The relevance and impact of MICA allele mismatching and MICA antibodies on renal transplantation outcome

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October 2011

A thesis submitted to University College London for the degree of Doctor of Philosophy

Declaration

I, Steven T. Cox, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: ……..

Date: ……..

This thesis is dedicated to my
late grandmother, Marjorie Bates.

Abstract

Even when kidney allografts are well matched for HLA antigens and anti-HLA antibodies are undetected, graft rejection still occurs. There is evidence of hyperacute rejection in the absence of HLA antibodies, implicating other alloantigens. Studies have shown that some patients with graft rejection or loss have antibodies specific for the highly polymorphic MHC class I-related chain A (MICA) antigens.

This thesis investigated whether mismatching MICA alleles associates with MICA antibody production and graft rejection, survival or dysfunction. Using commercial assays, MICA and HLA antibody screening of 442 recipients was performed and specificities were confirmed in a sub-group of 227 recipients using single antigen (SAg) multiplex technology. MICA antibody specificity was assigned using three independent SAg assays. In addition, MICA alleles of 227 recipients and donors were determined by development and application of DNA sequence based typing. Acute rejection (AR) was assessed by renal pathologists and classified as acute cellular rejection (ACR) or acute antibody-mediated rejection (aAMR). Graft function was assessed by estimated glomerular filtration rate (eGFR) and serum creatinine measurements.

Among the cohort of 442 recipients, 33 (7.5%) produced MICA antibodies, which correlated with ACR (P=0.03). Analysis of the MICA typed cohort revealed 17 patients (7.5%) had MICA antibodies and 13 (6%) developed MICA donor-specific antibodies (DSA). Multivariate analysis revealed MICA mismatching as a significant factor associated with the presence of MICA antibodies (P=0.009) and 14 mismatched MICA residues significantly correlated with MICA antibody production. MICA and HLA antibodies significantly associated with AR and MICA-DSA and HLA-DSA correlated with decreased graft function by univariate and multivariate analysis. To conclude, mismatching of specific MICA epitopes in renal transplantation is a mechanism leading to production of MICA antibodies and MICA-DSA that associate with AR and graft dysfunction.

Acknowledgements

Firstly I would like to thank my supervisor, Professor Alejandro Madrigal, for allowing me the time to pursue a PhD and supporting me throughout the process, I am very grateful. I am also immensely grateful to my supervisor, Ann-Margaret Little, whose guidance, support and advice from beginning to end was invaluable in maintaining my motivation.

This project would not have been possible without the co-operation of staff from the UCL Department of Nephrology. I would like to thank Henry Stephens for diligent and close support with manuscript preparation, Ray Fernando and Aliyye Karasu for friendly help, advice and technical assistance and all members of the team for guiding me through the technicalities of renal transplantation. I particularly appreciate the help provided by Mark Harber who enabled me to understand clinical aspects of transplantation and who provided important clinical data that was crucial for this thesis. I am also extremely grateful to Professor Alexander Howie for helpful discussions and detailed graft rejection data, and our collaborators - Professor Peter Stastny and Yizhou Zou for expert advice and Luminex testing.

I would like to thank Anthony Nolan Research Institute staff and students, past and present for their inspiration, support and friendship (and sometimes their blood!) throughout the years. I particularly thank fellow students Mehri, Daniel, Neema, Samir, Michelle, Martha and Isabela who shared a similar experience and often offered valuable advice. I am also grateful to Angus, Nush, Shem, Jennifer, Bronwen, Aurore, Richard and Jim for technical help and advice and to Hazel for organising the lab and keeping me 'safe'.

Finally, I thank my family and friends who have tolerated my long absences during this very demanding time in my life. In particular I thank my mother for her unwavering faith in my ability to succeed, and my brother Matthew for genuine interest in my project and the process of obtaining a PhD (got there in the end!). Lastly, to my late grandmother, 'Nan', for her knowledge and wisdom during my formative years, I hope this achievement would have made you proud.

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Abbreviations

aAMR	acute Antibody-Mediated Rejection
ACR	Acute Cellular Rejection
ADAM	A Disintregin And Metalloproteinase
ADCC	Antibody-Dependent Cellular Cytoxicity
APC	Antigen Presenting Cell
APS	Ammonium Persulphate
AR	Acute Rejection
AT1R	Angiotensin Type 1 Receptor
ATG	Anti-Thymocyte Globulin
BCA	Bicinchoninic Acid
BD	Behçet's Disease
BLCL	B-Lymphoblastoid Cell Line
BSA	Bovine Serum Albumin
CAD	Chronic Allograft Dysfunction
CAN	Chronic Allograft Nephropathy
CCD	Charge-Coupled Device
CD	Chronic Damage
CDC	Complement Dependent Cytotoxicity
CI	Confidence Interval
CKD	Chronic Kidney Disease
CNI	Calcineurin Inhibitor
CRP	C-Reactive Protein
CsA	Cyclosporin A
CTL	Cytotoxic T-Lymphocyte
CTS	Collaborative Transplant Study
DC	Dendritic Cell
DGF	Delayed Graft Function
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
ECACC	European Collection of Cell Cultures
DNA	Deoxyribonucleic acid
ESTDAB	European Searchable Tumour Database
dNTP	deoxyNucleotide TrisPhosphate
DSA	Donor Specific Antibody
EBV	Epstein-Barr Virus
EC	Endothelial Cell
ESRD	End-Stage Renal Disease
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FasL	Fas/Fas Ligand
FC	Flow Cytometry
FC-XM	Flow Cytometry crossmatch

FPLC	Fast Protein Liquid Chromatography
GS	Graft Survival
GvHD	Graft versus Host Disease
HAR	Hyperacute Rejection
HCMV	Human Cytomegalovirus
HEK	Human Embryonic Kidney (cell)
HI-FCS	Heat Inactivated Foetal Calf Serum
HLA	Human Leucocyte Antigen
HRP	Horse-Raddish Peroxidase
HSCT	Haematopoietic Stem Cell Transplantation
IFN	Interferon
IHIWC	International Histocompatibility and Immunogenetics Workshop and Conference
Ig	Immunoglobulin
IHC	Immunohistochemistry
IPTG	Isopropyl-β-D-thiogalactoside
IUB	International Union of Biochemists
IVIG	Intra-Venous Immunoglobulin
KIR	Killer-cell Inhibitory Receptor
LB	Luria Bertani
LD	Linkage Disequilibrium
LPS	Lipopolysaccharide
MBL	Mannose Binding Lectin
MDRD	Modification of Diet in Renal Disease
MFI	Mean Fluorescence Intensity
MICA	MHC class I-related Chain A
MICB	MHC class I-related Chain B
MHC	Major Histocompatibility Complex
MLE	Maximisation Likelihood Estimate
MMF	Mycophenolate Mofetil
NBR	Normalised Background Ratio
NC	Negative Control
NF-AT	Nuclear Factor of Activated T-cells
NDSA	Non-Donor Specific Antibody
NK	Natural Killer
OD	Optical Density
OR	Odds Ratio
OS	Overall Survival
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PE	Phycoerythrin
PEX	Plasma Exchange
PBS	Polybuffered Saline
PCR	Polymerase Chain Reaction
PRA	Panel Reactive Antibody
PRR	Pattern Recognition Receptors

Rae	Retinoic acid early inducible
RAET-1	Retinoic Acid Early Transcript-1
RNA	Ribonucleic acid
RSCA	Reference Strand-mediated Conformational Analysis
RT	Reverse Transcriptase
SAg	Single Antigen
SBT	Sequence Based Typing
SCr	Serum creatinine
SDS	Sodium Dodecyl Sulphate
Sf9	*Spodoptera frugiperda-9* (cell)
SFM	Serum-Free Medium
SSOP	Sequence-Specific Oligonucleotide Probing
SSP	Sequence-Specific Priming
STR	Short Tandem Repeat
TAP	Transporter Associated Protein
Taq	*Thermus aquaticus*
TBE	Tris-Boric acid-EDTA
TCR	T-Cell Receptor
TEMED	Tetramethylethylenediamine
Th	T-helper
TLR	Toll-Like Receptors
TM	Transmembrane Region
Tm	Melting temperature
TNF	Tumour Necrosis Factor
ULBP	UL16-Binding Protein
UNOS	United Organ Sharing scheme
WHO	World Health Organisation

CHAPTER 1

Introduction

1.1 The Human Immune System

The immune system in humans employs of a variety of mechanisms involving interactions between different cells and tissues in the body, with the aim of protecting the host from infections and cellular abnormalities. There are several levels of defence involved in preventing unwanted pathogens such as bacteria, viruses, parasites and fungi from entering the body and causing damage. The skin is the main barrier against infection and when penetrated, cells and factors of the innate immune system can quickly eradicate pathogens, as the first line of cellular defence. The next level of defence is provided by the adaptive immune system where cells can acquire 'memory' of their first encounter with their specific antigen and when presented with the same antigen again, can react with a fast and efficient response. The adaptive immune response has evolved to provide an army of lymphocytes, with each cell capable of recognising a separate antigen from an almost infinite variety. Therefore these specialist cells possess specificity and memory for any substance that is not a constituent of 'self' providing the capacity to eradicate most pathogens that can be encountered in a lifetime (Bonilla and Oettgen, 2010).

1.1.1 Innate Immunity

Innate immunity, as the name suggests, is present from birth and lasts for the lifetime of the individual. The term non-specific immunity can also be used, referring not only to cell-mediated defence against pathogens but also the physical and anatomical barriers that are designed to prevent entry of organisms into the body. The first physical defence that must be breached for a pathogen to establish an infection are the epithelial surfaces, such as the skin, which provide the first defence against infection with a tough and impenetrable barrier. The epithelial surfaces lining the body orifices of the respiratory, gastrointestinal and urogenital tracts are more vulnerable to infection. They are known as the mucosal surfaces or mucosa and are constantly bathed in mucous secreted by the epithelia forming a thick fluid layer containing glycoproteins, proteoglycans and enzymes that protect the epithelial cells and limit opportunistic infections (Knight and Holgate, 2003; M. W. Russell and Mestecky, 2010; Turner, 2009). For example, secretions such as tears and saliva

contain enzymes that have anti-bacterial properties, such as lysozyme (McClellan, 1997). To keep respiratory airways free from dust and microorganisms, specialised goblet-cells secret mucous, and their protruding cilia beat in a wave-like manner to move mucous and particles towards the oesophagus where they can then be swallowed (Knight and Holgate, 2003). Material that enters the stomach is also a potential source of infection however the acidic environment helps eliminate pathogens (Skillman and Silen, 1972). Thus, our body's physical and chemical barriers can defend us against the magnitude of pathogens present in the external environment. However, these barriers can be breached by physical damage, such as wounds or burns, allowing entry of pathogens into the body where they face the next level of defence: the inflammatory response.

Effector cells such as neutrophils, macrophages and dendritic cells (DC) have the primary function of identifying, ingesting and destroying microorganisms. Together, effector cells mediate the inflammatory response. The process by which particles or bacteria are engulfed is called phagocytosis, therefore neutrophils, macrophages and DCs can collectively be referred to as phagocytes. Macrophages are long-lived cells residing in infected tissues and are the first phagocytic cells to come into contact with an invading microorganism, secreting soluble proteins called cytokines that attract other cells such as neutrophils to the site of infection. Other factors are also secreted by macrophages and act as anti-bacterial agents that amplify the inflammatory response (Valledor *et al.*, 2010). Neutrophils are short-lived cells specialised for phagocytosis of pathogens and are the primary and most abundant cell in inflamed tissues. They are rapidly mobilised, can act in aerobic and non-aerobic conditions and often die at the site of infection giving rise to pus (Summers *et al.*, 2010). Cytokines secreted by phagocytes can induce local dilation of blood capillaries and change the adhesive properties of the vascular epithelium allowing phagocytes and other white blood cells to bind and migrate out of the blood into the inflamed tissue (Ley *et al.*, 2007). Infiltration of cells into inflamed tissues increases swelling, redness and dilation of blood vessels allowing leakage of plasma into the inflamed area (oedema) and factors secreted by effector cells can produce local pain. These physical features of inflammation have been known for a long time and are traditionally defined by the Latin: *calor, dolor, rubor* and *tumor*, meaning heat, pain, redness and swelling (White *et al.*, 2005).

Phagocytes can become activated by specific recognition of structures that are unique to pathogens through interaction of their Toll-Like Receptors (TLR) with microbial antigens such as lipopolysaccharide (LPS) (Trinchieri and Sher, 2007). There are an increasing number of TLRs being recognised in humans and are defined as pattern recognition

receptors (PRR) that recognise pathogen-associated molecular patterns or PAMPs such as LPS or deoxyribonucleic acid/ribonucleic acid (DNA/RNA) (Meylan *et al.*, 2006). As well as cell-bound PRR, soluble recognition molecules can enter sites of inflammation via infiltrating plasma as a result of oedema. One member of the pentraxin family of plasma proteins is C-reactive protein (CRP), which can bind to several different species of bacteria and fungi. A member of the collectin family of plasma proteins is mannose-binding lectin (MBL) and binds carbohydrates with a terminal mannose, typically found in microbial cell surface glycoproteins and glycolipids (Apostolopoulos and McKenzie, 2001). Therefore both CRP and MBL have specificity for pathogen-derived molecular patterns and have the function of 'opsonising' or coating the microorganism to allow the mechanism of complement activation. Complement is the name for a group of plasma proteins involved in an enzymatic cascade, initiated by the binding of complement component C1q to CRP or MBL on the surface of the microbe. Complement activation is a general effector mechanism of the immune system, responsible for antibody-mediated cell lysis associated with humoral adaptive immunity in addition to innate immunity (Tomlinson, 1993). Complement proteins bound to pathogens associate with specific complement receptors expressed by phagocytes, triggering their engulfment and elimination. The soluble complement fragments released during the enzymatic cascade enable recruitment of additional phagocytic effector cells to the site of inflammation, enhancing pathogen clearance (Tomlinson, 1993).

Another important cellular component of the innate immune response is a cell related to the lymphocyte called the Natural Killer (NK) cell. NK-cells are derived from the same lymphoid stem cell progenitor as T and B-cells and are characterised by their expression of CD56 and absence of the CD3 molecule found on all T-cells (Rees, 1990). They recognise infected or stressed cells and respond quickly and directly, killing them by secretion of potent inflammatory cytokines that mediate killing without prior activation, resulting in the term 'Natural Killer' (Kiessling *et al.*, 1975a; Kiessling *et al.*, 1975b). The effector functions of NK-cells are to kill infected or stressed cells and activate macrophages to destroy phagocytosed microbes by secreting interferon-gamma (IFN-γ). NK-cells contain protein granules that are released by exocytosis and act on adjacent cells. One of the granules, called perforin, initiates lysis by making a hole in the target cell through which other granules called granzymes can enter the cell cytoplasm and cause the cell to undergo apoptosis. NK-cells have several important roles in the defence against intracellular microbes and are able to directly kill virally infected cells in the early phase of viral infection. When effector T-cells are formed and arrive at the site of infection, they become

the main source of IFN-γ and cell-mediated cytotoxicity and secrete an inhibitory cytokine called IL-10, deactivating NK-cells (Biron *et al.*, 1999).

An important feature of NK-cells is their sensitivity to cells lacking HLA class I molecules, allowing them to identify virally infected cells that have escaped detection by T-cells, known as the 'missing-self hypothesis' (Karre *et al.*, 1986). Cell surface molecules expressed by NK-cells can induce activatory and inhibitory signals to regulate their activity and diverge into three groups called Killer-cell Immunoglobulin-like Receptors (KIR), the C-type lectin domain and Leucocyte Immunoglobulin-like Receptors (LIR) (Sawicki *et al.*, 2001). Activating signals must be blocked by inhibitory signals to prevent NK-cell activation which is achieved by the binding of KIRs to their HLA class I ligands. Deficiency of HLA class I molecules leads to NK-cell activation (Karre *et al*., 1986; Lanier, 2005; Vilches and Parham, 2002). Activating receptors on NK-cells also recognise ligands expressed on cells that have undergone stress, been infected by a virus or become malignantly transformed, activating NK-cells without the requirement of a second signal. One of the most studied NK-cell receptors is NKG2D and its ligands are structurally similar to HLA class I molecules (Bauer *et al.*, 1999; Wu *et al.*, 1999). In humans these ligands are MHC class I-related Chain A and B (MICA and MICB) and UL16-binding proteins (ULBP1-6) (Eagle and Trowsdale, 2007, Champsaur & Lanier, 2010). Among these, MICA has been the most widely studied as it displays considerable polymorphism, may be involved in autoimmunity and transplant rejection and has recently become recognised as an important molecule in tumour immunosurveillance (Salih *et al.*, 2002). MICA expression is up-regulated in response to stress as a danger signal to NK-cells, effectively marking the MICA-bearing cell for destruction by engagement of NKG2D and NK-cell degranulation (Bauer *et al*., 1999). Therefore NK-cells are important for the normal day-to-day surveillance of the body's cells, able to rapidly respond to changes that indicate viral infection or malignant transformation.

1.1.2 Adaptive Immunity

Just as the innate immune system relies on specialised cellular functions, the adaptive immune response has evolved complex and specialised mechanisms. The properties that make these cells distinct from innate immunity are their specificity for a particular antigen and their capacity for memory of antigenic encounters. The ability to remember a primary encounter with an antigen enables subsequent exposure to result in a much faster and more potent cytotoxic response (Bonilla and Oettgen, 2010). This role is carried out by

lymphocytes descended from a common lymphoid progenitor, called T-cells and B-cells; their names reflecting the different sites at which they undergo maturation: the Thymus (T-cells) or the Bone marrow (B-cells) (Prchal *et al.*, 1978). T-cells and B-cells can be characterised by their distinct cell surface markers. T-cells express CD3 in addition to either CD4 or CD8 molecules while B-cells are characterised by expression of CD19, CD20 and CD21. T-cells are further categorised as either the more common αβ or γδ-T-cell depending on the extracellular subunits of their T-cell receptor (TCR) and have slightly different functions (Kang and Raulet, 1997).

1.1.3 Generation of effector B-cells

Upon activation, B-cells differentiate into plasma cells and have a unique function in immunity, as they are the only cell type able to synthesise and secrete antibody. Antibodies are variable antigen-specific proteins known as immunoglobulins (Ig) that circulate the body as a major constituent of plasma in blood and lymph and specifically target their antigen by binding to it. There are five classes or isotypes of immunoglobulin: IgG, IgA, IgM, IgD and IgE that differ in their structure and effector functions. The binding of antibody renders the pathogen susceptible to other components and cells of the immune system, heralding its destruction. Each antibody has a unique specificity for a particular antigen covering a vast array of substances that may be encountered in a person's lifetime.

Antibodies are glycoproteins consisting of a basic unit of four polypeptide chains forming two identical heavy chains and two identical but smaller light chains that create a structure resembling the letter 'Y'. Polypeptide chains of different antibodies vary considerably in their amino acid sequence but this variability is concentrated in one area called the variable region. This variability is responsible for antigenic specificity and the heavy and light chain variable regions form the two antigen binding sites. The remaining part of the antibody does not differ and is called the constant region. Most genes are encoded by exons and introns that are readily transcribed and this is the case for the leader peptide and constant regions of Ig. However, the variable regions are encoded by two variable light chain and three variable heavy chain gene segments and rearrangement is required to produce an exon that can be transcribed. The light chain variable region is encoded by variable (V) and joining (J) gene segments and the heavy chain has an additional set of diversity (D) gene segments. The V, J and D gene segments have a number of variants and can be randomly rearranged by cutting and splicing the DNA, known as DNA recombination (Fanning *et al.*, 1996). This process occurs during the development of B-cells in the bone marrow and is

called somatic recombination. The unique rearrangement of V, D and J gene segments leads to the extreme diversity and specificity of immunoglobulin binding sites. Once the antibody molecule encounters its antigen, the specificity can be enhanced by single nucleotide changes within the variable region in a process known as somatic hypermutation (Weill and Reynaud, 1996). This final change in specificity ensures that only B-cells producing antibody with the strongest affinity will become antibody-secreting plasma cells (Hozumi and Tonegawa, 1976; Lefranc and Lefranc, 1980).

Naïve B-cells circulate the body and carry out immunosurveillance via their membrane bound antigen-specific IgM or IgD molecules. B-cells can become activated by an encounter with antigen and undergo somatic hypermutation and clonal expansion leading to the development of effector, plasma cells secreting antibody, and memory B-cells. The soluble IgM secreted by plasma cells is monoclonal and can inactivate antigens such as extracellular pathogens or soluble toxins by neutralisation, opsonisation and complement activation. Multiple epitopes can also be a target for B-cell receptors, giving rise to polyclonal antibodies against a single antigen. Memory B-cells remain in the lymphatic system and following a secondary exposure to the same antigen, can become plasma cells and instead of IgM, produce IgG by isotype switching, giving rise to long-term immunity to an antigen (Gowans and Uhr, 1966). Approximately 80% of serum Ig is in the form of IgG. B-cells can also be activated by synergistic interplay with another cell of the adaptive immune response, the T-cell (Gowans, 1966).

1.1.4 Generation of effector T-cells

T-cells are the main component of the adaptive immune response and although originating in the bone marrow, undergo education and maturation in the thymus. T-cells can perform cell-mediated immunity against intracellular pathogens including viruses, bacteria and parasites, and like B-cells generate memory as well as specificity. There are two broad subsets of T-cells based on their expression of cell-surface markers CD4 or CD8 and each have distinct functions. CD8+ T-cells are known as cytotoxic T lymphocytes (CTLs) and can directly kill or lyse cells that have been infected (Landegren *et al.*, 1982). CD4 expressing T-cells give 'help' to other cell types and are known as T-helper (Th) cells that can be divided into Th1, Th2 and Th17 subtypes based on differential phenotypic expression, cytokine secretion and the type of cells they help. Th1 cells can stimulate and activate macrophages and B-cells via cytokine secretion (Stout and Bottomly, 1989). Th2 cells can activate B-cells that have encountered their antigen via synergic recognition of an

antigenic peptide presented on the B-cell receptor, thus giving help to B-cells to differentiate into antibody-secreting plasma cells. Th2 cell effector function is carried out in the lymphoid tissues whereas Th1 and CD8 CTLs must migrate to the site of infection (Mosmann and Coffman, 1989a, b). Th17 cells are a new addition to the T-helper cell group, characterised by secretion of IL-17A with the role of mediating recruitment of neutrophils and macrophages to the site of infection (Bettelli *et al.*, 2007).

Recognition of antigen by T-cells is facilitated by the highly specific TCR. There are two types of TCR, the αβ and the γδ, the former being the most commonly expressed, while T-cells bearing γδ-TCR are relatively few (Kreslavsky and von Boehmer, 2010). The αβ-TCR is a membrane-bound glycoprotein heterodimer composed of α and β heavy chains with variable regions at their distal domains. The specificity of the TCR variable region is determined by gene segment rearrangement but does not interact with or recognise intact antigens, rather individual peptides (Lanier *et al.*, 1987). Therefore T-cells must be presented with their specific antigen via other cell types known as professional antigen presenting cells (APC), such as DCs or B-cells. The Human Leucocyte Antigen (HLA) gene in humans, located within the Major Histocompatibility Complex (MHC), encodes the cell surface molecules responsible for peptide presentation to T-cells. TCRs can only respond appropriately to an antigen if presented by an HLA molecule of the host, a phenomenon termed MHC restriction and originally observed by Zinkernagel and Doherty (Zinkernagel and Doherty, 1974, 1979).

