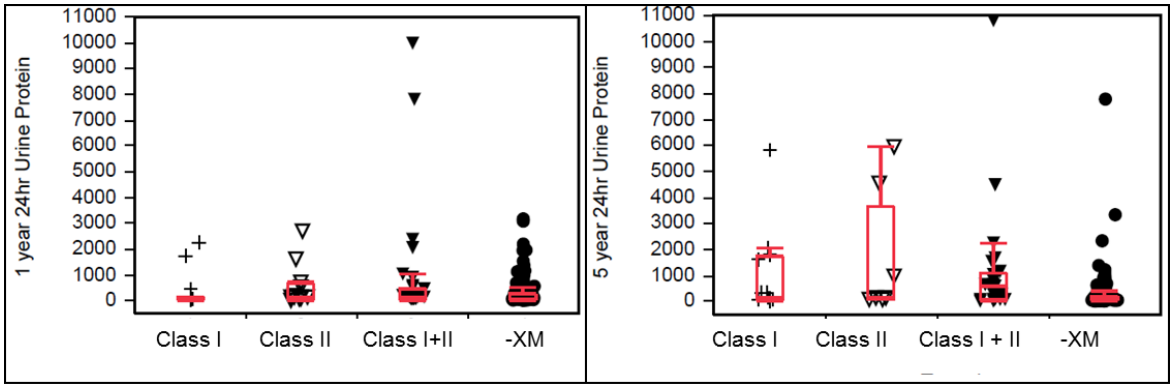
A. Renal function at 1 year

B. Renal function at 5 years



C. Proteinuria 1 year

D. Proteinuria at 5 years



4.3.5 Allograft Histology at 1 and 5 Years

Similar to functional data, allograft histology provides insight into the extent of injury in functioning allografts. At 1 year after transplantation, surveillance biopsies were obtained in 75.1% (142/189) of the –XMKTX; 77.4% (24/31) of the Class I only group; and 80.4% (45/56) of the Class II/I group. The entire dataset is presented in Table 4.2. At 1 year, chronic glomerulopathy (cg, score >0), a histologic finding associated with chronic antibody mediated injury, was more common in +XMKTX compared to –XMKTX (27.5% vs 3.5%, p<0.0001, Table 4.2). CG was more common in the Class II/I group compared to Class I only (37.8% vs. 8.4%, (p=0.009) at 1 year, and was only seen in Class II/I patients with DSA MFI>3000(n=50) at the time of transplantation. By 5 years, the prevalence of CG within the Class II/I group was not predicted by baseline MFI (MFI<3000 - 50% vs. 62.5%, p=0.34). In all biopsies, acute glomerulitis at 1 year was common in +XMKTx occurring in 44.4% of Class II/I patients, significantly higher than 20.9% of Class I patients (p=0.05) and 7.7% of –XMKTX patients. In contrast to acute glomerulitis, acute peritubular capillaritis (ptc) was not significantly different between Class I or Class II/I patients at 1 year (53.3% vs. 75%, p=0.14) but at 5 years this was significantly different (45.5% vs. 91.3%, p=0.003, Table 4.3). There was no difference in peritubular capillaritis between Class II/I patients with DSA MFI>3000 and those <3000 at 1 year (73.9% vs. 77.8%, p=0.82). Interstitial fibrosis (ci) was similar in all groups at 1 year (range 52.1 to 68.9%). By 5 years, the prevalence of cg had increased to 58.3% in the Class II/I group, 50.0% in the Class I group and was only 7.7% in the –XMKTX. In the +XMKTx cohort, the presence of CG at 1 year was associated with graft loss in 30.4% (n=7) of Class II/I patients at 5 years and 20% (n=1) Class I patient. In contrast, in the absence of CG at 1 year graft survival was >80% by 5 years in the +XMKTx cohort.

Table 4.2. All biopsy histological Banff scores from each cohort at 1 year biopsies

	12 month TOTAL -XMKTx			12 month TOTAL +XMKTx			12 month TOTAL Class I			12 month TOTAL Class II/I		
None	**g N=142**	92.3%	(n = 131)	**g N=69**	63.8%	(n = 44)	**g N=24**	79.2%	(n = 19)	**g N=45**	55.6%	(n = 25)
Mild		5.6%	(n = 8)		20.3%	(n = 14)		16.7%	(n = 4)		22.2%	(n = 10)
Mod-Sev		2.1%	(n = 3)		15.9%	(n = 11)		4.2%	(n = 1)		22.2%	(n = 10)
None	**i N=142**	82.4%	(n = 117)	**i N=69**	82.6%	(n = 57)	**i N=24**	83.3%	(n = 20)	**i N=45**	82.2%	(n = 37)
Mild		11.3%	(n = 16)		10.1%	(n = 7)		8.3%	(n = 2)		11.1%	(n = 5)
Mod-Sev		6.3%	(n = 9)		7.2%	(n = 5)		8.3%	(n = 2)		6.7%	(n = 3)
None	**t N=142**	76.1%	(n = 108)	**t N=69**	62.3%	(n = 43)	**t N=24**	75.0%	(n = 18)	**t N=45**	55.6%	(n = 25)
Mild		16.2%	(n = 23)		30.4%	(n = 21)		16.7%	(n = 4)		37.8%	(n = 17)
Mod-Sev		7.7%	(n = 11)		7.2%	(n = 5)		8.3%	(n = 2)		6.7%	(n = 3)
None	**v N=142**	100.0%	(n = 142)	**v N=69**	100.0%	(n = 69)	**v N=24**	100.0%	(n = 24)	**v N=45**	100.0%	(n = 45)
Mild		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)
Mod-Sev		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)
None	**ah N=131**	83.2%	(n = 109)	**ah N=64**	76.6%	(n = 49)	**ah N=22**	81.8%	(n = 18)	**ah N=42**	73.8%	(n = 31)
Mild		13.7%	(n = 18)		21.9%	(n = 14)		18.2%	(n = 4)		23.8%	(n = 10)
Mod-Sev		3.1%	(n = 4)		1.6%	(n = 1)		0.0%	(n = 0)		2.4%	(n = 1)
None	**cg N=142**	96.5%	(n = 137)	**cg N=69**	72.5%	(n = 50)	**cg N=24**	91.7%	(n = 22)	**cg N=45**	62.2%	(n = 28)
Mild		2.1%	(n = 3)		15.9%	(n = 11)		4.2%	(n = 1)		22.2%	(n = 10)
Mod-Sev		1.4%	(n = 2)		11.6%	(n = 8)		4.2%	(n = 1)		15.6%	(n = 7)
None	**ci N=142**	47.9%	(n = 68)	**ci N=69**	36.2%	(n = 25)	**ci N=24**	45.8%	(n = 11)	**ci N=45**	31.1%	(n = 14)
Mild		38.0%	(n = 54)		47.8%	(n = 33)		45.8%	(n = 11)		48.9%	(n = 22)
Mod-Sev		14.1%	(n = 20)		15.9%	(n = 11)		8.3%	(n = 2)		20.0%	(n = 9)
None	**ct N=142**	33.8%	(n = 48)	**ct N=69**	26.1%	(n = 18)	**ct N=24**	37.5%	(n = 9)	**ct N=45**	20.0%	(n = 9)
Mild		52.1%	(n = 74)		56.5%	(n = 39)		54.2%	(n = 13)		57.8%	(n = 26)
Mod-Sev		14.1%	(n = 20)		17.4%	(n = 12)		8.3%	(n = 2)		22.2%	(n = 10)
None	**cv N=142**	59.2%	(n = 84)	**cv N=69**	62.3%	(n = 43)	**cv N=24**	62.5%	(n = 15)	**cv N=45**	62.2%	(n = 28)
Mild		36.6%	(n = 52)		33.3%	(n = 23)		33.3%	(n = 8)		33.3%	(n = 15)
Mod-Sev		4.2%	(n = 6)		4.3%	(n = 3)		4.2%	(n = 1)		4.4%	(n = 2)
None	**mm N=19**	84.2%	(n = 16)	**mm N=11**	63.6%	(n = 7)	**mm N=1**	100.0%	(n = 1)	**mm N=10**	60.0%	(n = 6)
Mild		10.5%	(n = 2)		27.3%	(n = 3)		0.0%	(n = 0)		30.0%	(n = 3)
Mod-Sev		5.3%	(n = 1)		9.1%	(n = 1)		0.0%	(n = 0)		10.0%	(n = 1)
None	**C4d N=10**	90.0%	(n = 9)	**C4d N=60**	85.0%	(n = 51)	**C4d N=23**	100.0%	(n = 23)	**C4d N=37**	75.7%	(n = 28)
Mild		10.0%	(n = 1)		3.3%	(n = 2)		0.0%	(n = 0)		5.4%	(n = 2)
Mod-Sev		0.0%	(n = 0)		11.7%	(n = 7)		0.0%	(n = 0)		18.9%	(n = 7)
None	**ptc #**			**ptc N=47**	**31.9%**	**(n =15)**	**ptc N=15**	46.7%	(n = 7)	**ptc N=32**	25.0%	(n = 8)
Mild					**17.0%**	**(n =8)**		13.3%	(n = 2)		18.8%	(n = 6)
Mod-Sev					**51.1%**	**(n = 24)**		40.0%	(n = 6)		56.3%	(n = 18)

All histological scores are according to the Banff 2005 and then Banff 2007 classification for grading kidney transplant allograft biopsies [142, 148]. Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis. #The biopsies of –XMKTx were not re-examined to address 'ptc' in this cohort.

Table 4.3. All biopsy histological Banff scores from each cohort at 5 year biopsies

	60 month TOTAL -XMKTx			60 month TOTAL +XMKTx			60 month TOTAL Class I			60 month TOTAL Class II/I		
None	**g**	85.6%	(n = 89)	**g**	33.3%	(n = 12)	**g**	33.3%	(n = 4)	**g**	33.3%	(n = 8)
Mild	**N=104**	10.6%	(n = 11)	**N=36**	25.0%	(n = 9)	**N=12**	16.7%	(n = 2)	**N=24**	29.2%	(n = 7)
Mod-Sev		3.8%	(n = 4)		41.7%	(n = 15)		50.0%	(n = 6)		37.5%	(n = 9)
None	**i**	86.5%	(n = 90)	**i**	72.2%	(n = 26)	**i**	58.3%	(n = 7)	**i**	79.2%	(n = 19)
Mild	**N=104**	11.5%	(n = 12)	**N=36**	22.2%	(n = 8)	**N=12**	33.3%	(n = 4)	**N=24**	16.7%	(n = 4)
Mod-Sev		1.9%	(n = 2)		5.6%	(n = 2)		8.3%	(n = 1)		4.2%	(n = 1)
None	**t**	96.2%	(n = 100)	**t**	83.3%	(n = 30)	**t**	75.0%	(n = 9)	**t**	87.5%	(n = 21)
Mild	**N=104**	3.8%	(n = 4)	**N=36**	13.9%	(n = 5)	**N=12**	25.0%	(n = 3)	**N=24**	8.3%	(n = 2)
Mod-Sev		0.0%	(n = 0)		2.8%	(n = 1)		0.0%	(n = 0)		4.2%	(n = 1)
None	**v**	100.0%	(n = 104)	**v**	100.0%	(n = 35)	**v**	100.0%	(n = 12)	**v**	100.0%	(n = 23)
Mild	**N=104**	0.0%	(n = 0)	**N=35**	0.0%	(n = 0)	**N=12**	0.0%	(n = 0)	**N=23**	0.0%	(n = 0)
Mod-Sev		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)		0.0%	(n = 0)
None	**ah**	34.3%	(n = 35)	**ah**	13.9%	(n = 5)	**ah**	25.0%	(n = 3)	**ah**	8.3%	(n = 2)
Mild	**N=102**	41.2%	(n = 42)	**N=36**	44.4%	(n = 16)	**N=12**	50.0%	(n = 6)	**N=24**	41.7%	(n = 10)
Mod-Sev		24.5%	(n = 25)		41.7%	(n = 15)		25.0%	(n = 3)		50.0%	(n = 12)
None	**cg**	92.3%	(n = 96)	**cg**	44.4%	(n = 16)	**cg**	50.0%	(n = 6)	**cg**	41.7%	(n = 10)
Mild	**N=104**	1.0%	(n = 1)	**N=36**	13.9%	(n = 5)	**N=12**	16.7%	(n = 2)	**N=24**	12.5%	(n = 3)
Mod-Sev		6.7%	(n = 7)		41.7%	(n = 15)		33.3%	(n = 4)		45.8%	(n = 11)
None	**ci**	37.9%	(n = 39)	**ci**	8.3%	(n = 3)	**ci**	16.7%	(n = 2)	**ci**	4.2%	(n = 1)
Mild	**N=103**	46.6%	(n = 48)	**N=36**	61.1%	(n = 22)	**N=12**	58.3%	(n = 7)	**N=24**	62.5%	(n = 15)
Mod-Sev		15.5%	(n = 16)		30.6%	(n = 11)		25.0%	(n = 3)		33.3%	(n = 8)
None	**ct**	34.0%	(n = 35)	**ct**	8.3%	(n = 3)	**ct**	16.7%	(n = 2)	**ct**	4.2%	(n = 1)
Mild	**N=103**	50.5%	(n = 52)	**N=36**	61.1%	(n = 22)	**N=12**	58.3%	(n = 7)	**N=24**	62.5%	(n = 15)
Mod-Sev		15.5%	(n = 16)		30.6%	(n = 11)		25.0%	(n = 3)		33.3%	(n = 8)
None	**cv**	41.3%	(n = 43)	**cv**	20.0%	(n = 7)	**cv**	25.0%	(n = 3)	**cv**	17.4%	(n = 4)
Mild	**N=104**	37.5%	(n = 39)	**N=35**	45.7%	(n = 16)	**N=12**	41.7%	(n = 5)	**N=23**	47.8%	(n = 11)
Mod-Sev		21.2%	(n = 22)		34.3%	(n = 12)		33.3%	(n = 4)		34.8%	(n = 8)
None	**mm**	71.7%	(n = 33)	**mm**	52.6%	(n = 10)	**mm**	66.7%	(n = 4)	**mm**	46.2%	(n = 6)
Mild	**N=46**	26.1%	(n = 12)	**N=19**	26.3%	(n = 5)	**N=6**	16.7%	(n = 1)	**N=13**	30.8%	(n = 4)
Mod-Sev		2.2%	(n = 1)		21.1%	(n = 4)		16.7%	(n = 1)		23.1%	(n = 3)
None	**C4d**	92.3%	(n = 36)	**C4d**	78.8%	(n = 26)	**C4d**	83.3%	(n = 10)	**C4d**	76.2%	(n = 16)
Mild	**N=39**	0.0%	(n = 0)	**N=33**	6.1%	(n = 2)	**N=12**	16.7%	(n = 2)	**N=21**	0.0%	(n = 0)
Mod-Sev		7.7%	(n = 3)		15.2%	(n = 5)		0.0%	(n = 0)		23.8%	(n = 5)
None	**ptc #**	89.6%	(n = 60)	**ptc**	23.5%	(n = 8)	**ptc**	54.5%	(n = 6)	**ptc**	8.7%	(n = 2)
Mild	**N=67**	4.5%	(n = 3)	**N=34**	26.5%	(n = 9)	**N=11**	27.3%	(n = 3)	**N=23**	26.1%	(n = 6)
Mod-Sev		6.0%	(n = 4)		50.0%	(n = 17)		18.2%	(n = 2)		65.2%	(n = 15)

All histological scores are according to the Banff 2007 classification for grading kidney transplant allograft biopsies [142]. # The biopsies of –XMKTx were not re-examined to address 'ptc' in this cohort. Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis.

Table 4.4. Summary of Histologic Lesions Commonly Associated with anti-HLA Antibody

The overall incidence of histologic changes commonly-associated with anti-HLA antibody found on paired surveillance biopsies obtained at 1 and 5 years after transplantation are shown.

	Chronic glomerulopathy		**Acute glomerulitis**		**Peritubular capillaritis**	
	1 yr	**5 yr**	**1 yr**	**5 yr**	**1 yr**	**5 yr**
-XMKTx	4.2% (4/95)	7.4% (7/95)	7.4% (7/95)	14.7% (14/95)	Not done	Not done
+XMKTx	21.2% (7/33)	54.5% (18/33)	30.3% (10/33)	63.6% (21/33)	63.3% (19/30)	76.5% (24/32)
Class I	10% (1/10)	50% (5/10)	20% (2/10)	50% (6/10)	55.5% (5/9)	40% (4/10)
Class II/I	26.1% (6/23)	56.5% (13/23)	34.7% (8/23)	65.2% (15/23)	66.6% (14/21)	90.9% (20/22)

Class I 1 year peritubular capillaritis shows only 9 biopsies in the paired analysis, as the 1 year biopsy for 1 patient was unavailable for review for interpreting peritubular capillaritis

4.3.6 Paired Allograft Histology at 1 and 5 Years

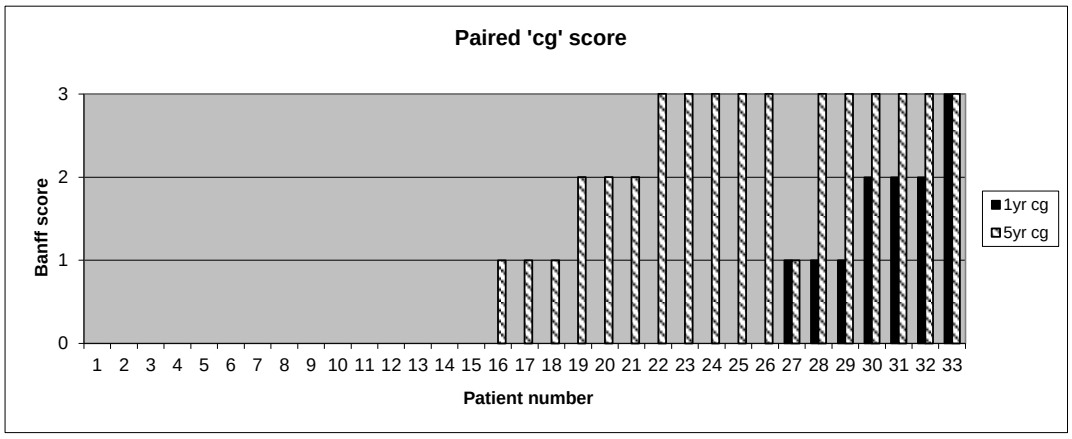
The data from the subset of patients who were biopsied at both 1 and 5 years (paired biopsies) might also yield data regarding the progression of antibody-mediated injury. Of the grafts that survived 5 years, paired surveillance biopsies at both 1 and 5 years were available for analysis in 56% (n=95) of the –XMKTx and 52% (n=33) +XMKTx. At 1 year, the prevalence of chronic glomerulopathy was 21.2% in +XMKTx and only 4.2% in –XMKTx (Table 4.3). This increased to 54.5% and 7.4% respectively by 5 years. At 5 years, chronic glomerulopathy was equally common in grafts in the Class I only group and in the Class II/I group. Importantly, slightly more than half of all +XMKTx patients (15/26) with no cg at 1 year also had no cg at 5 years (50% (5/10) of patients in the Class I group and 43.5% (10/23) of patients in the Class II/I group). Figure 4.4 shows the actual Banff scores in paired biopsies obtained at 1 and 5 years in +XMKTx. These data show that in addition to an increase in the prevalence of glomerulopathy between 1 and 5 years, the severity also increased in these patients with CG (Figure4.4A). However, at 5 years, 50% of the Class I only group and 43.5% of the Class II/I group had no evidence of chronic glomerulopathy.

The prevalence and severity of inflammatory changes associated with antibody-mediated injury (glomerulitis and peritubular capillaritis) increased from 1 to 5 years in +XMKTx (Figure 4.4B and Figure 4.4C). Interstitial fibrosis and tubular atrophy also was commonly seen, but was mild in many cases (Figure 4.4D and Figure 4.4E). See Tables 4.2 and 4.4 for all biopsy data.

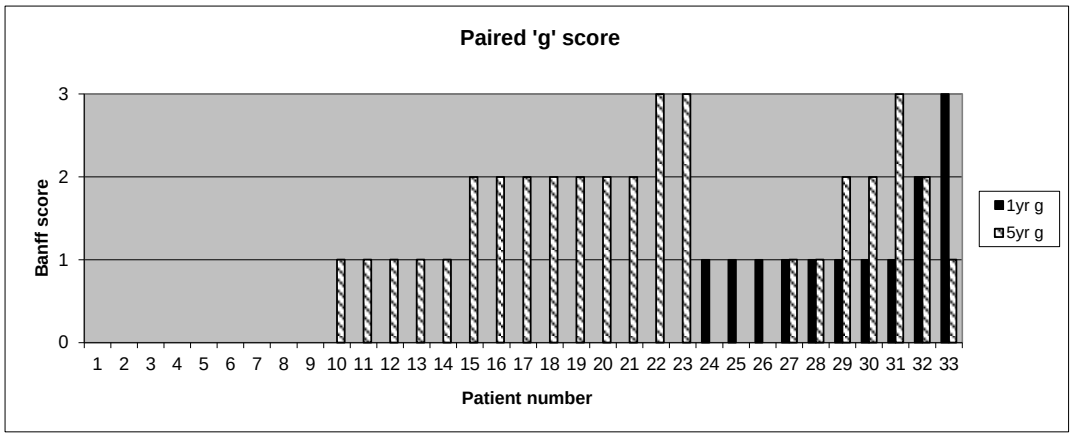
Figure 4.4.Detailed Histologic Score at 1 and 5 years in Paired Biopsies in +XMKTx.

Actual Banff scores at 1 and 5 years in paired biopsies in +XMKTx showing chronic glomerulopathy (Panel A); glomerulitis (Panel B), peritubular capillaritis (Panel C), interstitial fibrosis (Panel D) and tubular atrophy (Panel E).

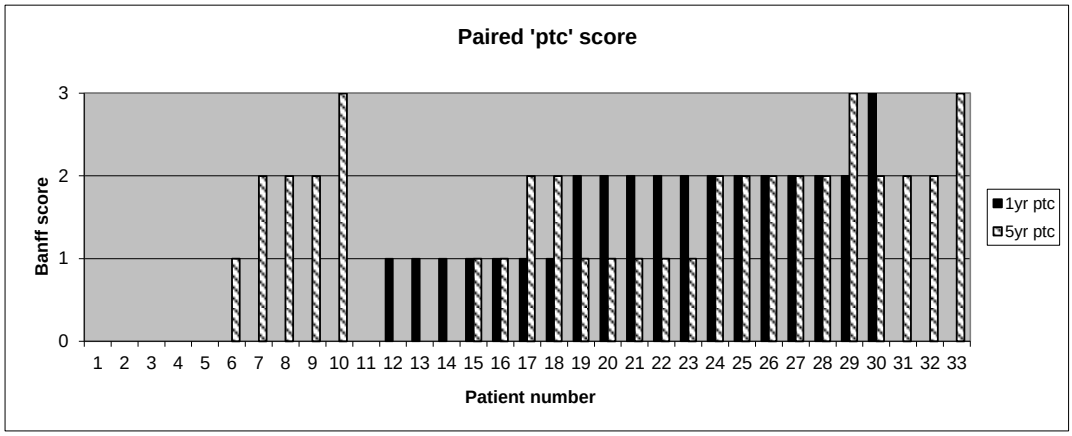
A. Chronic glomerulopathy



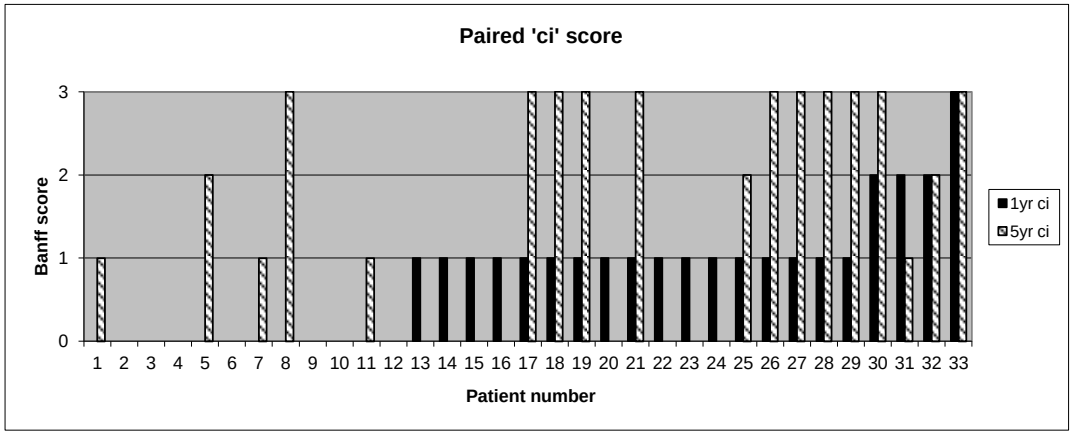
B. Glomerulitis



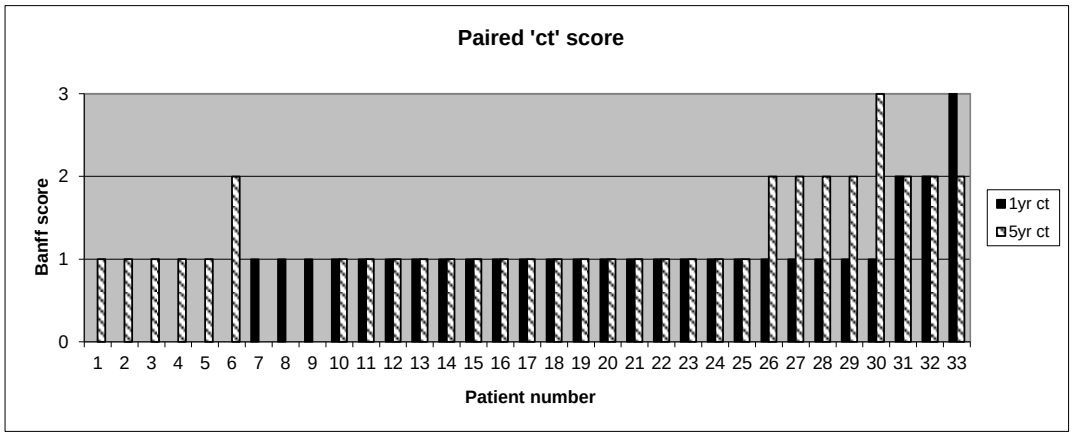
C. Peritubular capillaritis



D. Chronic interstitial fibrosis



E. Tubular atrophy



4.3.7 Outcomes of +XMKTx Excluded.

In addition to the 102+XMKTx studied above, we excluded 52 +XMKTx including: 8 lost to follow-up prior to 5 years; 12 CDC+ patients who did not achieve a negative CDC-crossmatch by the day of transplantation (5-year DCGS was 41.7% and all surviving grafts developed CG); 16 (1 CDC+, 15 FXM+) had no DSA detected by solid phase assay (the 5 year DCGS was 93.8% and none of the grafts developed CG); 5 had an MFI <1000 (the 5 year DCGS was 100%) and 11 had no pre-transplant sera stored for DSA testing (5 year DCGS was 62.3%).

4.4 Discussion

In this study, the actual 5-year outcomes of +XMKTx were retrospectively analysed. These results show 5-year patient survival of 83.5% were similar to those of HLA-incompatible kidney transplant recipients recently reported by the Johns Hopkins group [24]. The overall 5-year graft survival of 70.7% was similar to that of FXM+ living donors at the University of Maryland (70% in 19 patients followed to 5 years) and in deceased donors with donor-specific antibodies at Hôpital Saint-Louis de Paris (70% in 30 patients followed 5 years) [185, 252].

The current study supports prior studies showing that CDC+ allografts with high levels of anti-Class I antibody have a high rate of early graft loss [94]. However, our study suggests that the anti-HLA specificity may have an even greater impact on long-term graft survival. Most of the graft losses in the Class I only group occurred in the first year and the rate of graft loss between 1 and 5 years was similar to –XMKTx. In contrast, Class II DSA appears associated with a low rate of early AMR (15% in those with Class II only), yet a high rate of chronic injury with more than 40% of allografts

failing within the first 5 years after transplantation. Patients with antibody against both donor Class I and II (almost half of the +XMKTx cohort) had similar poor long-term outcomes as patients with Class II only. The rate of allograft loss is associated with the presence of CG at an earlier stage in the Class II/I group, compare with that of the Class I only group. The whole group histology at 1 year demonstrates 37.8% in Class II/I group, compared to only 8.3% in the Class I only cohort ($p<0.01$). Overall, if 1 year CG is present it is highly associated with graft failure at 5 years (42.1% vs 12%, $p<0.01$). The development of proteinuria in allografts at 1 year without histology evidence by light microscopy of CG was a good predictor of the development of CG at 5 years.

The cohort in this study was from the early "era" of the positive crossmatch transplant program at Mayo Clinic, USA. More recent data has shown a trend toward fewer graft losses in the first year after transplantation. This is likely due to many different protocol changes including improved DSA monitoring. However, the actual graft survival data presented here reflects the true incidence of chronic injury that occurs over time in +XMKTx—especially in patients with anti-Class II DSA.

The functional and histologic data provide important additional insight into the outcomes of +XMKTx. Chronic glomerulopathy was common at 1 year and increased over time in patients with anti-donor HLA antibody. Similar to the findings of Haas et al and Loupy et al, chronic inflammation (peritubular capillaritis and glomerulitis) also was extremely common at 1 and 5 years after +XMKTx [161, 253, 254]. An encouraging finding from the histology data was that approximately half of the biopsies at 5 years showed no evidence of transplant glomerulopathy suggesting that some grafts may avoid chronic antibody-mediated injury. Conversely, a worrisome finding is the increasing prevalence of inflammation and glomerulopathy from 1 to 5 years in the Class I only group suggesting that despite having survived 5 years, these grafts may still

be destined to fail from chronic injury. Since more grafts with Class II DSA were lost in the first 5 years after transplantation, the negative impact of Class II DSA on graft histology is potentially underestimated by the paired histologic data presented.

A limitation of this study is that post-transplant anti-donor antibody data were not available for sufficient number of patients to be analyzed in this study. Longitudinal studies correlating antibody levels and specificity with outcomes after +XMKTx are needed. In addition, the histologic data at 5 years should be interpreted with some caution because only 52% of eligible patients underwent paired surveillance biopsies.

Sensitization to HLA is a major problem affecting approximately 30% of wait-listed kidney transplant candidates. The results of this study have important implications in both informed consent for patients and for the development of protocols for the management of renal transplant candidates with anti-HLA antibodies. The data suggest that protocols designed to enhance the transplantation rate and outcomes of sensitized patients should consider the presence of antibody against donor Class II HLA in their algorithms and the sole reliance on a negative CDC crossmatch is insufficient. When available, both paired donation and acceptable mismatch programs are important and viable options for some sensitized patients. Most, if not all, sensitized candidates should be entered into a paired donor program for at least a short period of time. However, despite these efforts, many sensitized candidates will never be matched with a donor against whom they have no antibody [19]. Thus, a +XMKTx may be the only realistic option for transplantation. While many of the grafts in +XMKTx may fail within 5 years necessitating another kidney transplant, others will show no signs of chronic injury. Given the expected good outcome, +XMKTx patients with antibody against donor Class I only that are CDC- might be considered relatively low risk and thus

transplanted without a prolonged wait in the paired or acceptable mismatch programs. The same consideration also applies to +XMKTx patients with antibodies against Class II with MFI < 3000 who also comprise a lower-risk group within the +XMKTx population.

Finally, the study highlights the fact that chronic injury affects the majority of +XMKTx in the first five years leading to a very high rate of graft loss. Thus, these data clearly suggest that the prevention and treatment of chronic antibody-mediated injury should be a major focus of future research.

Chapter 5 Differences in Chronic Intragraft Inflammation between Positive Crossmatch and ABO Incompatible Kidney Transplants

This chapter has been published in Transplantation[255].

5.1 Introduction

While anti-donor antibody is present in both ABOiKTx and +XMKTx, their long-term outcomes appear to be quite different. Anti-HLA antibody, especially against donor Class II, is associated with a high rate of chronic injury which presents histologically as chronic transplant glomerulopathy (cg) and commonly progresses to graft loss [256]. In contrast, ABOiKTx rarely develop cg and appear to have long-term graft survival similar to that of transplants without anti-donor antibody [93, 125, 189-191]. The causes of these divergent outcomes are unclear, however, several mechanisms have been suggested including the development of resistance to antibody mediated injury in ABOiKTx termed accommodation [177].

In +XMKTx, recent studies have suggested that subclinical cellular infiltration by macrophages, neutrophils and lymphocytes in peritubular capillaries and glomeruli has a high correlation with chronic injury [147, 253]. Comparative data in ABOiKTx are lacking.

The aim of the current study was compare the 5 year outcomes of ABOiKTx and +XMKTx with a specific emphasis on impact of histologic findings of surveillance biopsies on late outcomes.

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5.2 Methods

This study was approved by the Institutional Review Board at the Mayo Foundation and Clinic, Rochester, Minnesota. All live donor kidney transplant recipients that took place between May 1999 and December 2006 were reviewed and separated into 3 categories according to baseline anti-donor antibody status including: 1) Blood group incompatible (ABOi) recipients with negative crossmatch; 2)Positive crossmatch (+XMKTx) recipients—i.e. those with donor-specific alloantibody (DSA) at high enough levels to achieve a positive crossmatch (either T and/or B cell flow cytometric crossmatch as described in chapter 4) against their living donor at baseline; and 3) Conventional kidney transplants that were both ABO blood group compatible and had a negative crossmatch at the time of transplant (conventional KTx). Recipients who were both ABOi and +XMKTx were excluded (n=4).

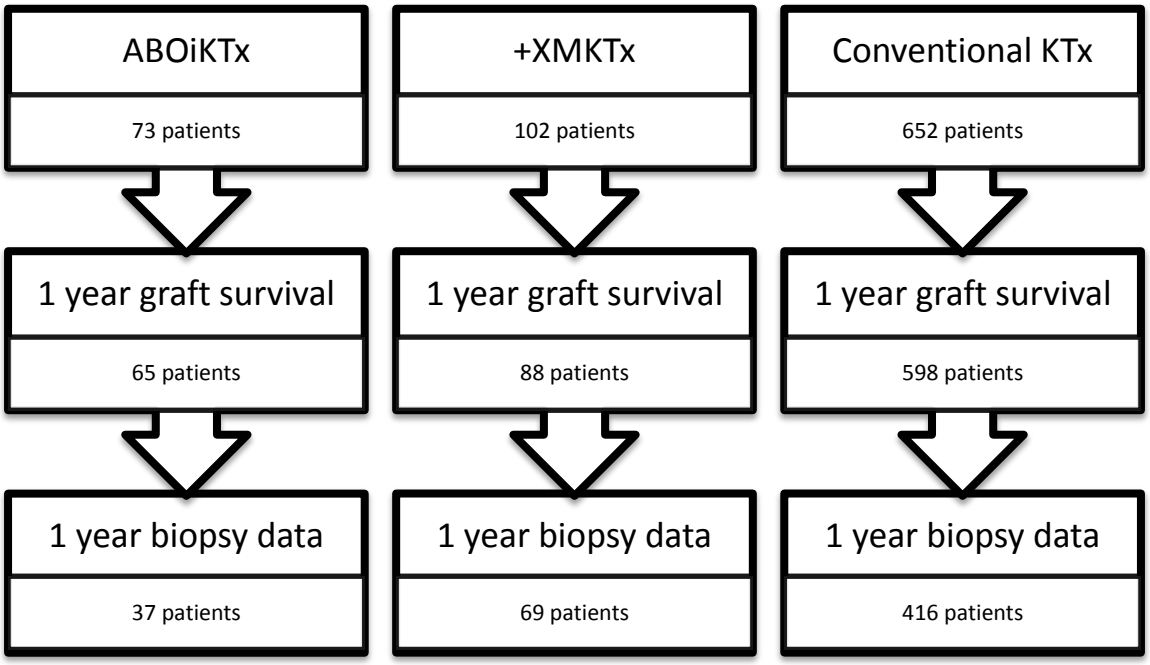
During this time period, an ABO incompatible kidney transplant was offered to all candidates whose only living donor was blood group incompatible. The cutoff for accepting recipients in this setting was an anti-blood group titer of ≤ 1:512. A positive crossmatch kidney transplant was offered to any sensitized patient who had a positive crossmatch against their living donor. The cutoff for accepting recipients in this setting was the ability to achieve a negative T cell AHG crossmatch on the day of transplantation.

5.2.1 Populations

Within these cohorts, patients were included for further analysis if they had a surveillance biopsy performed at 1 year following day of transplant (median days (Q1-Q3) 371 [361-393]). Of the seventy three ABOiKTx performed, 65 patients survived 1 year and there were 56.9% (37/65) had protocol biopsies performed at 1 year. One

hundred and two +XMKTx were reviewed which had been performed during the same time period and 75.0% (69/92) had a 1-year protocol biopsies performed. Finally, as a control cohort, 652 ABO compatible, -XMKTx living donor kidney recipients transplanted during the same time period were included of which 67.4% (416/617) patients had protocol biopsies performed at 1year. These populations are shown in Figure 5.1.

Figure 5.1. Population of transplant recipients in each antibody category included in this study who were available to be studied at 1 year allograft survival.



5.2.2 Antibody Measurement

Baseline (i.e. prior to any therapy) anti-HLA antibody levels were determined at the time of transplantation using a combination of cytotoxicity assays (T cell anti-human globulin CDC crossmatch) and T cell and B cell flow cytometric crossmatch (TFXM and BFXM) as previously described [69, 70, 257]. The solid-phase, single antigen bead assay

was not available during the entire study period, so for some patients, the determination of the presence of donor-specific alloantibody (DSA) was determined retrospectively using LABscreen (One Lambda, Canoga Park, CA).

Anti-blood group antibody was determined using isohemagglutination using AHG [191]. Single antigen bead testing was not carried out on the –XMKTx or ABOiKTx recipients at time of transplant.

5.2.3 Immunosuppression and Desensitization

All patients received induction immunosuppression with rabbit polyclonal anti-lymphocyte antibodies (Thymoglobulin™, Sangstadt, Menlo Park, CA, USA; 1.5mg/kg per day 4–10 doses), tacrolimus (Prograf™, Fujisawa, Deerfield, IL, USA), mycophenolate mofetil (750–1000 mg twice daily) (Cellcept™, Roche, Nutley, NJ, USA) and prednisone (tapering doses reaching 5 mg daily by month 3). The dose of tacrolimus was adjusted to achieve trough levels of 10–12 ng/mL (High-performance Liquid Chromatography/ Tandem Mass Spectrometry technique) during the first 4 months after transplantation and 6–8 ng/mL thereafter.

In addition to the conventional immunosuppression described above, recipients of ABOiKTx underwent pre-transplant conditioning and post-transplant management using a protocol reported previously [191]. The pre-conditioning consisted of plasma exchange (PE) followed by low-dose intravenous immunoglobulin (IVIG, 100 mg/kg after each PE (Gamimune 10%:Bayer Pharmaceutical, Elkhart, IN, USA or Venoglobulin-S-10%, Alpha Therapeutic Corporation, Los Angeles, CA, USA)) with a goal of achieving an anti-donor blood group antibody titer ≤1:8 on day of transplant.

Recipients of +XMKTx with a positive T-cell AHG-CDC at baseline underwent pre-transplant conditioning using a protocol described previously which is similar to that

described for ABOiKTx recipients [144, 186]. The goal of PE/IVIG in these patients was to achieve a negative T-cell AHG-CDC crossmatch on the day of transplantation. Individuals with a negative CDC but positive FXM received IVIG (Venoglobulin-S 10%, Alpha Therapeutic Corporation or Gamimune N, 10%, Bayer Biological, Elkhart, IN) at 2 g/kg immediately prior to transplantation, and did not undergo PE. This cohort has been previously well described [144, 257].

5.2.4 Biopsy and Clinical Outcomes

Patients underwent surveillance biopsies at 1 and 5 years after transplantation. A percutaneous ultrasound guided 18 gauge biopsy gun (Bard, Murray Hill, NJ) was used. Biopsies were interpreted using Banff 1995 criteria. Peritubular capillaritis (PTC) scores were reviewed by a single renal pathologist blinded to the clinical information using recent Banff 2007 criteria for both the +XMKTx and the ABOiKTx cohorts [142, 148].

Biopsy data were classified according to each category of the Banff criteria where a score of 0 was classified “none” ; a score of 1 as “mild” and a score greater than 1 as “moderate to severe”. Graft failure was defined as the need for maintenance dialysis therapy or the need for retransplantation. Glomerular filtration rate (eGFR, $ml/min/m^2$) was estimated using the 4 variable Modification of Diet in Renal Disease (MDRD) equation [258].

5.2.5 Statistical Analysis

Analyses were performed using JMPv9. (SAS, Cary, NC, USA) using the Kruskal–Wallis/ Wilcoxon rank-sum test, Pearson and Student’s t-tests according to the nature and distribution of the data compared. Shapiro-Wilk test was used to assess normal distribution of these data. Differences in patient and graft survival were calculated by

the log-rank test. Unless otherwise indicated, data are expressed as proportions or as mean ± standard deviation or median and range. Univariate and multivariate analysis was performed using a logistical regression model. Data for analysis of these patient cohorts was censored at 1st January 2012.

5.3 Results

5.3.1. Study Population

The study population included 73 ABOiKTx, 102 +XMKTx and 652 Conventional KTX (i.e. ABO compatible/-XMKTx) living donor kidney recipients. All patients were at least 5 years after the day of transplantation surgery. The demographics of the three groups are compared in Table 5.1. Compared to +XMKTx, ABOiKTx had increased recipient age (50.6 ± 14.5 vs. 46.3 ± 12.0, p=0.029); fewer females (35.6% vs. 69.6%, p<0.001); older donor age (45.1 ± 11.2 vs. 40.0 ± 13.0, p=0.048); more pre-emptive transplants (60.3% vs. 18.6%, p<0.001) and fewer re-transplants (17.8% vs. 39.2%, p=0.003). Patients in the ABOiKTx and +XMKTx groups were on dialysis longer prior to transplant (Respective median(Q1-Q3) time 18(11-52) months vs. 23(11-52) months, p=0.231) compared to Conventional KTx (Median (Q1-Q3) 9(4-20) months; ABOiKTx p=0.004 and +XMKTx p<0.001, respectively).

Table 5.1. Demographics and immunological profile in the 3 cohorts of transplant recipients in this study

	Conventional KTx	+XMKTx	ABOiKTx			
	652	102	73	Con vs. ABOi	Con vs. +XM	ABOi vs. +XM
Age (years) Mean±StDev	51.1 ± 14	46.3 ± 12	50.6 ± 14.5	0.862	<0.001	0.029
Gender(Female) %(n)	43.7% (285)	69.6% (71)	35.6% (26)	0.271	<0.001	<0.001
Ethnicity=Caucasian %(n)	92.9% (606)	96.1% (98)	93.2% (68)	0.948	0.237	0.387
Retransplant %(n)	14.7% (96)	39.2% (40)	17.8% (13)	0.423	<0.001	<0.001
ABDR mismatch Median (Q1-Q3)	3 (2-4)	3 (3-5)	3 (2-5)	0.381	<0.001	0.141
Pre-emptive %(n)	48.2% (314)	18.6% (19)	60.3% (44)	0.050	<0.001	<0.001
Dialysis time (months) Median (Q1-Q3)	9.1 (4.0-19.9)	23.1 (11.1-52.2)	18 (9.8-35.8)	0.004	<0.001	0.231
Living donor %(n)	100% (652)	100% (102)	100% (73)	n/a	n/a	n/a
AHGCDC Dilution Median (Q1-Q3)		4 (1-64)				
CDC negative %(n)		59.8% (61)				
CDC positive %(n)		40.2% (41)				
TFXM Channel Shift Mean±StDev		187 ± 124				
BFXM Channel Shift Mean±StDev		330 ± 93				
Anti-Class I antibody only %(n)		35.3% (36)				
Anti-Class II antibody only %(n)		19.6% (20)				
Both anti-Class I & anti-class II antibody %(n)		45.1% (46)				
Baseline Class I MFI Median (Q1-Q3)		7378 (2342-12363)				
Baseline Class II MFI Median (Q1-Q3)		2735 (0-10616)				
Recipient Blood group O %(n)	39.1% (255)	46.1% (47)	65.8% (48)	<0.001	0.182	0.010
Donor Blood group A %(n)	27.6% (180)	28.4% (29)	57.5% (42)	<0.001	0.863	<0.001
Follow up (days) Mean±StDev	2891 ±968	2488 ± 911	2795 ± 1043	0.034	<0.001	0.228
Donor age (years) Mean±StDev	43.5 ± 11.6	41 ± 11.9	45.1 ± 11.2	0.221	0.048	0.043

* comparison made between each group using Student's t-test for parametric data; Wilcoxon rank-sum test for non-parametric continuous data and between each group using Pearson chi-squared analysis for categorical data.

5.3.2. Antibody Data

The ABOiKTx cohort consisted of mainly blood group “O" recipients (48/73, 66.6%) of which 32 (68.7%) received a blood group “A” donor, 10 of which were A2 donors. At baseline, the median anti-blood group antibody titer in ABOiKTx was 1:64 (range 1:1 - 1:512). In the +XMKTx group, 40.2% (n=41) had a positive CDC crossmatch at baseline and 59.8% (n=61) had a negative CDC crossmatch, but a positive flow cytometric crossmatch with a channel shift >200. The mean B flow cytometric channel shift was 330 ± 93 and the mean T flow cytometric channel shift was 187 ± 124. In the +XMKTx group, 35.3% (n=36) had antibody against donor Class I HLA only, 19.6% (n=20) had antibody against donor Class II HLA only and 45.1% (n=46) had antibody against both donor Class I and Class II HLA. The different outcomes of these +XMKTx based on target HLA class specificity have been reported previously [144, 186].

5.3.3. Patient and Graft survival

Patient survival at 5 years was not significantly different between the 3 groups: ABOiKTx (88.9%), +XMKTx (86.3%); and Conventional KTx (87.9%); (ABOiKTx vs. +XMKTx, p=0.165) (Figure 5.2a). Actual 5 year death-censored graft survival (DCGS) was higher in ABOiKTx compared to the +XMKTx (79.5% and 70.7%, p= 0.03), but both were lower than Conventional KTx (86.7%) (Figure 5.2b).

Importantly, in grafts surviving at least 1 year, the subsequent death censored graft survival (DCGS) between 1 and 5 years after transplantation was similar in the ABOiKTx and Conventional KTx (89.2% vs. 92.9%, p=0.155), and higher than +XMKTx (89.2% vs. 77.6%, p=0.03; Figure 5.2c). A calculation of these data is that the rate of graft loss in ABOiKTx between 1 and 5 years was 1.7%/year in Conventional KTx, 2.6%/year in ABOiKTx and 5.8%/year in the +XMKTx.

Figure 5.2 Five year outcomes for Patient and Graft Survival

Conventional KTx are solid lines (——); +XMKTx are dotted lines (⋯) and ABOiKTx are dashed lines (---). Patient survival (Figure 5.2A) is comparable between groups while actual 5-year death-censored graft survival rates in antibody incompatible kidney transplants are inferior to conventional KTx (Figure 5.2B). While overall survival in ABOiKTx is similar at 5 years to +XMKTx, ABOiKTx allografts that survive to 1 year have significantly better outcome than +XMKTx (Figure 5.2C).

Figure 5.2A- Actual Patient Survival

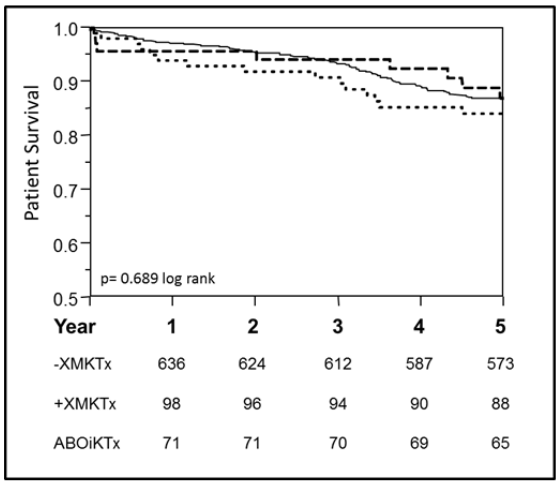


Figure 5.2B - Actual 5 –Year Death-Censored Renal Allograft Survival

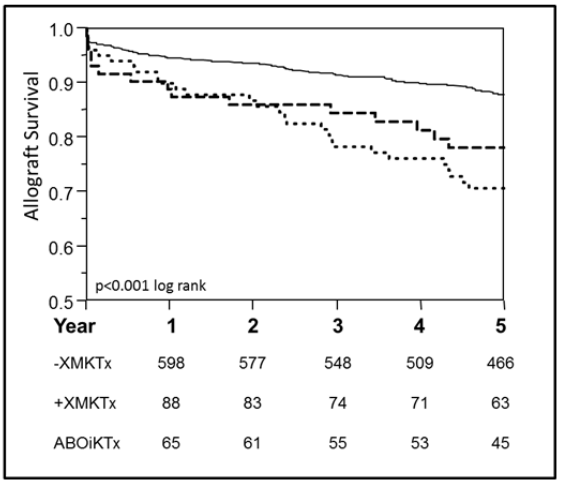
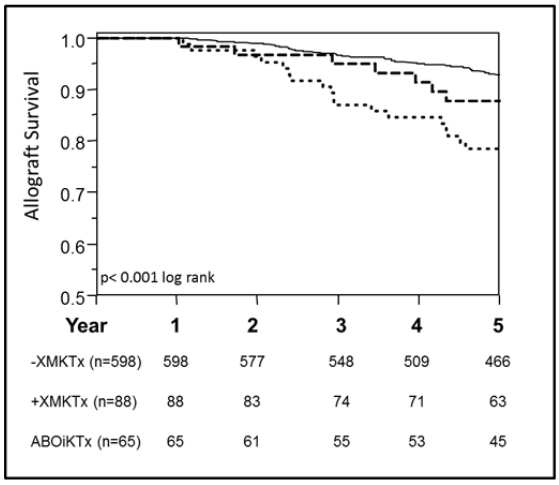


Figure 5.2C - Actual 5 –Year Death-Censored Renal Allograft Survival for all those who survived 1 year



At baseline, data for transplant recipients was assessed using logistic regression to identify risk factors for 5 year allograft failure using the following variables: Pre-emptive transplantation, Age at Transplantation, Age of Donor, ABDR mismatch, Re-transplantation, Diabetes, Female Gender; Caucasian Ethnicity. The significant univariate factors are shown in Table 5.2A. The factors with $p<0.1$ were then used in a logistic regression analysis and results are shown in Table 5.2B. Increased Age at Transplant, Increased Age of Donor and Increased Length of time on dialysis were related to 5 year allograft failure.