1.1.5 The Major Histocompatibility Complex

MHC molecules are essential for ensuring that the appropriate T-cell class is activated by stimulation with a particular source of infection. There are two types, or classes, of MHC molecules known as MHC class I and MHC class II, with each presenting peptides to CD8 and CD4 T-cells, respectively. CD8 T-cells recognise peptides of intracellular origin presented by MHC class I molecules and CD4 T-cells present peptides from antigens of extracellular origin via MHC class II molecules. Specific interactions between CD4 or CD8 glycoproteins and MHC class I or II occur when a TCR recognises its specific peptide and MHC molecule. This involvement of CD4 and CD8 with immune recognition gives them a role as co-receptors (McMichael, 1980).

MHC class I and class II molecules have similar three-dimensional structures comprised of differing elements. The MHC class I molecule has a transmembrane heavy α-chain non-covalently complexed with a protein called β_2-microglobulin. The three extracellular domains of the heavy chain are called α-1, α-2 and α-3, with the folding of the α-1 and α-2 domains forming the peptide-binding region, which is supported by β2-microglobulin (Bjorkman *et al.*, 1987). The α-3 domain provides the binding site for CD8 co-receptors (Norment and Littman, 1988). MHC class II molecules comprise two transmembrane α and β heavy chains each having two extracellular domains. The distal α and β domains form the peptide binding groove and the β2-domain nearest the cell membrane provides a binding site for CD4 co-receptors (Stern and Wiley, 1994).

Proteins derived from bacteria or viruses as well as proteins derived from self-molecules must be processed and presented as peptides by MHC class I and II molecules in order to be recognised by a TCR. Proteins from within the cell are processed by the MHC class I pathway and extracellular proteins by the MHC class II pathway as illustrated and described in Figure 1.1.1.

HLA class I and class II molecules also differ by the size of peptide that can be bound. Peptides presented by HLA class I molecules are around nine amino acids in length and bind the HLA molecule via anchor residues at either end of the peptide. Peptides associated with class II molecules are generally longer, between 10-34 amino acids, and are fixed by multiple anchor residues. Openings at either end of the binding groove allow extended lengths of peptide to protrude (Madden, 1995).

The TCR is capable of recognising an infinite number of peptides by rearrangement of gene segments in a similar manner to antibody hypervariable regions. In addition, thymic education attempts to ensure that immune responses are not inappropriately generated against self-antigens by a mechanism known as peripheral tolerance (Walker and Abbas, 2002). Immune responses against pathogenic or non-self antigens can also be enhanced by the conformation of peptides bound to HLA molecules and depending on the HLA molecule, a different repertoire of peptides may bind. These attributes, acquired through polymorphism of HLA molecules, have occurred through evolution and natural selection as a mechanism of ensuring that during pandemic infections, some individuals will remain infection-free (Bodmer, 1975; Parham *et al.*, 1989; Piazza *et al.*, 1980).

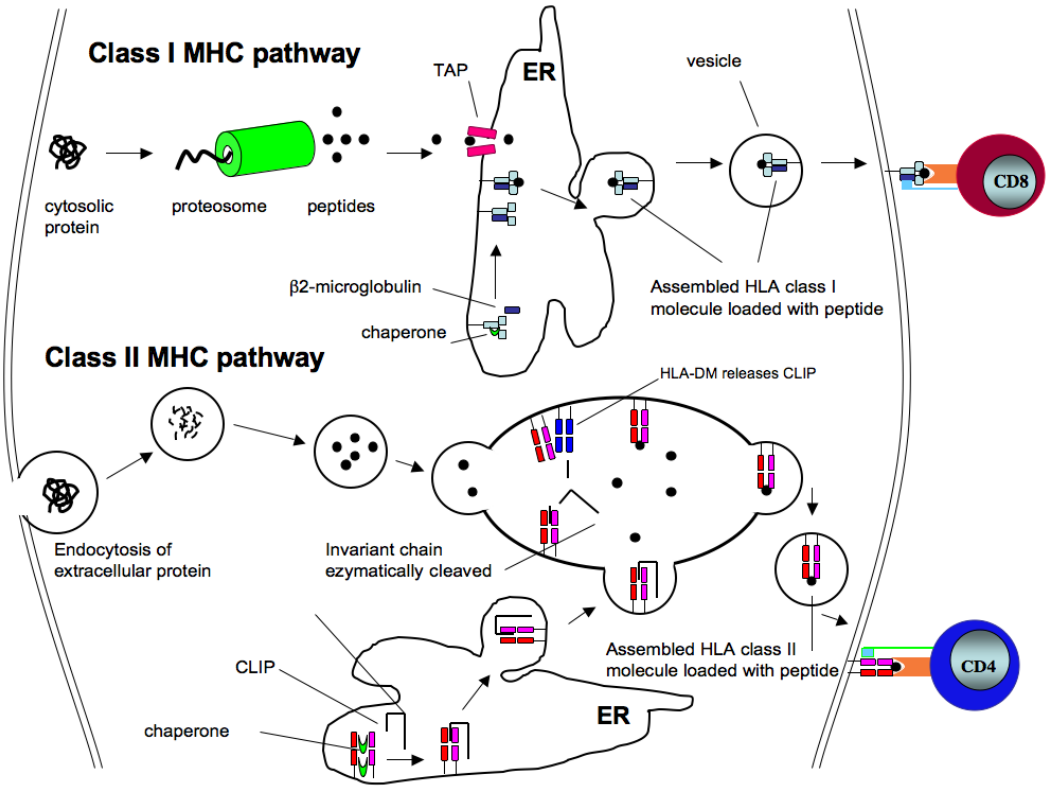


Figure 1.1.1: Antigen processing and presentation by MHC class I and class II molecules. Peptides presented by HLA class I molecules are produced by proteosomal digestion of intracellular components, including pathogens, within the cytosol of cells and are shuttled to the Endoplastic Reticulum (ER) by a protein called the Transporter associated with Antigen Presentation (TAP). Within the ER, MHC class I molecules are transcribed and stabilised by chaperone molecules before binding with β2-m. Peptides derived from proteosomal digestion of proteins associate with newly formed HLA class I molecules making them stable. Complexes are transported in vesicles to the cell surface to engage with CD8+ TCRs. By contrast, HLA class II peptides are derived from extracellular antigens engulfed by the cell in a process called endocytosis and transported inwards inside vesicles. Increasing acidity activates proteases and hydrolases within the vesicle, degrading the antigen and producing peptides from the proteins and glycoproteins. Inside the ER, newly transcribed class II molecules are stabilised by chaperone molecules and are prevented from binding peptides by association with an invariant chain. The class II:invariant chain complex then leaves the ER in vesicles where the invariant chain is degraded by enzymes, activated in changing pH, leaving a small fragment called CLIP in the peptide binding groove. The vesicle then fuses with a vesicle containing peptides where a vesicle membrane protein, HLA-DM releases CLIP. Peptides then become bound to HLA class II molecules and the complexes are carried to the cell surface by outward-bound vesicles where they present their peptide to CD4+ T-cells (Trombetta and Mellman, 2005).

1.1.6 HLA Polymorphism

The MHC molecules and other proteins involved in antigen processing and presentation, are encoded by the MHC genes situated on the short arm of chromosome 6 (6p21.3) (Ziegler *et al.*, 1990). The striking feature of HLA class I and II molecules is their extensive variability between individuals, termed polymorphism. HLA class I molecules are encoded by HLA-A, B and C genes and class II molecules by HLA-DR, DQ and DP genes as shown in Figure 1.1.2.

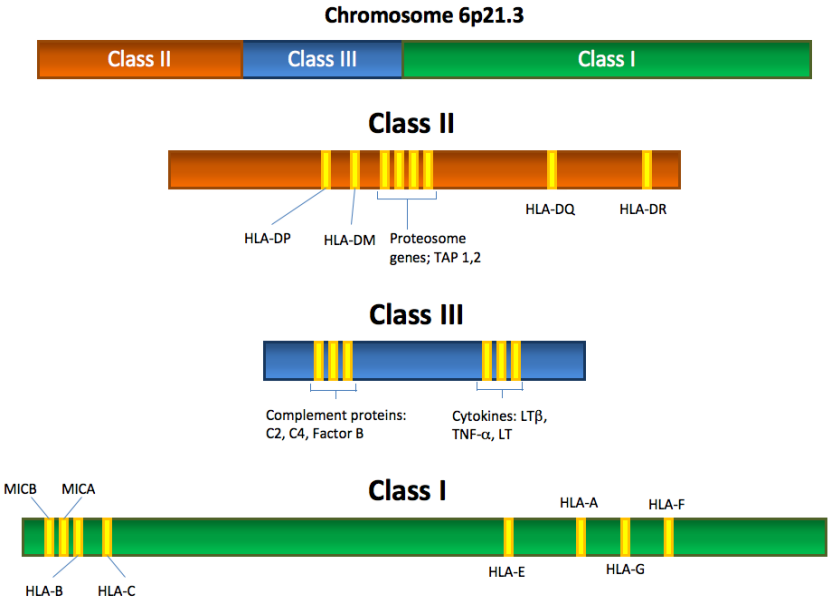


Figure 1.1.2: Genomic organisation of the MHC region. The human MHC region encompasses 3.6 Megabases on chromosome 6 and contains over 200 gene loci including all HLA genes and many immune regulatory genes. HLA-A, B and C genes are encoded within the class I region and HLA-DR, DQ and DP genes within the class II region. (Klein and Sato, 2000).

HLA class I and II molecules are encoded by inherited genes and ensure diversity by two mechanisms. For each loci, two genes are expressed, one inherited from the mother and the other from the father, thus a total of twelve different HLA molecules can be expressed from the six classical HLA gene loci. The second mechanism of generating diversity of the MHC is by genetic polymorphism that arises from changes in the nucleotide structure of HLA gene loci and hence the amino acid structure can be altered. This has resulted in the evolution of thousands of HLA allelic variants making the HLA genes the most polymorphic genetic system to be discovered. Currently (IMGT/HLA Release 3.4.0 April 2011) there are over 6400 HLA alleles, and those encoded by the classical HLA loci are detailed in Table 1.1.1 (Robinson *et al.*, 2001).

Table 1.1.1: The allelic repertoire of classical HLA genes[1]

HLA class I loci	Alleles	HLA class II loci	Alleles
HLA-A	1601	HLA-DRB	1027
HLA-B	2125	HLA-DQB1	153
HLA-C	1102	HLA-DPB1	149

[1]IMGT/HLA release 3.4.0, April 2011

Extensive polymorphism of HLA alleles ensures that most individuals will inherit a different allele from each parent and therefore become heterozygous for all their HLA genes. This is known as the heterozygote advantage as there is a greater chance that infection can be overcome with two versions of an HLA molecule, able to present slightly different peptides (Hughes and Nei, 1988). Genetic variation of HLA alleles has evolved through positive selection resulting in differences that are concentrated in the regions involving peptide binding and interaction with the TCR (α-1 and α-2 regions of the class I loci and the β-1 region of class II gene products). The combination of alleles that can be inherited on each chromosome 6 is called a haplotype and heterozygous individuals have two haplotypes.

1.2 Solid Organ Transplantation

Human organ transplantation is one of the most remarkable developments in medical science in recent times, providing a routine life-enhancing procedure and facilitating emergency life-saving intervention. The first successful living-related donor kidney transplant between identical twins took place in 1954, performed by Dr. Joseph E. Murray at Peter Bent Brigham Hospital in Boston, USA. The transplant was from Ronald Herrick into his identical twin Richard who lived for a further eight years. Murray became a winner of the 1990 Nobel Prize along with E.D. Thomas "for their discoveries concerning organ and cell transplantation in the treatment of human disease" (Murray, 1994). An increasing understanding of the immunological mechanisms of graft rejection and the development of drugs to inhibit them has brought about a revolution in organ transplantation.

An organ transplant or 'graft' has several classifications. An autograft is from one site on an individual to another, for example a skin graft. An isograft (also called syngeneic graft) is between genetically syngeneic individuals, i.e., identical twins. An allograft is between members of the same species but who are genetically different, for example siblings and finally, a xenograft is between different species. The main two categories used are autograft and allograft and these treat or cure many conditions as listed in Table 1.2.1. It was recognised over a 100 years ago that autografts and isografts would succeed whereas allografts failed (Karamehic *et al.*, 2008). Modern transplantation dates back to World War II when many burned airforce pilots were treated by attempting skin grafts, which was a complete failure (Medawar, 1948). Peter Medawer described the inflammatory reaction that was observed as rejection. "Skin grafts between genetically unrelated individuals will

undergo necrosis and fall off in seven to ten days" (Medawar, 1956). A repeat transplant using the same pair rejected more rapidly and experiments in mice and rabbits led to the conclusion that the 'accelerated' rejection of a second graft can be adoptively transferred by lymphocytes and that graft rejection exhibits both memory and specificity (Medawar, 1956). Other observations forming evidence of rejection being an immunological phenomenon are infiltration of lymphocytes and monocytes as seen in biopsies of rejected allografts.

Table 1.2.1: Types of solid organ transplantation and conditions they are used to treat

Transplanted Organ	**Reason**
Kidney	End-stage renal failure
Lung, heart/lung	Pulmonary hypertension, cystic fibrosis
Liver	Cirrhosis, cancer, biliary atresia
Heart	Cardiomyopathy, congenital heart disease
Cornea	Dystrophy, keratitis
Pancreas or islets	Diabetes
Small bowel	Cancer
Skin	Burns

1.2.1 The immune response in organ transplantation

There are three stages involved in the immune response to alloantigens. The first stage is the recognition of alloantigens presented to naïve host T-cells either directly or indirectly. The second stage is proliferation and activation of primed alloreactive T-cells and the third stage is allograft destruction by effector cells. Figure 1.2.1 depicts and describes the three stages of the alloreactive response via the direct and indirect pathways.

Direct allorecognition occurs when donor APCs, mainly DCs migrate to the lymph nodes and spleen where they stimulate CD4+ and CD8+ T-cells directly via donor MHC molecules on the surface of the DC (Rogers and Lechler, 2001). Indirect allorecognition is where host DCs engulf donor antigens (class I or class II and also minor histocompatibility antigens) that have been shed from the graft and after processing present them as peptides to CD4+ T-cells in the context of self-MHC (of the recipient) thus stimulating host CD4+ T-cells leading not only to cell-mediated immunity but also antibody production by B-cells (Baker *et al.*, 2001; Gokmen *et al.*, 2008).

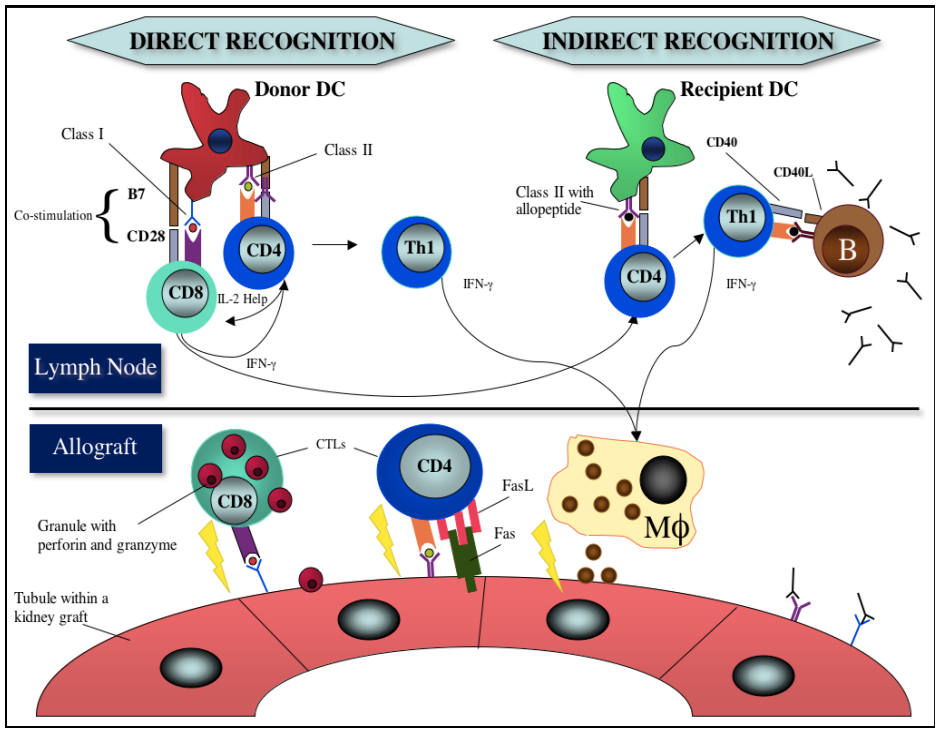


Figure 1.2.1: Diagrammatic representation of cellular events leading to an alloimmune response. Antigen is recognised directly or indirectly by T-cells interacting with donor or recipient DCs, activating effector cells. Activated cytotoxic CD8 cells recognising foreign MHC class I migrate to the allograft where the release of perforin and granzyme granules kills cells. Effector CD4 cells recognising foreign MHC class II kill graft cells by the interaction of Fas/FasL. Activated macrophages release cytotoxic granules, and antibodies to graft antigens cause activation of complement. Modified from Le Moine *et al*., 2002 (Permission granted by Wolters Kluwer Health).

The interaction of the TCR with antigen has high specificity but low affinity requiring a second signal, or co-stimulation, before T-cell activation can occur. Stimulation of the TCR without the second signal results in the T-cell becoming anergised (Matzinger, 1999). Co-stimulatory molecules are expressed on activated donor and recipient APCs. B7 and CD40 ligands interact with CD28 and CD40L respectively on the T-cell membrane resulting in the induction of several intracellular signals. Interaction of CD28 with B7 results in production and secretion of IL-2 by the CD4+ T-cell recognising intact donor class II molecules via the direct pathway and donor-derived MHC peptides via the indirect pathway (Gokmen *et al*., 2008). Either way, IL-2 activates host CD8+ T-cells that recognise class I peptides of the donor presented by donor DCs via direct allorecognition. The CD8+ cells become activated and secrete IFN-γ that acts on CD4+ T-cells involved in direct and indirect allorecognition, leading to a Th1 response. Alloreactive B-cells interact with Th1 CD4+ cells, arising from the indirect pathway, and produce alloantibodies (Le Moine *et al.*, 2002; Pettigrew *et al.*, 1998). The end result is the production of effector cells. CD4+ and CD8+ T-cells recognise donor antigens and proliferate in response to IL-2. The production of IFN-γ by

the activated T-cells activates macrophages and antigen recognition by B-cells together with help from activated CD4+ T-cells leads to the proliferation of plasma cells secreting alloantibodies. IFN-γ also up-regulates the expression of donor MHC on the allograft creating more targets for tissue destruction (Le Moine *et al*., 2002).

Alloreactive T-cells migrate out of the lymph node to the site of the graft, guided by a chemo-attractant gradient of chemokines released from the graft mainly due to ischaemia and reperfusion injury (inflammation) but also by infiltrating alloreactive cells. The T-cells are now cytotoxic and can to kill their targets by two main mechanisms, perforin/granzyme and Fas/Fas-ligand (FasL). CD8+ cytotoxic T-cells synthesise and release perforin and granzyme granules (Berke, 1995), leading to apoptosis. Fas/FasL interaction is the most important mechanism for cytotoxic CD4+ T-cells and results in the death inducing signal complex leading to target cell apoptosis. Activated macrophages release toxic molecules, for example nitric oxide and tumour necrosis factor-alpha (TNF-α) which also kills target cells. Alloreactive B-cells engulf the Ig and HLA complex and present the peptides via their class II molecule to CD4+ Th1 cells originating from the indirect pathway. The co-stimulatory signal is provided by interaction of CD40L expressed on the Th1 cell and CD40 expressed on the B-cell. Th1 cells give help to the B-cell in the form of IL-2 to mature into plasma cells and produce antibodies, which opsonise the target cell (Chinen and Buckley, 2010). The subsequent binding of IgM and IgG1 activates the complement cascade, giving rise to the membrane attack complex and target cell apoptosis. Additionally, NK-cells may bind IgG1 with their Fcγ (CD16) receptor on antibody-coated cells. The cross-linking of NK-cells and Fcγ causes perforin/granzyme-mediated NK cytotoxicity, known as antibody-dependent cellular cytotoxicity (ADCC). Cytokines and factors, released by all effector cells, attract other inflammatory cells to the site, leading to more immune-mediated damage (Le Moine *et al*., 2002).

1.2.2 Classification of Allograft Rejection

Rejection may occur at any time following a transplant and the patient must be monitored to detect problems and given treatment accordingly. The classification of rejection into early, short-term and long-term reflects the differing immunological mechanisms involved.

Hyperacute rejection

Hyperacute rejection (HAR) occurs rapidly, following re-vascularisation of an allograft, caused by preformed antibodies to the graft present in the serum of the patient. Anti-HLA antibodies that occur in response to prior blood transfusions, multiple pregnancies, previous transplants or antibodies against the ABO blood group system all have the potential to cause HAR (McAlack *et al.*, 1987; Rego *et al.*, 1987). HAR damages the endothelial cell lining of the blood vessels. Leakage of cells and fluid causes aggregation of platelets that block the microvasculature, leading to ischaemia (restriction in blood supply) and loss of function (Olszewski and Lukasiewicz, 1973). HAR can be avoided by performing ABO blood group matching, HLA antibody screening and crossmatching (described later).

Accelerated Rejection

Accelerated rejection can occur within days or weeks of an organ transplant. If a graft is given to someone who has been pre-sensitised to antigens expressed on the organ, a secondary reactivation of memory B and T-cells can occur, leading to an accelerated or antibody-mediated rejection response. This can occur even if the crossmatch is negative, although crossmatching significantly decreases the risk (Anderson and Newton, 1975).

Acute rejection

Acute cellular rejection (ACR) occurs within a few weeks or months following a transplant and early diagnosis is important to allow treatment. Most immunosuppressive therapy is aimed at preventing T-cell responses to allografts. The incidence of ACR is around 5-10% in the first year of transplantation for patients without pre-formed HLA antibodies (Colvin, 2007). Characteristic histological features of ACR are accumulation of mononuclear cells in the interstitium with inflammation of the tubules and sometimes arteries. Mononuclear cells, mainly CD4+ and CD8+ T-cells, are found in the interstitial space around tubules and cause graft damage (Colvin, 2007). Infiltration of T-cells and macrophages into the tubular epithelium is called tubulitis and is a characteristic lesion for ACR diagnosis.

Acute antibody-mediated rejection (aAMR) is now widely accepted as a separate rejection mechanism (Colvin, 2007) and antibodies directed against donor antigens cause approximately 25% of AR episodes. Risk factors are pre-formed HLA antibodies, the degree of HLA mismatching and decreased immunosuppression (non-compliance). In addition, aAMR is resistant to all immunosuppression regimes and is therefore difficult to

treat (Lorenz *et al.*, 2004). Acute AMR usually occurs together with ACR, but can occur independently, effecting targets other than HLA on endothelial cells such as ABO blood group antigens. The symptoms for aAMR are similar to ACR and a biopsy is required to confirm diagnosis. The histological pattern for diagnosis shows necrosis of graft vessel walls with acute inflammation with accumulation of neutrophils and monocytes in peritubular and glomerular capillaries as shown in Figure 1.2.2 (Racusen *et al.*, 2003).