5.3.4. Renal Allograft Function

Estimated GFR spanned a wide spectrum in all 3 groups at both 1 and 5 years after transplantation. The mean eGFR was similar in all 3 groups at 1 year (Figure 5.3A) including Conventional KTx (49.4±15.5ml/min); ABOiKTx (51.4±13.4 ml/min, p=0.30 vs. Conventional KTx) and +XMKTx (49.3ml/min, p=0.15 vs. ABOiKTx). In patients whose allograft survived 5 years, using a paired comparison analysis, the mean eGFR between 1 and 5 years in the ABOi group remained stable (52.8+12.8ml/min to 51.7+15.0ml/min, p=0.47) with similar function to the Conventional KTx (50.1±15.1 ml/min to 48.1±16.9 ml/min, p=0.78). In contrast, the mean eGFR declined in the +XMKTx group (51.4±16.7 ml/min to 44.4±19.6 ml/min, p=0.01). In addition, at 1 and 5 years, the proportion of patients with an eGFR <40 ml/min was higher in the +XMKTx group compared to the ABOiKTx group (1 year 32.8% vs. 17.0%, p=0.04 and 5 year 48.8% vs. 19.2%%, p<0.01). At 5 years (Figure 5.3B), in surviving transplants the mean eGFR was significantly lower in the +XMKTx group compared to ABOiKTx (44.8 ± 19.6 ml/min vs. 51.7 ± 15.0 ml/min, p=0.04) although the difference with Conventional KTx (48.5 ± 16.5ml/min, p=0.09) did not reach statistical significance. Thus, at 5 years, the

+XMKTx group had experienced a higher number of graft losses and the eGFR was lower in the grafts still functioning.

Table 5.2. Table of Variables included in the Analysis for 5 year graft failure.

Table 5.2A. Univariate Analysis

	B	S.E.	Wald	df	Sig.	OR	95% C.I.for OR	
							Lower	Upper
Age At Transplant (per year of age)	**-0.03**	**0.01**	**13.37**	**1.00**	**<0.001**	**0.97**	**0.96**	**0.99**
Donor Age (per year of age)	**0.03**	**0.01**	**13.91**	**1.00**	**<0.001**	**1.03**	**1.02**	**1.05**
Pre-emptive	**-0.69**	**0.21**	**10.44**	**1.00**	**0.001**	**0.50**	**0.33**	**0.76**
Dialysis Time (per month of dialysis)	**0.01**	**0.00**	**8.90**	**1.00**	**0.003**	**1.01**	**1.00**	**1.02**
ABDR mismatch (per mismatch unit)	**0.18**	**0.07**	**7.80**	**1.00**	**0.005**	**1.20**	**1.06**	**1.37**
Re-transplanted vs. 1st transplant	**0.55**	**0.24**	**5.45**	**1.00**	**0.020**	**1.73**	**1.09**	**2.74**

Table 5.2B.Multivariate Analysis

	B	S.E.	Wald	df	Sig.	OR	95% C.I.for OR	
							Lower	Upper
Age At Transplant (per year of age)	**-0.02**	**0.01**	**4.86**	**1.00**	**0.027**	**0.98**	**0.96**	**1.00**
Donor Age (per year of age)	**0.04**	**0.01**	**10.83**	**1.00**	**0.001**	**1.04**	**1.02**	**1.07**
Pre-emptive	**0.01**	**0.00**	**7.67**	**1.00**	**0.006**	**1.01**	**1.00**	**1.02**
Dialysis Time (per month of dialysis)	-18.88	28151.01	0.00	1.00	0.999	0.00	0.00	
ABDR mismatch (per mismatch unit)	0.17	0.09	3.52	1.00	0.060	1.19	0.99	1.43
Re-transplanted vs. 1st transplant	0.58	0.32	3.30	1.00	0.069	1.79	0.96	3.34
Constant	-3.32	0.76	19.18	1.00	0.000	0.04		

B= coefficient of the constant; S.E. = standard error around B; df=degrees of freedom; OR= Odds Ratio of Allograft Failure at 5 years., CI= Confidence Interval. Analysis performed using logistic regression. Variables were included in multivariate analysis if $p<0.1$ in univariate analysis

5.3.5. Acute Rejection Rates

Given that anti-HLA immune responses are thought to be T cell dependent, it is possible that differences in the incidence and severity of cellular rejection in the sensitized patients might contribute to the differences in the late outcomes. To address this, I examined in detail all biopsies (n=2544) in the 3 groups prior to their 1 year surveillance biopsy: these were undertaken per protocol or for indication for graft dysfunction (Table 5.3). There was no significant difference between all 3 groups for cellular rejection (+XMKTx vs. –XMKTx 5.8% [n=6/102] vs. 14.2% [n=93/652], p=0.45; ABOiKTx vs. –XMKTx 9.6% [n=7/73] vs. 14.2% [n=93], p=0.40 and +XMKTx vs. ABOiKTx 5.9% [n=6] vs. 9.6% [n=7], p=0.92).

The severity of the cellular rejection in the three groups appeared to be similar and "v" lesions were similar in biopsies at 4 months in the 3 groups (Conventional KTx 1%, +XMKTx 1% and ABOiKTx 0%). Thus, pure cellular rejection as defined by the current Banff classification does not explain the differences in outcome. While there were higher rates of AMR in both +XMKTx (33.3%, n=34) and ABOiKTx (31.5%, n=23) than Conventional KTx group (1.5%, n=10, p<0.001 for both groups), mixed cellular and humoral rejections might explain the differences in allograft outcomes. In all groups, the incidence of mixed cellular and humoral rejection was rare—even in the +XMKTx with AMR .The prevalence of interstitial infiltration ('i') and/or tubulitis ('t') with AMR was similar in Conventional KTx (20.0%) and +XMKTx (17.6%) and lower in the ABOiKTx group although did not reach statistical significance (4.3%, p=0.15 and p=0.13 respectively).Thus, the phenotype of mixed rejection does not equate with poor allograft survival.

Figure 5.3. Renal Allograft Function (eGFR) at 1 and 5 years in Surviving Kidney Transplants.

Allograft function is not significantly different at 1 year (A), however at 5 years the +XMKTx had inferior eGFR compared to both Conventional KTx and ABOiKTx (B). Mean eGFR (MDRD) is shown by the green line. Analysis made by Student's t-test.

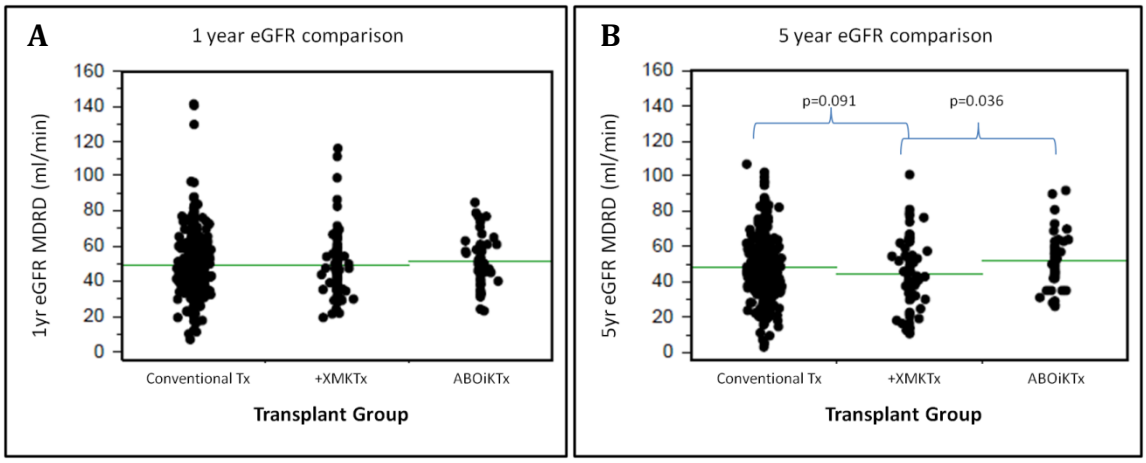


Table 5.3. Incidence of acute rejection episodes for patients in each cohort

Biopsy scores according to Banff criteria in the first year following transplant classified for rejection, type of rejection and cellular component of AMR. The highest Banff score was used for each patient in the first year for rejection episodes if multiple biopsies were performed. This demonstrates a higher number of patients in the antibody incompatible groups with biopsies with rejection (+XMKTx 39.2% and ABOiKTx 42.9% compared to Conventional KTx 15.7%). There were similar rates of cellular rejection rates in each cohort.

	Conventional KTx	+XMKTx	ABOi
Patient number	652	102	73
Number of patients biopsied % (n)	94.9% (619)	100% (102)	95.9% (70)
Number of Biopsies	1651	553	340
Biopsies/patient (Median [Q1-Q3])	3[2-3]	5[4-6]	5[4-6]
Percentage of patients with rejection on biopsy (n)	15.7% (103)*	39.2% (40)*	41.1% (30)*
Total Cellular Rejection % (n)	14.2% (93)*	5.9% (6)*	9.6% (7)*
Total Antibody Mediated Rejection % (n)	1.5% (10)*	33.3% (34)*	31.5% (23)*
Highest Banff score of rejection % (n)			
Total Cellular Rejection of rejection biopsies	90% (93)†	15% (6)†	23.3% (7)†
AR0	35.9% (37)†	2.5% (1) †	6.7% (2) †
AR1	39.8% (41) †	10% (4) †	16.7% (5) †
AR2	14.6% (15) †	2.5 (1) †	0 †
Antibody Mediated Rejection of rejection biopsies	9.7% (10) †	85% (34) †	76.7% (23) †
of AMR, interstitial inflammation % (n)	10% (1) †	2.9% (1) †	4.3% (1) †
of AMR, tubulitis % (n)	0 †	11.8% (4) †	0 †
of AMR, tubulitis and interstitial inflammation	10% (1) †	2.9%(1) †	0 †

AR0 = subclinical rejection; AR1 = Banff 1 rejection AR2 = Banff 2 rejection

*= of all patients in each group; †= of all patients with biopsies in each group.

5.3.6. Protocol Surveillance Biopsies

A goal in the transplant programme was to perform protocol surveillance biopsies on all functioning grafts at 1 and 5 years after transplantation, but lack of consent, the need for chronic anticoagulation, and other medical reasons decreased the biopsy rate. Thus, in the +XMKTx group, 78.4% (69/88) had biopsies at 1 year and 63.5% (40/63) at 5 years. In the ABOiKTx group, 56.9% (37/65) had biopsies at 1 year and 55.5% (25/45) at 5 years. In the Conventional KTx group, 69.6% (416/598) had biopsies at 1 year and 60.1% (280/466) at 5 years.

A summary of the histological differences in the surveillance biopsies of the surviving allografts are shown in Table 5.4. At 1 year (Table 5.4A), +XMKTx demonstrated higher rates of microcirculatory inflammation as evidenced by glomerulitis (g) and peritubular capillaritis (ptc) at 3 months have been associated with early chronic injury in +XMKTx [163]. In both ABOiKTx and Conventional KTx, the prevalence of microcirculation injury was low and generally similar. In the Conventional KTx there was 20% vs. 73.7% in +XMKTx, ($p=0.045$) and in the ABOiKTx 27.8% vs. 73.7% in +XMKTx, ($p<0.001$). At 1 year, the prevalence of glomerulitis was 36.2% in +XMKTx compared to only in 2.7% ABOiKTx and 6.0% in Conventional KTx ($p<0.001$ for both compared to +XMKTx). Similarly at 5 years, there was significantly higher rates of antibody-mediated injury in the +XMKTx, than the other 2 groups (Table 5.4B).

A lack of inflammation on renal biopsy was associated with improved graft survival in all groups. Using a cumulative microcirculation score (addition of glomerulitis and peritubular capillaritis), the transplants were stratified into no inflammatory infiltrate (score 0); mild infiltration (cumulative score 1-3) and heavy infiltrations (cumulative score >3) [152]. There were lower microcirculation scores in ABOiKTx group than +XMKTx group ($p<0.001$) shown in Table 5.5.

Table 5.4. Differences in the surveillance biopsies scores of the surviving allografts at 1 year and then 5 years respectively

Table 5.4A – Summary of Surveillance Biopsies at 1 year. Increased microcirculatory inflammation in antibody incompatible compared to Conventional KTx, but significantly greater in +XMKTx. C4d was not done routinely in the early transplant programme, thus under-represented at 1 year biopsies.

		Conventional KTx (n=416)	+XMKTx (n=69)	ABOiKTx (n=37)	Con/ ABOi*	Con/ + XM*	+XM/ ABOi*
G	None	94.0% (n=391)	63.8% (n=44)	97.3% (n=36)	0.403	<0.001	<0.001
	Positive	6.0% (n=25)	36.2% (n=25)	2.7% (n=1)			
Cg	None	96.9% (n = 403)	71.0% (n = 49)	88.9% (n = 32)	0.018	<0.001	0.026
	Positive	3.1% (n=13)	29.0% (n=20)	11.1% (n=4)			
C4d	None	94.7% (n = 18)	84.1% (n = 53)	16.7% (n = 1)	<0.001	0.21	<0.001
	Positive	5.3% (n=1)	15.9% (n=10)	83.3% (n=5)			
ptc #	None	80.0% (n = 4)	27.7% (n = 18)	72.2% (n = 26)	0.918	0.045	<0.001
	Positive	20.0% (n=1)	73.3% (n=47)	27.8% (n=10)			

* comparison made between groups using Pearson chi-squared analysis

Data not cumulative to total number of biopsy was not available for analysis

The biopsies of Conventional KTx were not re-examined to address 'ptc' in this cohort.

Table 5.4B - 5 year Histology - Increased microcirculatory inflammation and chronic injury in +XMKTx. No significant differences in ABOiKTx to Conventional KTx at 5years, except the activation of complement with increased C4d deposition in allografts.

		Conventional KTx (n=280)	+XMKTx (n=40)	ABOiKTx (n=25)	Con/ ABOi*	Con/ + XM*	+XM/ ABOi*
g	None	90.4% (n = 253)	30.0% (n = 12)	92.0% (n = 23)	0.762	<0.001	0.001
	Positive	9.6% (n=27)	70% (n=28)	8% (n=2)			
i	None	88.9% (n = 249)	74.4% (n = 29)	76.0% (n = 19)	0.055	0.01	0.884
	Positive	11.1% (n=31)	25.6% (n=10)	24.0% (n=6)			
ah	None	37.3% (n = 103)	15.0% (n = 6)	40.0% (n = 10)	0.539	0.001	0.005
	Positive	62.7% (n=173)	85.0% (n=34)	60.0% (n=15)			
cg	None	92.4% (n = 257)	42.5% (n = 17)	88.0% (n = 22)	0.457	<0.001	<0.001
	Positive	7.6% (n=21)	57.5% (n=23)	12.0% (n=3)			
ci	None	38.0% (n = 106)	12.5% (n = 5)	52.0% (n = 13)	0.123	0.002	<0.001
	Positive	62.0% (n=173)	87.5% (n=35)	48.0% (n=12)			
ct	None	28.1% (n = 78)	10.0% (n = 4)	40.0% (n = 10)	0.125	0.013	0.003
	Positive	71.9% (n=200)	90.0% (n=36)	60.0% (n=15)			
cv	None	38.0% (n = 106)	20.5% (n = 8)	32.0% (n = 8)	0.676	0.017	0.129
	Positive	62.0% (n=173)	79.5% (n=31)	68.0% (n=17)			
C4d	None	94.3% (n = 83)	81.1% (n = 30)	22.2% (n = 4)	<0.001	0.02	<0.001
	Positive	5.6% (n=5)	18.9% (n=7)	77.8% (n=14)			
ptc #	None	91.1% (n = 123)	33.3% (n = 10)	92.3% (n = 12)	0.863	<0.001	0.001
	Positive	8.9% (n=12)	66.7% (n=20)	7.7% (n=1)			

* comparison made between groups using Pearson chi-squared analysis

Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis

The biopsies of Conventional KTx were not re-examined to address 'ptc' in this cohort.

Table 5.5. Microcirculation scores of different transplant groups in biopsies at 1 and 5 years post transplantation.

MIC scoring was done in the available biopsies at each time period where both glomerulitis and peritubular capillaritis were recorded. Severity of microcirculation injury was not scored in the Conventional KTx group and is therefore not shown.

		XM POS	ABOi	+XM/ ABOi*
1yr MIC score	0	26.2% (n=17)	70.6% (n=24)	<0.001
	1	46.2% (n=30)	29.4% (n=10)	
	2	27.7% (n=18)	0	
5yr MIC score	0	13.3% (n=4)	92.3% (n=12)	<0.001
	1	56.7% (n=17)	0	
	2	30.0% (n=9)	7.7% (n=1)	

Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis

* comparison made between groups using Wilcoxon rank-sum test treating Banff score as continuous data

At 1 year chronic glomerulopathy (cg) was much more common in +XMKTx, occurring in 29.0% (n=20) of biopsies compared to only 11.0% (n=4) of ABOiKTx (p=0.026) and 3.1% (n=13) of Conventional KTx (p<0.001 for both compared to +XMKTx). The severity of the 'cg' was much higher in the +XMKTx group, in which 14.5% (n=10) had moderate to severe 'cg', whereas no ABOiKTx had this degree of severity (Table 5.6A). At 1 year, chronic glomerulopathy was more common in ABOiKTx (11.1%) compared to Conventional KTx (3.1%, p=0.018).

These differences in inflammation and chronic injury persisted and increased on 5 year biopsies (Table 5.6B). The prevalence of peritubular capillaritis was 66.7% in +XMKTx compared to only 7.7% in ABOiKTx and 8.9% in Conventional KTx (p<0.001 for both compared to +XMKTx). Similarly, at 5 years the prevalence of glomerulitis was 70.0% in +XMKTx compared to only in 8.0% ABOiKTx and 9.6% in Conventional KTx (p<0.001 for both compared to +XMKTx). Chronic glomerulopathy was much more common in +XMKTx at 1 year occurring in 57.5% of biopsies compared to only 12.0% of ABOiKTx and 7.6% of Conventional KTx (p<0.001 for both compared to +XMKTx). In contrast to the 1 year data, the prevalence of chronic glomerulopathy was similar in ABOiKTx and Conventional KTx. Peritubular capillaritis on 1 year protocol biopsy correlated with chronic glomerulopathy on the 5 year biopsy in both the ABOiKTx (p=0.009) and +XMKTx (p=0.003).

Table 5.6. Overall histological Banff scores for biopsies at 1year and 5years

Overall histological Banff scores for all 1 year biopsies

	Table 5.6A 1 year biopsies	Conventional KTx (n=416)	+XMKTx (n=69)	ABOiKTx (n=37)	Con/ ABOi*	Con/ + XM*	+XM/ ABOi*
g	None	94.0% (n = 391)	49.3% (n = 34)	97.3% (n = 36)	0.403	<0.001	<0.001
	Mild	4.6% (n = 19)	27.5% (n = 19)	2.7% (n = 1)			
	Mod-Sev	1.4% (n = 6)	23.2% (n = 16)	0.0% (n = 0)			
i	None	84.6% (n = 352)	84.1% (n = 58)	91.9% (n = 34)	0.274	0.902	0.3
	Mild	8.7% (n = 36)	8.7% (n = 6)	2.7% (n = 1)			
	Mod-Sev	6.7% (n = 28)	7.2% (n = 5)	5.4% (n = 2)			
t	None	79.6% (n = 331)	62.3% (n = 43)	83.8% (n = 31)	0.537	0.004	0.032
	Mild	12.5% (n = 52)	30.4% (n = 21)	10.8% (n = 4)			
	Mod-Sev	7.9% (n = 33)	7.2% (n = 5)	5.4% (n = 2)			
v	None	99.8% (n = 415)	100.0% (n = 69)	100.0% (n = 37)	0.772	0.688	1
	Mild	0.2% (n = 1)	0.0% (n = 0)	0.0% (n = 0)			
	Mod-Sev	0.0% (n = 0)	0.0% (n = 0)	0.0% (n = 0)			
ah	None	81.0% (n = 319)	75.8% (n = 50)	83.8% (n = 31)	0.689	0.355	0.372
	Mild	16.2% (n = 64)	22.7% (n = 15)	13.5% (n = 5)			
	Mod-Sev	2.8% (n = 11)	1.5% (n = 1)	2.7% (n = 1)			
cg	None	96.9% (n = 403)	71.0% (n = 49)	88.9% (n = 32)	0.018	<0.001	0.026
	Mild	1.7% (n = 7)	14.5% (n = 10)	11.1% (n = 4)			
	Mod-Sev	1.4% (n = 6)	14.5% (n = 10)	0.0% (n = 0)			
ci	None	45.9% (n = 191)	36.2% (n = 25)	52.8% (n = 19)	0.591	0.148	0.182
	Mild	39.2% (n = 163)	49.3% (n = 34)	30.6% (n = 11)			
	Mod-Sev	14.9% (n = 62)	14.5% (n = 10)	16.7% (n = 6)			
ct	None	30.5% (n = 127)	26.1% (n = 18)	38.9% (n = 14)	0.374	0.267	0.164
	Mild	55.0% (n = 229)	56.5% (n = 39)	44.4% (n = 16)			
	Mod-Sev	14.4% (n = 60)	17.4% (n = 12)	16.7% (n = 6)			
cv	None	57.7% (n = 239)	62.3% (n = 43)	60.0% (n = 21)	0.784	0.428	0.793
	Mild	35.3% (n = 146)	33.3% (n = 23)	34.3% (n = 12)			
	Mod-Sev	7.0% (n = 29)	4.3% (n = 3)	5.7% (n = 2)			
C4d	None	94.7% (n = 18)	84.1% (n = 53)	16.7% (n = 1)	<0.001	0.21	<0.001
	Mild	5.3% (n = 1)	1.6% (n = 1)	0.0% (n = 0)			
	Mod-Sev	0.0% (n = 0)	14.3% (n = 9)	83.3% (n = 5)			
ptc #	None	80.0% (n = 4)	27.7% (n = 18)	72.2% (n = 26)	0.918	0.045	<0.001
	Mild	0.0% (n = 0)	16.9% (n = 11)	22.2% (n = 8)			
	Mod-Sev	20.0% (n = 1)	55.4% (n = 36)	5.6% (n = 2)			

* comparison made between groups using Wilcoxon rank-sum test treating Banff score as continuous data. Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis. . #The biopsies of Conventional KTx were not re-examined to address 'ptc' .

. Overall histological Banff scores for all 5 year biopsies

	Table 5.6B 5 year biopsies	Conventional KTx (n=280)	+XMKTx (n=40)	ABOiKTx (n=25)	Con/ ABOi*	Con/ + XM*	+XM/ ABOi*
g	None	90.4% (n = 253)	30.0% (n = 12)	92.0% (n = 23)	0.762	<0.001	0.001
	Mild	6.8% (n = 19)	25.0% (n = 10)	8.0% (n = 2)			
	Mod-Sev	2.9% (n = 8)	45.0% (n = 18)	0.0% (n = 0)			
i	None	88.9% (n = 249)	74.4% (n = 29)	76.0% (n = 19)	0.055	0.01	0.884
	Mild	9.3% (n = 26)	20.5% (n = 8)	20.0% (n = 5)			
	Mod-Sev	1.8% (n = 5)	5.1% (n = 2)	4.0% (n = 1)			
t	None	94.3% (n = 264)	87.5% (n = 35)	92.0% (n = 23)	0.649	0.101	0.564
	Mild	5.4% (n = 15)	10.0% (n = 4)	8.0% (n = 2)			
	Mod-Sev	0.4% (n = 1)	2.5% (n = 1)	0.0% (n = 0)			
v	None	100.0% (n = 280)	100.0% (n = 39)	100.0% (n = 25)	1	1	1
	Mild	0.0% (n = 0)	0.0% (n = 0)	0.0% (n = 0)			
	Mod-Sev	0.0% (n = 0)	0.0% (n = 0)	0.0% (n = 0)			
ah	None	37.3% (n = 103)	15.0% (n = 6)	40.0% (n = 10)	0.539	0.001	0.005
	Mild	39.1% (n = 108)	40.0% (n = 16)	44.0% (n = 11)			
	Mod-Sev	23.6% (n = 65)	45.0% (n = 18)	16.0% (n = 4)			
cg	None	92.4% (n = 257)	42.5% (n = 17)	88.0% (n = 22)	0.457	<0.001	<0.001
	Mild	2.2% (n = 6)	10.0% (n = 4)	8.0% (n = 2)			
	Mod-Sev	5.4% (n = 15)	47.5% (n = 19)	4.0% (n = 1)			
ci	None	38.0% (n = 106)	12.5% (n = 5)	52.0% (n = 13)	0.123	0.002	<0.001
	Mild	44.4% (n = 124)	60.0% (n = 24)	40.0% (n = 10)			
	Mod-Sev	17.6% (n = 49)	27.5% (n = 11)	8.0% (n = 2)			
ct	None	28.1% (n = 78)	10.0% (n = 4)	40.0% (n = 10)	0.125	0.013	0.003
	Mild	54.3% (n = 151)	62.5% (n = 25)	52.0% (n = 13)			
	Mod-Sev	17.6% (n = 49)	27.5% (n = 11)	8.0% (n = 2)			
cv	None	38.0% (n = 106)	20.5% (n = 8)	32.0% (n = 8)	0.676	0.017	0.129
	Mild	42.3% (n = 118)	48.7% (n = 19)	64.0% (n = 16)			
	Mod-Sev	19.7% (n = 55)	30.8% (n = 12)	4.0% (n = 1)			
C4d	None	94.3% (n = 83)	81.1% (n = 30)	22.2% (n = 4)	<0.001	0.02	<0.001
	Mild	1.1% (n = 1)	5.4% (n = 2)	0.0% (n = 0)			
	Mod-Sev	4.5% (n = 4)	13.5% (n = 5)	77.8% (n = 14)			
ptc #	None	91.1% (n = 123)	33.3% (n = 10)	92.3% (n = 12)	0.863	<0.001	0.001
	Mild	5.2% (n = 7)	30.0% (n = 9)	7.7% (n = 1)			
	Mod-Sev	3.7% (n = 5)	36.7% (n = 11)	0.0% (n = 0)			

* comparison made between groups using Wilcoxon rank-sum test treating Banff score as continuous data. Biopsy data that is not cumulative to total number of biopsy means that the biopsy material was not available for analysis. . #The biopsies of Conventional KTx were not re-examined to address 'ptc' .

At 1 year, almost all of the other Banff scores were similar among the 3 groups, including acute indices (interstitial inflammation, i; vascular inflammation, v;) and chronic indices: interstitial fibrosis (ci); tubular atrophy (ct) and arteriolar hyalinosis (ah). The one exception was an increased prevalence of acute tubulitis (t) in the +XMKTx at 1 year (37.6%) compared to the ABOiKTx (16.2%, p=0.032) and conventional KTx (19%, p=0.004). In contrast to the 1 year findings, at 5 years, the +XMKTx generally showed more interstitial inflammation, but no differences in tubulitis. Chronic indices in the +XMKTx at 5 years were higher than those in ABOiKTx and Conventional KTx further suggesting the presence of chronic injury. For example, interstitial fibrosis (ci) was present in 87.5% of +XMKTx compared to 48% in ABOiKTx (p=0.001) and 62% of Conventional KTx (p=0.002). In addition, 27.5% of +XMKTx had moderate-to-severe interstitial fibrosis compared to 8.0% ABOiKTx (p<0.001) and 17.6% Conventional KTx (p=0.002).

Special mention should be made of the C4d staining of protocol biopsies. This immunofluorescent stain was not performed routinely on all biopsies during this entire time period—especially in conventional KTx. For +XMKTx and ABOiKTx, C4d testing became the standard of care toward the end of the study and thus was performed more commonly on 5 year biopsies. Interestingly, the prevalence of C4d deposition was much more common in ABOiKTx even at 5 years (77.8%) than in +XMKTx (18.9%, p<0.0001) despite a higher prevalence of chronic inflammation and glomerulopathy in the +XMKTx group.

5.3.7 Correlation between Antibody titre and histology

There was no association between starting titre and microcirculatory injury histology on 1-year or 5-year biopsies, when categorized into >1:16, >1:64 or >1:128 categories. Distribution of starting titers and titers at 1 year can be seen in Figure 5.4.

5.3.8 Correlation between Histology and Outcomes

In all 3 groups, the presence of chronic glomerulopathy at 1 year was correlated with graft failure (OR 9.6, 4.2-21.3) and peritubular capillaritis was also associated with graft failure (OR 3.2, 1.03-12.0). Figure 5.5 A and B depict the difference in rate of graft loss with and without chronic glomerulopathy on the 1-year surveillance biopsy. Even in the absence of chronic glomerulopathy at 1 year, the +XMKTx patients have significantly higher rate of graft loss over time.

The presence of chronic glomerulopathy at 1 and 5 years in the +XMKTx group was associated with reduced eGFR at both time points (1 yr CG 40.0±9.7ml/min vs.1yr No CG 51.7±18.4ml/min p=0.001 and 5yr CG 38.8±14.9ml/min vs. 5yr No CG 57.2±15.5ml/min p<0.001)–whereas ABOiKTx did not experience a decline in allograft function when chronic glomerulopathy was present at 5 years. The presence of chronic damage in 1 year biopsies was associated with significantly reduced function in the Conventional KTx group, but did not affect the other 2 groups (Table 5.7A). The presence of peritubular capillaritis at 1 year was not associated with a change in eGFR between 1 or 5 years in either ABOiKTx or +XMKTx cohort and did not predict an eGFR <40ml/min.

While there was significantly more chronic injury (5 yr ci/cv/ah) in the +XMKTx cohort compared the other cohorts, this was not associated with worse function in the allograft, as it was in the Conventional KTx group (Table 5.7B), although care needs to

be taken in interpretation given the already higher rates of graft loss in the +XMKTx cohort. This could therefore be confounded by a degree of survivor bias.

Early AMR was significantly associated with the development of cg at 1 year in the ABOiKTx cohort (p=0.001), but not in the +XMKTx cohort (p=0.74). There was no association with AMR and peritubular capillaritis in either group.

Figure 5.4. Comparison of anti-ABO titers at baseline and 1 year post transplantation.

There is a reduction in titers between baseline and 1 year post transplantation in the ABOiKTx patients in both immediate (IM) and antihuman globulin (AHG) techniques for anti-A/B titer measurement. The graph demonstrates titers expressed in number of dilutions. Median values of titer were 1:4 (AHG) or 1:8 (AHG) at 1 year, compared to 1:16 (IM) or 1:64 (AHG) at baseline.

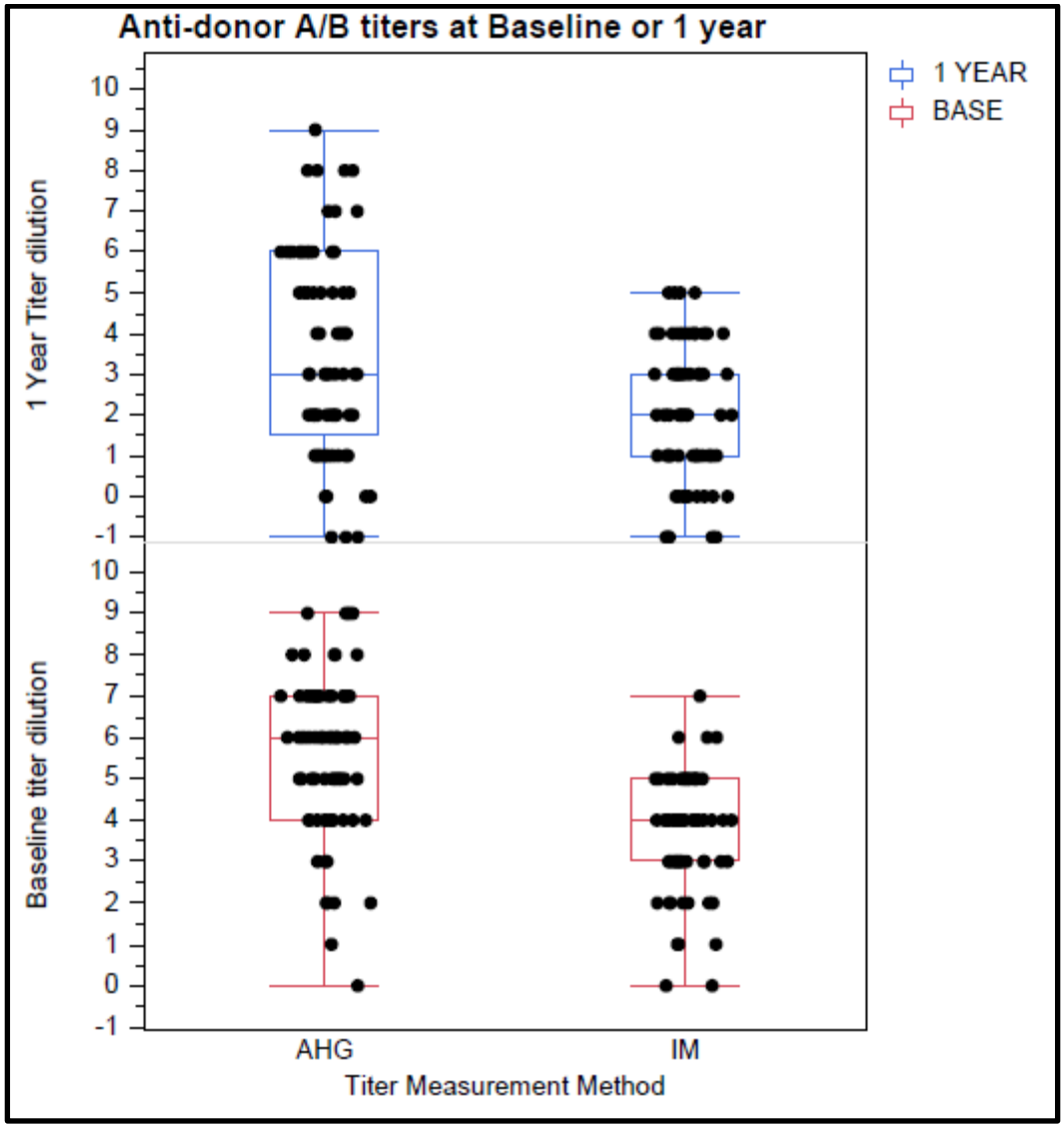


Table 5.7. Association between biopsy findings and eGFR at 1 and 5 years.

Table 5.7A - 1 year eGFR with 1 year surveillance biopsy results for each cohort.

1 year	ah	No ah	P value	cg	No cg	P value	ci	No ci	P value	ct	No ct	P value	cv	No cv	P value
Conventional (n=416)	47.8 ± 14.7	49.7 ± 13.8	0.303	44.2 ± 14.9	49.6 ± 13.8	0.218	47.2 ± 14.6	52.1 ± 12.4	<0.001	48.4 ± 14.6	51.7 ± 11.8	0.014	48.3 ± 15.7	50.3 ± 12.2	0.168
+XMKTx (n=69)	48.6 ± 18.6	47.3 ± 16.2	0.809	39.8 ± 9.5	51.7 ± 18.4	0.001	47.9 ± 20.3	49.0 ± 9.5	0.759	48.3 ± 19.1	48.3 ± 10.4	0.995	49.4 ± 16.9	47.7 ± 17.5	0.691
ABOiKTx (n=37)	52.5 ± 14.4	48.7 ± 13.0	0.565	40.8 ± 6.7	50.3 ± 13.6	0.055	48.5 ± 14.0	49.8 ± 12.9	0.763	47.8 ± 12.5	51.4 ± 14.6	0.461	51.7 ± 14.2	48.2 ± 12.6	0.465

Table 5.7B - 5 year eGFR with 1 year surveillance biopsy results for each cohort.

5 year	ah	No ah	P value	cg	No cg	P value	ci	No ci	P value	ct	No ct	P value	cv	No cv	P value
Conventional (n=250)	48.4 ± 16.7	51.9 ± 13.9	0.076	36.9 ± 11.0	50.4 ± 15.7	0.001	45.2 ± 13.6	56.7 ± 16.6	<0.001	47.1 ± 15.5	51.2 ± 17.2	0.036	47.8 ± 15.9	52.4 ± 15.3	0.025
+XMKTx (n=40)	45.6 ± 17.3	50.7 ± 17.9	0.578	37.4 ± 12.6	57.9 ± 15.6	0.001	45.1 ± 17.8	52.9 ± 13.2	0.292	43.0 ± 18.6	46.1 ± 20.1	0.658	47.0 ± 18.9	45.4 ± 12.2	0.786
ABOiKTx (n=25)	51.5 ± 14.9	51.8 ± 8.8	0.96	56.0 ± 23.3	51.0 ± 11.4	0.747	48.8 ± 13.0	54.5 ± 12.4	0.278	52.9 ± 11.1	46.6 ± 13.4	0.191	50.7 ± 10.7	53.9 ± 17.5	0.67

ah- arteriolar hyaline thickening; cg-allograft glomerulopathy; ci-interstitial fibrosis; ct-tubular atrophy; cv-vascular fibrous intimal thickening

eGFR calculated using MDRD equation – expressed as Mean ± StDev and compared using Student's t-test

Figure 5.5. Longer term DCGS for transplant recipients with and without 'CG' on 1 year biopsy.

Figure 5.5A. No Chronic glomerulopathy

Kaplan-Meier Graft survival for patients who had 1 year biopsies without chronic glomerulopathy (cg) on 1 year surveillance biopsy demonstrated +XMKTx had significantly worse allograft survival than other 2 groups (p=0.003. log-rank test).

Solid line = Conventional KTx; Dotted line = ABOiKTx; Mixed line = +XMKTx.

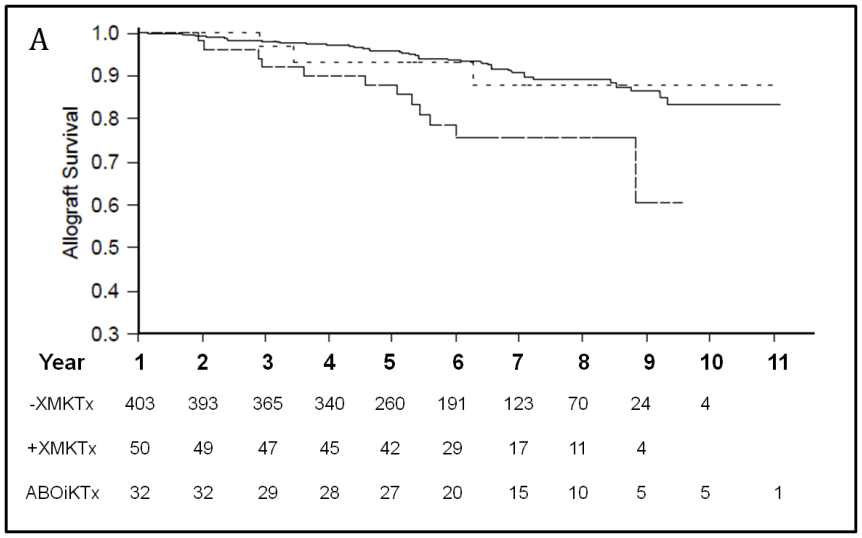
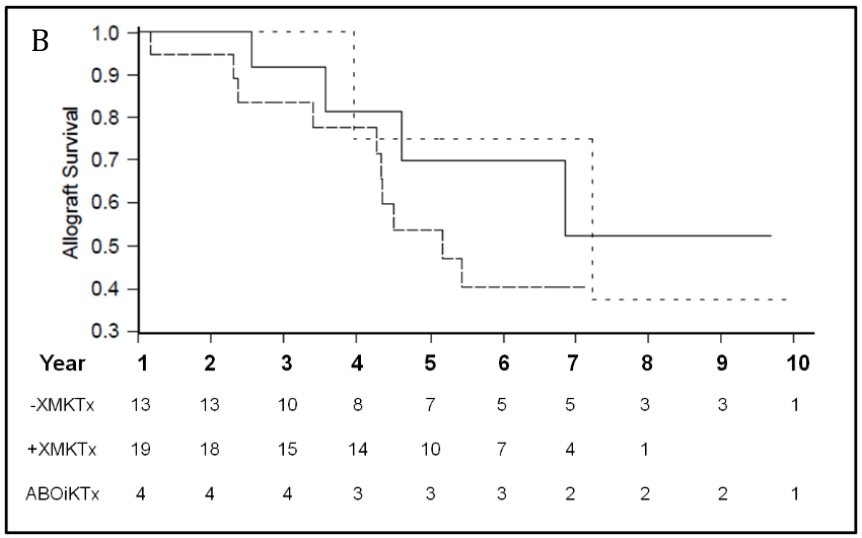


Figure 5.5B. Chronic glomerulopathy on the 1 year biopsy

In the presence of cg on 1 year biopsy, there is no difference in allograft survival between groups (p=0.470 log-rank test).



5.4. Discussion

The current study validates previous studies and extends our understanding of the outcome of kidney transplants in patients with anti-donor antibody. While some studies have demonstrated inferior outcome in +XMKTx compared to Conventional KTx and other studies have demonstrated equivalent outcome in ABOiKTx compared to Conventional KTx [72, 144, 259], no study has compared the outcomes of these 3 cohorts together in the same centre. The current study shows that the 5-year graft survival and renal function of ABOiKTx and Conventional KTx were superior to those of +XMKTx. In addition, the rate of graft loss between 1 and 5 years after transplantation—a measure of chronic injury—was similar and low in Conventional transplants (1.7%/year) and ABOiKTx (2.6%), but was significantly higher in +XMKTx (5.8%/ year).

In addition to the increased rate of graft loss in +XMKTx, data from this study suggest that surviving grafts also demonstrate more chronic injury compared to ABOiKTx and Conventional KTx. These data show however that ABOiKTx have a higher rate of early graft loss in the first year compared to Conventional KTx, but chronic injury is low despite the presence of antibody and complement activation as judged by C4d staining. +XMKTx showed lower mean eGFR and a higher percentage of patients with an eGFR<40 ml/min. +XMKTx had a higher prevalence of glomerulitis and peritubular capillaritis, which correlated with the presence and/or later development of chronic glomerulopathy.

Taken together, these data support the paradigm that chronic injury in +XMKTx follows a progression involving early evidence of peritubular capillaritis and glomerulitis followed by the development of chronic glomerulopathy months to years later which finally may result in declining renal function progressing to graft loss [163].

This study also presents new data showing that other forms of chronic injury such as fibrosis, tubular atrophy and arteriolar hyalinosis also increase in +XMKTx by 5 years after transplantation. Previous data have suggested an association with DSA and intimal thickening vasculopathy, which may explain some of these changes [260].

Finally, this study reemphasizes the lack of association between C4d deposition and chronic injury late after kidney transplantation. C4d deposition in the peritubular capillaries was extremely common in ABOiKTx at 5 years (77.8%), yet chronic injury was absent. In contrast, C4d deposition was much less common in +XMKTx at 5 years (18.9%) but evidence of inflammation and chronic injury was more common.

The light microscopic data presented in this study are similar to findings from gene expression studies performed on these cohorts of patients. In these studies, +XMKTx recipients demonstrated increased expression of inflammatory markers including cell-specific markers such as CD68, CD3 and CD20 [182]. These changes were present even in grafts that showed little signs of cellular infiltration at the light microscopic level. In contrast, ABOiKTx recipients did not show increased expression of inflammatory genes when compared to conventional kidney transplants [261].

These studies shed new light on the pathobiology of chronic renal allograft injury in recipients with anti-donor antibody at the time of transplantation. For example, the fact that chronic inflammation occurs commonly in the absence of C4d deposition and that C4d deposition does not necessarily lead to inflammation both suggest that this inflammation is not critically dependent on complement activation in the allograft. The fact that a recent study showing that peritubular capillaritis develops in +XMKTx recipients receiving terminal complement blockade with eculizumab further supports this hypothesis [136].

An active protective process termed “accommodation” has been hypothesized to prevent antibody-mediated injury despite complement deposition in ABOi renal allografts. Indeed, the lack of inflammation could be yet another manifestation of “accommodation” in which the graft protects itself from injury [177]. Anti-apoptotic genes (HO-1, Bcl-2, Bcl-xl and Bax) are upregulated in certain experimental models of accommodation [262, 263], but in previous studies increased expression of these genes classically associated with accommodation were not identified in some of the same grafts studied here [261].

Anti-blood group antibodies are predominantly IgG2 and others have suggested that differences in antibody isotype class may explain differences in rejection and non-rejection after ABOiKTx [66, 214]. These studies have involved very few patients and focused on early graft loss. However, it is possible that antibody class differences could impact on chronic injury rates. In the current study, a detailed analysis of antibody class types was not performed.

The differences in inflammation between +XMKTx and ABOiKTx may be more fully explained by a different mechanism. The immune response against carbohydrate antigens such as blood groups involves primarily antibodies and does not lead to activation of antigen presenting cells and T cells. In contrast, the response to protein antigens such as HLA proteins commonly involves both a cellular and humoral immune response. Thus, allosensitized recipients likely possess not only anti-HLA antibodies, but also a variety of alloreactive cell types including memory T cells and activated dendritic cells. When viewed from this perspective, antibody against donor HLA is only one aspect of the immune response that may or may not be important for the development of peritubular capillaritis and glomerulitis. The fact that this inflammation appears relatively independent of complement activation in the graft is consistent with

the possibility that this process may be only partially dependent on the presence of anti-donor antibody. The persistent presence of the allograft could allow for continued activation of these and other immune cells. This might explain why the prevalence of peritubular capillaritis and glomerulitis increases over time even when antibody levels in the serum decrease and C4d staining is absent [248]. This hypothesis not only may help to explain the differences in cellular infiltration and chronic injury observed in the current study, but also suggests that strategies to prevent and/or treat these processes should address the cells involved and not the antibody.

Chapter 6 Early Antibody Mediated Rejection Despite Inhibition of Terminal Complement

This chapter has been published in Transplant International [264].

6.1 Introduction

The Mayo Clinic Kidney transplant group recently demonstrated that terminal complement blockade with eculizumab, a humanized monoclonal antibody with high affinity for C5, decreased early acute antibody mediated rejection (eAMR) in positive crossmatch kidney transplantation (+XMKTx). The incidence of clinically-significant eAMR in patients treated post-transplant with eculizumab was 7.7% (2/26) compared to 41.2% (21/51, p=0.003) in a historical control group treated with a plasma exchange (PE) based protocol that was similar in both groups [136].

The goal of the current study was to investigate the possible causes of eAMR in the first month after +XMKTx in patients treated with eculizumab. Eculizumab had blocked haemolytic assays of complement activity with therapeutic levels in all 26 eculizumab treated patients in the first month after transplantation. Thirteen patients developed high levels of IgG DSA and even C4d+ biopsies which would have likely been associated with eAMR without terminal complement blockade, yet protocol surveillance biopsies in the majority of these patients did not show evidence of eAMR. Importantly, IgM DSA was detected in 4 patients after transplantation including both cases of clinical eAMR, the only case of subclinical eAMR and one case without eAMR who developed chronic injury in the first year after transplantation. These data suggest a possible role of anti-donor IgM alloantibody in the pathogenesis of eAMR in patients treated with terminal complement blockade.

6.2 Methods

6.2.1 Patient population

These studies were carried out using protocols approved by the Institutional Review Board of the Mayo Foundation and Clinic. Twenty-six consecutive +XMKTx patients were studied between 2008 and 2010 who received C5-inhibition (Eculizumab) as part of a PE-based desensitization protocol [136]. Study patients had samples taken at baseline, on the day of transplant and on post-operative days (POD) 7, 14 and 28. The primary endpoint of the original study and of this report was the incidence of biopsy-proven, clinically-significant, eAMR in the first 28 days after transplantation.

6.2.2 Desensitization Protocol

As described by Stegall et al, recipients with baseline positive B-cell flow cytometric crossmatch (BFXM) channel shift greater than 200 and less than 450 were included in the eculizumab protocol [136]. IgG DSA specificity was identified using LABscreen (One Lambda, Canoga Park, CA) on a Luminex platform with levels expressed as the mean fluorescence intensity (MFI). The protocol included pre-transplant PE if the BFXM was greater than 300 in order to reduce the pre-transplant BFXM to less than 300; low dose intravenous immune globulin (IVIG, 100 mg/kg) therapy was administered with PE; antithymocyte globulin and triple therapy of tacrolimus, mycophenolate mofetil and prednisolone [136]. PE consisted 1.0 plasma volume exchanges performed using the COBE Spectra version 7.0 software (CaridianBCT, Lakewood CO, USA) with acid citrate dextrose solution A (ACD-A) as the anticoagulant. Replacement fluid was 5% albumin (27). Eculizumab was given on day of transplant and day 1 (1200 mg and 600 mg respectively) and weekly for the first 4 weeks and continued until the BFXM was less

than 200. No protocol post-operative PE was performed after the first 2 patients; however, 2 patients did receive PE as treatment of eAMR.

6.2.3 IgM DSA Assay

Anti-HLA IgM was identified retrospectively using serum samples which were collected prior to desensitization with plasma exchange; prior to transplant (Day 0); and on POD 7; 14 and 28 in patients treated with eculizumab. This was done using a modification of the commercially available single antigen bead IgG detection assay (LABscreen, One Lambda, Canoga Park, CA, USA). In brief, 20µL serum and 3µL single antigen beads of each class specificity were incubated for 30minutes in the dark, washed, incubated with the 100µL secondary PE-conjugated anti-human IgM antibody diluted at 1:100 with luminex wash buffer(Goat Anti-Human IgM (µ) R-PE, Invitrogen Corporation, Camarillo, CA 93012) for 30minutes in the dark, washed and then re-suspended before reading. IgG results were obtained at the same time points for these samples using the clinically approved manufacturer's protocol. The positive control anti-HLA IgM control serum was a kind gift of Karen Nelson, Puget Sound Blood Center. The positive control for the IgM assay produced an MFI of >8000 each assay. An MFI >1000 was considered positive as in the IgG assay.

In order to deduce IgM specific binding, dithiothreitol (DTT, Sigma, St Louis, MO) was used to treat samples to cleave the disulphide bonds (ratio 1:1 sample volume to 0.01 M DTT, incubated at 37 ºC for 15minutes). Assessment of IgG and IgM was carried out according to the technique described above for the undiluted and post-dithiothreitol and post-dilution samples for both isotypes.

Sera was tested initially on twelve patients at all time points where complete sera samples were available, but extended to 6 further patients at time of biopsy on day

7 and day 14 who had higher levels of IgG DSA. IgG3 HLA antibodies were tested in all patients by Terasaki Foundation using LABscreen kits and a secondary anti-human IgG3 PE-conjugated antibody (IgG3 clone HP6050, Southern Biotech, Birmingham, AL) [265].

6.2.4 Efficacy of C5 inhibition

The efficacy of eculizumab therapy was assessed using two in-vitro assays including: 1) serum drug levels using a validated enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab (PK levels) and; 2) activity as determined by the ability of the eculizumab-treated patient's serum to lyse chicken erythrocytes in a validated total human serum–complement hemolytic assay (PD levels) [266, 267]. These methods are described below.