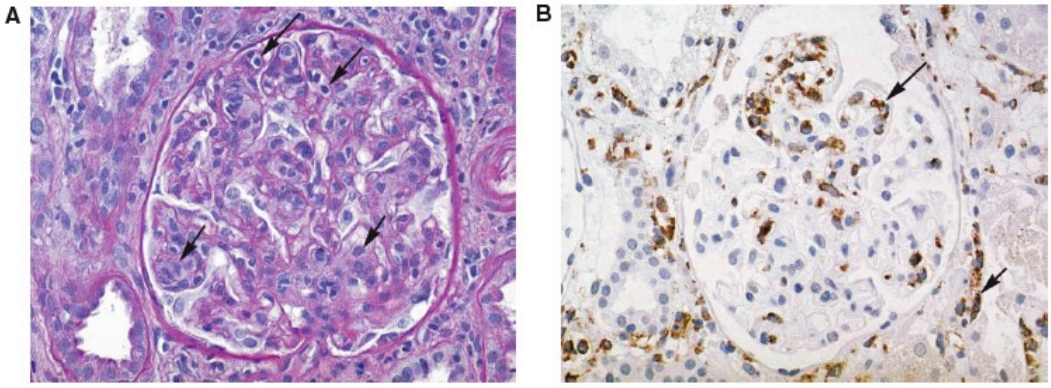


Figure 1.2.2: Histological features of antibody-mediated rejection. A. Glomerulitis showing infiltrating monocytes and swollen endothelial cells in capillaries. B. Immunoperoxidase staining of monocytes and macrophages (top arrow) and peritubular capillaries (lower arrow) magnification 400x. Racusen *et al*., 2003 (Permission for reproduction granted by John Wiley and Sons).

Classic signs and symptoms of AR include swelling and tenderness of the allograft. In the case of renal transplantation, decrease in renal function can be diagnosed by a decrease in urine volume and estimated glomerular filtration rate (eGFR) or increasing blood urea, nitrogen and creatinine levels (Torpey *et al.*, 2010, p. 237).

Chronic Allograft Dysfunction

Chronic rejection of renal allografts can involve either or both cellular and antibody-mediated mechanisms, occurs over months to years and can be recognised by fibrosis and vascular abnormalities with loss of graft function occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than AR and has become a major cause of allograft loss. Many processes, both immunological and non-immunological, are thought to be involved. The predominant cause of chronic rejection, leading to organ failure, is arterial occlusion as a result of proliferation of intimal smooth muscle cells (Vathsala, 2005). This process is called transplant arteriopathy and is often seen in failed cardiac and renal allografts. Initially called chronic rejection, this condition has been renamed chronic allograft dysfunction (CAD)

(Racusen *et al.*, 1999) and can occur within six months to a year after transplantation with progressive dysfunction occurring over many years.

Other histological features of CAD are transplant glomerulopathy, peritubular capillaropathy, transplant arteriopathy, interstial fibrosis and tubular atrophy. These lesions, and their severity in renal biopsies, form the basis of diagnosis of CAD as detailed by the 2003 Banff Classification of Renal Allograft Pathology (Racusen *et al.,* 1999, 2003). Transplant glomerulopathy is characterised by duplication of the glomerular basement membrane and most cases are associated with the presence of donor-specific HLA class II antibodies with approximately 30-50% demonstrating C4d deposition in the peritubular capillaries (Colvin, 2007; Lorenz *et al*., 2004). Histological proof of antibody-mediated rejection has become possible by the introduction of the C4d stain by Feucht (Feucht, 2003). C4d is an inactive component of the classical complement pathway and remains in the tissue for several days after AMR. C4d deposition is visualised using immunofluorescence as intense ring pattern staining in the majority of peritubular capillaries as shown in Figure 1.2.3. An alternative method using immunoperoxidase can be used although this is less sensitive. Transplant glomerulopathy revealed by histological changes and C4d deposition by circulating donor-specific antibodies is diagnostic of CAD.

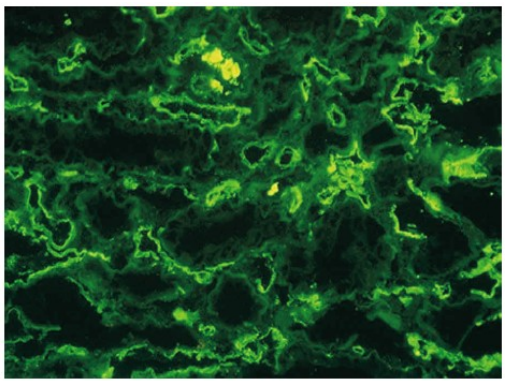


Figure 1.2.3: Immunofluorescence method of C4d deposition detection. Immunofluorescence shows peritubular capillary staining of C4d as bright ring patterns, indicating AMR. Racusen *et al*., 2003 (Permission for reproduction granted by John Wiley and Sons).

Duplication or lamination of the basement membrane, as seen in glomeruli of patients with transplant glomerulopathy, may also be seen in peritubular capillaries. When damaged endothelium repairs itself, it forms a new basement membrane layer and multilamination occurs by repeated injury caused to the graft by alloantibody. This can occur sub-clinically and only become apparent following CAD or chronic rejection (Lerut *et al.*, 2007).

Interstitial fibrosis and tubular atrophy is not specific for rejection but is an important histological feature of CAD. Formerly, this lesion was known as chronic allograft nephropathy (CAN) but this definition is now removed from the Banff system (Solez *et al.*, 2007). Evidence of these changes indicates donor-specific AMR or T-cell-mediated rejection.

1.2.3 Prevention and treatment of immunological allograft rejection

Screening and Crossmatching

ABO blood group testing is performed uniformly for all transplants to prevent HAR. However it is possible to perform transplants from ABO incompatible donors when an otherwise suitable live donor is found. In this situation there would be time to plan and prepare the recipient who would require immunodepletion of anti-ABO antibodies by a process known as plasmapheresis (Thielke *et al.*, 2007).

Patients awaiting organ transplantation are screened for the presence of preformed antibodies reactive with allogeneic HLA molecules. These antibodies may have arisen from previous pregnancies, transfusions or transplants and are a risk factor for hyperacute or acute vascular rejection, both mediated by antibodies. There are a number of different tests used to screen organ transplant recipients for the presence of HLA specific antibodies. Complement-Dependant Cytotoxicity (CDC) utilises panels of volunteer sera with known HLA antibodies and can define specificity and distinguish complement binding IgG and IgM, but may not be sensitive enough to define all specificities that can potentially be recognised by the recipient. ELISA (Enzyme-linked immunosorbent assay) can also be used to detect HLA antibody and specificity directed against immobilised, affinity purified HLA molecules. Finally, flow cytometry (FC) and Luminex bead technology, are methods similar to ELISA but more sensitive and can detect specific antibodies against donor antigens. According to British Transplantation Society guidelines, patients should be screened every three months and following any sensitisation event, for example blood transfusion (www.bts.org.uk/transplantation/standards-and-guidelines/).

If a potential donor is identified, the crossmatch test will determine whether or not the patient has antibodies that will react specifically with cells of the donor organ. Crossmatching is performed pre-transplant in a similar manner to the detection of HLA antibody specificity with the CDC test, except the patient's serum is only tested for reactivity with the particular donor's lymphocytes. The introduction of CDC-

crossmatching dramatically reduced the incidence of HAR (Patel and Terasaki, 1969) however, up to 20% of patients still experienced early antibody-mediated AR. This suggested that the CDC-crossmatch did not detect all DSA leading to the introduction of more sensitive techniques, including prolonging incubation times (Amico *et al.*, 2008). The flow-cytometry crossmatch (FC-XM) became available in the 1980's and involves incubating donor lymphocytes with recipient serum and the presence of antibody reactive with these cells is detected by FC. A positive CDC-crossmatch reflects high titre DSA that may cause HAR and is contraindication to transplant. However, a positive T-cell FC-XM with a negative CDC-crossmatch may only indicate increased risk of acute antibody-mediated rejection. As only B-cells express HLA class II, a B-cell positive, T-cell negative FC-XM indicates donor specific HLA-DR, DQ or DP antibodies may be present. If B-cell and T-cell FC-XM are both positive then the recipient has HLA class I and possibly class II alloantibodies (Tait *et al.*, 2009).

Alloantigens not expressed by B-cells or T-cells such as molecules displayed on renal endothelial surfaces, may also be targets of antibodies produced by the graft recipient. As a consequence, T-cell and B-cell FC-XM cannot detect these non-HLA antibodies and current BTS guidelines do not require their identification. However, methods are being developed to enable detection of antibodies reactive with donor endothelial cell antigens in organ transplant recipients as discussed further at the end of this chapter.

HLA Matching

The perfectly matched donor and recipient would be syngeneic, for example monozygotic twins, however this is rare and major and minor histocompatibility differences between the donor and recipient usually exist. For kidney transplants from deceased donors it is only necessary to match for broad HLA antigens and can be achieved in a few hours by serological methods or PCR-SSP, prior to harvesting the organ from the donor. In practice, matching is only performed for HLA-A, -B and –DR based on criteria for allocation of donor organs where a score is given for the number of mismatched HLA antigens. For example a completely matched donor and recipient would have the score 0-0-0 and a recipient and donor with a score of 2-2-2 would have all HLA-A, B and DR antigens mismatched. Often the most suitable recipient is not a complete HLA match and compromises are made to allow usage of all available organs.

Matching for HLA antigens has a beneficial effect on graft survival (GS) by reducing or eliminating one or more arms of the immune response to alloantigens. Direct and indirect

activation of T-cells responsible for ACR is dependent on differences in the structure of class I and class II molecules expressed on the allograft and by the recipient. As HLA matching is only performed for HLA-A, B and DR, differences at other loci such as HLA-C, HLA-DQ and HLA-DP may occur. Additionally, minor histocompatibility (H) mismatches will exist between related and unrelated individuals and may cause ACR, although more slowly and less intense. For example, cellular responses to proteins encoded on the male Y chromosome, known as H-Y antigens can occur in females with a graft from a male.

Patients who require more than one graft are complicated by the additional risk of sensitisation to HLA and other antigens expressed by their first graft and may require closer matching of a subsequent graft. HLA matching data from the United Organ Sharing scheme (UNOS) show that 25% of re-grafted renal transplant patients had no HLA mismatches (Cecka, 1998). However, this means the patient will have to wait longer for a suitable kidney with good HLA matching and some patients with rare HLA types may never find a suitably matched donor. For these patients it may be possible to match only for HLA-DR and compensate with heavier immunosuppression (Opelz, 1996) although this does increase the risk of further sensitisation if the graft fails.

Immunosuppression

The strategies used in clinical practice to avoid or delay rejection are known as immunosuppression. Immunosuppression is the major approach for the prevention and management of organ rejection and can be tailored to the patient's needs. Different combinations of immunosuppressive drugs can be used at three main stages before and after transplantation. The first stage is induction therapy, given before the transplant as prophylaxis against early AR. The combination of drugs taken routinely by patients following an organ transplant is the second stage, known as maintenance immunosuppression and can be adjusted by dose. In the event of AR episodes, increased dose or different drugs can be used (third stage).

Drugs that inhibit or kill T-cells are the most widely used, the most important of which is cyclosporine. Cyclosporin A (CsA) is a fungal macrolide produced by soil organisms (Borel and Kis, 1991) and inhibits the transcription of genes by T-cells that encode cytokines, most notably IL-2. CsA binds with high affinity to a ubiquitous cellular protein called cyclophilin; the complex of CsA and cyclophilin binds to and inhibits the enzymatic activity of the protein phosphatase, calcineurin. Calcineurin function is required to activate

the transcription factor NF-AT (nuclear factor of activated T-cells) thus blocking the transcription of IL-2 and other cytokines, which abrogates IL-2 dependent growth and differentiation of T-cells (Walsh *et al.*, 1992). FK506, now known as tacrolimus, is another fungal macrolide with immunosuppressive properties similar to CsA (Tanaka *et al.*, 1987). Tacrolimus, and its binding protein FKBP, has the ability to bind calcineurin and inhibit its action, blocking the transcription of IL-2 by activated T-cells. Tacrolimus is less toxic than CsA and is most frequently used in liver transplant recipients and in cases where CsA has not properly controlled kidney rejection (Fung *et al.*, 1991; Shapiro *et al.*, 1991).

Another type of immunosuppressive drug is the antibiotic rapamycin (also called sirolimus), which has a different mode of action but its principal effect is to inhibit T-cell proliferation. Rapamycin binds FKBP in a similar manner to tacrolimus but the rapamycin and FKBP complex does not inhibit calcineurin but binds to another molecule called MTOR (mammalian target of rapamycin). The mechanism inhibiting T-cell proliferation is not fully understood but may regulate a protein kinase that participates in cell cycle control. Combination of cyclosporin A and rapamycin blocks IL-2 synthesis and IL-2 driven proliferation, and are powerful inhibitors of T-cell responses (Vathsala, 2005).

Metabolic toxins inhibit the maturation of lymphocytes from immature precursors and also kill proliferating mature T-cells that have been stimulated by alloantigens. The first such drug to be developed for the prevention and treatment of rejection was azathioprine. This drug is still used but is toxic to precursors of lymphocytes in the bone marrow and enterocytes in the gut. The newest and most widely used drug in this group is mycophenolate mofetil (MMF). MMF is metabolised to mycophenolic acid, which blocks a lymphocyte-specific isoform of inosine monophosphate dehydrogenase, an enzyme required for *de novo* synthesis of guanine nucleotides. There are few toxic effects because MMF selectively inhibits the lymphocyte-specific isoform of this enzyme (Mele and Halloran, 2000). MMF is now routinely used in combination with cyclosporin A to prevent AR (Chadban *et al.*, 2008).

Polyclonal T-cell depleting antibodies known as anti-thymocyte globulin (ATG) target CD3 CD4, CD8, HLA class I, HLA class II, cytokine receptors and adhesion molecules. The ubiquitous targeting of ATG has numerous effects on immunosuppression but can also lead to T-cell activation (Mohty, 2007). ATG is used for induction therapy in 35% of kidney and 60% pancreas transplants in the USA but is used less often in the UK (Torpey et al., 2010, p. 150). Antibodies that react with TCRs and deplete or inhibit T-cells are used for

induction therapy and to treat AR episodes. A mouse monoclonal antibody called OKT3 is the most widely used and is specific for human CD3. OKT3 acts either as a lytic antibody by activating the complement system to eliminate T-cells, or opsonises T-cells for phagocytosis. Another antibody in clinical use targets CD25, the α-subunit of the IL-2 receptor. There are two types of this drug called basiliximab (chimeric monoclonal antibody) and daclizumab (humanised monoclonal antibody) and is used for induction therapy, preventing T-cell activation by blocking IL-2 binding to activated T-cells, or depleting CD25-expressing activated T-cells by mechanisms similar to OKT3 (Waid *et al.*, 1991). Alemtuzumab or Campath 1H® is a monoclonal antibody specific for CD52 expressed on T-cells, B-cells, NK-cells, monocytes and macrophages. CD52+ cells are rapidly cleared from the circulation either by complement-mediated lysis or removal of opsonised cells in the lymphoid organs (Torpey et al., 2010, p. 154).

Anti-inflammatory drugs are also routinely used for the prevention and treatment of graft rejection, the most powerful of which are corticosteroids. These natural hormones and their analogues block the synthesis and secretion of cytokines, including TNF-α and IL-1, by macrophages as well as lymphocytes and DCs. This reduces graft endothelial cell activation and recruitment of inflammatory cells. Corticosteroids may also block other effector mechanisms of macrophages, such as the generation of prostaglandins, reactive oxygen intermediates and nitric oxide. Table 1.2.2 summarises the immunosuppressive drugs used and their mode of action.

The challenge facing the clinical transplantation team is to balance the individual needs of each patient with drug efficacy and toxicity to provide adequate protection from AR and prevent early graft loss. For many years 'triple therapy' using CsA, azathioprine and corticosteroids was the gold standard immunosuppressive protocol for all solid organ transplantation. The introduction of newer drugs such as tacrolimus and MMF and widespread use of induction therapy drugs such as OKT3 have further decreased AR however, nephrotoxic effects of calcineurin inhibitors such as CsA, increased cardiovascular risk and development of malignancy associated with immunosuppression have also become important considerations. An effective immunosuppression protocol should provide prophylaxis against AR and maintenance immunosuppression sufficient to prevent immunological graft damage and also limit toxic effects of drugs by using lower doses or alternative products.

Table 1.2.2: Methods of immunosuppression in clinical use

Drug	Use and Mode of Action
Cyclosporin A and Tacrolimus	Induction and maintenance therapy. Blocks transcription of genes by T-cells that encode cytokines, most notably IL-2.
Azathioprine	Induction and maintenance therapy. Blocks proliferation of lymphocyte precursors by preventing DNA synthesis.
Mycophenolate mofetil (MMF)	Induction, maintenance and AR therapy. Blocks lymphocyte proliferation by inhibiting guanine nucleotide synthesis in lymphocytes.
Rapamycin (Sirolimus)	Maintenance therapy. Binds MTOR preventing T-cell proliferation.
Anti-thymocyte globulin (ATG)	Induction therapy. Polyclonal antibody that targets and depletes cells via numerous surface receptors including CD3, CD4, CD8 and HLA molecules.
OKT3	Induction and AR therapy. Monoclonal antibody. Depletes T-cells by binding to CD3 and promoting phagocytosis or complement-mediated lysis.
Basilizimab/Daclizumab	Induction therapy. Monoclonal antibody. Targets CD25 on activated T-cells and binding prevents IL-2 driven expansion of alloreactive T-cells.
Alemtuzumab (Campath 1H®)	Induction therapy. Monoclonal antibody targeting CD52 expressed on T-, B- and NK-cells, monocytes and macrophages resulting in depletion by phagocytosis or complement-mediated lysis.
Corticosteroids	Induction, maintenance and AR therapy. Reduces inflammation by inhibiting cytokine secretion.

1.2.4 Other factors associated with rejection and morbidity

Mechanisms that cause rejection can be due to either immunological or non-immunological clinical factors, they may also be early or late events after transplantation. Despite advances in immunosuppressive therapy, 10-30% of kidney allografts are affected by AR episodes, although organ loss in the first few months has become rare due to improved procedures (Pascual *et al.*, 2002). The challenge remaining is overcoming chronic allograft failure.

In renal transplantation, the type of donor kidney used is one of the most important factors determining long-term allograft survival. Organs from living donors lead to improved short and long-term outcomes by avoiding the cold-ischaemic injury that occurs during the time between organ retrieval and reperfusion (Bryan *et al.*, 2001). There is also an avoidance of long waiting lists and progressive disease in the patient. Donor age is known to have a considerable influence on GS and nullifies the effect of HLA-matching with donors over 60 years (Arnol *et al.*, 2008; Koka and Cecka, 1990).

Later in the post-transplant course, there may be problems with inadequate graft function and malignancy. A particular problem is post-transplant lymphoproliferative disorder which is a lymphoma resulting from the reactivation of Epstein Barr virus (EBV) (Colleoni *et al.*, 2000). This is more of a problem when an EBV positive donor organ is given to an EBV negative recipient (Le Moine *et al*., 2002). Primary problems in all transplants post-operatively include wound infection, early rejection, bleeding and graft thrombosis (Vathsala, 2005). Conditions such as co-morbid diabetes mellitus, hypertension, hyperlipidaemia or the toxic effects of immunosuppressive drugs are also factors, along with those described above, associated with chronic allograft failure in solid organ transplantation (Pascual *et al*., 2002). As the liver has the ability to regenerate itself, chronic allograft failure is not such a problem.

Sub-optimal immunosuppression can lead to long-term chronic allograft failure as a result of inadequate dosage by the clinician or non-compliance of the patient with the immunosuppressive drug regime (Vathsala, 2005). In addition to chronic allograft failure, some of these factors (as well as smoking and obesity) are also responsible for the exacerbation of pre-existing conditions such as cardiovascular disease, which is the major cause of patient death with a functioning allograft. Other causes of death with a functioning allograft are infections (Le Moine et al., 2002) and malignancy (Agraharkar *et al.*, 2004). Figure 1.2.4 summarises many of the factors involved in CAD.

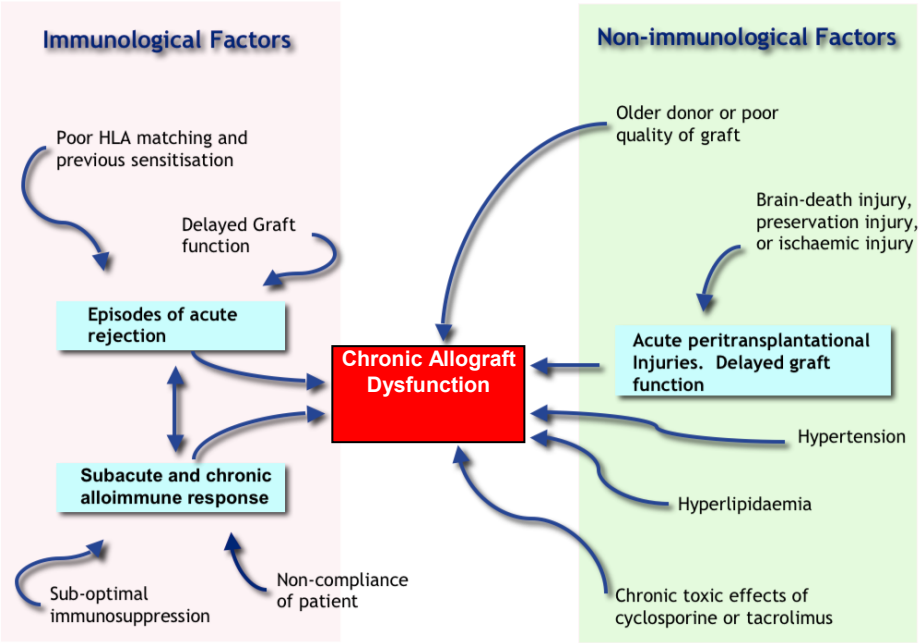


Figure 1.2.4: Immunological and non-immunological factors contributing to late allograft loss due to CAD. Modified from Le Moine *et al*., 2002 (Permission granted by Wolters Kluwer Health).

1.2.5 Antibodies and their role in transplantation

The extent to which antibodies are implicated with graft destruction and rejection has been the centre of an on-going debate for many decades. During Medawer's experiments, he attempted to prove a link between rejection of allografts with the production of antibodies (Medawar, 1948) and in 1950, Peter Gorer took this work further and established an association of tumour graft destruction with the production of alloantigen-specific antibodies (Gorer, 1950). However, it was not proven at this time that antibodies caused any injury to the graft and some investigators questioned whether graft destruction was actually an immunological mechanism. A few years later, it was found that cells, not antibodies, were responsible for graft destruction and rejection (Mitchison, 1958) and since then most attempts at limiting the immune response to transplanted organs have focussed on suppression of cellular immunity. Modern immunosuppression targeting T-cell function shows improved short-term GS of 88-95% in the first year of renal and cardiac transplantation (Colvin and Smith, 2005).