6.2.4.1 Drug levels (PK) ELISA binding assay

For detection of recombinant Fab binding to human C5, F96 PolySorp microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 0.1 mg/well human C5 (Quidel, San Diego, CA, U.S.A.) at a concentration of 2 mg/ml in 0.1 M Na,CO_3, pH 9.6. The plates were then washed three times with 100 µl/well wash buffer (PBS containing with 0.5% (v/v) Tween 20) and blocked with 100 µl/well blocking buffer (PBS supplemented with 1% (w/v) bovine serum albumin, fraction V and 0.5% (v/v) Tween 20) at 37°C for 1 hr. The plates were again washed three times with wash buffer and incubated with 50 µl/well blocking buffer, containing 30 ng/ml murine 5G1.1 mAb, plus the indicated concentrations of inhibitor, at 37°C for 2 hr. The plates were again washed three times with wash buffer, and incubated with 50 µl/well blocking buffer, containing peroxidase conjugated goat anti-mouse IgG Fc antibody, at a 2000- fold dilution (Sigma, St. Louis, MO, U.S.A.), at 37°C for 1 hr. After three final washes, the plate was developed with 50 µl/well substrate buffer (0.05 M phosphate-citrate buffer, pH 5.0, containing 0.3mg/ml

sodium perborate and 0.4 mg/ml σ-phenylenediamine dihydrochloride). Reactions were stopped by the addition of 50 µl/well 1 M sulfuric acid and quantified using a Bio-Rad model 3550 plate reader set at 490 nm.

6.2.4.2 Haemolytic assay

Haemolytic assays to determine the complement activity in samples were performed as described above on frozen serum samples that were rapidly thawed at 37°C immediately before the assay. Human serum was diluted to 20% vol/vol with gelatin veronal-buffered saline buffer (GVB 2+: 0.1% gelatin, 141 mM NaCl, 0.5 mM MgCl2, 0.15 mM CaCl2, 1.8 mM sodium barbital [Sigma Chemical Co., St. Louis, MO]) buffer and added (50 µl/well) to the rows of the same 96-well plate such that the final concentration of human serum in each well was 10%. The plate was then incubated at room temperature for 30 min while chicken erythrocytes (Lampire Biological Laboratories, Piperville, PA) were washed five times with 1 ml of GVB 2+ buffer and re-suspended to a final concentration of 5 x 10^7/ml in GVB 2+ buffer. 4 ml of the chicken erythrocytes were sensitized by the addition of an anti-chicken red blood cell polyclonal antibody (0.1% vol/vol, Intercell Technologies, Hopewell, NJ), and the cells were incubated at 4°C for 15 min with frequent vortexing. The cells were then washed twice with 1 ml of GVB 2+ buffer and re-suspended to a final vol of 2.4 ml in GVB 2+ buffer. 30µl aliquots of chicken erythrocytes (2.5 x 106 cells) were added to the plate human serum samples and mixed well. Then these were incubated at 37°C for 30 min. Each plate contained two additional wells of 30 µl of identically prepared chicken erythrocytes, one incubated with GVB 2+ buffer alone (negative control) as a control for spontaneous haemolysis and the other containing 0.1% nonyl phenoxypolyethoxylethanol (NP- 40 [Sigma]) serving as a control for 100% lysis. The

plate was then centrifuged at 1,000 g for 2 min and 85 µl of the supernatant transferred to a new 96-well plate. Haemoglobin release was determined at OD 415 nm using a microplate reader (Bio-Rad Laboratories, Richmond, CA), and the percent haemolysis was determined using the following formula:

Percent haemolysis = 100x (OD sample - OD negative control)/(OD 100% lysis - OD negative control)

6.2.5 C1q binding of IgG DSA

The Bio-C1q assay is a modification of the commercially available method and was performed as described in [268]. It detects both IgM and IgG. Briefly, 10 µl of serum, absorbed with beads (Spherotech, Lake Forest, IL), was spiked with 10 µl (0.1 mg/ml) purified human C1q (Sigma) biotinylated in house (Bio-C1q) and incubated with 2.5 µl of LabScreen Single Antigen Bead (SAB) for 30 min at room temperature (rt) followed by 10 µl of phycoerythrin-conjugated streptavidin. After an additional 20 min rt incubation, the beads were washed X2 with wash buffer and analysed on a Luminex instrument. Data were analyzed by HLA Fusion software. All reagents and software except absorption beads and Bio-C1q were obtained from One Lambda, Inc..

6.2.6 Criteria for AMR and Histologic Assessment

Renal allograft biopsies were obtained percutaneously using ultrasound guidance, and submitted for routine light microscopy, immunofluorescence for C4d, and electron microscopy. All biopsies were reviewed by a pathologist and AMR was diagnosed using standard Banff criteria in combination with graft dysfunction (increase in serum creatinine ≥0.3 mg/dL over nadir) [142].

6.2.7 Statistical Analysis

Data were analyzed using JMP 9 (SAS, Cary, NC). Continuous normally distributed data were expressed as mean ± standard deviation, non-parametric data by median and range, and nominal data by counts and percentages The data were compared using a Student's *t*-test for normally distributed data, otherwise non-parametric tests were applied (Wilcoxon rank-sum test,). Normality of distribution of variables was tested using Shapiro-Wilk test. A p value of <0.05 was considered significant.

6.3 Results

These patients demographics have been described previously in published data by Stegall et al [136]. Table 6.1 displays the demographics of the 26 patients in this study cohort. All 26 +XMKTx recipients received eculizumab for at least 1 month post-transplant—the duration of the primary endpoint of the original study.

Two patients developed acute clinical eAMR in the first month and one had subclinical eAMR on a 1 week protocol biopsy. None of the other patients showed evidence of eAMR on biopsy (all patients underwent at least 1 protocol biopsy during the first month). Three patients developed graft dysfunction and their biopsy did not demonstrate eAMR. Renal function in these patients improved without specific DSA-reduction therapy. No episodes of eAMR were diagnosed in months 1 to 12. All patients had functioning grafts at 1 year after transplantation

6.3.1 Antibody-Mediated Rejection Episodes

Two patients who met criteria for eAMR (Table 6.2) had graft dysfunction with the classic triad of AMR (one on post-operative day (POD) 7 and another on POD 14) that included: 1) a biopsy consistent with antibody-mediated damage on light microscopy

(Banff 97 Level II or III AMR); 2) C4d+ staining of the peritubular capillaries and 3) circulating DSA by both B flow cytometric crossmatch and total IgG DSA.

The first patient that developed eAMR had a surveillance biopsy on day 4 which was C4d negative and showed only mild acute tubular injury without inflammation or other features of AMR. On day 7, the serum creatinine rose from 2.1 mg/dl to 2.7 mg/dl in 24 hours and the biopsy demonstrated glomerular thrombi, endothelial swelling, mesangiolysis and glomerular neutrophil margination with acute tubular injury and the peritubular capillaries showed diffuse dim staining for C4d. Electron microscopy showed endothelial swelling with loss of fenestration and peritubular capillary endothelial cell swelling. Treatment consisted of PE (daily for 12 days), ongoing eculizumab (600 mg after each PE session) and low dose IVIG. The serum creatinine decreased to 1.9 mg/dl by day 21. This patient, at one year and two years of follow up, had creatinine values of 1.7 mg/dl and 2.0 mg/dl respectively (eGFR MDRD 34ml/min and 28ml/min).

The second patient with eAMR had a surveillance biopsy done on day 7 which did not show any evidence of tissue injury or inflammation by light microscopy, although there was diffuse C4d deposition in the peritubular capillaries. Electron microscopy did show segmental endothelial cell swelling of glomerular and peritubular capillaries. On POD 11, the serum creatinine rose to 1.3 mg/dl and on day 14 a biopsy was obtained that demonstrated glomerular microthrombi, interstitial hemorrhage; glomerulitis and peritubular capillaritis in addition to diffuse C4d deposition in the peritubular capillaries. Electron microscopy showed diffuse swelling of endothelial cells in glomerular and peritubular capillaries (Figure 6.1). Treatment consisted of PE (9 days), low dose IVIG and continued eculizumab (600 mg after each PE). The 1 year and 2 year

serum creatinine measurements were 1.5 mg/dl and 1.6 mg/dl (eGFR 38ml/min and 35 ml/min), respectively.

A third patient met criteria for "subclinical AMR" who had high levels of DSA (BFXM >360), C4d+ staining and a biopsy consistent with antibody-mediated injury (peritubular capillaritis and glomerulitis on POD 7), but did not develop graft dysfunction during the first 28 days after transplantation, and did not receive additional therapy.

Table 6.1. The Baseline Characteristics of the Eculizumab Study Cohort

	Eculizumab Cohort N=26
Age (years) Mean±StDev	47.8 ± 12.3
Gender (Female) n(%)	21(80.8%)
Ethnicity=Caucasian n(%)	26(100%)
Retransplant n(%))	13 (50%)
ABDR mismatch Mean±StDev	3.7 ± 1.3
Pre-emptive n(%)	4 (15.4%)
Dialysis time (months) Median (Q1-Q3)	21.0 (15-32)
Living donor n(%)	26(100%)
BFXM Channel Shift Mean±StDev	333 ± 112
BFXM >360 Channel Shift n(%)	10 (38.5%)
Anti-Class I antibody only n(%)	9 (34.6%)
Anti-Class II antibody only n(%)	3 (11.5%)
Both anti-Class I & anti-class II antibody n(%)	14 (53.8%)
Baseline Class I MFI Mean±StDev	7175 ± 4808.
Baseline Class II MFI Mean±StDev	8899 ± 3521
Patients with Pre-transplant apheresis	19 (73.1%)
Pre-Transplant Apheresis. Mean±StDev	6.7 ± 3.3

Data expressed as Mean ± St Dev for normally distributed data, tested using Shapiro–Wilk test, otherwise expressed as median (Q1-Q3)

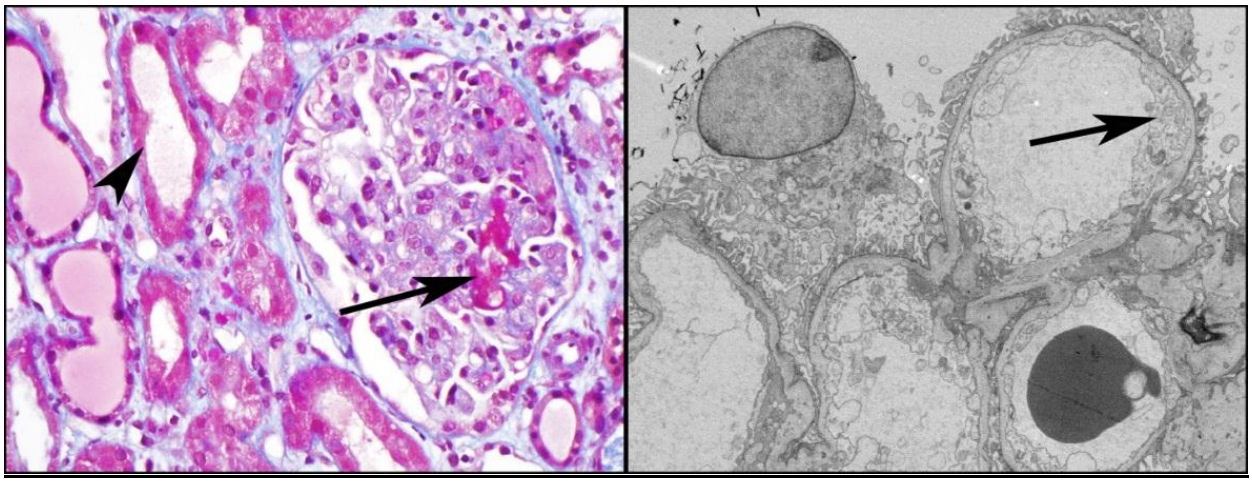
Table 6.2 Post-Transplant Clinical Characteristics of patients with eAMR and those without eAMR

	N	Graft Dysfunction[1]	DSA[2]	C4d Deposition	Histologic Injury
AMR	2	2	IgG and IgM	Yes	Yes[3]
Subclinical AMR	1	No	IgG and IgM	Yes	Yes[6]
No AMR	23				
Normal Histology Low DSA[4]	15	2	IgG	11/15	None
Normal Histology High DSA[5]	8	1	IgG	7/8	None

[1]Increase in serum creatinine ≥0.3 mg/dl (27micromol/L) over baseline during the first month. [2]DSA is a positive BFXM and/or DSA by solid phase assay that is either IgG or IgM. [3]Glomerular microthrombi, mesangiolysis and/or peritubular capillaritis. [4]Low DSA means highest post-transplant BFXM <360. [5]High DSA means highest post-transplant BFXM ≥360. [6]Capillaritis only

Figure 6.1. Histology of eAMR in patient with eculizumab.

Histological evidence of early AMR in Eculizumab-treated patients with IgM DSA. By light microscopy, a glomerulus shows a thrombus (arrow) and acute tubular injury (arrowhead)(left panel). By electron microscopy, glomerular endothelial cells are enlarged (arrow) and show loss of fenestrations and microvillous change.



6.3.2 Eculizumab Levels

Serum eculizumab drug levels were therapeutic (>25μg/ml) in all patients and the hemolytic assay was blocked (<20% haemolysis), indicating effective complement blockade. There was no significant difference in either drug level or hemolytic lysis score between the 3 patients with rejection and the 23 patients without rejection (see Figure 6.2).

6.3.3 DSA Levels and AMR

It has previously been shown that high DSA levels after +XMKTx correlate highly with the development of acute AMR in patients not treated with eculizumab [136]. In the current eculizumab study, DSA levels were allowed to increase without PE. Eculizumab treatment did not appear to prevent the development of high levels of DSA in that in the first month 38.5% (10/26) of eculizumab treated patients develop a BFXM channel shift >360—a level associated with AMR in 100% of historical controls from Stegall et al (see Figure 6.3)[136]. Two of the three cases of AMR (one clinical and one subclinical) had a BFXM >360 and in the other the highest BFXM was 303. This patient's highest IgG DSA was against Cw7 which can be poorly expressed on lymphocytes. Thus, 8 patients treated with eculizumab who did not develop rejection would have been predicted to have AMR based on historical data. In addition, all 10 of the biopsies were C4d+ (with high levels of DSA - IgG DSA SAB, median 10453, Q1 3480-Q3 11710; BFXM median 445, Q1 409-Q3 544) consistent with antibody-dependent complement activation of the allograft.

A more detailed analysis of DSA levels by Ig type is presented in Figure 6.4. This shows that high levels of DSA were present at some time point in the first month after transplantation in both rejecting and non-rejecting patients. Specifically, high levels of

total IgG (as commonly measured in the LABscreen SAB assay), IgG3 and C1q+ binding were detected in both groups and none were associated with the development of eAMR (Comparison were not significant with p=0.279; p=0.799 and p=0.711 respectively).

Figure 6.2. Eculizumab drug levels and complement blockade assay results in the cohort

Hemolytic assays showed almost complete blockade (left panel) and eculizumab levels were therapeutic (right panel) in all patients including at the time of AMR in the two patients who developed AMR (open circles) and non-rejections (closed diamonds). Actual levels expressed as median (Q1-Q3).

Table A		Serum Haemolytic Assay (% haemolysis)			
		Week 0	Week 1	Week 2	Week 4
Rejection	Median (Q1-Q3)	95 (93-97)	9 (0-18)	1 (0-2)	28 (1-56)
No Rejection	Median (Q1-Q3)	82.5 (31-130)	2.5 (1.3-5.8)	1 (0-3)	1 (0-2)

Table B		Eculizumab Levels (µg/ml)			
		Week 0	Week 1	Week 2	Week 4
Rejection	Median (Q1-Q3)	0 (0-0)	110.1 (92.8-127.3)	166.4 (147.8-185)	154.8 (43.2-266.4)
No Rejection	Median (Q1-Q3)	0 (0-0)	111.5 (83.0-182.3)	131.7 (92.5-182.6)	162.5 (107-193.5)

Figure 6.3. Eculizumab does not prevent the development of high levels of alloantibody after transplantation

Figure 6.3A. BFXM Channel Shift of patients following treatment with Eculizumab with a high DSA level (BFX>360) in the first month after transplant. Rejecter in Red Dashed line(- - - -); Continuous lines are non-rejecters(___); Black Dotted line is the subclinical rejection patient(…….).

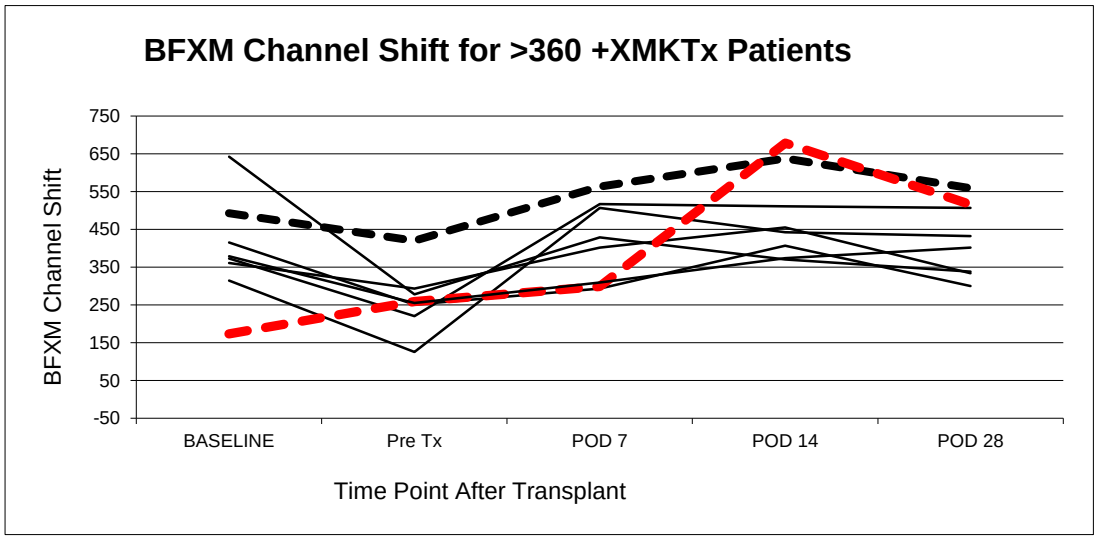
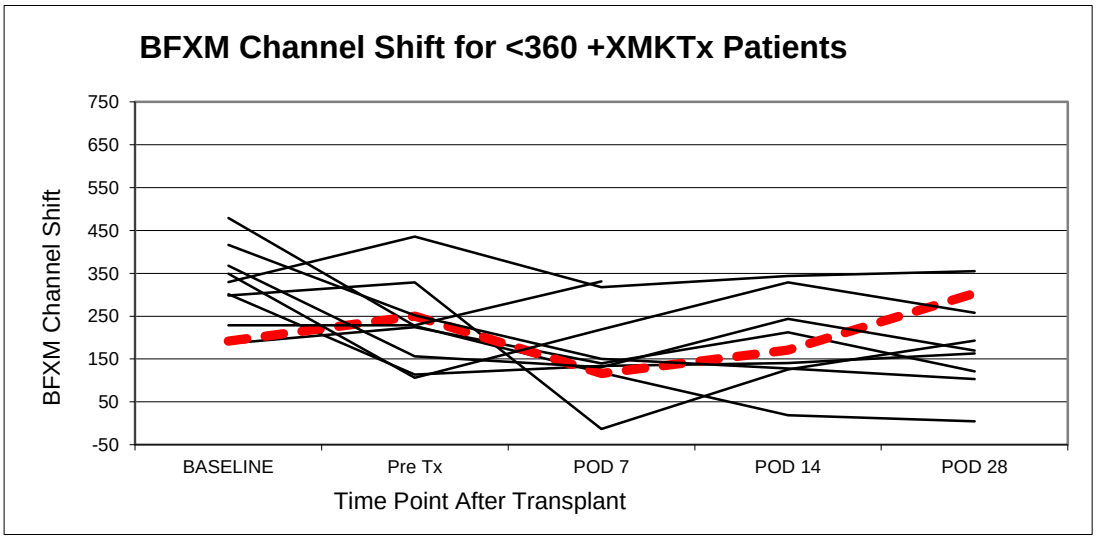


Figure 6.3B. BFXM following transplant with BFXM<360. Patient with rejection had indistinguishable levels from rest of cohort. Rejecter in dashed line(- - - -); Continuous lines are non- rejecters(___).

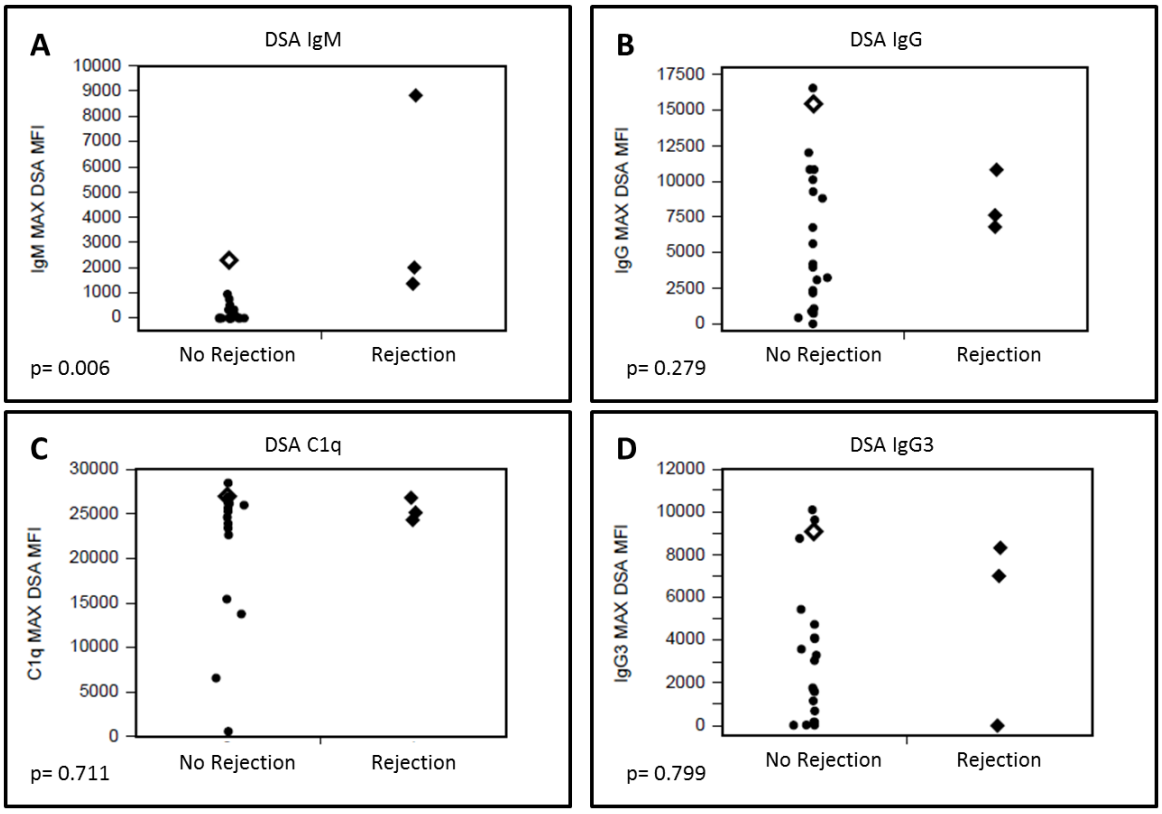


In contrast, IgM DSA was detected in only 4 patients after transplantation including: the two patients with eAMR (maximum MFI levels of 1721 against HLA-B8 and 8816 against HLA-DR7), the patient with subclinical eAMR (maximum MFI = 1997 against HLA-DQ8) and a patient who had normal biopsies throughout (maximum MFI = 2320 against HLA-DR53). Comparison between rejecters and non-rejecters demonstrated a significantly higher IgM DSA MFI (median 1997 [1344-8816] vs. 0[0-2320], p=0.006 Wilcoxon rank-sum test). Interestingly, this 4th patient also had persistently high DSA that necessitated continued treatment with eculizumab for 1 year according to study protocol. This patient developed transplant glomerulopathy on a 7 month biopsy and his allograft failed at 15 months after transplantation.

Patient #1 had increased levels of IgM DSA (anti-B8 and anti-Cw7) at day 7 correlating with eAMR. Plasma exchange started on Day 7 rapidly reversed the eAMR episode and led to reduced IgM DSA. IgG DSA continued to rise despite the resolution of AMR both clinically and on follow-up biopsy. Patient #2 showed an increase in IgM DSA at day 7 correlating with early peritubular capillaritis and then subsequent developed clinical AMR on day 14. Again, the eAMR episode was easily treated with plasma exchange and IgM quickly was reduced while IgG DSA continued to rise. These data are shown in Figures 6.5A and 6.5B. Figure 6.5C shows a DSA pattern typical of a patient with persistently high IgG DSA (MFI>8,000) without IgM DSA who did not develop eAMR. There was no significant difference in IgG3 DSA levels over the first 1 month after transplant in rejecters and non-rejecters (Figure 6.5D).

Figure 6.4. Comparison of levels of anti-donor antibody during the first month post transplantation.

This demonstrates significantly higher levels in rejecters for IgM DSA (A), but no difference in IgG DSA (B); C1q DSA (C) or IgG3 DSA (D). Patients with Rejection are with full diamonds, the one patient with no rejection, but accelerated transplant glomerulopathy is in open diamond, the non-rejecters are in closed circles.



Non-parametric data compared using Wilcoxon rank-sum test.

Figure 6.5. Anti-HLA antibody patterns in patients who developed early AMR and one without AMR.

Figure 6.5A. AMR Patient #1 DSA Levels

Increased levels of IgM DSA (anti-B8 and anti-Cw7) at day 7 correlating with early AMR (solid arrow). Plasma exchange started on Day 7 rapidly reversed the AMR episode and led to reduced IgM DSA but IgG DSA continued to rise.

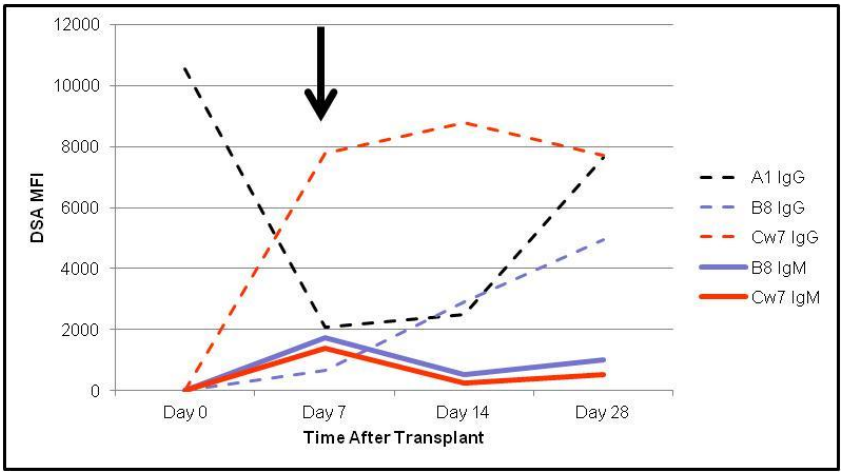


Figure 6.5B. AMR Patient #2 DSA Levels.

Increase in IgM DSA at day 7 correlating with early peritubular capillaritis (dashed arrow) and then subsequent early AMR (solid arrow) in patient 2. Again, the AMR episode was easily treated with plasma exchange and IgM quickly was reduced while IgG DSA continued to rise.

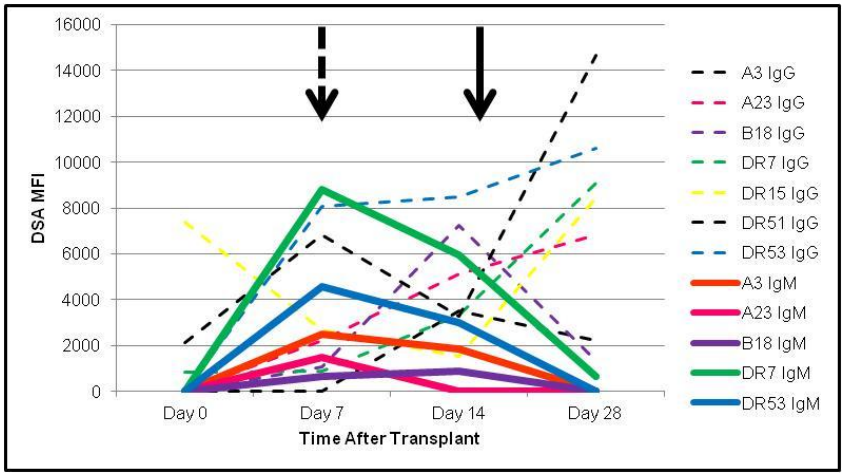


Figure 6.5C. High levels of IgG DSA without IgM DSA in a patient who did not develop AMR. Typical DSA pattern in a patient with persistently high IgG DSA and C4d deposition on surveillance biopsy who did not develop AMR and did not have IgM DSA.

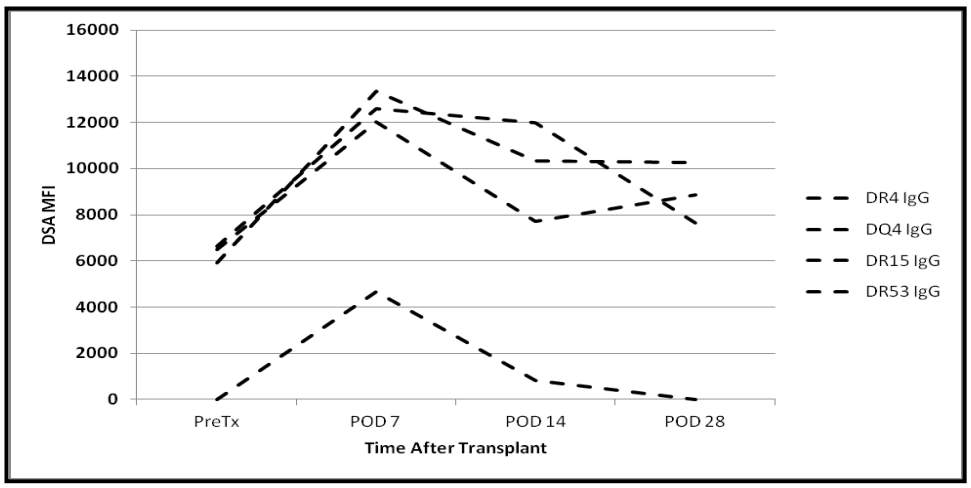
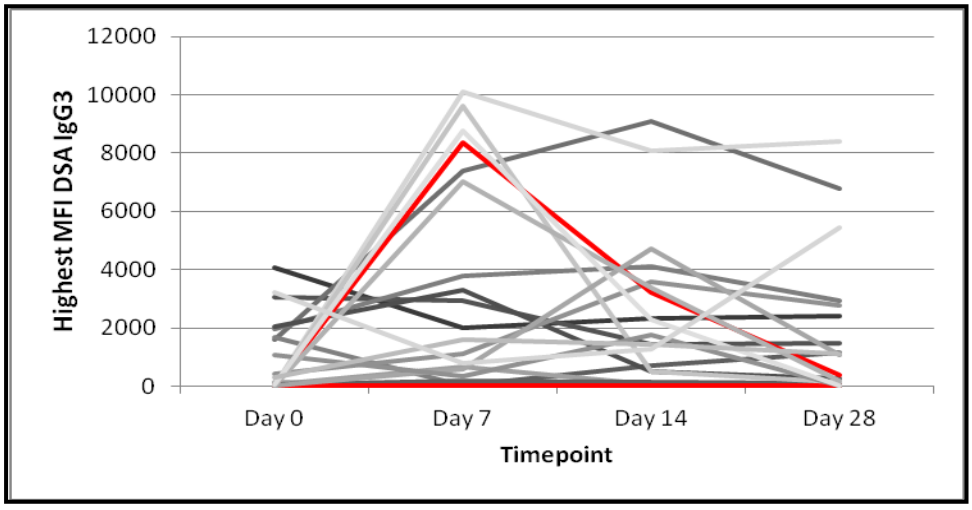


Figure 6.5D. Levels of IgG3 in patients with AMR and without AMR do not differentiate. The level of IgG3 DSA subclass does not determine early AMR (red lines) against no early AMR (grayscale lines) plotted for each patient within the first month post transplant.



6.4 Discussion

Terminal complement inhibition significantly reduces the rate of acute clinical AMR in +XMKTx patients with high levels of DSA and evidence of complement deposition in the allograft, which suggests that most cases of AMR are C5-dependent [136]. However, despite C5 inhibition being therapeutic and functionally adequate, 2 +XMKTx recipients who received eculizumab developed clear-cut, clinically-significant AMR and another developed subclinical AMR. All 3 of these patients had IgM DSA detected and only 1 of 23 patients without AMR had IgM DSA. That the 2 episodes of eAMR were reversed easily with PE which more effectively removed IgM without reducing IgG levels further supports the concept that IgM DSA might be a possible causative agent in early AMR independent of C5. Importantly, high levels of total IgG DSA and complement-binding DSA (IgG3 subclass and C1q+) were commonly present in recipients who did not have eAMR further supporting the concept that complement binding IgG was not the mediator of eAMR in eculizumab-treated patients.

These data do not suggest that IgM is the only possible mechanism of AMR in eculizumab-treated patients. Further studies of larger numbers of patients might reveal other possible mechanisms. For example, it is possible that high levels of DSA might be sufficient to cause forme fruste AMR in some patients via either direct endothelial cell activation or via infiltration of cells via FcγR binding to IgG bound to the allograft. Yet the data from the current study did not suggest either of these mechanisms were a common cause of early clinical AMR.

IgM will not be the major cause of most AMR in +XMKTx recipients not treated with eculizumab, where in the absence of terminal complement blockade, it is likely that IgG DSA is the major cause and its blockade is the major reason for the reduced incidence of AMR in eculizumab treated patients. However, IgM DSA may be capable of causing

eAMR in specific situations. Techniques to identify IgM in serum have varied over time and the resulting conclusions from these studies have ranged from the irrelevance of IgM to a protective mechanism to being associated with poor outcomes [269-273]. More recently, IgM DSA binding complement was associated with AMR without IgG DSA in cardiac transplantation [157]. One of the factors that may have contributed to this confusion is that different methods have been used to identify IgM and few have been able to identify HLA specificities clearly [55, 60, 129, 274, 275].

IgM commonly has been associated with early B cell responses, but IgM DSA production can be long-lived similar to that of IgG production and can be produced in a memory B cell response [276, 277]. In the current study, 15.4% (4/26) had IgM DSA and all had the same specificity as the IgG DSA. The coexistence of IgM and IgG with the same HLA specificity has been previously reported [278].

One patient in this study had increased IgM after transplantation, but did not develop eAMR. The reason for the lack of AMR in this patient is unclear, but underlies the fact that not all IgM may be capable of causing eAMR. While this patient went on to develop chronic AMR by 1 year, the current study cannot clearly address the role of IgM in chronic injury because C5-independent processes may contribute to chronic injury. It has been previously shown that a BFXM channel shift > 360 post-transplant was associated with AMR with a sensitivity of 100% and a specificity of 95.4% in +XMKTx who develop AMR [70]. Since, approximately 1/3 of all transplanted patients in the eculizumab-treated patients in the current study had these levels, and it would have been expected that they develop AMR with high DSA, yet only 1/26 did. The other case had a relatively low BFXM, but high IgG DSA by single antigen beads against an unusual HLA-Cw7 which is known to be variably expressed on lymphocytes. The IgG3 DSA was not detectable for this allele on solid phase assay, but had high levels of C1q binding.

Interestingly, in this case, the increase in IgM DSA preceded the development of IgG DSA, and the episode of AMR occurred when the IgM DSA was rising.

It is possible that DSA can injure the allograft via complement-independent mechanisms. For example, anti-HLA antibody can directly activate endothelial cells in vitro without complement and NK cells may attach to membrane-bound IgG via their Fcγ receptor. However, these mechanisms do not explain why some patients with high levels of IgG DSA developed AMR and others did not [279].

IgM might cause AMR via several mechanisms. For example, IgM has a higher affinity for C3 (not blocked by eculizumab) and this may significantly increase the local effect of C3 and its breakdown products [280]. The increased number of C3 binding sites on IgM compared to IgG leads to increased inflammation and tissue damage sufficient to cause histologic changes of AMR and graft dysfunction and altered monocyte infiltration [281, 282]. C3a, an anaphylatoxin, increases inter-endothelial cell dilatation and this increase gives rise to the pro-thrombotic process. The exposure of inter-endothelial gap junctions leads to increased intravascular coagulation on endothelial surfaces due to the increased expression of tissue factor and plasminogen activator inhibitor type I via complement activation [283]. C3a also leads to neutrophil adhesion, chemotaxis and cellular allograft infiltrate [284]. Local tissue injury leads to increased C3 production, further increasing the local inflammation and cellular infiltrate [285]. C3b produced binds to endothelium and attracts leukocytes which express complement receptors [286]. C3b also amplifies the alternate complement pathway, as C3b bound to tissue is not inhibited by regulatory proteins [287]. The increase in C3b activates C3 convertase, as C3b becomes an active protein, thus increasing the presence of C3a in the allograft [288]. Furthermore, IgM- mannose binding lectin (MBL) complexes can initiate cell injury. Without the presence of C1q, it has been demonstrated that binding of MBL can

initiate cell injury and lead to increased C3 deposition. This would increase the cell injury through the C3 pathway described above, in addition to the classical pathway [289].

These preliminary data suggest that there is a possible role of IgM DSA in the pathogenesis of AMR in patients treated with terminal complement blockade. These data also suggest a testable hypothesis in that monitoring IgM DSA and preemptive PE might further reduce AMR in eculizumab-treated patients.

Chapter 7 Conclusion

Antibody incompatible kidney transplantation allows transplantation in a select group of patients to improve clinical outcomes compared to remaining on dialysis. Whilst the outcomes are inferior to antibody compatible live donor transplantation, there is a role for antibody incompatible transplantation; however, the risks and benefits need to be explained to patients. This thesis provides more information to guide clinical decisions.

7.1 Summary of main research findings and limitations

The assessment of anti-ABO specific antibodies can be measured with good reproducibility by a single user in agglutination and a defined protocol using haemagglutination. This allowed comparison between centres for ABO titres and demonstrated a wide range of local titre results in centres which was associated with more antibody removal therapy. There was a similar distribution of ABO titres in the major centres when compared using the central assay. Comparative studies would be possible in these cohorts; however, the treatment needs to be guided by a standardised assay, so that treatment variations are based on clinical parameters and not assay variability.

The development of a synthetic assay on microspheres has yielded a sensitive, but not specific assay; however, the same trisaccharide structures on the SPR platform demonstrate binding specificity. The use of different oligosaccharides was limited to the availability of oligosaccharide provided by Dextra Ltd. There were intellectual property restrictions on obtaining tetrasaccharides with the 8-carbon linker to be able to compare different binding characteristics between the tri- and tetrasaccharides. Further work would develop the SPR assay to differentiate binding characteristics of similar ABO titres, and the impact on clinical events, including antibody removal and rejection.

In the ABOUT-K study, the rate of early antibody-mediated rejection was low and a larger cohort is needed to be able to correlate assays with clinical events.

The clinical outcome data in the ABOUT-K study demonstrated that ABOiKTx can be performed safely and effectively with equivalent outcomes to ABO compatible live kidney donation transplantation. There was a greater incidence of acute rejection in this cohort than is widely reported; however this did not affect allograft survival. The rate of viraemia and associated transplant viral nephropathy is in keeping with published data, but needs further investigation to determine whether the therapy given or the nature of ABO incompatibility increases the risk of viral nephropathies, particularly BK nephropathy. The prospective assessment of data and local anti-ABO specific antibodies was limited by local adherence to protocol and compliance with study protocol from local transplant centres. The ABOUT-K study cohort was a descriptive cohort, and an equivalent control cohort from the same centres for ABOcKTx would provide a good comparative group for data analysis.

A major risk factor for allograft loss in positive crossmatch (HLAiKTx) transplantation was the presence of anti-Class II antibody at time of transplantation and the onset of early antibody-mediated damage on histology before there was a change in allograft function. Patients with anti-Class I DSA had equivalent 5-year allograft survival compared to the –XMKTx control cohort, and thus could be an acceptable DSA in live donor exchange, while avoiding Class II DSA. The Mayo Clinic antibody incompatible data may not be transferable to other centres, as patients were referred from different centres across the USA which would not transplant these patients due to high immunological risk. This creates a selection bias of highly sensitized patients. The analysis of this clinical cohort from the Mayo Clinic describes a cohort of patients with heterogeneous treatment regimens during which the experience guided further

development in techniques for +XMKTx. The retrospective nature of the analysis of serum for DSA informs assessment, however the continual development of the single antigen bead assay leads to difficulties in application of these data to current populations. The development of the assay has led to increases in the number of specificities to HLA antigens (for example Cw and DP) able to be identified over time and thus may reveal different antibody data on more recent single antigen bead kits. Furthermore, the recent assays also assess C1q binding for each DSA for which the current dataset had not been measured. The presence of C4d staining as part of the diagnosis for antibody-mediated rejection is not associated with the same cellular infiltration in ABOiKTx compared to +XMKTx recipients. The cellular infiltration is neither associated with the activation of the classical complement cascade nor the degree of early rejection in these cohorts. The use of eculizumab in patients in Chapter 6 was given under pharmaceutical sponsorship of this study. There would be significant financial implications for using this drug in a larger cohort of patients and thus use of this as a routine protocol therapy is unlikely to be implemented. The clinical effectiveness of reducing early AMR is apparent, but it does demonstrate that despite preventing acute changes, the chronic infiltrate associated with DSA still occurs. Glomerulitis and capillaritis occur earlier with IgM DSA present; by removing IgM with plasma exchange it may be possible to test this hypothesis for reducing early tissue injury after transplantation.

In conclusion, this thesis demonstrates that despite antibody incompatible kidney transplantation carrying a higher risk than ABO compatible live donor kidney transplantation, there are sub-populations of patients that will benefit from antibody incompatible transplants, whilst other higher risk patients may benefit from alternative pathways to transplantation.

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Appendix

8.1 Solutions and Buffers

8.1.1 Luminex

Preparation of PBS

Preparation of PBS solution		
PBS	150mM	NaCl (MW 1.71g)
	25mM	Phosphate (MW 0.78g)
Adjusted to pH 7.4	5M NaOH	
De-ionised water		Total Volume 200ml

Preparation of Luminex Wash Buffer (LWB):

PBS (As prepared above)	95ml
Fetal Calf Serum (4.9%), (F6178 Sigma. UK)	4.9ml
Sodium Azide (0.1%), (438456, Sigma. UK)	0.1ml

Non-denaturaturing Lysis buffer

Stock	Need	Use	
1M TRIS-HCL pH 8	20mM	20ml	
5M NaCl	137mM	27.4ml	
100% Glycerol	10%	100ml	
100% NP-40	1%	10ml	+838.6ml Distilled Water
0.5M EDTA	2nM	4ml	
Store for up to 6months at 4°C			

PVA and PVP formation

For suppression of non-specific binding, after Waterboer et al [224].

PVA	0.5% Polyvinylalcohol (P8136, Sigma Aldrich)	0.5g	1000ml PBS
PVP	0.8% Polyvinylpyrrolidone (PVP-360 Sigma Aldrich)	0.8g	1000ml PBS
PVX	1: 1 ratio of PVA and PVP	50ml PVA	50ml PVP

For each solution, agitated until no residual components are seen.

Marvel Preparation

Marvel prepared at 5% w/v in Distilled Water.

Serial dilutions of 5%; 2.5% and 1.25% were made in distilled water.

8.1.2 Flow Cytometry

Flow Cytometry Diluent prepared as per Luminex Wash Buffer

PBS (As prepared above)	95ml
Fetal Calf Serum (4.9%) (F6178 Sigma. UK)	4.9ml
Sodium Azide (0.1%) (438456, Sigma. UK)	0.1ml

8.1.3 Surface Plasmon Resonance

SPR Running Buffer

This was prepared using the following chemicals. After mixing, the solution was de-aerated using a suction filter before running on the SPR machine.

Constituent	Volume	Final Concentration in 1L
2M NaCl	75ml	0.15M
0.5mM HEPES	20ml	0.01mM
0.5M EDTA	6.3ml	3.15mM
Tween 20	5ml	0.5%

In order to conjugate trisaccharides to the SPR chip, the trisaccharides were dissolved in 1M Boric acid (Sigma, UK) was made using 15g in 250ml of PBS and adjusting the pH to pH 8.5 using 1M NaOH

8.2 Reagents

Sigma

P8136-250g Poly(vinyl alcohol)

PVP360-500g Polyvinylpyrrolidone

E6383 *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride

56485-250mg N-Hydroxysulfosuccinimide sodium

S7148 Type AB, frozen liquid (Sigma)

F1641-2ML Goat Anti-Human IgG (γ-chain specific), F(ab′)2 fragment–FITC antibody

P5413 Poly Ethylene Glycol (PEG)

Southern Biotech

1140-09	Rat anti-mouse IgM R-phycoerythrin (R-PE) conjugate
9042-09	Mouse Anti-Human IgG R-phycoerythrin (R-PE) conjugate
9022-09	Mouse Anti-Human IgM R-phycoerythrin (R-PE) conjugate
9080-02	Mouse anti-human IgG2 FITC
9210-09	Mouse anti-human IgG3 R-PE
9190-01	Mouse anti-human IgG4 purified

DAKO

X0902 Rabbit Serum

Millipore

NI – BIOSCOT® Core Anti-A (clone Birma-1) FFMU

NJ – BIOSCOT® Core Anti-B (clone LB-2) FFMU

Chemiblock™ - SUPERCHEMIBLOCK HETEROPHILE BLOCKING AGENT (10.5mg/ml)

Luminex

L100-S011-01	SeroMAP™ Microspheres

BIORAD

171-506021	Bio-Plex COOH Bead # 21, 1 ml
176-5011	ProteOn GLC Sensor Chip
176-5012	ProteOn GLM Sensor Chip
170-5013	GLH Sensor Chip ProteOn System
176-6010	ProteOn Sample Vials
176-2410	ProteOn Amine Coupling Kit
176-2810	ProteOn Chip Normalization Sol
100405	Anti-A1 Lectin

AbD Serotec

LNK073PERCP LYNX RAPID PerCP ANTIBODY CONJUGATION KIT

MACS

130-093-113 Anti-human IgA – APC

130-093-189 Anti-human IgG1 - APC

Dextra Laboratories

Blood Group A Series

L305 Blood Group A trisaccharide

GalNAcα1-3Gal 2 | Fuc α 1

$C_{20}H_{35}NO_{15}$ FW 529.50 CAS: 49777-13-1

L306 Blood Group A trisaccharide amine derivative

GalNAcα1-3Galβ-1-O$(CH_2)_7$CONH$(CH_2)_2NH_2$ 2 | Fucα1

Blood Group B Series

G323 Blood Group B Trisaccharide

Galα1-3Gal 2 | Fucα1

$C_{18}H_{32}O_{15}$ FW 488.44 CAS: 49777-14-2

G324 Blood Group B trisaccharide amine derivative

Galα1-3Galβ-1-O$(CH_2)_7$CONH$(CH_2)2NH_2$ 2 | Fucα1

$C_{28}H_{52}N_2O_{16}$ FW 687.73

Blood Group H (O) Series

L205 Blood Group H Disaccharide

Fucα1-2Gal - $C_{12}H_{22}O_{10}$ FW 326.30 CAS: 16741-18-7

Publications

Estimation of kinetic rate constants from surface plasmon resonance experiments

Neil D. Evans [*] **David Lowe** [**] **David Briggs** [***]
Robert Higgins [****] **Andrew Bentall** [†] **Simon Ball** [†]
Daniel Mitchell [**] **Daniel Zehnder** [**] **Michael J. Chappell** [*]

[*] *School of Engineering, University of Warwick, Coventry CV4 7AL (e-mail: neil.evans@ warwick.ac.uk).*
[**] *Clinical Sciences Research Institute, Medical School Building, University of Warwick, Coventry CV4 7AL*
[***] *NHS Blood and Transplant, Birmingham B15 2SG*
[****] *Transplant Unit, University Hospitals Coventry and Warwickshire, Coventry CV2 2DX*
[†] *University Hospital Birmingham, Birmingham B15 2TN*

Abstract: In order to characterise antibody binding characteristics it is necessary to determine reaction constants from quantitative measurements of the process. Surface plasmon resonance (SPR) provides convenient real-time measurement of the reaction that enables subsequent estimation of the reaction constants. Two models are considered that represent the binding reaction in the presence of transport effects. One of these models, the effective rate constant approximation, can be derived from the other applying a quasi-steady state assumption. Uniqueness of the reaction constants with respect to SPR measurements is considered via a structural identifiability analysis. It is shown that the effective rate constant model is unidentifiable, unless the analyte concentration is known, while the full model is structurally globally identifiable provided association and dissociation phases are considered. Both models provide comparable estimates for the unknown rate constants for a commercial anti-A monoclonal IgM experiment.

Keywords: Biomedical systems, structural identifiability, parameter estimation, surface plasmon resonance, surface-volume reactions, binding affinity

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Five-Year Outcomes in Living Donor Kidney Transplants With a Positive Crossmatch

A. Bentall[a,e], L. D. Cornell[b], J. M. Gloor[c], W. D. Park[a], M. J. Gandhi[d], J. L. Winters[d], M. F. Chedid[a], P. G. Dean[a] and M. D. Stegall[a,*]

[a] *Division of Transplantation Surgery, von Liebig Transplant Center, Mayo Clinic, Rochester, MN*
[b] *Division of Anatomic Pathology, von Liebig Transplant Center, Mayo Clinic, Rochester, MN*
[c] *Division of Nephrology and Hypertension, von Liebig Transplant Center, Mayo Clinic, Rochester, MN*
[d] *Division of Transfusion Medicine, von Liebig Transplant Center, Mayo Clinic, Rochester, MN*
[e] *Renal Institute of Birmingham, Queen Elizabeth Hospital, Birmingham, UK*
**Corresponding author: Mark D. Stegall, stegall.mark@mayo.edu*

Renal transplant candidates with high levels of donor-specific anti-HLA antibodies have low transplantation rates and high mortality rates on dialysis. Using desensitization protocols, good short-term outcomes are possible in "positive crossmatch kidney transplants (+XMKTx)", but long-term outcome data are lacking. The aim of the current study was to determine *actual* 5-year graft outcomes of +XMKTx. We compared graft survival and the functional and histologic status of 102 +XMKTx to 204 −XMKTx matched for age and sex. Actual 5-year death-censored graft survival was lower in the +XMKTx group (70.7% vs. 88.0%, $p < 0.01$) and chronic injury (glomerulopathy) was present in 54.5% of surviving grafts. Graft survival was higher in recipients with antibody against donor class I only compared with antibody against class II (either alone or in combination with class I) (85.3% vs. 62.6%, $p = 0.05$) and was similar to −XMKTx (85.3 vs. 88.0%, $p = 0.64$). Renal function and proteinuria ranged across a wide spectrum in all groups reflecting the different histological findings at 5 years. We conclude that when compared to −XMKTx, +XMKTx have inferior outcomes at 5 years, however, almost half of the surviving grafts do not have glomerulopathy and avoiding antibodies against donor class II may improve outcomes.

Key words: Antibody-mediated rejection, donor-specific antibodies, HLA-sensitized patients, Long-term outcomes, positive crossmatch kidney transplant

Abbreviations: AMR, antibody-mediated rejection; BFXM, B cell flow cytometric crossmatch; CDC, complement-dependent cytotoxicity; eGFR, estimated glomerular filtration rate; +XMKTx, positive crossmatch kidney transplants; −XMKTx, negative crossmatch kidney transplants; TFXM, T cell flow cytometric crossmatch.