The first study of antibodies in renal patients following graft loss was published in 1968 (Morris *et al.*, 1968) and revealed 38% of recipients with failed grafts had HLA antibodies. In 1970, a strong association of circulating donor HLA-specific antibodies and chronic allograft arteriopathy was found (Jeannet *et al.*, 1970). Later experimental mouse cardiac allografts showed that passive transfer of alloantibodies induced the development of chronic allograft arteriopathy but B-cell deficient mice did not develop these lesions (P. S. Russell *et al.*, 1997).

The percentage of patients with detectable HLA antibodies before graft rejection has increased over time with improved techniques for their detection. Using FC, 95% of patients who rejected a kidney were found to have HLA antibodies (Harmer *et al.*, 1995). In 2002, Lee and Terasaki published a study of 139 renal transplant recipients who were followed up for eight years and regularly tested for HLA antibodies using ELISA. Although this is a rather small study, the authors found all 29 patients who developed chronic rejection produced HLA antibodies before graft failure (P. C. Lee *et al.*, 2002).

The association of HLA antibodies with rejection is evident but does not prove that antibodies are causing rejection and so the argument has continued to the present day. The difficulty was visualising the HLA antibodies histologically using the complement product C3 as a marker, which was not easy to detect in renal tissues. A breakthrough was made in the 1990s with the advent of C4d deposition in grafts as a marker of complement fixation (Feucht *et al.*, 1993) and was followed by the discovery that C4d deposition is usually associated with donor-specific antibodies (A. B. Collins *et al.*, 1999). This improvement in diagnosis of antibody-mediated rejection together with advances in methods to detect antibodies with greater sensitivity and specificity, has led to resurgence in the study of antibody-mediated allograft rejection.

There have been many studies investigating whether a relationship exists between the production of HLA antibodies and graft failure. The problem is that the results are extremely variable ranging from 1.6 to 60% (Akalin and Pascual, 2006) of patients with detectable HLA antibodies post-transplant, probably relating to differences in methods used to detect antibodies. This is complicated further by some centres measuring DSA and others only detecting panel reactive antibodies (PRA). To complicate matters even further, there is considerable variation in the patient populations that are studied, for example some centres use randomly selected patients while others may select only patients with acute or chronic rejection. The time of sample collection and testing also varies.

The assays used to detect antibodies are discussed more fully later, but fall into two main groups. The CDC is a cellular assay that detects HLA antibodies present in the patient's serum by reactivity with a panel of cells collected from cell donors with common and rare HLA types (PRA). Antibodies against HLA class I or II can be detected by using either T or B-cells, respectively and results are reported as %PRA, for example if the patient's serum reacts with 20 of 40 different cells, the PRA would be 50%. Other tests are known as solid-phase assays and use purified or recombinant HLA antigens fixed to a solid surface. The ELISA was the first solid-phase assay to detect HLA antibodies with greater sensitivity than CDC but sensitivity was considerably increased with the introduction of FC beads, coated with HLA antigens. Beads used for antibody detection may be coated with many different HLA antigens for use in a screening assay, or an individual antigen can be used with a specific-coloured bead to identify the exact specificities of the antibodies in a patient's serum (Luminex™). These sensitive techniques have the advantage of detecting specificities undetected by CDC and as such are not a risk for HAR but may have implications for aAMR in the early transplant period. Sensitive antibody detection is also useful to detect the rise of *de novo* HLA antibodies after transplantation. These tests are however restricted to detecting anti-HLA antibodies in routine practice but other non-HLA antibodies may also be clinically relevant.

1.2.6 HLA antibodies and graft rejection

HLA antibodies present before transplantation

Most studies aimed at identifying HLA antibodies as a risk factor for acute and chronic rejection focus on *de novo* antibody production post-transplant, but it has been found that pre-formed HLA antibodies can also affect GS. In an analysis conducted for the Collaborative Transplant Study (CTS) (Susal and Opelz, 2002), 4136 recipients of kidneys from deceased donors from 28 centres were tested pre-transplant by ELISA for HLA class I and II antibodies and GS at two years was analysed. The study revealed pre-sensitisation to either HLA class I or II was not a risk factor for graft loss whereas having both types of antibody associated with graft rejection ($P<0.001$). This was confirmed by the CTS in another series of 5315 patients where, as before, patients who simultaneously produced both classes of HLA antibody had an increased risk of graft failure ($P<0.001$), however association of donor-specific HLA antibodies was not investigated (Susal *et al.*, 2009). In another, albeit smaller study (Q. Zhang *et al.*, 2005b) 20 of 49 patients (41%) were sensitised to HLA antigens pre-transplant, seven had class I antibodies, four had class II and

nine had both types as determined by ELISA. The presence of class I and/or class II HLA antibodies was significantly associated with aAMR (P=0.005), but not ACR. The donor-specificity of pre-existing HLA antibodies was not investigated, although presumably the presence of HLA antibodies specific for donor antigens would have been a contraindication to transplant. It is possible that patients with pre-existing HLA antibodies who developed AR had non-donor-specific antibodies that cross-reacted with donor antigens. Alternatively, these patients may have been more likely to develop HLA antibodies and rejection was associated with *de novo* production of donor-specific HLA antibodies.

Post-transplant *de novo* HLA antibody production

Studies have also investigated whether the type of HLA antibody, class I or class II, developed *de novo* associates with acute or chronic rejection. In an early study (Martin *et al.*, 1987) it was demonstrated using CDC-PRA that most *de novo* antibodies were anti-HLA class I (50%) and 36% had both classes I and II. Shortly after, a study by Halloran (Halloran *et al.*, 1992) investigated HLA class I antibodies by CDC in 64 patients within three months of transplant. They found that all the patients with *de novo* class I antibodies developed AR compared to 41% of those with no antibodies. Moreover, those with class I antibodies had more severe rejection that occurred earlier with a greater incidence of graft loss.

In Zhang's study (Q. Zhang *et al*., 2005b) *de novo* HLA antibodies were detected by ELISA and the results show 12 of 49 patients developed class I antibodies (24%), eight (16%) developed class II antibodies and 10 (20%) developed both classes of HLA antibody and *de novo* HLA antibodies significantly associated with aAMR and ACR. Often cited is a paper published by Worthington (Worthington *et al.*, 2003) where 60% of patients with HLA antibodies developed them before rejection, and again the majority had class I antibodies. An association was also found between the post-transplant production of HLA antibodies detected by ELISA and AR and graft loss in another study (Fernandez-Fresnedo *et al.*, 2003). These authors found 62.5% of patients with HLA antibodies (predominantly class I) developed aAMR and all but one of the patients with HLA class I antibodies lost their grafts due to AR. In a study of chronic rejection (P. C. Lee *et al*., 2002) 139 patients were followed-up for eight years and tested for HLA antibodies using ELISA. Among 29 patients with chronic rejection, 14 developed *de novo* antibodies, all had class I antibodies and 86% had class II antibodies. Details were not provided of HLA class I and class II mismatching by Lee *et al*, but in the study by Worthington and colleagues and that of Fernandez-Fresnedo *et al*, class I mismatching was more frequent. This may indicate that

the higher frequency of class I antibodies detected post-transplant reflects increased HLA class I mismatching and not that class I antibodies are more likely to be produced. Alternatively, as discussed further below, HLA class I antibodies may be produced earlier than class II antibodies, accounting for higher frequencies.

Pelletier and colleagues (Pelletier *et al.*, 2002) used FlowPRA beads to detect HLA antibodies, revealing a different pattern to that emerging from the literature. In this study pre-transplant sera was also tested and 18% of 277 recipients developed *de novo* HLA antibodies. However, contrary to previous studies, most of these patients produced HLA class II antibodies only (68%) and multivariate analysis revealed that production of class II antibodies and not class I was significantly associated with chronic rejection independent of AR. Unfortunately it was not possible to deduce from this paper whether there was less HLA class I mismatching. Another study (Campos *et al.*, 2006) also found association of HLA class II antibodies (ELISA) with chronic graft loss, although pre-transplant sera were not tested. In this study of 512 first kidney recipients, sera were tested 4.4 years median time post-transplant. Twenty (4%) had class I HLA antibodies, 55 (11%) had class II and 16 (3%) had both, however details of mismatching was not provided. Univariate analysis showed that female gender, pregnancies and blood transfusions were associated with class I antibodies and deterioration in graft function was associated with class II antibodies. Using multivariate analysis, HLA class II antibodies were an independent risk factor for CAD.

A very large study of 4943 kidney graft recipients from 45 centres was carried out as part of the 14th International Histocompatibility and Immunogenetics Workshop and Conference (IHIWC) (Ozawa *et al.*, 2007). In total, 27.2% developed HLA antibodies after more than six months post-transplant detected by ELISA, Flow beads or Luminex beads, and the method of detection did not significantly influence the results. A separate analysis of HLA class I and II antibodies showed association with chronic rejection was highest when both antibody types were present (9.7%), 7.1% with class II only and 6.1% with class I only.

Donor-specific HLA antibody production

In a study of 263 renal allograft recipients (Supon *et al.*, 2001) the authors used a screening ELISA and a more specific ELISA to detect DSA (HLA-A, B, C, DR and DQ) and observed significant association between the presence of DSA against class II HLA antigens and acute and chronic rejection. Additionally, all five patients with both class I and class II HLA-DSA lost their grafts. Worthington (Worthington *et al*., 2003) used the same methods to detect HLA-DSA and graft recipients were categorised as failure (previous

graft loss) or functioning. All were negative for HLA antibodies before transplantation and sera was taken at regular intervals for follow-up. Among the 112 patients in the failure group, 51% produced DSA compared to 1.6% of 123 patients with functioning grafts ($P<0.0001$). In 60% of the HLA antibody positive recipients, antibody specificities were defined before graft failure with seventeen having class I-DSA, fourteen class II-DSA and three with both HLA class I and II-DSA. These authors also noted a difference in time taken to develop either class I-DSA or class II-DSA post-transplant, as class I-DSA were produced at a mean time of 627 days compared to 1542 days for class II-DSA.

One of the first studies to use beads coated with recombinant HLA single antigens (SAg) was published by Zhang and co-workers (Q. Zhang *et al*., 2005b). In addition to ELISA screening and DSA identification for HLA class I and II antibodies, the authors also used the Labscreen SAg HLA Class I Luminex assay (One Lambda). Forty-nine patients were prospectively monitored for the development of HLA-DSA and eight (16%) developed aAMR, eleven (22.4%) were diagnosed with ACR and all patients were negative by CDC and FC-XM. Sensitisation prior to transplant was revealed in 20 of the 49 patients (41%) among whom seven (35%) had class I, four (20%) had class II and nine (45%) had both antibody types. There was a significant correlation of pre-transplant class I and/or class II HLA antibodies with aAMR ($P=0.005$) but no association was found with ACR. Serial analysis of post-transplant sera from the 49 patients showed 22.4% developed DSA whilst 39% of patients had non-donor-specific antibodies (NDSA). Among those who developed DSA, five had class I-DSA, three had class II-DSA and three had both class I and class II-DSA. There was a highly significant correlation with HLA-DSA and both aAMR and ACR ($P<0.001$) although seven of the eleven patients with ACR had concomitant aAMR, two patients with HLA-DSA had ACR only. Patients who had only NDSA did not associate with aAMR or ACR.

The concept that HLA-DSA are dangerous and NDSA are harmless was taken further by Hourmant (Hourmant *et al.*, 2005) who investigated 1229 renal graft recipients by prospective annual screening for HLA-DSA during a period of five years using Luminex and ELISA screening followed by ELISA HLA SAg testing. The screening results showed 16.8% had HLA antibodies post-transplant and 5.5% of patients developed HLA-DSA. NDSA were detected in 11.3% and appeared between 1 and 5 years whereas DSA were detected between 5 and 10 years. In multivariate analysis, HLA-DR mismatching, prior sensitisation and AR significantly associated with the development of both DSA and NDSA with patients producing HLA antibodies having lower GS and inferior function.

A recent study (P. C. Lee *et al.*, 2009) described SAg Luminex testing for class I and class II HLA antibodies in two groups of renal graft recipients, with a follow-up of twelve years. One group of 25 recipients had graft failure due to chronic rejection and a total of 230 sera were tested while the other group had functioning grafts and 305 sera were tested. Sixty percent of the failure group developed HLA antibodies within one year compared to none with functioning grafts and ten-year GS was 27% versus 80%. It was also found that HLA class I antibodies were produced sooner at a median of 6.6 months and associate with rapid graft loss, compared to class II antibodies which appeared by a median of 12.5 months and associated with chronic graft loss. Interestingly, a study of patient's early biopsies or biopsies for clinical indication of rejection found that early biopsies did not associate with HLA antibodies but *de novo* HLA class II-DSA associated with late biopsies and subsequent graft failure (Hidalgo *et al.*, 2009).

Lachmann (Lachmann *et al.*, 2009) included 1014 deceased donor kidney recipients for analysis of chronic graft loss and HLA antibodies. Thirty percent were found to have HLA antibodies post-transplant and a third of these patients had HLA-DSA associating with significantly lower GS of 49% versus 83% in the HLA antibody negative group. This study showed that HLA-NDSA also associated with reduced GS of 70% versus 83%.

Gaston (Gaston *et al.*, 2010) also investigated late graft failure and association with HLA-DSA and included C4d deposition analysis. A total of 173 renal graft recipients with new onset late kidney graft dysfunction were divided into four groups: C4d+ with and without HLA-DSA and C4d- with and without HLA-DSA. Among the 173 patients, 68 (39%) demonstrated significant C4d staining whereas 105 (61%) had negative C4d staining. Among those who were positive for HLA-DSA (41%) using SAg Luminex (One Lambda), 40 stained positive for C4d and 31 were negative. Altogether, 99 of the 173 patients (57%) with graft dysfunction had C4d, DSA or both

The relevance of HLA antibodies detected post-transplant

The main finding by all the studies discussed above is that HLA antibodies are more frequent in graft recipients who experience aAMR (or defined as AR) and/or chronic dysfunction, and both conditions associate with reduced GS. In the majority of studies investigating *de novo* production of HLA antibodies, HLA class I antibodies are implicated with AR. In Halloran's study (Halloran *et al*., 1992) he stated that HLA class I antibodies are injurious to the graft but class II antibodies are of little or no consequence since class II antigen expression is limited. However, the study by Worthington (Worthington *et al.*, 2001) found that class I antibodies generally led to rapid graft failure whereas class II antibodies were more associated with chronic graft deterioration. This was evidenced by the differential time taken by each class of antibody to lead to graft failure and agrees with Pelletier (Pelletier *et al*., 2002) and Hidalgo's findings (Hidalgo *et al*., 2009). It is probable Halloran did not detect significant association with class II antibodies because of the time-point for antibody testing. The relatively short follow-up period of six-months may not have allowed sufficient time for class II sensitisation and hence did not appear significant.

With improvements in the detection of HLA antibodies due to the introduction of solid-phase assays such as ELISA and fluorescent bead technology, the identification of antibodies specific for donor HLA antigens has become possible and is carried out routinely in many laboratories. What is less clear is how DSA are defined and the relevance of antibody detected in the periphery. While many studies find significant associations with the presence of HLA-DSA and graft dysfunction compared to those without DSA, some studies also show that NDSA can also affect graft outcomes. As HLA antibodies recognise specific epitopes on HLA antigens, antibody cross-reactivity has been a problem since the beginning of histocompatibility testing by serology. This has led to the establishment of cross-reactive groups or CREGs based on epitopes that are shared between different HLA antigens and, if mismatched, could lead to an alloresponse. Therefore, HLA antibodies classed as NDSA may in fact be able to react with epitopes expressed on the graft by recognising a particular epitope and therefore able to cause rejection. Alternatively, a theory often used to explain non-detection of peripheral HLA antibodies prior to rejection that can then be detected after nephrectomy, is that alloantibodies are fixed to the graft where they are causing damage. In this case patients undergoing rejection may not have detectable HLA-DSA because they are concentrated in the graft whereas NDSA are not and may be detected in the periphery.

What is not provided by all of the earlier studies and most studies from the mid-1990's, is actual proof that the antibodies are the cause of the rejection and not the result. This can be determined with the use of Feucht's C4d assay (Feucht *et al*., 1993) but requires the availability of biopsy samples and highly skilled preparation and analysis by clinical pathologists. The presence of both circulating DSA and evidence of C4d deposition is a requirement of the Banff criteria for classification of chronic dysfunction (Racusen *et al*., 2003). Several studies have, however, used C4d deposition analysis in conjunction with DSA detection, including the one described above (Gaston *et al*., 2010). However, the results of many other studies show conflicting and inconclusive results. This could be due to differences in interpretation of results, the different methods used – immunofluorescence or immunohistochemistry, or other technical difficulties. Now that C4d deposition testing is used routinely in diagnosis, a clearer correlation with antibody-mediated rejection may be possible in the near future.

Overall, it is evident HLA antibodies are involved, at least in part, in the pathogenesis of rejection and that the initial targets of graft destruction leading to early graft loss are HLA class I antigens. These antibodies may be pre-formed, *de novo*, donor-specific or non-donor-specific as all have been implicated in aAMR. HLA class II antibodies also associate with AR but tend to appear later than class I, perhaps due to less ubiquitous expression of HLA class II antigens, and are often associated with chronic or late graft failure following many years. Finally, most studies show co-production of HLA class I and II antibodies are predictive of a poorer prognosis.

Treatment of antibody-mediated rejection

Rejection of grafts by alloantibodies cannot be treated in the same way as cellular rejection and treatment is usually much less effective. One approach is to remove antibodies from the circulation to eliminate further antibody-mediated injury in a process known as plasma exchange (PEX). PEX requires daily treatment together with solid-phase detection of DSA until levels begin to fall and must be accompanied by intravenous immunoglobulin (IVIG) administration. PEX also removes complement components, which further decreases humoral rejection. An alternative approach is HLA-DSA neutralisation using high dose IVIG (Jordan *et al.*, 2003) and intense immunosuppression using MMF in place of azathioprine or CsA has been shown to reduce antibody levels (Dudley *et al.*, 2005). Immunoadsorption with protein A was evaluated in a randomised trial and it was found that aAMR was reversed in all five cases whereas four of five patients without immunoabsorption lost their grafts (Bohmig *et al.*, 2007). Furthermore, it has also been

demonstrated that MICA antibodies (discussed below) can be removed by immunoadsorption with protein A (Yao *et al.*, 2011). Once antibodies are successfully removed, treatment with Rituximab (anti-CD20 monoclonal antibody) can be effective by eliminating B-cells and limiting re-synthesis, although this treatment is expensive and plasma cells (CD20-) are unaffected, therefore DSA production is not blocked (Torpey et al., 2010, p. 254). However, none of these treatments have been shown to be completely effective and success depends on the titre of antibody before treatment.

Antibody responses directed against non-HLA antigens

In a report published by Terasaki (Terasaki, 2003) he stated that 38% of graft failures were due to non-HLA, 18% to HLA and 43% to non-immunological factors. This implies that targets other than HLA antigens may also be involved in antibody-mediated graft injury. The clinical importance of non-HLA antigens is emphasised by reports of HAR of HLA-identical sibling transplants (Amico *et al*., 2008) and suggests that non-HLA antibody targets are important in the pathogenesis of organ rejection. Many of these putative antigens are tissue-specific such as vimentin, which is expressed on renal tubular and stromal cells. Autoimmune responses to vimentin associate with acute and chronic rejection of kidney and heart allografts (Jurcevic *et al.*, 2001) and antibody-mediated responses to vimentin have been shown to associate with the generation of vimentin-specific autoreactive CD8 T-cells (Barber *et al.*, 2004). Other non-HLA autoantibodies include antinuclear, antinucleoprotein, anti-DNA and anti-cytoplasmic antibodies among others (Nakano *et al.*, 2007). Another non-HLA target of antibodies may be the angiotensin type 1 receptor (AT1R), which is a receptor for angiotensin II in the glomerulus, regulating arterial blood pressure and salt balance and is also expressed in other areas of the kidney. Studies have shown that removal of AT1R antibodies by PEX improved renal function and GS (Dragun *et al.*, 2005).

Another target of alloantibodies that has emerged over the past ten years is the non-classical MICA and MICB antigens. The genes that encode these molecules are not only co-dominantly expressed, as with HLA, but are also highly polymorphic and have emerged as important targets for rejection in kidney, heart and lung transplantation. Furthermore, recipients with MICA antibodies can be identified and associate with rejection or reduced GS, as revealed by the results of many studies and discussed fully in the next section.

1.3 MICA antibody prevalence and its significance in solid organ transplantation

In 1994 two groups of researchers independently described a new family of genes encoded within the MHC and related to HLA class I genes called MHC class I-related chain A and B (MICA and MICB) (Bahram *et al.*, 1994) or Perth Beta block transcript 11 (PERB11) (Leelayuwat *et al.*, 1994). The World Health Organisation (WHO) nomenclature committee for factors of the HLA system decided on the more descriptive name adopted by Bahram and colleagues as official designation. The genes encode molecules that are strikingly similar to HLA class I, however MICA and MICB do not associate with β2M or bind or present peptides to T-cells. MICA and MICB are ligands for the NK-cell activatory receptor NKG2D and expression is up-regulated due to stress such as infection and malignancy (Bauer *et al.*, 1999). Furthermore, MICA and MICB gene products are highly polymorphic with 76 MICA and 31 MICB alleles (Release 3.4.0, April 2011) currently listed on the IMGT/HLA database (Robinson *et al.*, 2001).

1.3.1 Genetic organisation and structure of MIC gene products

The MICA and MICB genes are situated within the MHC region of the genome on chromosome 6p21.3 centromeric to HLA-B in the class I region (as shown in Figure 1.3.1).

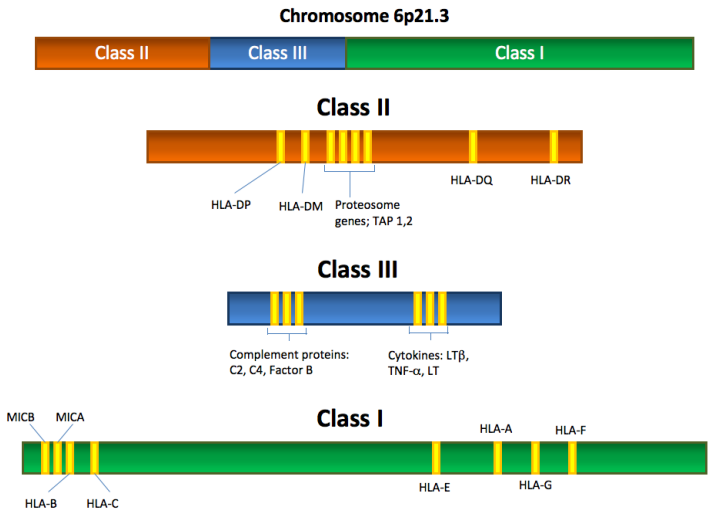


Figure 1.3.1: Genomic organisation of the MHC region. The human MHC region encompasses 3.6 Mb on chromosome 6, contains over 200 gene loci including all HLA genes and many immune regulatory genes. MICA and MICB are situated centromeric to HLA-B in the class I region. (Klein and Sato, 2000).