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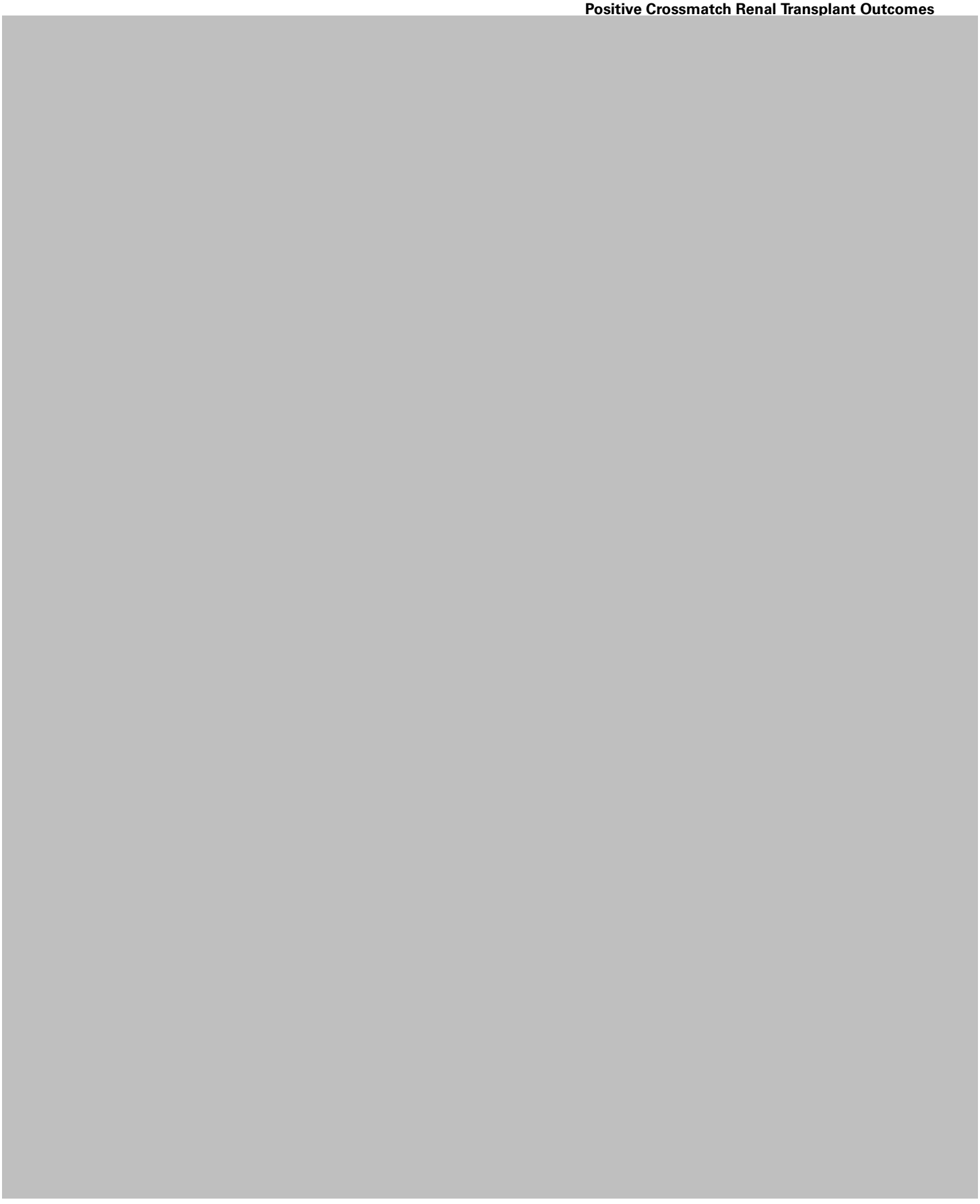
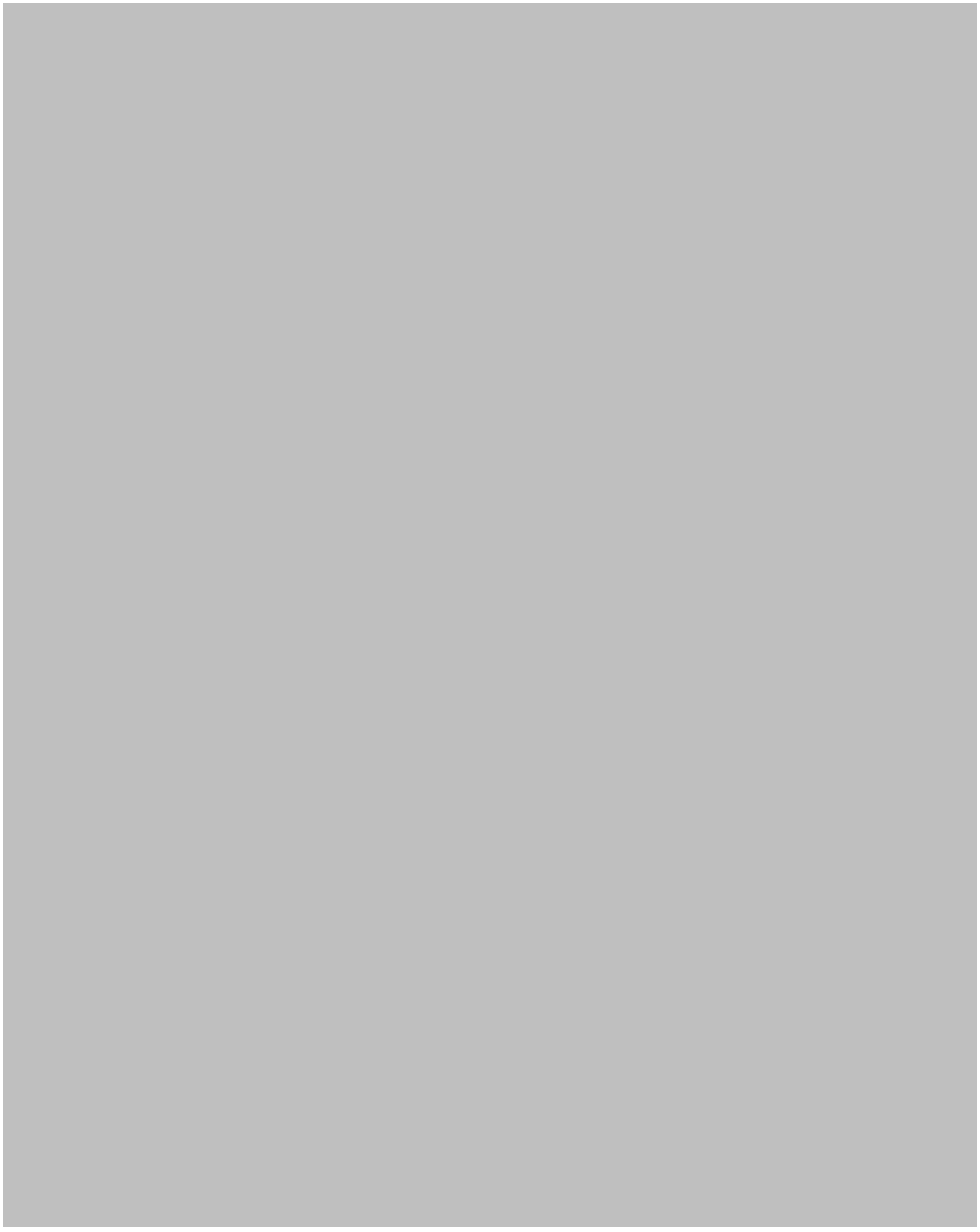
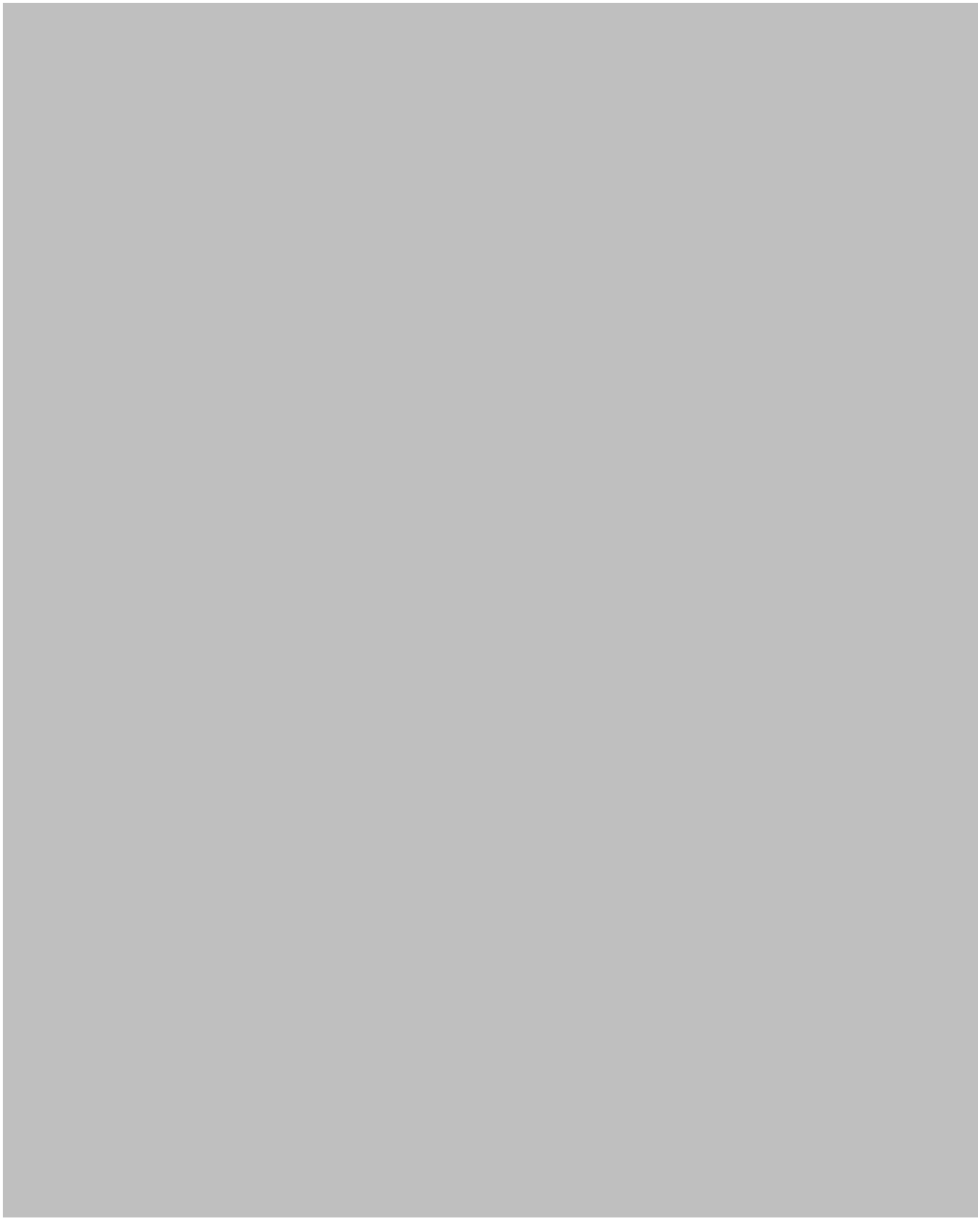
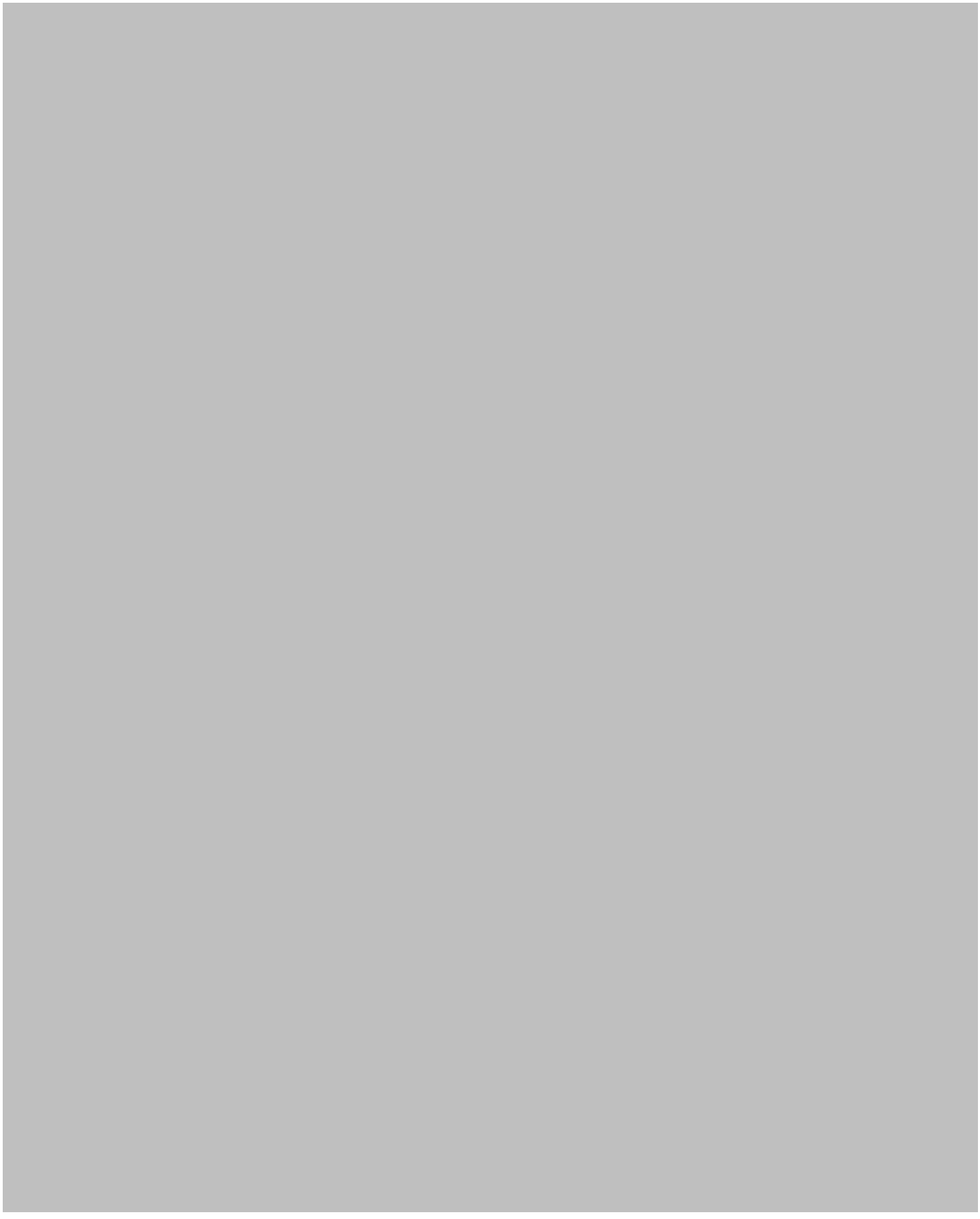
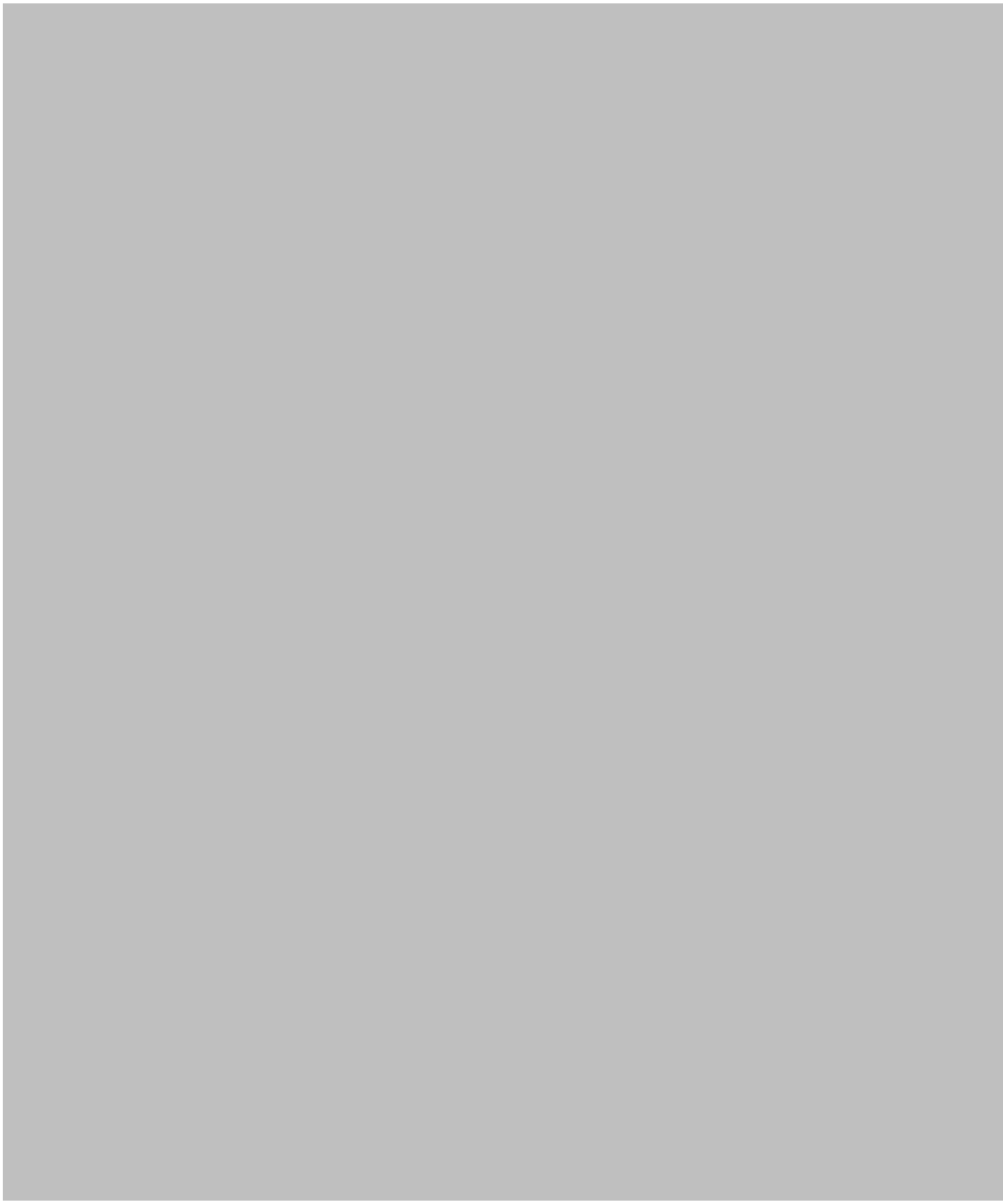
Introduction