The MIC family consists of two expressed genes, MICA and MICB, and five pseudogenes, MICC-MICG. MICA is in very close proximity to HLA-B with a distance of only 46.4 Kb (Shiina *et al.*, 1999) between the two genes resulting in a very strong linkage disequilibrium effect between MICA and HLA-B alleles. The MICA gene has 30% homology to HLA class I genes, spans 11.7 Kb and comprises six exons separated by five introns with intron 1, between exons 1 and 2, being the largest (Bahram, 2000). The MICA coding sequence of 1383 nucleotide base pairs is transcribed as a 383 amino acid polypeptide of 43 kDaltons including the leader peptide (Bahram *et al*., 1994) consisting of three extracellular domains α1-α3 encoded by exons 2-4 (Figure 1.3.2), a transmembrane region encoded by exon 5 and a cytoplasmic tail region encoded by exon 6. The MICA crystal structure was discovered by collaboration between Strong and Spies and revealed structural differences between MICA and HLA class I molecules (P. Li *et al.*, 1999). As can be seen in Figure 1.3.2, the structure of MICA is very similar to HLA class I but the α-2 helix is disordered and flexible and the resulting groove is not suited for peptide binding.

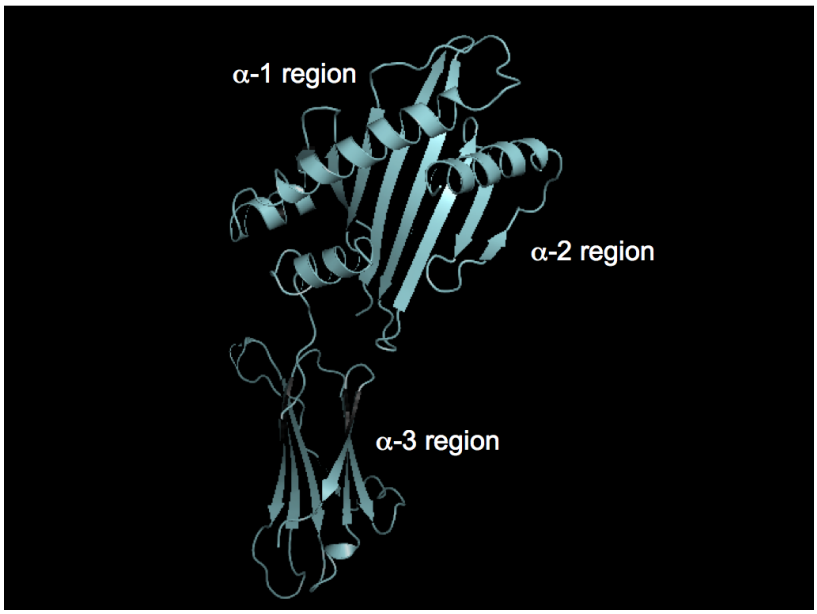


Figure 1.3.2: Ribbon model derived from X-ray crystallographic analysis of the extracellular domains of the MICA molecule. The three alpha regions are indicated with α-3 nearest the cell membrane.

In further contrast to HLA class I molecules, the platform formed by the α1 and α2 regions of the MICA molecule does not face outwards from the cell as with HLA class I, but is flipped over by an angle of 113.5° and points downwards towards the cell membrane, exposing its underside to the extracellular space. However, when MICA engages NKG2D,

the flexible α2 helix becomes ordered by a further two alpha-helical turns and the α1 and α2 domains flip back 96° (P. Li *et al*., 1999). The crystal structure for MICB has also been elucidated and shows structural similarity with MICA (Holmes *et al.*, 2002), having 83% amino acid homology (Bahram, 2000). Although also a ligand for NKG2D, MICB is less polymorphic than MICA and does not display GCT triplet variation in exon 5 that distinguishes many MICA allelic variants and subsequently attention has focussed more specifically on the MICA gene.

1.3.2 MICA/B expression on cells and tissues

Compared with the wide expression of HLA molecules on most cells and tissues, MICA and MICB expression is limited. Bahram and colleagues examined different cell lines for the presence of MICA and MICB messenger RNA (mRNA) and found that expression was restricted to fibroblasts and epithelial cells (Bahram *et al*., 1994). Groh *et al* discovered that MICA is constitutively expressed at high levels in the gastrointestinal epithelium and postulated that its expression may be induced by cellular stress (Groh *et al.*, 1998). Zwirner and colleagues demonstrated by Western blot analysis that freshly isolated monocytes, keratinocytes and umbilical vein endothelial cells express MICA molecules but freshly isolated CD4+ and CD8+ T-cells and CD19+ B-cells do not (Zwirner *et al.*, 1999). These authors found that MICA does not associate with β2M or become up-regulated by IFN-γ and by using FC, also discovered that there were differences in surface expression of MICA on endothelial cells and fibroblasts compared with keratinocytes and monocytes.

Molinero (Molinero *et al.*, 2006) showed that CD4+ and CD8+ T-cells express MICA molecules when activated by IL-2 but as Zwirner found, was not expressed on resting T-cells. However, using confocal microscopy they found low-level surface expression, which they concluded might indicate a safeguard mechanism to protect them from NK-cell attack, especially during a T-cell dependent immune response. Once the final phase of the immune response is completed due to antigen exhaustion, activated T-cells need to be removed and this is when expression of MICA could contribute to their elimination by NK-cells.

Using a Northern blot technique, Schrambach (Schrambach *et al.*, 2007) performed a total body tissue scan of both MICA and MICB transcription which clearly showed that with the exception of the central nervous system, both genes are widely transcribed and therefore possibly translated and membrane-bound. MICA and MICB are upregulated in situations

of stress and in malignancy; therefore expression in any tissue appears to be a signal for destruction by NK-cells.

1.3.3 Polymorphism and evolution of MICA and MICB genes

A feature of MICA and MICB genes is the high degree of polymorphism and although MICA and MICB polymorphism is not as extensive as HLA class I, the allelic repertoire continues to grow. In contrast to the polymorphism displayed by class I molecules, which is concentrated around the location of the peptide-binding groove, all three extracellular α1-α3 domains of MICA and MICB are polymorphic with the greatest variability in the α2 domain, encoded by exon 3. The allelic variation of MICA is generated by single amino acid substitutions resulting in dimorphism (except residues 156 and 251) as shown in Figure 1.3.3.

α1 Domain

Residue:	6	14	24	26	36
MICA*001:	Arg	Trp	Thr	Val	Cys
Variant:	Pro	Gly	Ala	Gly	Tyr

α2 Domain

Residue:	90	91	105	114	122	124	125	129
MICA*001:	Leu	Gln	Arg	Gly	Leu	Thr	Lys	Met
Variant:	Phe	Arg	Lys	Arg	Val	Ser	Glu	Val

Residue:	142	151	156	173	175	176	181
MICA*001:	Val	Met	His	Lys	Gly	Val	Thr
Variant:	Ile	Val	Leu/Arg	Glu	Ser	Ile	Arg

α3 Domain

Residue:	206	208	210	213	215	221	230	251
MICA*001:	Gly	Tyr	Trp	Thr	Ser	Val	Trp	Gln
Variant:	Ser	Cys	Arg	Ile	Thr	Leu	Ser	Arg/Glu

Residue:	256	268	271
MICA*001:	Arg	Ser	Pro
Variant:	Ser	Gly	Ala

Figure 1.3.3: Amino acid variation of the extracellular domains of the MICA molecule. Updated from Stephens, 2001 (Permission granted by Elsevier).

Polymorphisms are also found in the transmembrane and cytoplasmic tail region of the MICA molecule, in particular, exon 5 sequences show alleles can vary in the number of GCT repeats with either four, five, six, seven, eight, nine and ten repetitions, a feature not shared by MICB. One group of alleles (mostly MICA*008 variants) has a nucleotide insertion of 'g' after the second of five GCT repeats resulting in a premature stop codon and

truncated trans-membrane region. The number of GCT repeats is important in distinguishing particular alleles as extracellular domains can be identical with alleles only differing by their GCT triplet number. These features are designated A4, A5, A6, A7, A8, A9, A10 and A5.1 (g insertion) and their identification are critical for allele level MICA genotyping (discussed later).

Choy and colleagues attempted to resolve the high polymorphism of MICA by suggesting a possible polyphyly of MICA evolution (Choy and Phipps, 2003). The authors show that MICA alleles branch into two lineages, which they designated LI and LII, when a phylogenetic tree is constructed using a common 821-nucleotide MICA sequence across exons 2-4. The four variable nucleotide positions in exon 4 distinguishing the two lineages are at positions 684, 685, 697 and 713 and encode polymorphisms in the α3 domain. Position 684 is synonymous and substitutions at nucleotide positions 685, 697 and 713 lead to amino acid substitutions at resides 206, 210 and 215. MICB alleles appear to be closely related to the LII lineage because they encode amino acids at residues 206, 210 and 215 that are homologous to MICA LII alleles. Concerning polymorphism in exon 5, A5.1 and A6 alleles are found in both MICA lineages but A4, A7, A8, A9 and A10 are only found in lineage LI and A5 is specific for LII. Variation of GCT triplet repeats within both lineages suggests that further polymorphism occurred after they diverged. Phylogenetic studies of the MICA gene in non-human primates have so far revealed that most species have either MICA/MICB or MICD/MICE and rarely both (Cattley *et al.*, 1999; Doxiadis *et al.*, 2007). Furthermore, the MIC gene of the gorilla (*Gorilla gorilla*) shares homology with MICA-LI and MIC sequences of the pygmy chimpanzee (*Pan paniscus*) and the chimpanzee (*Pan troglodytes*) are related to MICA-LII (Choy and Phipps, 2003).

These authors proposed three ancestral types of MIC genes: MICA-L1, MICA-LII/MICB and MICD/MICE and one or more of these types could have been acquired by different primate species and evolved into the existing MICA and MICB alleles. The authors go on to say that MICA-LI evolved into human MICA-LI alleles and gorilla MIC, MICA-LII/MICB evolved into human MICA LII and MICB and non-human primate MICA LII, MICB and MICA/MICB alleles. MICD/MICE evolved into human MICD and MICE pseudogenes and non-human primate MICD and MICE genes. This hypothesis for the evolution of MIC genes may explain the variable allelic frequencies in different human populations. MICA-LII alleles such as MICA*008 and MICA*009 are prevalent in European, North American, Brazilian, Korean, Thai, Moroccan and Japanese populations, whereas MICA*002 is MICA-LI lineage and the most frequent allele in several American

Indian populations. This may suggest that MICA alleles are polyphyletic, having evolved from two distinct ancestral types (Oliveira *et al.*, 2008).

1.3.4 MICA alleles and disease association

Figure 1.3.1 shows the MICA gene in very close proximity to HLA-B and HLA-C and the products of these genes are associated with various disorders, mostly autoimmune. Since HLA-B (and therefore HLA-C) alleles are in linkage disequilibrium with inherited MICA alleles it was thought that MICA polymorphism might affect recognition through NKG2D expressed on γδ T-cells, which are involved in autoimmunity (Bahram, 2000). Many diseases that were known to be associated with HLA-B and HLA-C alleles were investigated for MICA polymorphism association. However, most studies focussed on the GCT triplet repeat number in exon 5 of the MICA gene rather than the allele defined by the extracellular regions of the molecule.

One of the first diseases studied was Ankylosing spondylitis (AS), which is known for its association with HLA-B27. Among the first published studies was by Goto and colleagues (Goto *et al.*, 1997) who studied MICA polymorphism in Caucasoid (European) patients with AS and found highly significant association with MICA-A4 alleles (four GCT repeats in exon 5). Two separate studies (Ricci-Vitiani *et al.*, 2000; Yabuki *et al.*, 1999) later confirmed this in a similar population. MICA*007 and *010 were found to be highly significantly associated with AS in Japanese patients (Tsuchiya *et al.*, 1998) but only HLA-B27 was associated in a study of Caucasoid, African and Asian patients (Martinez-Borra *et al.*, 2000). A more recent study (Amroun *et al.*, 2005) found juvenile AS was associated with a polymorphism at residue 129 where both haplotypes expressed methionine as opposed to valine. This polymorphism is thought to be associated with strong (valine) or weak binding (methionine) with NKG2D (Steinle *et al.*, 2001).

Behçet's disease (BD) has also been found by numerous studies to be associated with MICA alleles with 6 GCT repeats (A6) or MICA*009 but HLA-B51 is also highly correlated. The first published study was of Japanese BD patients (Mizuki *et al.*, 1997) where association was found with A6 and HLA-B51, however a significant number of B51 negative patients also had MICA-A6, suggesting an independent and primary role of MICA and association with BD. However, a later study (Mizuki *et al.*, 2000) involving microsatellite analysis of genes clustered around HLA-B in Japanese, Greek and Spanish patients, found only HLA-B51 was significantly associated with BD and the authors

concluded that association with other genes was the result of linkage disequilibrium. In 2002, a study of MICA association with BD in Korean patients found significant association with MICA-A6, which they claimed was independent of HLA-B51 (Park *et al.*, 2002) but Mizuki *et al* confirmed their findings of no association in a study of Turkish patients where stratification analysis revealed B51 alone was associated with BD (Mizuki *et al.*, 2007).

Other diseases investigated for association with MICA alleles are psoriasis, insulin-dependent diabetes mellitus, Addison's disease, Kawasaki disease, ulcerative colitis, Chrohn's disease, coeliac disease, Grave's disease and leprosy. The problem is that usually there is an HLA association, the numbers of patients studied is often low and very few studies are confirmed in the same population or confirm the same MICA specificity responsible. Thus, there is still a lack of clear evidence for primary association of MICA alleles with any of these diseases.

1.3.5 Role of MICA in innate immunity

MICA and MICB together with ULBP1-6 in humans are all ligands for the C-type lectin-like NKG2D receptor found on all NK-cells, γδ and αβ CD8+ T-cells and NKT-cells (Eagle and Trowsdale, 2007, Champsaur & Lanier, 2010). ULBP1-6 genes are also referred to as Retinoic Acid Early Transcript-1 in humans (RAET-1I, 1H, 1N, 1E, 1L, 1G, respectively) and these genes are polymorphic, particularly RAET-1E (Radosavljevic *et al*., 2002; Antoun *et al*., 2010). Mice do not have MIC genes but express proteins homologous to RAET1 called Retinoic acid early inducible (Raeα-ε), H60 and MULTI-1 (Krmpotic *et al.*, 2005; Champsaur & Lanier, 2010). There may be a certain amount of redundancy associated with NKG2D ligands as 3.8% of Japanese people have the MICA/MICB null haplotype and even homozygotes are healthy, indicating that other NKG2D ligands compensate their function (Komatsu-Wakui *et al.*, 1999).

NKG2D recognises its ligand in association with a transmembrane signalling adaptor protein, DAP-10 (Wu *et al*., 1999) and activates perforin and granzyme based cytotoxicity in NK-cells or co-stimulates CD8+ T-cells (Groh *et al.*, 2001). NKG2D ligands are upregulated on cells that are stressed by viral infection or malignant transformation (Groh *et al*., 1998) targeting them for destruction.

NK-cell-mediated lysis evasion by tumour cells

Wide expression of MICA and MICB has been found on biopsy specimens from carcinomas of the lung, breast, kidney, ovary, prostate, and colon (Groh *et al.*, 1999) and it is thought that expression of NKG2D ligands is important to activate anti-tumour responses of NK and T-cells. Groh and colleagues (Groh *et al.*, 2002) found that compared to CD8+ T-cells from healthy subjects, patients with malignancies had decreased NKG2D expression on CD8 T-cells by 60-70%. Substantially reduced expression of NKG2D was also observed on CD56+ NK-cells and γδ T-cells. The authors found that sera from patients with tumours had detectable amounts of soluble MICA (sMICA) whereas in healthy control sera it was absent. These results suggested that tumour-derived sMICA in peripheral blood of individuals with cancer caused downregulation of NKG2D. This hypothesis was confirmed by experiments showing that sera from individuals with MIC-positive tumours, but not negative controls, downregulated NKG2D expression on CD8+ T-cells among normal PBMCs even when sMICA was undetectable by ELISA. Therefore, tumours may be able to evade immune detection by shedding of MICA (and possibly MICB), which also leads to downregulation of NKG2D on effector cells, reducing their ability to target MICA on the tumour.

The phenomenon termed MICA shedding was eventually explained as the result of proteolytic cleavage (Salih *et al.*, 2002) and Kaiser and colleagues showed that on the surface of tumour cells, MICA associates with Endoplasmic Reticulum protein 5 (ERp5), which usually assists in the folding of nascent proteins inside cells (Kaiser *et al.*, 2007). Pharmacological inhibition of thioreductase activity and ERp5 gene silencing revealed that cell-surface ERp5 function was required for MICA shedding. ERp5 and the α3 domain of membrane anchored MICA formed transitory mixed disulphide complexes and soluble MICA was released after proteolytic cleavage near the cell membrane, involving a large conformational change to allow cleavage by ADAM (a disintregin and metalloproteinase) proteases (Waldhauer *et al.*, 2008).

During an immune response to a tumour, various cytokines are produced by immune and non-immune cells, as well as by the tumour itself. It has been demonstrated that IFN-α and γ downregulate expression of the NKG2D ligand H60 on mouse sarcomas (Bui *et al.*, 2006) and IFN-γ downregulates MICA on human melanoma cell lines (Schwinn *et al.*, 2009). IFN-γ has also been to shown to downregulate NKG2D on human NK-cells (C. Zhang *et al.*, 2005a). Thus, high levels of IFN-γ can downregulate both NKG2D and its ligands but

can also upregulate HLA class I molecules on tumour cells, thereby inhibiting NK-cells via their inhibitory class I receptors. Another cytokine that can downregulate both NKG2D and its ligands is TGF-β as shown with mouse models (Dasgupta *et al.*, 2005) and in cancer patients (J. C. Lee *et al.*, 2004). Substantial amounts of TGF-β can be found in the sera of cancer patients and cultured cancer cell-lines (Eisele *et al.*, 2006). Additionally, studies have shown that regulatory T-cells are a source of TGF-β, which modulates their immunosuppressive function (Horwitz *et al.*, 2008).

Soluble MICA levels were originally reported as elevated in the sera of patients with breast and lung cancer (Groh *et al*., 2002) and subsequently many other types of cancer including leukaemia (Salih *et al.*, 2003), pancreatic carcinomas (Marten *et al.*, 2006) and colon carcinoma (Doubrovina *et al.*, 2003). This has led to the use of sMIC as diagnostic biomarkers for tumour progression (Yokoyama, 2002). Studies have now confirmed that sMICA can be used as a diagnostic marker for cancer in the early stages and sMICB levels correlate with advanced cancer and metastasis (Holdenrieder *et al.*, 2006).

NK-cell-mediated lysis evasion by viruses

Human cytomegalovirus (HCMV) is a member of the *Herpes-viridae* family and is prevalent around the world with an incidence of between 50-100% (Sissons *et al.*, 2002). HCMV infection in healthy individuals is asymptomatic but can result in severe disease and mortality in immunocompromised individuals (Ho, 2008). HCMV can downregulate expression of HLA class I molecules, evading lysis by CD8+ T-cells (Barnes and Grundy, 1992) but this renders the cell vulnerable to NK-cell lysis due to a lack of HLA class I inhibitory ligands. HCMV has evolved several mechanisms in an attempt to prevent lysis by NK-cells including expression of molecules that engage with inhibitory NK receptors and downregulation of ligands for NK-cell activatory receptors (Wilkinson *et al.*, 2008).

Infection by HCMV results in upregulation of NKG2D ligands such as MICA, MICB and ULBP1-6. Thus, vulnerability to NK-cell attack is further increased and HCMV has evolved mechanisms to downregulate these NKG2D ligands. It was found that the HCMV glycoprotein UL142 modulates expression of full-length MICA proteins but does not affect the truncated MICA*008 molecule or MICB (Chalupny *et al.*, 2006). Another HCMV protein, UL16, has been shown to selectively prevent expression of MICB and ULBP1/2 by retaining these molecules in the ER (Cosman *et al.*, 2001). Therefore most NKG2D ligands, except MICA*008 are prevented from being expressed on the cell surface, abrogating NK-cell-mediated lysis.

The *Adenoviride* virus family are associated with acute pharyngitis, conjunctivitis, diarrhoea and cystitis in immunocompromised individuals. In particular, immunosuppressed paediatric transplant recipients can develop systemic spread of adenovirus that significantly affects morbidity and mortality (McSharry *et al.*, 2008). Adenoviruses have been demonstrated to encode functions to promote evasion of both adaptive and innate immune responses (Burgert *et al.*, 2002) and the E3 early transcription unit has been associated with these immunomodulatory functions (Lichtenstein *et al.*, 2004). E3/10K is an E3 protein that has been shown to impair cell surface expression of HLA class I molecules by retaining them in the ER (Andersson *et al.*, 1987) and, as with HCMV, adenovirus-infected cells become susceptible to NK-cell-mediated lysis. A group headed by Gavin Wilkinson were interested in the role of NK-cells in controlling adenovirus infections. They investigated immediate-early adenovirus (E1A) gene expression, which is known to induce cell surface expression of NKG2D ligands on infected cells (McSharry *et al*., 2008). We collaborated with these authors on this study and I determined high-resolution MICA genotyping for cell lines used in the experiments. The results showed that infection with wild-type adenovirus increases synthesis of MICA and MICB proteins, but their expression is actively suppressed by retaining immature MICA and MICB molecules in the ER. Furthermore, by examining a range of cell lines and adenoviruses with mutated E3 genes, E3/19K was identified as the gene responsible. Unlike UL142, which does not affect MICA*008 expression in HCMV infection, E3/19K can sequester a broad range of MICA allelic variants, including MICA*008.

These mechanisms indicate that polymorphism of MICA and MICB may have been generated by selective pressure. Not only is MICA*008 the only allele with a truncated cytoplasmic region but it is the most frequent allele in most populations (discussed in the next section). It seems likely that expression of MICA*008 confers resistance against HCMV and possibly other viruses by promoting NK-cell lysis via NKG2D in contrast to other MICA allelic variants. However, other viruses such as the adenovirus can prevent cell surface expression of a broad range of MICA allelic variants, including MICA*008. These studies highlight important roles for MICA in innate immunity and it is possible that other, unknown mechanisms involving NKG2D ligands in innate and adaptive immunity have generated the polymorphism of MICA and MICB genes.

1.3.6 MICA allele frequencies in different populations

Several studies have investigated MICA allele frequencies in different populations and although not as extensive as HLA population studies, these reports give an insight into differential MICA allele frequencies around the world. Figure 1.3.4 shows frequencies of MICA alleles in nine different populations. The first of these studies (Petersdorf *et al.*, 1999) revealed the most frequent MICA allele in North American Caucasoids was MICA*008 with a frequency of 55%. The second most frequent allele was MICA*002 (13%) followed by MICA*004 (6%). This contrasts with MICA allele frequencies in South American Indian tribes where MICA*002 is most prevalent with a frequency of 42% followed by MICA*027 (32%) and MICA*010 (21%) (Y. Zhang *et al.*, 2002). Similar frequencies of MICA*010 are found in Koreans (19%) (Sohn *et al.*, 2009), Thais (18%) (Romphruk *et al.*, 2001) and Chinese Han (19%) (Tian *et al.*, 2010) while most other populations except Japanese, with MICA*010 frequency of 11% (Ota *et al.*, 1997), have frequencies under 10%. The MICA*010 molecule has a proline at residue 6, resulting in loss of cell surface expression as it suggested that proline at this residue interferes with protein folding (Z. Li *et al.*, 2000).