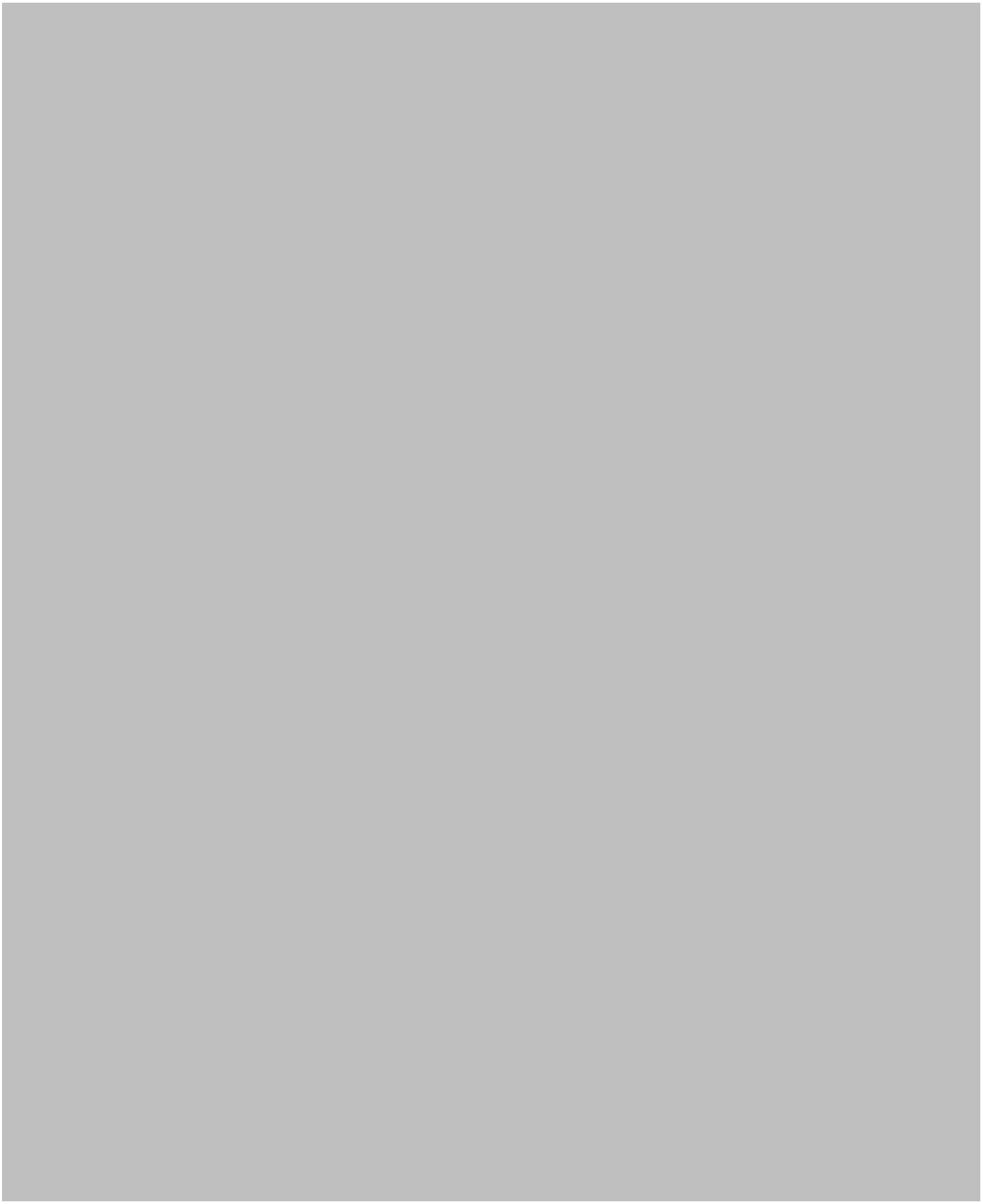


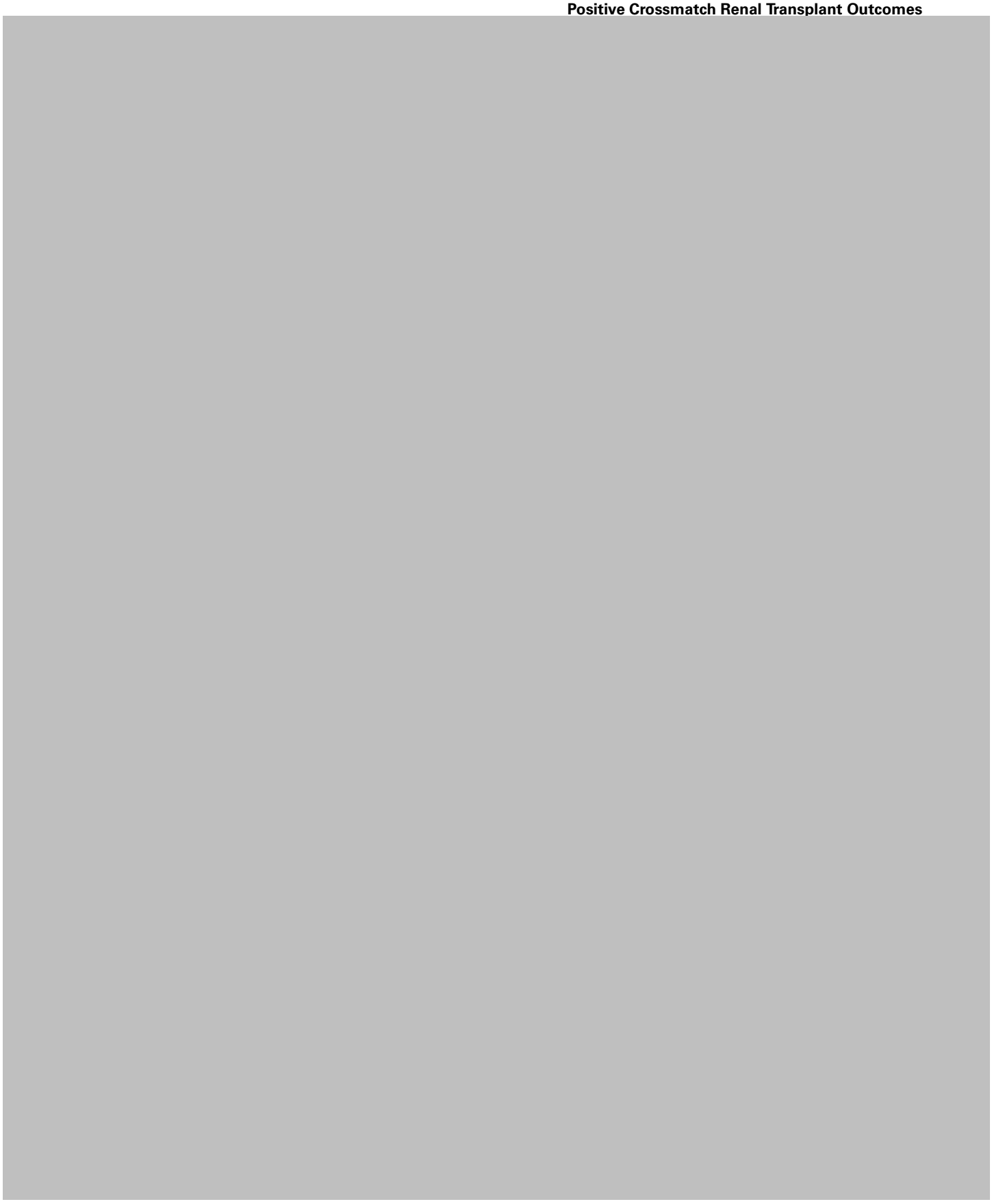


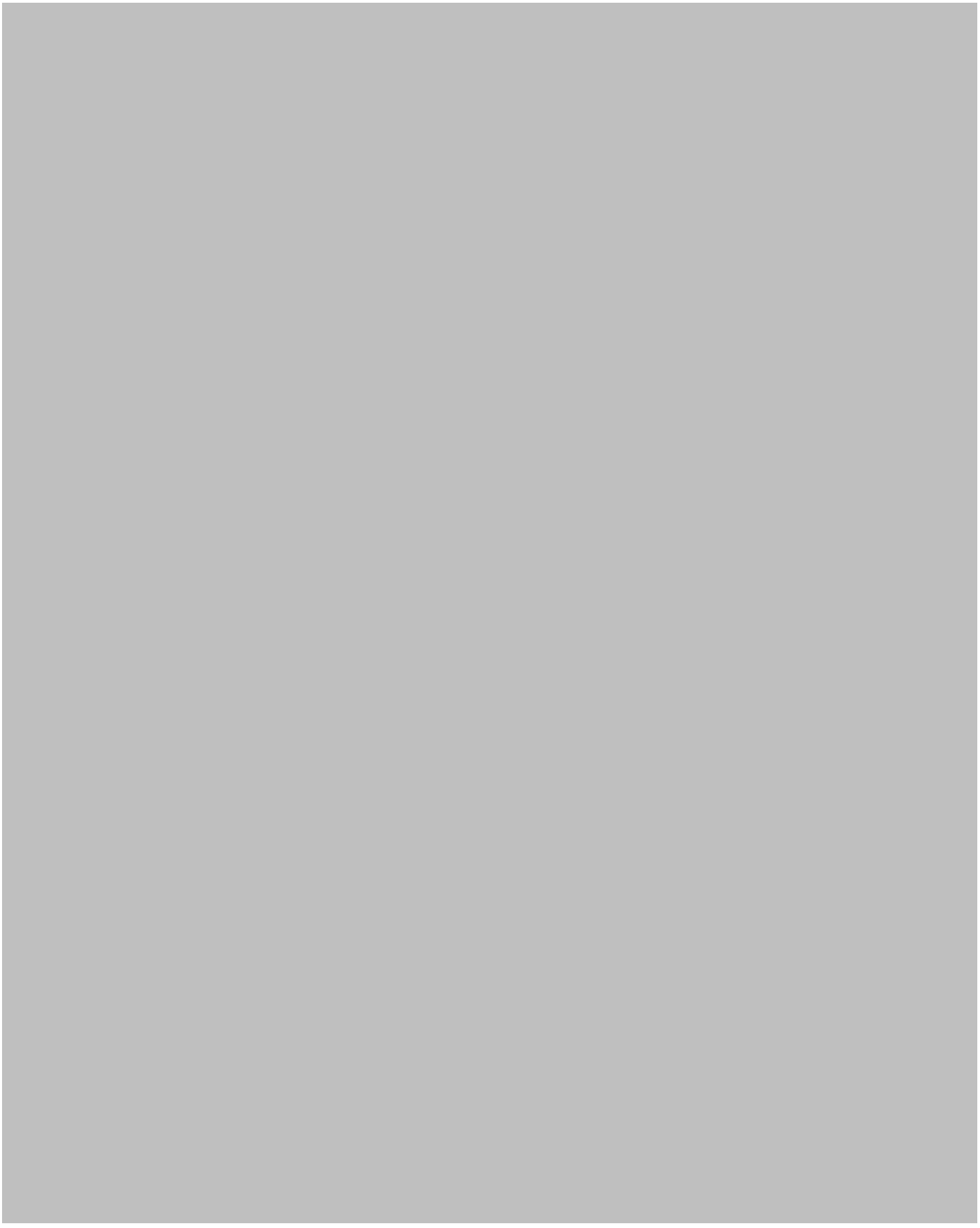












Differences in Chronic Intragraft Inflammation Between Positive Crossmatch and ABO-Incompatible Kidney Transplantation

Andrew Bentall,[1,2] Loren P. Herrera,[1,3] Lynn D. Cornell,[3] Manuel A. Moreno Gonzales,[1] Patrick G. Dean,[1] Walter D. Park,[1] Manish J. Gandhi,[4] Jeffrey L. Winters,[4] and Mark D. Stegall[1,5]

Background. ABO-incompatible kidney transplantations (ABOiKTxs) seem to have better long-term outcomes than positive crossmatch kidney transplantations (+XMKTxs).

Methods. This study aimed to assess the differences in chronic injury on histologic findings on 1- and 5-year surveillance biopsies and the clinical outcomes in living-donor kidney transplantations performed between May 1999 and November 2006 including 102 +XMKTxs, 73 ABOiKTxs, and 652 conventional KTxs.

Results. Although 5-year patient survival was similar between groups, graft loss between 1 and 5 years was similar in ABOiKTx (2.6% per year) and conventional KTx (1.7% per yr), and both were lower than that of +XMKTx (5.8% per year). At 5 years, renal function was similar in ABOiKTx and conventional KTx, and both were higher than that of +XMKTx, which had higher rates of inflammation and chronic glomerulopathy on both 1- and 5-year biopsies. Despite having evidence of less chronic injury, ABOiKTx showed a higher rate of intragraft complement activation (C4d deposition) at 5 years compared with +XMKTx (77.8% vs. 18.9%, $P<0.001$).

Conclusion. These data suggest that +XMKTxs have high rates of chronic inflammation at 1 and 5 years after transplantation, which may explain the higher rates of graft loss and lower renal function compared with other factors such as anti–donor antibody or intragraft complement deposition.

Keywords: ABO incompatible, HLA antibodies, Positive crossmatch, Chronic histologic injury, Kidney transplant.

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[1] Division of Transplantation Surgery, Mayo Clinic, Rochester, MN.
[2] Renal Institute of Birmingham, Queen Elizabeth Hospital, Birmingham, United Kingdom.
[3] Department of Anatomic Pathology, Mayo Clinic, Rochester, MN.
[4] Division of Transfusion Medicine, Mayo Clinic, Rochester, MN.
[5] Address correspondence to: Mark D. Stegall, M.D., Division of Transplantation Surgery and von Liebig Transplant Center, Mayo Clinic, Rochester, MN, U.S.A., Mayo Clinic, 200 First Street, SW Rochester, MN 55905.

E-mail: stegall.mark@mayo.edu

A.B. participated in the research design, writing of the paper, performance of the research, and data analysis. L.P.H. participated in the performance of the research and data analysis. L.D.C. participated in the research design, writing of the paper, and performance of the research. M.A. M.G. participated in the performance of the research and data analysis. P.G.D. participated in research design, writing of the paper, and performance of the research. W.D.P. participated in the writing of the paper, performance of the research, and data analysis. M.J.G. participated in the research design, writing of the paper, and performance of the research. J.L.W. participated in research design and performance of the research. M.D.S. participated in the research design, writing of the paper, performance of the research, and data analysis.

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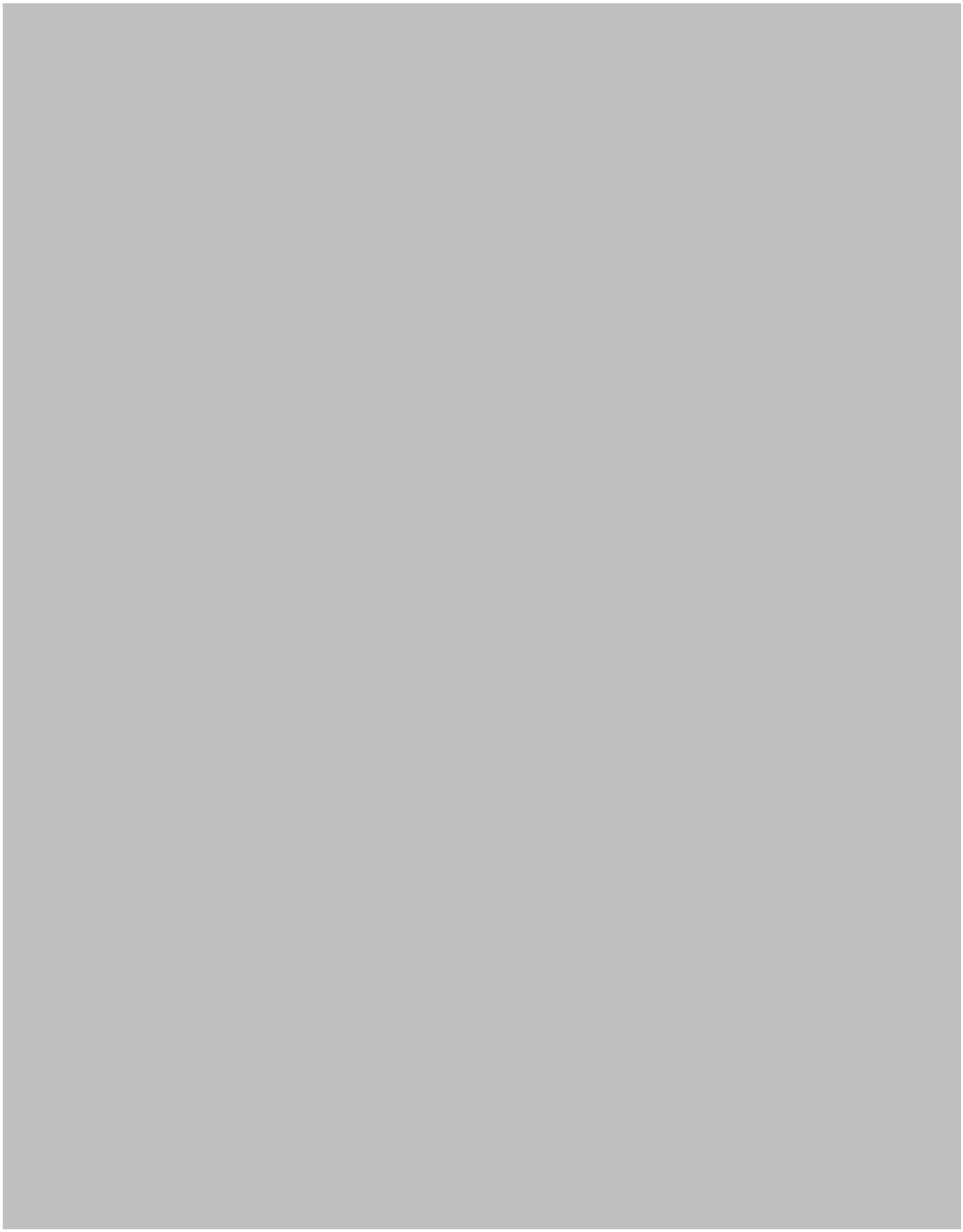

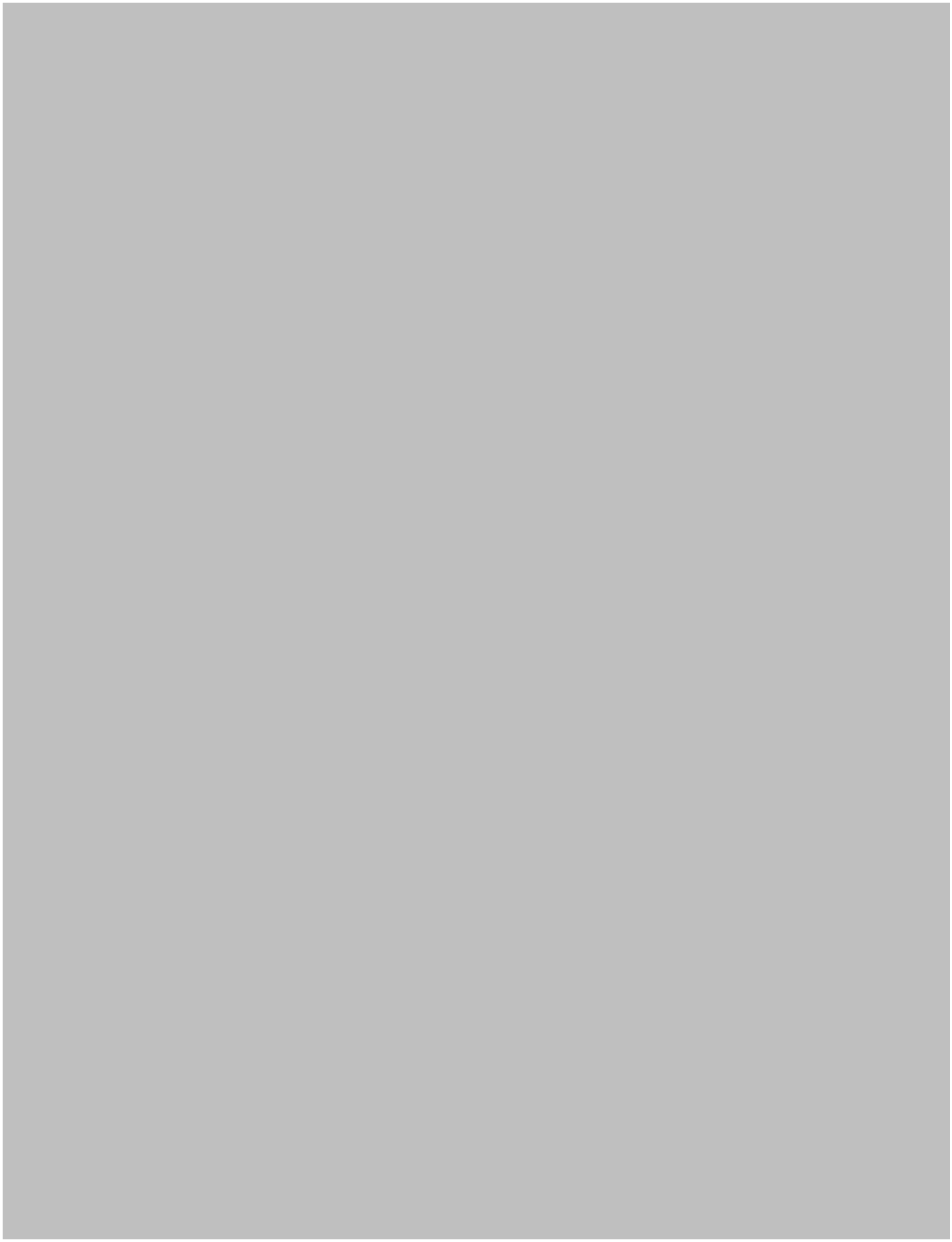

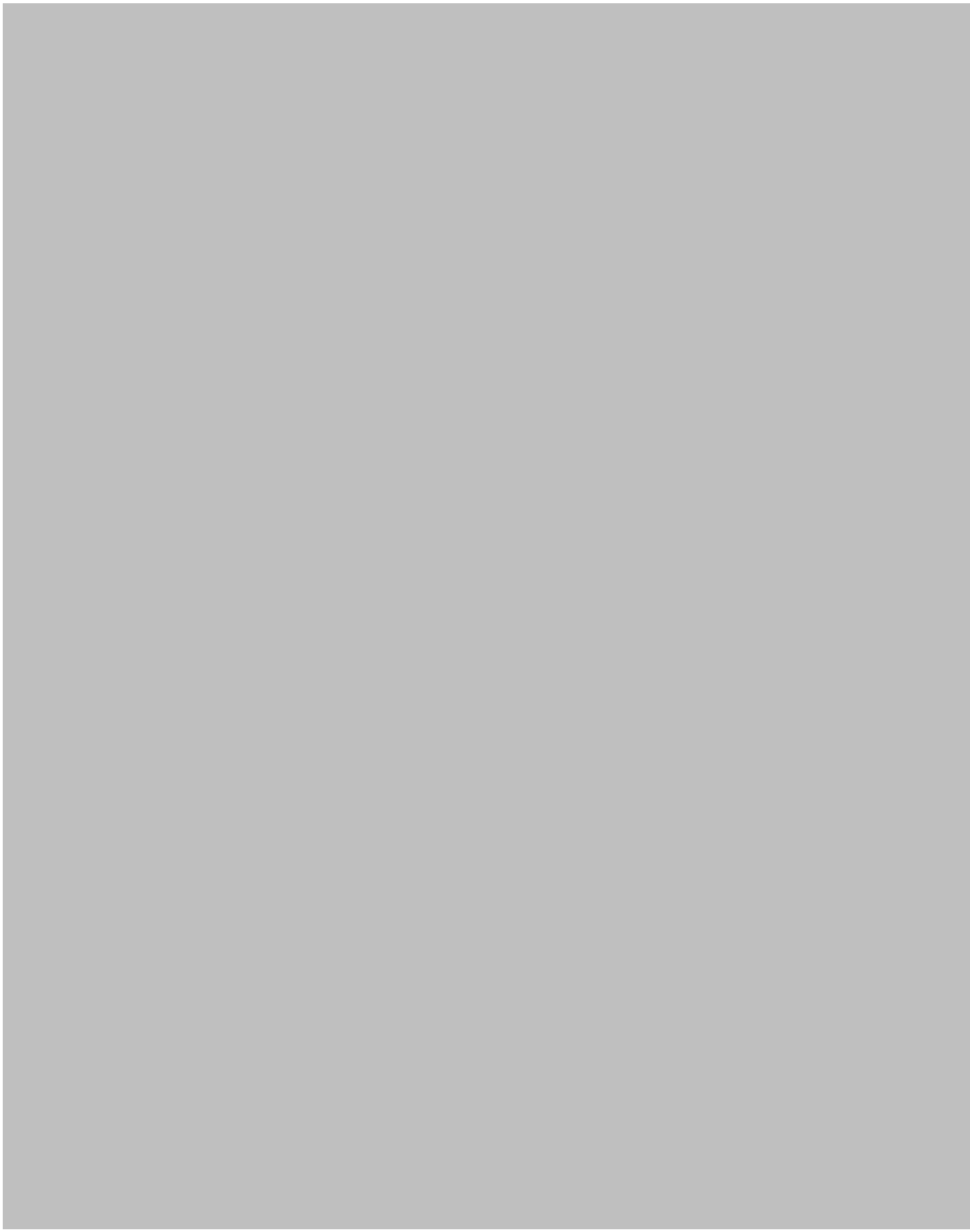

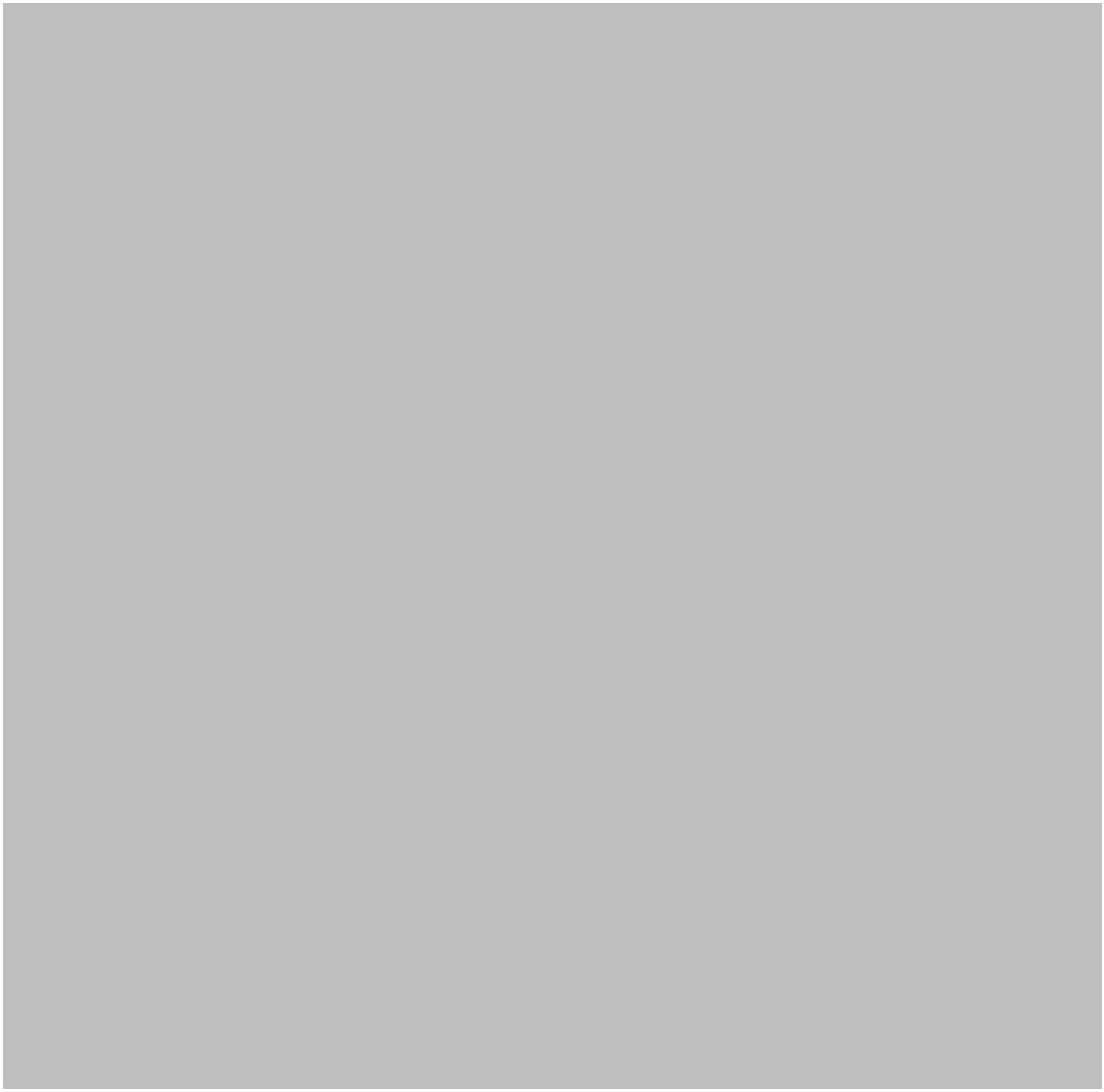

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ORIGINAL ARTICLE

Antibody-mediated rejection despite inhibition of terminal complement

Andrew Bentall,[1,2] Dolly B. Tyan,[3] Flavia Sequeira,[3] Matthew J. Everly,[4] Manish J. Gandhi,[5] Lynn D. Cornell,[6] Han Li,[1] Nicole A. Henderson,[5] Suresh Raghavaiah,[1] Jeffrey L. Winters,[5] Patrick G. Dean[1] and Mark D. Stegall[1]

1 Division of Transplantation Surgery, William J. von Liebig Transplant Center, Mayo Clinic, Rochester, MN, USA
2 Renal Institute of Birmingham, Queen Elizabeth Hospital, Birmingham, UK
3 Histocompatibility, Immunogenetics & Disease Profiling Laboratory, Department of Pathology, Stanford University School of Medicine, Palo Alto, CA, USA
4 Terasaki Foundation, Los Angeles, CA, USA
5 Division of Transfusion Medicine, William J. von Liebig Transplant Center, Mayo Clinic, Rochester, MN, USA
6 Division of Anatomic Pathology, William J. von Liebig Transplant Center, Mayo Clinic, Rochester, MN, USA

Keywords
antibody-mediated rejection, anti-HLA antibodies, complement, IgM, kidney transplantation, sensitized recipients.

Correspondence
Mark D. Stegall MD,
Division of Transplantation Surgery, von Liebig Transplant Center, Mayo Clinic, 200 First Street, SW Rochester, MN 55905, USA.
Tel.: +1 507 266 2812;
fax: +1 507 266 2810;
e-mail: stegall.mark@mayo.edu

Conflicts of interest
MDS has research contracts with Alexion Pharmaceuticals and Millennium Pharmaceuticals. LDC has research contracts with Alexion Pharmaceuticals. DBT receives royalties from One Lambda for C1q license. All other authors have no conflicts of interest.

Part of the clinical study: Dosing Regimen of Eculizumab Added to Conventional Treatment in Positive Cross Match Living Donor Kidney Transplant.

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Summary

Terminal complement blockade has been shown to decrease the incidence of early acute antibody-mediated rejection (eAMR) in the first month after positive cross-match kidney transplant recipients, yet some patients still develop eAMR. The current study investigated possible mechanisms of eAMR despite eculizumab treatment. Of the 26 patients treated with eculizumab, two developed clinical eAMR and another patient developed histologic signs of eAMR without graft dysfunction ('subclinical eAMR'). Twenty-three did not have histologic injury on early surveillance biopsies. All 26 patients had therapeutic levels of eculizumab and showed complete blockade of complement in hemolytic assays. High levels of donor-specific alloantibody (DSA) including total IgG, IgG3, and C1q+ DSA were present in patients with and without eAMR, and none correlated well with eAMR. In contrast, IgM DSA was present in only four patients after transplantation: the two patients with clinical eAMR, one patient with subclinical AMR, and one patient without eAMR ($P = 0.006$ correlation with eAMR). Both clinical eAMR episodes were easily treated with plasma exchange which removed IgM more completely and rapidly than IgG, resulting in normalization of function and histology. These data suggest a possible role of antidonor IgM DSA in the pathogenesis of eAMR in patients treated with terminal complement blockade (ClinicalTrials.gov Identifier: NCT00670774).