South American Indians are the only studied population not to have a frequency of MICA*008 above 10% and instead express MICA*027 which is identical in the extracellular domains but differs from MICA*008 in the transmembrane region, where MICA*027 has the A5 GCT repeat and MICA*008 has A5.1, with an insertion of ‘g’ leading to truncation of the transmembrane region. Previous studies determined that South American Indians have restricted HLA polymorphism (Erlich *et al.*, 1997; Monsalve *et al.*, 1998; Tsuneto *et al.*, 2003) possibly due to a genetic bottleneck created by disease outbreak following population of the American continent by European settlers. This is also true for MICA polymorphism as 95% of South American Indian MICA alleles consist of just three variants. MICA molecules may have a role in defence against pathogens as they have been shown to be upregulated following infection with HCMV (Groh *et al.*, 2001) and polymorphism may have evolved to circumvent immune evasion strategies by viruses. It is thought that modern American Indians migrated from North to South America and could have encountered different pathogens along the way, creating a MICA allelic repertoire that was better suited to these pathogens. Additionally, viruses introduced by European settlers may have had a more recent impact. The similarity between MICA alleles expressed by

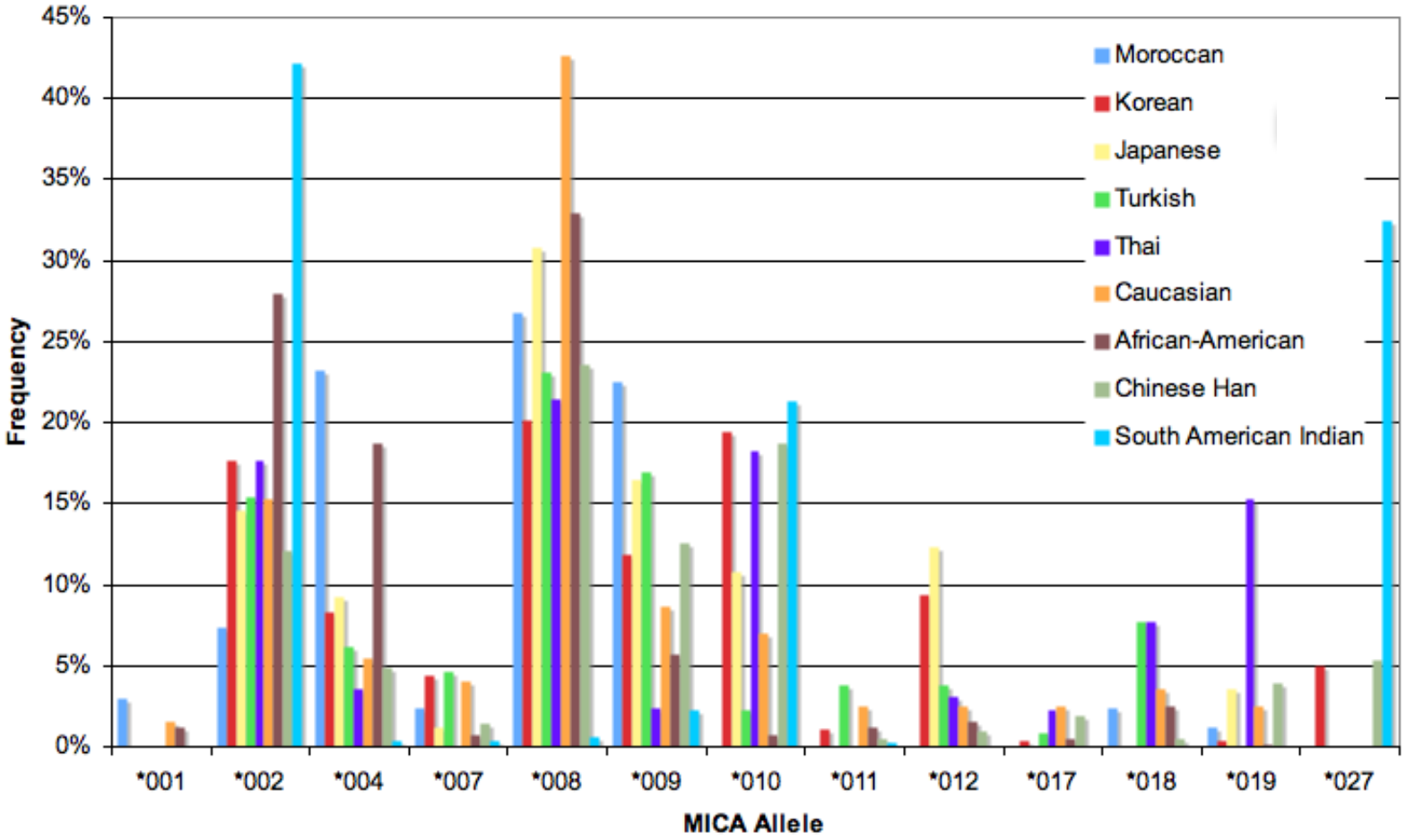


Figure 1.3.4: Common MICA allele frequencies in nine different populations: Moroccan (Piancatelli *et al.*, 2005), Korean (Pyo *et al.*, 2003), Japanese (Komatsu-Wakui *et al.*, 1999), Turkish (Mizuki *et al.*, 2007), Thai (North Eastern) (Romphruk *et al.*, 2001), Caucasian (Petersdorf *et al.*, 1999), African-American (Petersdorf *et al.*, 1999), Chinese Han (Zhu *et al.*, 2009) and South American Indian (Y. Zhang *et al.*, 2002).

South American Indians and Asian populations (Korean, Thai, Chinese Han, Japanese) gives credence to the hypothesis that South American Indian tribes originated from individuals who crossed the Bering Strait from Asia (Hoffecker *et al.*, 1993; Parham and Ohta, 1996). With the exception of South American Indians, most populations studied are dominated by MICA*008 expression with the next most frequent allele being MICA*002 followed by MICA*009. However, African-Americans are characterised by high frequencies of MICA*002, 004 and 008, comprising 70% of their MICA alleles, suggesting that diversity may have been driven as populations moved out of Africa, populating the world. Most other MICA alleles in all populations are detected with a frequency of less than 5%.

1.3.7 MICA antibodies and solid organ transplantation

Transplantation is the treatment of choice for patients with end-stage renal disease (ESRD) and heart, liver, lung and pancreatic diseases, among others, facilitating a return to near normal health and extending the life expectancy of graft recipients. Improvements in immunosuppressive therapy, aimed at limiting the effects of T-cell-mediated immune responses to the graft, have increased overall GS (Meier-Kriesche *et al.*, 2004) and reduced acute rejection (AR). However, rejection due to antibody-mediated graft damage arising from B-cell responses to mismatched HLA antigens remains a problem. The production of post-transplant, *de novo* DSA to HLA antigens is associated with acute and chronic allograft rejection (Trpkov *et al.*, 1996). Despite renal transplant rejection being strongly associated with HLA antibodies, some 11-20% of patients without HLA antibodies develop CAD (P. C. Lee *et al*., 2002). Furthermore, HAR can occur in the absence of HLA antibodies, thus implicating other non-HLA alloantigens (Brasile *et al.*, 1986) including the highly polymorphic MICA and MICB antigens also encoded by genes within the human MHC (Bahram *et al*., 1994).

It was first suggested that MICA antigens could be a target for graft destruction in solid organ transplantation, after expression of MICA antigens on the surface of endothelial cells was demonstrated (Zwirner *et al*., 1999). This was followed by evidence that antibodies in patient's serum could specifically react with different recombinant MICA molecules in an ELISA technique, thus polymorphisms of MICA could be recognised by allo-specific antibodies (Zwirner *et al.*, 2000). Methods for genotyping MICA alleles were also being developed and included Sequence-Based Typing (SBT) (Katsuyama *et al.*, 1999),

Reference Strand-mediated Conformational Analysis (RSCA) (Perez-Rodriguez *et al.*, 2000) and Polymerase Chain Reaction - Sequence-Specific Oligonucleotide Priming (PCR-SSOP) (Y. Zhang *et al.*, 2001). MICA was found to be expressed on renal and pancreatic allograft biopsies (Hankey *et al.*, 2002), confirmed as a target for CDC with both mouse MICA monoclonal antibodies and human alloantibodies, providing a method to detect MICA antibodies (Zou *et al.*, 2002) and confirmed to be co-dominantly expressed like HLA molecules (Molinero *et al.*, 2002b) providing evidence that MICA expression in graft tissues could lead to antibody-mediated lysis.

Once established that MICA antigens and antibodies may play a role in rejection of solid organ grafts, several studies were carried out investigating the relevance of MICA antibodies in patient's serum and correlation with rejection. A study of 69 patients with acute renal rejection and 70 with no rejection showed a significant correlation with graft loss and MICA-specific antibodies pre- and post-transplant (Sumitran-Holgersson *et al.*, 2002). In 2005, a serial ten-year follow up of HLA and MICA antibody production prior to kidney graft failure was carried out (Mizutani *et al.*, 2005) and 95% of the 39 patients who rejected their grafts had HLA or MICA antibodies, compared to 58% with functioning grafts ($p<0.01$). This was followed by another study from the same authors, revealing that among 34 recipients with functioning transplants and with HLA antibodies, 24% had MICA antibodies and 19% of 32 patients without HLA antibodies produced MICA antibodies. Among 46 patients who lost grafts with HLA antibodies, 26% also produced MICA antibodies, and among 27 failed patients without HLA antibodies, 37% were positive for MICA antibodies. This study indicated that MICA antibodies detected pre-transplant could have a role in aAMR (Mizutani *et al.*, 2006b).

Until 2006, methods used to detect MICA antibodies were based on CDC using transfected cell lines expressing different MICA antigens with the advantage of mammalian cell expression in the native form compared to recombinant MICA antigen production using bacterial expression. The latter method was employed to produce recombinant MICA molecules for use in an ELISA assay and were manufactured 'in-house' often using only recombinant MICA*008 or a few allelic variants. In 2006, One Lambda launched a new Luminex™ bead SAg assay using eleven MICA antigens, and also included two screening beads for MICA antibodies with their HLA antibody screening kit, free of charge. In addition, Peter Stastny's group in Dallas, USA, developed their own Luminex™ bead assay (Zou *et al.*, 2006b) using recombinant MICA molecules produced in insect cells, a procedure that produces molecules more similar to mammalian products, compared to

molecules produced by bacteria. The application of the solid-phase assay, as with HLA antibody detection, provided a reliable, convenient way to detect MICA antibodies and resulted in many more studies investigating the effect of MICA antibodies on graft outcome.

Zou and co-workers published a study of MICA antibody production in renal transplant recipients, detected using a Luminex assay produced in-house utilising recombinant MICA*001, 002, 004, 008, and 009 from insect cell transfection (Zou *et al*., 2006b). They found 25% of 85 recipients awaiting a subsequent transplant produced MICA antibodies, as did 23% of 66 patients transplanted within four years. Among 59 acid eluates from nephrectomy specimens of patients who had immunological rejection, 19% contained MICA antibodies. Among those, six recipients were positive for HLA antibodies and five were not, suggesting an independent role for MICA antibodies. Mizutani and colleagues used Luminex methods to detect HLA antibodies and a transfected mammalian cell line expressing eight different MICA alleles to detect MICA antibodies with CDC (Mizutani *et al.*, 2006a). They found 65% of 63 patients who rejected grafts had HLA antibodies and 52% produced MICA antibodies, compared to 45% of those with functioning grafts who produced HLA antibodies and 21% with MICA antibodies. In all, 92% of patients with either type of antibody had graft failure compared to 70% of those with functioning grafts, suggesting involvement of HLA, MICA or both antibodies in chronic rejection. However, these are small studies and although the results are significant, they only give an indication that MICA antibodies are causing rejection.

As part of the 13th and 14th IHIWC, 1329 recipients of renal grafts from deceased or living donors from 21 participating centres, were tested for HLA and MICA antibody production (Terasaki *et al.*, 2007). Only recipients who did not produce HLA antibodies pre-transplant (pre-transplant testing for MICA antibodies was not performed) and who survived for more than six months were included in this study. HLA antibodies were detected with either CDC, ELISA or Luminex and MICA antibodies were detected using eight different recombinant MICA molecules produced in HMY2.C1R cells, isolated and coated on Luminex beads. At one year post-transplant, survival for 1329 patients with no HLA antibodies was 96% compared to 94% survival for 344 patients with HLA antibodies and 83% survival for 33 patients with MICA antibodies. After four years of engraftment, survival among 806 patients who were negative for HLA antibodies after one year of transplantation was 81% compared to 58% for 158 recipients with HLA antibodies and 72% for 69 patients with MICA antibodies. Multivariate analysis at both time-points revealed

MICA antibodies were significantly and independently associated with reduced GS, providing strong evidence for involvement of MICA antibodies with graft rejection.

Using ELISA to detect post-transplant HLA and MICA antibodies in 185 renal graft recipients, significantly reduced two-year GS for patients with MICA antibodies was found, particularly if both HLA and MICA antibodies were detected, where survival was only 17% compared to 89% of those with no antibodies (Panigrahi *et al.*, 2007a). In addition, patients with only MICA antibodies or only HLA antibodies had significantly reduced survival of 71%.

A study was performed to investigate a relationship between MICA antibody production and heart allograft rejection in 44 recipients (Suarez-Alvarez *et al.*, 2007). MICA antibodies were detected using both MICA transfected cell lines in a CDC assay and a commercial assay that had just been released by One Lambda using Luminex beads to detect MICA*001, 002, 004, 007, 008, 009, 012, 017, 018 and 019. MICA antibodies were detected in 11 (25%) patients by CDC and seven (16%) by Luminex assay. Nine patients had rejection and testing by CDC showed 61% had rejection and were positive for MICA antibodies compared to five (14%) with no rejection. Analysis by Luminex revealed 55.5% of rejecters were positive for MICA antibodies compared to 6% with no rejection. These authors also included MICA allele typing for donors and recipients where the recipient was positive for MICA antibodies by either CDC or Luminex assay and found that all patients with MICA antibodies and AR had MICA-DSA although five patients also had auto-antibodies. This study was limited by the small number of patients, nevertheless it was the first study to show correlation of MICA-DSA with AR.

A study of 1910 pre-transplant serum samples from cadaveric renal transplants was carried out in a multi-centre study organised by the CTS (Zou *et al.*, 2007). This study has been much discussed and was the first large investigation to correlate MICA sensitisation pre-transplant with poorer GS. It was found that 217 patients of 1910 (11.4%) had MICA antibodies and their one-year GS rate was 88.3% compared to 93% in the group with no MICA antibodies. Among those who received their first renal graft, survival was even lower with 87.8% GS compared with 93.5% for those without MICA antibodies. Separate analysis of 326 patients with 0 or 1 HLA-A, -B or DR mismatches also showed that recipients with MICA antibodies had poorer survival of 83.2% compared to 95.1% of those with no MICA antibodies. However, no MICA allele typing was performed for patients or donors and no association with donor-specificity could be made and as this was a study of

pre-transplant sera, the relative contribution of *de novo* MICA antibodies could not be made.

Amico (Amico *et al*., 2008) used MICA typing of renal graft recipients who were positive for MICA antibodies detected by MICA LABScreen SAg Luminex (One Lambda). One out of ten patients with early aAMR had a definitive MICA-DSA and two had possible MICA-DSA and taken together the results showed that of 433 patients the incidence of early aAMR due to MICA-DSA was only 0.7%, leading the authors to conclude that early graft loss due to non-HLA-DSA was rare. A study of nineteen renal transplant recipients, who fulfilled the criteria for aAMR, including C4d deposition, also investigated involvement of MICA-DSA (Alvarez-Marquez *et al.*, 2009). The authors found four patients had MICA-DSA antibodies and were C4d-positive (21%) compared to 3 of a control group of 39 patients who were C4d-negative (7.7%) and although not significant suggested a trend for MICA antibodies associating with aAMR. Additionally, two of the patients had only MICA-DSA detected, indicating a primary association independent of HLA antibodies although, again, the numbers of patients are low. Another study (Gautier *et al.*, 2009) included MICA typing of 43 recipients of third renal transplants and testing for MICA antibodies using LABScreen MICA SAg Luminex (One Lambda) on the day of transplant and after one year. Patients with two MICA mismatches were more frequent in the group of patients with AR (40%), however patients with zero or one MICA mismatch also experienced graft losses and MICA-DSA were equally associated with functional and failed grafts. This could potentially have been a very interesting study as they typed all patients and donors, not just those positive for MICA antibodies, and could have examined the nature of the mismatches between those who produced *de novo* MICA antibodies and those that did not. Unfortunately this was missing from the study, as was the use of statistical methods.

Up until around 2009, the specificity of MICA antibodies and epitopes recognised by MICA antibodies had received very little attention. Evidence from studies using techniques to detect MICA antibodies show, overall, a polyspecific response to MICA antigens with sometimes donor-specific antibodies detected among them. Many MICA alleles share particular polymorphisms and it could be hypothesised that MICA antibodies recognise polymorphisms associated with one or more alleles. Duquesnoy (Duquesnoy *et al.*, 2008) determined, with the use of a computer algorithm, that there were 38 potential eplets (immunogenic groups of amino acids) and correlated shared polymorphisms with MICA antibody profiles detected in sera with LABScreen MICA SAg Luminex (One Lambda).

This hypothesis was tested empirically by employing absorption and elution experiments using MICA transfected fibroblasts to sequentially absorb MICA antibodies followed by testing with MICA SAg Luminex (Zou *et al.*, 2009). This analysis revealed several MICA residues recognised by MICA antibodies including a group of amino acids in the α3 domain (exon 4) separating MICA antigens into two groups corresponding to the MICA-LI and LII ancestral MICA lineages proposed by Choy (Choy and Phipps, 2003).

Suarez-Alvarez and colleagues combined a clinical study of MICA antibody production in deceased donor renal transplantation with MICA epitope analysis (Suarez-Alvarez *et al.*, 2009a). In this study of 161 recipients tested post-transplant, 30 (18.6%) developed MICA antibodies and 8/27 (30%) had AR compared with 13/98 (13%) without MICA antibodies ($P<0.05$). Using LABScreen MICA SAg Luminex (One Lambda), they determined by epitope mapping with a synthesised library of overlapping peptides from the extracellular domains of MICA molecules, nine antigenic regions reactive with MICA antibodies in patient's serum. These regions included sites common to all MICA antibodies and also polymorphisms, confirming the conclusions of Duquesnoy and Zou's studies.

Li *et al* used a novel technique to detect *de novo* HLA and MICA antibodies in fifteen patients after renal transplantation (L. Li *et al.*, 2010). Pre and post-transplant serum were profiled using the Invitrogen Protoarray® Human Protein Microarray platform containing 5056 nonredundant human proteins, purified from insect cells. They found *de novo* MICA antibodies in eleven of the 15 transplant patients. The mean MICA antibody signal intensity was higher in recipients with AR and C4d+ compared to those who were C4d- with AR. Additionally, integrative genomics predicted localisation of MICA antigen to the glomerulus in the normal kidney, confirmed by immunohistochemistry showing MICA staining of the podocytes within the glomeruli along with the presence of infiltrating monocytes, B-cells, T-cells and NK-cells. They concluded that MICA antibody-mediated immune responses occurred irrespective of graft rejection and MICA antibody levels increase in aAMR but not with cellular rejection, therefore serial measurement of MICA antibodies may detect a significant rise in antibody titre that could occur before or during aAMR.

Recently, Lemy and colleagues (Lemy *et al.*, 2010) studied MICA antibody production in sera from 494 healthy controls and 597 patients with chronic kidney disease (CKD) stage V among them, 425 received kidney transplants (tested pre-transplant). MICA antibodies were detected using LSA-MIC (Gen-Probe), a new addition to the commercially available

MICA SAg Luminex assays. This study is interesting because it highlights many of the deficiencies that still remain in this area of research, including epidemiology and specificity of MICA antibodies and impact on AR and GS. The prevalence of MICA antibodies in healthy subjects, immunising events leading to MICA antibody production and donor-specificity of MICA antibodies are also far from clear and Lemy sought to address these issues. Among 425 CKD patients who received transplants, univariate analysis revealed male gender, younger age, haemodialysis, transfusion, pregnancy, previous graft and presence of HLA antibodies all associated with MICA antibody production. Factors remaining significantly associated with MICA antibodies after logistic regression analysis were blood transfusions, previous transplantation and females with two or more pregnancies. However, Zou and colleagues found no significant association with blood transfusion and MICA antibodies in 1242 renal graft recipients, although five transfusions were required for categorisation as 'transfused' compared to only one in Lemy's study (Zou *et al*., 2007). These authors found MICA antibodies at a frequency of 4.6% among 494 healthy controls, 14.9% in 597 CKD patients and 13.9% of 425 transplant recipients, although in this group only 3% had both HLA and MICA antibodies. MICA antibodies were also more frequent in men rather than women in the cohort as a whole (14% versus 7%) and also among individual groups, and patients were more likely to have HLA antibodies in addition to MICA antibodies as previously observed (Zou *et al*., 2007).

Regarding sensitisation to MICA antigens, a third of patients with MICA antibodies and CKD stage V had no sensitisation events identified, implicating unknown mechanisms and the higher frequency of MICA antibodies in males rather than females is intriguing. Additionally, 20% of CKD patients who were typed for MICA alleles using an SSOP method (One Lambda) had MICA antibodies that were auto-reactive, a rare finding with HLA antibodies (Morales-Buenrostro *et al.*, 2008) but also observed with MICA antibodies in the study of heart transplant recipients by Suarez-Alvarez and colleagues (Suarez-Alvarez *et al*., 2009a).

Compared to Zou's study (Zou *et al*., 2007), there was no significant association of pre-transplant MICA antibodies with overall one-year GS found by Lemy. In fact, Lemy's study found those with MICA antibodies had a better ten-year GS compared with those with no MICA antibodies. As re-transplantation was the only independent significant factor associated with graft loss, the authors also analysed a group of patients with primary grafts. Again, graft recipients with MICA antibodies had slightly better, but not significant, GS.

Recent evidence has emerged of association of MICA antibodies with chronic rejection, known as bronchiolitis obliterans syndrome (BOS) in lung transplantation (Angaswamy *et al.*, 2010). Angaswamy and colleagues analysed sera from 80 lung transplant recipients for MICA antibodies using LABScreen and MICA SAg Luminex and HLA antibodies using FlowPRA beads (One Lambda). Development of either only MICA antibodies or MICA and HLA antibodies significantly correlated with the development of BOS ($P<0.01$). The same research group, in another published study, also found significant association with MICA antibody production and the development of cardiac allograft vasculopathy in heart transplantation (n=95) (Nath *et al.*, 2010). A finding from both studies was that HLA-DSA preceded the development of MICA antibodies. In the lung transplant study, HLA antibodies were detected 7.6 ± 4.7 months post-transplant and MICA antibodies were detected at 10 ± 3.5 months after transplantation. Heart transplant recipients developed antibodies sooner; HLA-DSA were detected 2.7 ± 1.4 months post-transplant and MICA antibodies emerged by 6.5 ± 2.1 months post-transplant. An explanation for this could be that binding of HLA antibodies within the graft may increase expression of MICA antigens and allo-reactivity leading to MICA sensitisation.

A recent study of 68 heart transplant recipients (Pavlova *et al.*, 2009) for association of HLA and MICA antibodies with aAMR and ACR found that recipients with pre-transplant HLA-DSA had significantly lower aAMR-free survival of 50% compared with 90% for patients with no HLA antibodies and 92% when recipients had HLA-NDSA ($P=0.005$). There was no significant association of HLA antibodies with ACR. However, patients with pre-transplant MICA antibodies (n=9, 14%) did not significantly associate with aAMR or ACR, although a trend was associated with aAMR ($P=0.06$). A previous study of 491 heart transplant recipients tested pre-transplant also found no significant correlation of MICA antibodies with AR, or decreased three and five-year GS (Smith *et al.*, 2009). The outcome for GS, excluding patients with HLA antibodies, was similar to the findings of Lemy *et al*, in that patients with MICA antibodies had better survival compared to MICA negative recipients after one and five years (89% and 83% versus 72% and 64%; $P=0.051$). A study of MICA antibody production in liver allograft recipients also found no significant association with rejection or any other clinical parameters (Uzunel *et al.*, 2008). Histological analysis revealed that MICA is not normally expressed on liver cells and its expression is not induced during rejection episodes. However, patents were found who only produced MICA antibodies after transplantation and there was a trend for MICA antibodies to associate with aAMR, therefore further studies of liver transplants are required.

Despite all of these studies investigating association of MICA antibodies with graft rejection and survival, there is still no strong evidence implicating these antibodies with worse outcomes. Moreover, it is still unclear how people become sensitised to MICA antigens, although the evidence does suggest that previous transplantation is one factor, it is not known if MICA mismatching of donors and recipients is the cause, as is the case with HLA antibodies, or whether certain mismatches are more detrimental than others. Some studies find association with MICA antibody production and pregnancy but many studies also find that more males have MICA antibodies than females (Lemy *et al*., 2010; Smith *et al*., 2009; Zou *et al*., 2007) and this is not usually the case with HLA antibodies where more females are sensitised. It is also unclear if blood transfusion was a source of MICA immunisation with Zou finding no association compared to HLA sensitisation but in the study by Lemy, there was significant association of MICA antibodies with transfusion however, differences in study design may have influenced the results as already mentioned. It is also interesting that Lemy and colleagues found a third of their patients with MICA antibodies had no known sensitisation event for MICA antibody production and 4.6% of 494 healthy controls also had MICA antibodies with a higher percentage being male, suggesting pregnancy is not the main reason for MICA sensitisation in healthy subjects. The association of previous transplants with MICA antibody production is also not convincingly demonstrated and reports are conflicting. In the study of 161 renal graft recipients (Suarez-Alvarez *et al*., 2009a), no statistical significance was correlated with MICA antibodies and in the large CTS study of pre-transplant MICA antibodies in renal graft recipients (Zou *et al*., 2007) there was also no association with re-transplantation and production of MICA antibodies. However, Lemy (Lemy *et al*., 2010) found that 27% of 74 patients with a previous renal transplant had MICA antibodies compared with 11% of 351 patients not previously transplanted and was highly significant ($P<0.001$). Many other studies do not make the comparison, therefore previous transplantation as a MICA sensitising event has not been proven, although it seems likely that allo-responses to mismatched MICA antigens is a factor leading to development of MICA antibodies.

Most studies of MICA antibodies in transplant recipients concentrate on association with acute or chronic graft rejection but, with the exception of two meta-studies, most studies are relatively small in terms of numbers of patients. Interpretation of data from these studies is complicated by differences in study design such as testing for MICA antibodies pre- or post-transplant, deceased or living donors and many other factors as is the case with studies investigating association of HLA antibodies with graft outcomes. Similarly, the method of

testing for MICA antibodies may also affect the results from these studies, as there was no international serum exchange scheme to establish reference sera standards for MICA antibody detection. Most studies since 2006 have used the Luminex SAg assay from One Lambda but the reliability of this method has not been evaluated and some centres report unexpectedly high frequencies of MICA*019 detection that is not detected when another method is used (Zou and Stastny, 2009). In addition studies have demonstrated the presence of autoantibodies to MICA antigens using the One Lambda assay, even when patients do not have an autoimmune disease. Lemy's study also found unexpectedly high frequencies of MICA autoantibodies using the SAg assay from Gen-Probe (Lemy *et al*., 2010). A possible reason for detection of these antibodies is that they could be cross-reactive with other MICA antigens and Zou and colleagues have demonstrated that this can be the case (Zou *et al*., 2009) alternatively, denatured antigens on beads used in the assay may expose cryptic epitopes that are recognised by these antibodies. It would be beneficial for future studies to verify their results with another method, as there are now currently two commercial MICA SAg Luminex assays available.

The studies by Zou (Zou *et al*., 2007) and Lemy (Lemy *et al*., 2010) are similar in design with pre-transplant testing for MICA antibodies, but the outcomes are broadly dissimilar in terms of GS. Although there are differences in numbers of patients between the two studies, it is surprising that Lemy found better survival when patients were positive for MICA antibodies, albeit insignificantly. However, analysis of immunosuppression protocols between the two studies showed that Lemy's group of patients were more heavily immunosuppressed. A third of the patients in Zou's study received induction therapy compared to nearly all patients in the Lemy study, the predominant calcineurin inhibitor was cyclosporine A in Zou's study and tacrolimus in Lemy's study and two thirds of patients in Zou's study were given mycophenolic acid compared to nearly all of Lemy's patients. Therefore the incidence and impact of MICA antibodies could be reduced in patients who are more heavily immunosuppressed. If this can be proven in well-controlled large studies, a case could be argued for MICA allele and antibody testing in transplantation as changes to immunosuppression may affect the outcome. Perhaps this putative effect of immunosuppression was also the reason why there was no association with MICA antibodies found in the study by Smith and colleagues (Smith *et al*., 2009).

To evaluate the impact of MICA allele mismatching and the production of MICA antibodies in renal transplantation it is necessary to MICA type all patients and donors included in the study and not just patients who are found to have MICA antibodies. This

could enable definition of mismatches associating with development of MICA antibodies. Also, as with HLA antibodies, it is important to show that MICA antibodies are actually causing rejection, which can be achieved with C4d immunohistochemistry and has been demonstrated by some studies. However, the problem facing all researchers in this field currently is the method of detecting MICA antibodies, as none can be verified with reference serum standards and all methods can produce false negative or positive results. Using more than one assay to detect MICA antibodies and verify the results may enable a better understanding of their involvement in rejection of organ allografts.

1.4 Methods for detection of MICA alleles and MICA antibodies

Following the discovery of the MICA and MICB genes and the finding that their products are polymorphic, efforts were made to produce typing methods for allele identification and, later, to identify the presence and specificity of anti-MICA antibodies in patients sera. Although MICB antibodies may also be involved in transplant rejection, the less polymorphic nature of MICB has led to attention being focussed mainly on MICA antibodies. Methods have evolved along the same lines as HLA allelic typing and antibody identification as the similar structure and genomic organisation of MICA coding regions means similar methods can be employed. There are, however, some major differences associated with MICA compared to HLA. Firstly, unlike HLA where for each locus exons are the same length, exon five of MICA genes is not only polymorphic but also differs in length due to varying numbers of GCT triplets encoding different numbers of alanine repeats in the transmembrane (TM) region. Secondly, MICA has limited tissue distribution compared to HLA and, importantly, is not expressed on peripheral CD4+ or CD8+ T-cells eliminating serological analysis as a method to detect antigenic polymorphism. Furthermore this also means that the traditional crossmatch CDC or FC assays cannot be used, however, methods have been developed to circumvent these obstacles.

1.4.1 MICA allele genotyping methods

Methods based on exon 5 GCT repeat polymorphism

MICA allele polymorphism became the focus of disease association and population frequency studies shortly after the discovery of the MICA gene and discrimination of alleles was initially based on exon 5 polymorphisms, in particular the number of GCT repeats. In 1997 Ota published a report detailing a method for determining the size of exon 5 fragments

in homozygous and heterozygous DNA samples to discriminate the known exon 5 polymorphisms at that time (A4, A5, A5.1, A6 and A9) and used the data to determine MICA allele frequencies in different populations (Ota *et al*., 1997). For this assay, an automated DNA sequencer was used and the first generation of these machines used a slab polyacrylamide gel as the resolution medium. Software was available from the manufacturers (Genescan, ABI, CA) to resolve differences in nucleotide length by a minimum of one nucleotide. To generate fragments for Genescan analysis primer pairs were designed flanking exon 5 in intron 4 and intron 5, with the 5' primer (intron 4) labelled with a fluorescent dye (6-Fam) as shown in Figure 1.4.1.

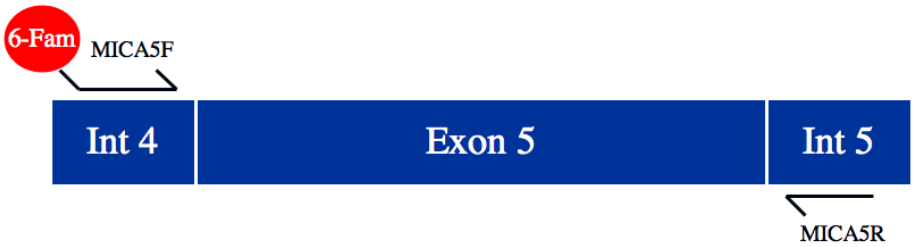


Figure 1.4.1: PCR Amplification strategy to generate fluorescently labelled fragments for size analysis using Genescan software and an automated ABI 377 sequencer (Applied Biosystems). (Ota *et al*., 1997).

The PCR fragments were then purified and loaded onto a denaturing polyacrylamide gel alongside a fluorescent size marker and PCR products from B-cell lines of known size defined by nucleotide sequencing of exon 5. The results generated by the Genescan software represent the number of base pairs (bp) in the DNA fragment and were compared to predetermined sizes for the different number of GCT repeats as shown in Table 1.4.1.

Table 1.4.1: Relative sizes of exon 5 in different MICA alleles

MICA microsatellite	Cell line or Nationality	Size (bp)
A4	BTB[a]	179
A5	MLF	182
A5.1	CGM1[a]	183
A6	MOU[a]	185
A7	Spanish[b]	188
A8	Italian[c]	191
A9	YAR[a]	194
A10	25/1506[d]	197

[a](Ota *et al*., 1997); [b](Rueda *et al*., 2002); [c](Gambelunghe *et al*., 2006); [d](Perez-Rodriguez *et al*., 2000).

Sequence Based Typing (SBT) of MICA alleles

DNA sequencing is the determination of the sequential order of deoxynucleotide triphosphates (dNTPs; dATP, gGTP, dCTP and dTTP) in a given sample of genomic DNA (gDNA) or coding DNA (cDNA). Sequencing methods have now been automated but are still based on the chain-termination method described by Sanger (Sanger *et al.*, 1977). DNA sequences can be obtained from DNA vectors such as plasmids and cosmids and also from gDNA or cDNA that is amplified by PCR to produce a template. The template is then mixed with a sequencing primer designed to cover the region of interest in either the 5' to 3' (sense) direction or 3' to 5' (antisense) direction. Fluorescently labelled dNTPs called dideoxynucleotides (ddNTPs) and non-fluorescently labelled dNTPs are added to build the new DNA, which is synthesised by DNA polymerase. This process is called cycle sequencing and during each round, primer anneals to the template, is extended by incorporation of dNTPs and chain elongation is terminated when a ddNTP is incorporated. The DNA is then denatured (by heating) producing an extension product and the original template and the process begins again, finally producing differing lengths of DNA that can be sorted into the correct order by electrophoresis. This process is shown if Figure 1.4.2.

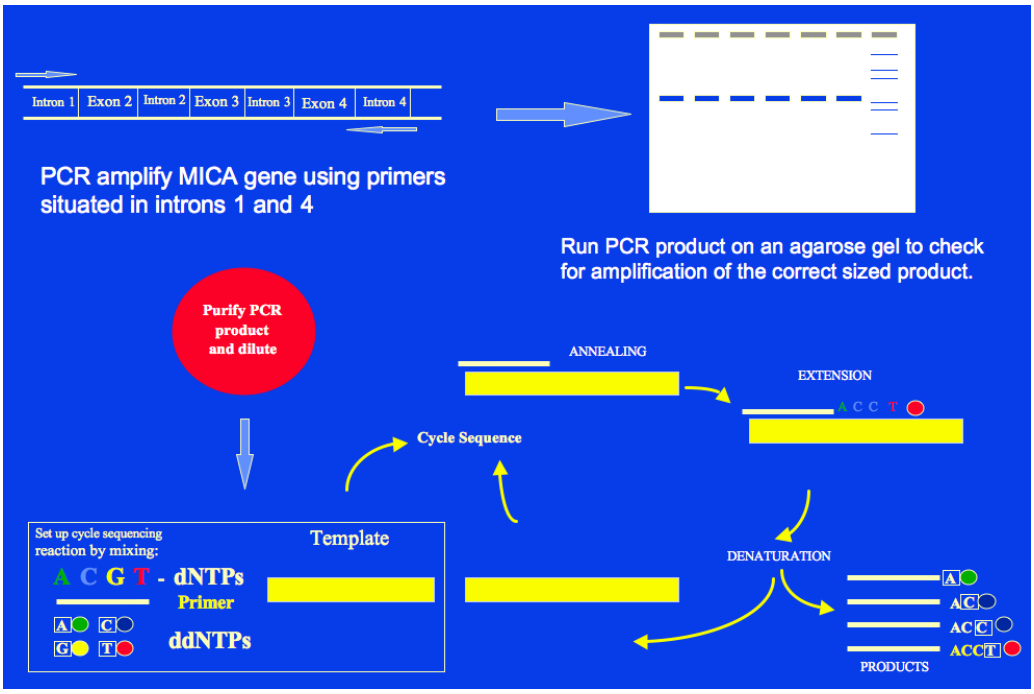


Figure 1.4.2: Diagram showing stages involved in PCR-SBT of the MICA gene. The region of interest is amplified by PCR, purified and added to DNA primers, dNTPs, ddNTPs and DNA polymerase and subjected to several rounds of cycle sequencing. DNA sequence is determined by electrophoresis where differing sizes of product have different mobilities.

Methods for SBT of MICA alleles have been described where either exons 2-4 (Katsuyama *et al*., 1999) or exons 2-5 are amplified (Visser *et al.*, 1999; Zwirner *et al*., 2000) and primers were designed for bidirectional sequencing of the polymorphic exons. As with HLA typing, codominant expression of MICA alleles means that at polymorphic nucleotide positions, there can be two different nucleotides detected as shown in Figure 1.4.3.

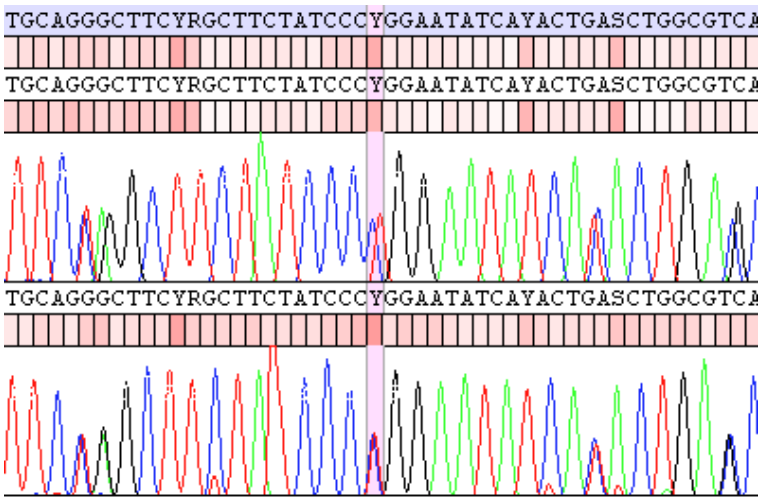


Figure 1.4.3: Sequence electropherogram showing sense and antisense sequence from exon 4 of the MICA gene. Two peaks may be seen at polymorphic nucleotide positions representing a different nucleotide for each MICA allele.

International Union of Biochemistry (IUB) codes are assigned at these positions, for example nucleotides C and T together are given the code 'Y'. It is necessary to use a computer algorithm to deduce the possible alleles present and the allele type is sometimes ambiguous, resulting in more than two allele types being possible due to sharing of polymorphic nucleotides. Sequencing more exons can resolve this further, however, with MICA allele typing, exon 5 cannot be sequenced in heterozygous samples as differing number of GCT repeats mean the sequences will become out of sync and therefore unreadable. This can be overcome by sequencing exons 2-4 using SBT and sizing of exon 5 to reduce ambiguous allele combinations. If exon 5 is included in the PCR fragment, further ambiguities can be resolved. For example MICA*002 and *052 have identical exon 5 GCT repeats are identical in exons 2-4 but differ by one nucleotide towards the end of exon 5 that can be determined by manual analysis. Using a combination of sequencing exons 2-5 and sizing of exon 5, almost allele level typing for MICA can be achieved, except MICA*009 and *049 cannot be distinguished unless exon 6 is also included and MICA*00801 and *00804 can only be resolved by amplification and sequencing of exon 1.

MICA typing using conformational analysis

A method that can achieve allele level typing for MICA and simultaneously incorporate analysis of exon 5 is Reference Strand-mediated Conformational Analysis (RSCA), and was developed as a tool for HLA allele identification (Arguello *et al.*, 1998; Arguello and Madrigal, 1999) and later adapted for MICA allele typing (Perez-Rodriguez *et al.*, 2000). The principle of this technique as applied to HLA-A typing is shown in figure 1.4.4. An automated DNA sequencer is required.

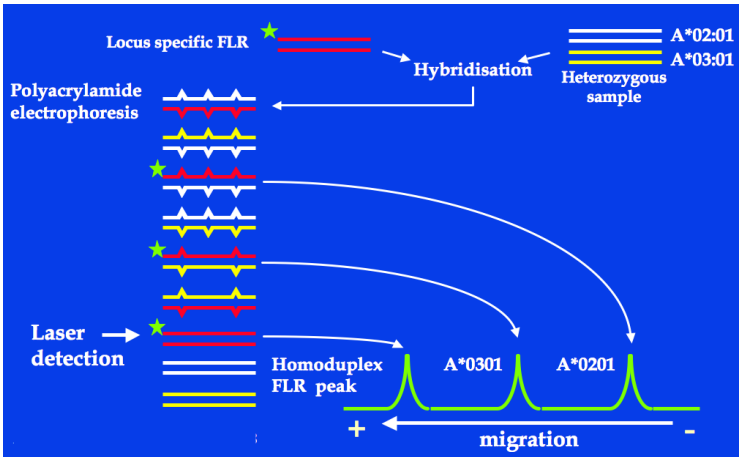


Figure 1.4.4: Reference Strand-mediated Conformational Analysis (RSCA). Sample DNA and a fluorescently labelled reference DNA are heated to denature then allowed to cool. Heteroduplexes are formed by reannealing of sense or antisense strands obtained from sample or reference DNA. The fluorescently labelled reference strand can anneal to its complimentary strand (homoduplex) or with one of each of the alleles, resulting in three peaks being detected by a laser.

The incorporation of a fluorescent reference ensures that only three peaks will be detected by the laser and confusing multiple bands seen with other conformational techniques are avoided. Alleles are identified as they have unique mobilities in polyacrylamide gel electrophoresis (PAGE) with a particular reference and can be compared to a ladder comprised of alleles of known type. RSCA is a useful technique as novel alleles can be detected as well as known alleles. A disadvantage is that mobilities for each allele have to be pre-determined using cell line DNA, which may not be available for every allele. An example of the output generated by the software is given in Figure 1.4.5.

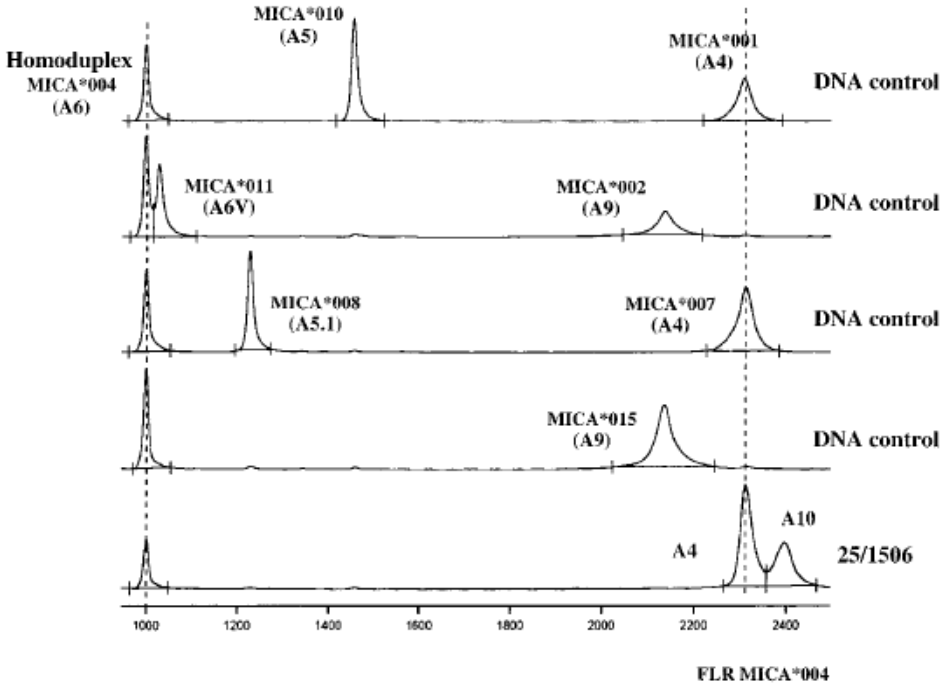


Figure 1.4.5: Detection of novel MICA alleles using RSCA. The sample 25/1506 in the bottom lane was analysed by RSCA and showed a different mobility in the gel when hybridised with fluorescent reference MICA*004. One peak in this sample corresponded with MICA*001 and the other was unknown. Sequence analysis of exon 5 revealed a novel allele with ten GCT repeats. Perez-Rodriguez *et al*., 2002 (Permission for reproduction granted by John Wiley and Sons).

MICA typing using PCR and Sequence Specific Primers (SSP)

PCR-SSP utilises amplification primers specifically designed to anneal with known polymorphisms within the MICA gene. It was also originally developed for HLA allele typing (Olerup and Zetterquist, 1992) and later developed for MICA typing (R. W. Collins *et al.*, 2002; Stephens *et al.*, 1999). Primers are designed so that polymorphic target nucleotides are situated on the 3' end of the primer and an amplicon will only be generated if the specific polymorphism is present. Resolution of possible allele combinations is improved with the addition of further primer pairs to cover all known polymorphisms within the gene. To ensure a negative result is because the nucleotide mutation is not detected, a control 'housekeeping' gene is amplified from the same sample. Therefore if the housekeeping gene is amplified and the specific amplicon is not generated, the polymorphism is not present. All primer reactions are loaded onto an agarose gel, resolved by electrophoresis and visualised by ethidium bromide staining and photography. SSP is popular because it is rapid, results can be achieved in two hours and only basic molecular biology laboratory equipment is required. However, only polymorphisms that are known can be detected unless a novel allele generates anomalous results due to unexpected positive or negative reactivity. Point mutations that create novel polymorphic positions are not

detected with this method unless they affect primer binding. Furthermore, manual reaction setup and gel loading renders this technique less suited to testing large numbers of individuals and can be expensive due to the large number of PCR reactions required.

MICA typing with PCR and Sequence Specific Oligonucleotide Primers (SSOP)

SSOP was another method developed for HLA allele typing (Saiki *et al.*, 1986) and later developed for MICA typing (Mendoza-Rincon *et al.*, 1999; Y. Zhang *et al*., 2001). Firstly, PCR of exons 2-5 of the MICA gene is carried out for each sample. For high-throughput this is usually done in a 96-well PCR tray so that a robot, or some other system, can directly transfer the amplified DNA to the membranes after being heat denatured. The membranes are cut to size to allow room for the 96 'blots' and one membrane is used for each DNA probe which will bind to complimentary sequences in the DNA. Once the DNA has dried onto the membranes they are placed in a UV cross-linker, which covalently binds the DNA to the membrane so that it cannot be washed off. Each membrane is coated in a solution containing the probe, which is known as hybridisation. In most protocols, the probe is labelled with digoxygenin-ddUTP (DIG-ddUTP) so that hybridisation can be detected later, and excess probe is removed by washing. The hybrids are detected with an antibody-enzyme conjugate, anti-DIG-alkaline phosphate and visualised by adding a substrate which reacts with the alkaline phosphate to produce a chemiluminescent signal which can be detected by autoradiography film. The result is a pattern of reactions or positive signals that can be interpreted to give the combination of alleles present. It is important to include controls in each batch of samples to ensure the procedure is working correctly, positive controls are DNA samples with well-defined MICA types, expected to react with the probes used and negative controls detect contamination. The level of resolution is dependent on the number of probes utilised.

This technique has the advantage of being suited to high-throughput analysis, for example, population studies of MICA allele frequencies or where large numbers of individuals are screened for disease association studies. A major limitation is with results analysis, which can be difficult and tedious. Currently, the only available commercial assay for MICA typing is based on this technique and overcomes many of the difficulties associated with the traditional method. The commercial assay is based on Luminex™ X-MAP technology (Dunbar, 2006) and developed by One Lambda. The Luminex X-MAP system is a multiplexed microsphere-based suspension array platform capable of analysing and reporting up to 100 different reactions in a single reaction tube. PCR amplicons are generated, as with other MICA typing assays, by PCR, except one primer has been

biotinylated. The amplicons are denatured and mixed with the bead suspension where hybridisation of DNA complimentary to the probe coated on the bead takes place. Detection of hybridised amplicons is enabled with the use of streptavidin-phycoerythrin (PE reporter fluorochrome) and complex formation with biotin. Sample analysis is performed using a flow cytometer, which has two lasers, one that detects the fluorescence of the bead (identifies the probe) and the other to detect the reporter fluorochrome (positive hybridisation). Computer software is used to generate reports and eliminates complicated analysis and risk of human error.

1.4.2 MICA antibody detection methods

MICA antibody detection CDC

As MICA is not normally expressed on peripheral blood lymphocytes, the traditional CDC test cannot be used either to detect MICA antibodies in patient serum or to perform a CDC-crossmatch. However, cells can be used for MICA antibody detection if DNA encoding MICA molecules is transfected into a B-lymphoblastoid cell line (BLCL). HMY2.C1R cell line does not express MICA antigens on the cell surface and can be transfected by electroporation with MICA cDNA encoding extracellular, transmembrane and cytoplasmic domains. Co-transfection of a gene allowing resistance to the antibiotic drug G418 permits growth of cells that have incorporated the transfected gene while non-transfected cells die. Individual MICA alleles are transfected into separate cultures of HMY2.C1R cells to produce of panel of cells expressing single MICA antigens that can be used in a CDC assay as depicted in Figure 1.4.6.

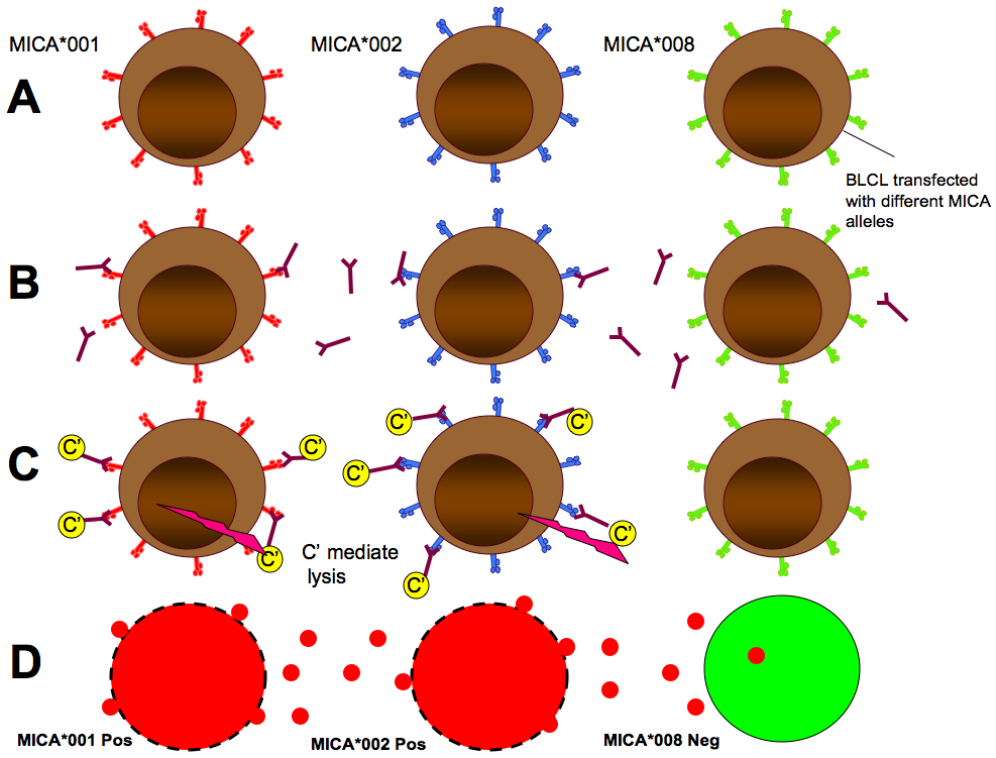


Figure 1.4.6: MICA antibody detection using CDC. In this example, three MICA alleles have been transfected into BLCLs – MICA*001, 002 and 008. A. Cells are placed into individual wells of a 'Terasaki' micro-well plate and B. patient serum is added. C. Following incubation, complement is added to the wells causing a membrane attack complex where antibody is bound. D. Lysis (positive result) is visualised with a fluorescent dye that stains dead cells red while live cells appear green by fluorescent microscopy.

MICA antibody detection using ELISA

As CDC requires live, viable cells, a method where MICA antigen is immobilised on a solid support (solid phase) is preferable. Also, antibody-mediated graft damage (anti-HLA) in transplantation has been reported, in the absence of a positive result by CDC, indicating more sensitive techniques are required. The easiest way to achieve this is to produce recombinant MICA molecules representing the extracellular portion of the antigen recognised by antibodies. Soluble recombinant MICA molecules can be produced using bacterial, insect cell or mammalian cell systems with bacteria producing the largest amount of antigen at the lowest cost and mammalian cells producing the least protein and is the most expensive system to develop. Insect cell transfection and recombinant molecule production falls somewhere between the other two in terms of cost and amount of antigen produced.

Purified, recombinant MICA (rMICA) molecules are adhered to a solid support, usually a 96-well plastic plate. When patient serum is added, only antibodies specific for the

particular MICA antigen will bind, thus avoiding non-specific binding that can occur with CDC. If MICA specific antibodies are present in the patient's serum they can be detected bound to the antigen by addition of a secondary anti-human IgG antibody labelled with biotin (or some other detection system). Biotin strongly binds to streptavidin, which is conjugated with horse-raddish peroxidase (HRP), an enzyme that is catalysed by the addition of a substrate to produce a colour reaction. The intensity of colour produced, and hence the amount of antibody present in patient serum, is measured by spectrophotometry and recorded as optical density (OD). The ODs are compared with MICA positive and negative controls to determine a cut-off value for assigning a positive reaction. A disadvantage with this method is that it takes many hours to perform, involves numerous washes and incubations and requires several hundred microlitres of serum, depending on the number of MICA alleles tested. However, it is more sensitive than CDC, detecting the presence of antibodies at much lower concentrations, and the information gained is useful for clinicians especially when donor-specific antibodies are detected. The main stages involved in performing an ELISA to detect MICA antibodies are shown in Figure 1.4.7.

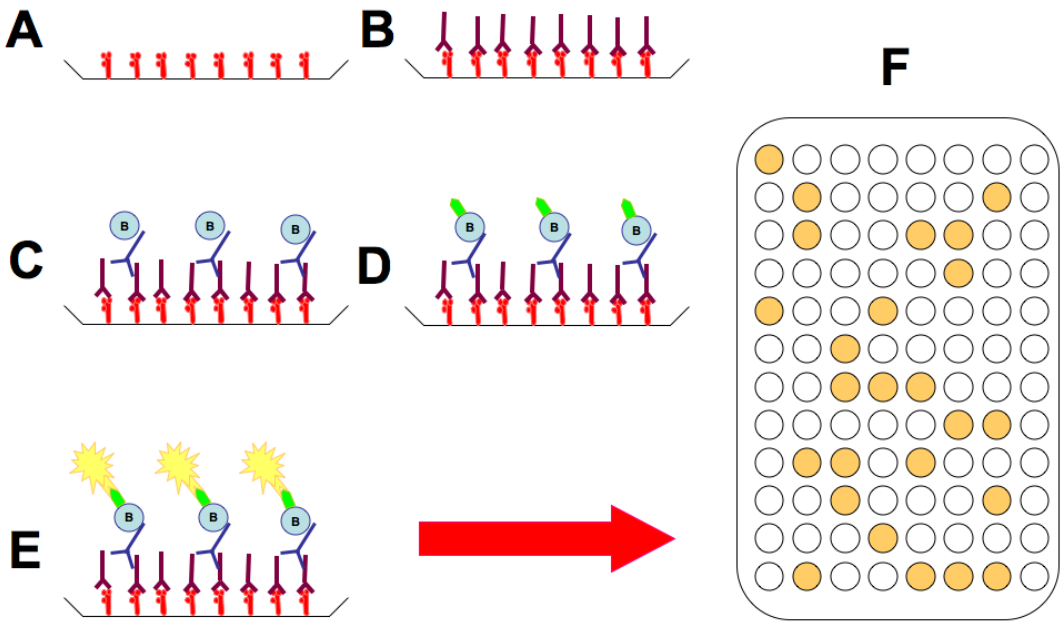


Figure 1.4.7: MICA antibody detection using ELISA. A. rMICA molecules are adhered to the surface of a 96-well plate. B. Patient serum is added and allowed to incubate with the antigens. C. Anti-human IgG conjugated with biotin is added. D. Streptavidin/Horse-raddish peroxidase (enzyme) is then added which binds with biotin, if present. E. A substrate is added and is catalysed by the enzyme to produce a colour reaction. F. An automatic ELISA plate reader (spectrophotometer) measures the optical density of each well, which is used to deduce positive and negative reactions relating to the presence or absence of MICA-specific antibodies in patient serum.

MICA antibody detection using flow cytometry

FC can be used as a platform for solid-phase antibody detection by utilising a series of polystyrene microspheres (beads) containing a mixture of two fluorochromes of varying concentration, embedded within the bead giving them unique fluorescence intensity. This technology was developed by the Luminex Corporation (TX, USA) and adopted originally by One Lambda (CA, USA) as a solid-phase support for recombinant HLA molecules in order to detect HLA antibodies. The Luminex bead method for antibody detection (referred to henceforth as 'Luminex') is discussed at length in Chapter 4. Briefly, soluble rMICA, or MICA molecules cleaved from the surface of cells, are attached to the beads as either a mixture of antigens on one bead or individual SAgs on separate beads. If single-antigen beads are used they can be conveniently mixed together and assayed as a multiplex in a single well of a 96-well plate. Beads are incubated with patient serum, followed by washing and the addition of a PE-labelled anti-human antibody. When the beads are run through a flow cytometer, the two lasers detect the differential fluorescence intensity of the beads and also the presence of PE, thereby identifying which antigen is on the beads and whether there is a positive reaction with patient serum. The output from the instrument is in the form of mean fluorescence intensity (MFI) and adjusted using a negative control MFI and a positive control can be included to ensure the assay performed correctly. Empirical testing using reference standard sera can establish cut-off values for assigning a positive reaction, however, sera standards for MICA testing are currently unavailable.

Luminex has many advantages over alternative methods of antibody detection and has transformed the histocompatibility laboratory. Multiplexing allows analysis of multiple analytes in one tube therefore many individual tests can be performed at once, saving time and manpower while at the same time increasing sensitivity up to 10% compared to ELISA methods (Tait *et al*., 2009). Luminex can easily distinguish between HLA class I and II antibodies and it is now possible to detect antibodies directed at antigens of loci such as HLA-DRB3, -DRB4, -DRB5, -DQB1 and DPB1 which are expressed independent of HLA-DRB1. It is also possible to detect antibodies reactive with HLA-C antigens, completing the repertoire of potentially clinically relevant HLA antibodies.

In 2006, One Lambda introduced a Luminex SAg assay to detect MICA antibodies and for the first time many more laboratories were able to investigate the clinical relevance of these antibodies in transplantation. This was followed by the release of a second commercial Luminex MICA SAg assay by Tepnel Lifecodes (now produced by Gen-Probe, CA, USA) in 2008, setting the scene for future research into the effects of MICA antibodies in acute

and chronic allograft rejection. The principle of the Luminex antibody detection assay is depicted in Figure 1.4.8.

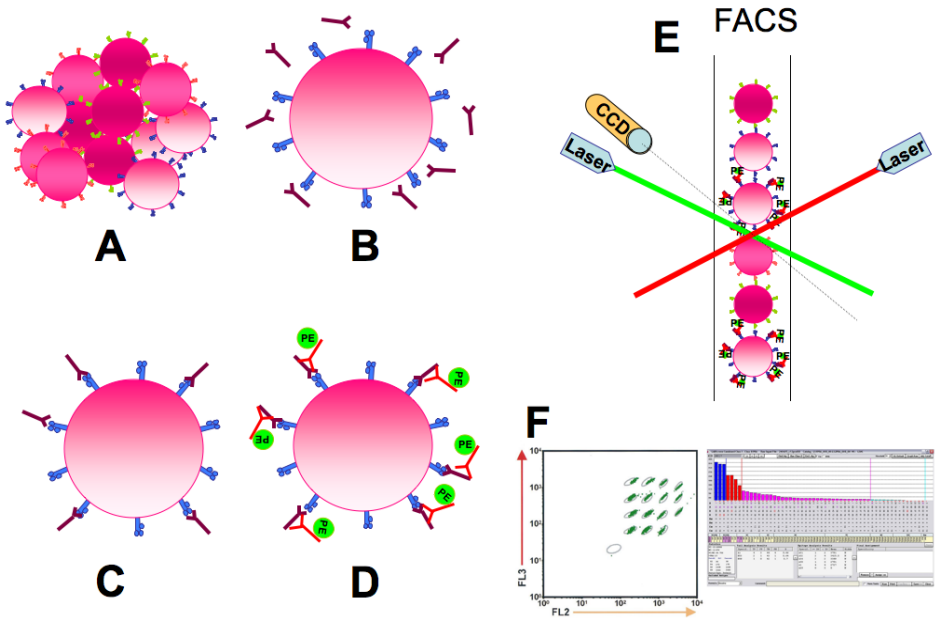


Figure 1.4.8: Principle of the Luminex single antigen assay to detect MICA antibodies.
A. Single rMICA antigens are attached to beads with specific fluorescent properties and mixed together. B. Patient serum is mixed with pooled Luminex beads and allowed to incubate. C. Antibodies in patient serum, specific for individual MICA antigens, bind to the MICA molecule. D. Anti-human IgG conjugated with PE is added and binds with MICA antibodies, if present. E. Flow cytometry is performed for each patient being tested. Two lasers detect the presence of antibody (PE) and the bead specificity (bead fluorescence) and a charge-coupled device (CCD) camera detects the emitted light. F. Individual beads form distinct groups (gates) based on their fluorescence and computer software calculates results and produces a report.

Detecting MICA antibodies in patient and donor crossmatching

MICA antigens are not expressed on peripheral blood lymphocytes rendering the traditional crossmatch unsuitable for detecting clinically relevant non-HLA antibodies that are expressed on endothelial cells. These include angiotensin II type I receptor (Dragun *et al*., 2005), vimentin, MICA and MICB (Q. Zhang and Reed, 2010). A commercially available endothelial cell crossmatch assay is now available.

The XM-ONE® (Absorber, Stockholm, Sweden) endothelial cell (EC) crossmatch assay kit contains magnetic beads for EC isolation from peripheral blood of the donor that target the TIE-2 receptor expressed on ECs and also T- and B-cells. Once isolated, the cells can be incubated with patient sera, as in the traditional crossmatch assay, followed by detection of bound patient antibody with anti-human IgG antibody conjugated with PE and FACS

analysis. Lymphocytes and endothelial cells can be separated by size using forward scatter and side scatter and gated, allowing separate analysis and, therefore, T- and B-cell and EC crossmatch can be performed simultaneously (Alheim *et al.*, 2010). If the lymphocyte crossmatch is negative but EC crossmatch is positive, potentially harmful antibodies to donor EC antigens are present in the patient's serum, however, they could be directed against any alloantigen expressed on the EC, including MICA. If the T- or B-cell crossmatch is positive it is impossible to tell if non-HLA antibodies are present as ECs also express both HLA class I and II antigens. In either case, a positive result could be a contraindication to transplant. A multicentre evaluation of this assay has been carried out and it was found that 24% of patients had antibodies to EC antigens and among these patients, 46% had rejection episodes compared to 12% without such antibodies. These results suggest that patients who would normally be crossmatch negative are at risk of rejection if non-HLA antibodies directed against EC antigens are present (Breimer *et al.*, 2009). The principle of this technique is illustrated in Figure 1.4.9

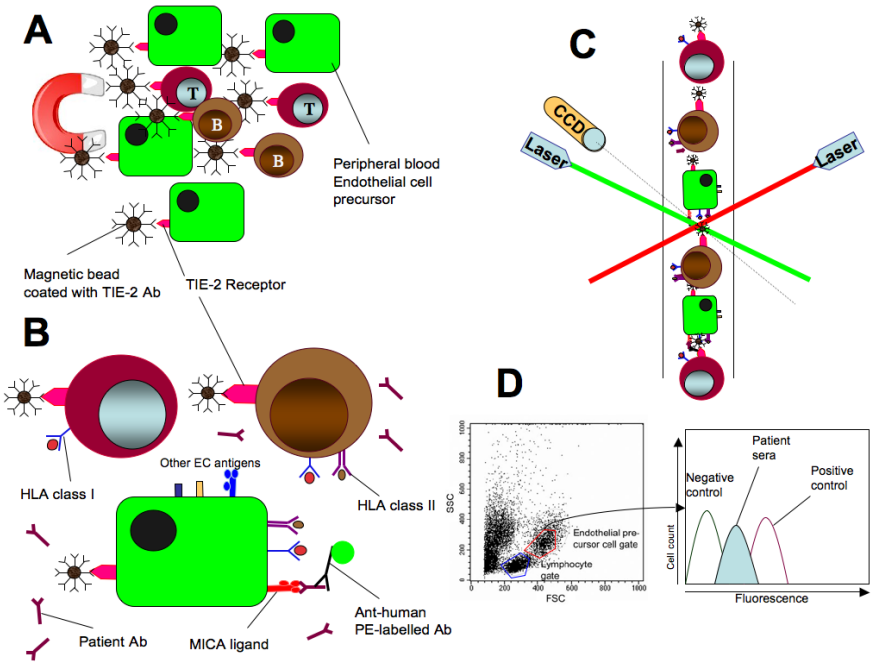


Figure 1.4.9: XM-ONE® crossmatch assay to detect non-HLA antibodies directed against EC antigens. A. Magnetic isolation of EC and lymphocytes from peripheral blood, targeting the TIE-2 receptor. B. Cells are incubated with patient serum followed by antihuman IgG labelled with PE. C. Flow cytometry analysis detects bound antibodies. D. Separate analysis of the EC gate and lymphocyte gate determines result.

1.5 Aims of this Thesis

Renal transplantation can transform the lives of patients with ESRD and improvements in immunosuppressive therapy have limited the effects of T-cell mediated alloimmunity, reducing AR. However, rejection due to antibody-mediated graft damage arising from B-cell responses to mismatched HLA antigens remains a problem and the production of *de novo* HLA-DSA post-transplant is associated with acute and chronic graft rejection. Despite renal transplant rejection being strongly associated with HLA antibodies, 11-20% of patients without HLA antibodies develop chronic allograft rejection and furthermore, HAR can occur in the absence of HLA antibodies, implicating other non-HLA alloantigens. A possible candidate is the highly polymorphic MICA antigen as expression can be upregulated by stress on endothelial cells within renal grafts and has been shown to associate with acute and chronic graft rejection.

The mechanisms by which individuals develop MICA antibodies are largely unknown but MICA sensitisation can possibly be induced by pregnancy and blood transfusion, however it is has not been clearly shown that mismatching of MICA alleles in transplantation results in the production of antibodies. Therefore, the aims of this thesis were to develop an SBT strategy to genotype patients and their donors for MICA alleles at high resolution and develop methods for detection of MICA antibodies in recipients.

Following establishment of assays for MICA allele typing and MICA antibody detection, the aim was to determine whether mismatching of MICA alleles results in the production of *de novo* MICA antibodies directed against MICA antigens of the donor. A correlation with AR determined by biopsy analysis was investigated for patients who developed *de novo* MICA antibodies or had pre-existing MICA antibodies and compared to recipients without MICA antibodies. Statistical analysis to determine MICA antibody association with five-year GS and function assessed by eGFR measurement after one, two and three years post-transplant was carried out. In addition, longitudinal analysis of MICA antibody production was performed to investigate changes in MICA antibody levels over time. Some studies suggest that the presence of soluble MICA proteins in patient serum may have an immunomodulatory role and protect patients from graft rejection mediated by MICA antibodies and this was also investigated.

CHAPTER 2

Materials and Methods

The materials and methods described in this chapter were used to perform the research work referred to throughout the chapters of this thesis.

2.1 Study Cohorts

2.1.1 Recruitment and ethical approval

All renal patients included in this study provided written consent for diagnostic testing for the presence of alloantibodies that may be a contraindication to transplant. The analysis of data in this study did not require any additional patient samples or consent.

2.1.2 Specimen collection

Genomic DNA, extracted from whole blood, and serum samples were collected from patients who received a renal graft or with ESRD and treated at the UCL Department of Nephrology, The Royal Free Hampstead, London. Specimens were stored at the Anthony Nolan Histocompatibility Laboratories and available for testing, relevant to this study.

2.1.3 Healthy adult controls

Sera and DNA from normal healthy individuals, who had donated blood for testing to join The Anthony Nolan Trust volunteer hematopoietic stem cell donor register, were anonymized and used for quality control validation of the methods and approved by the Anthony Nolan UK Medical and Scientific Advisory Committee in 2008. Additionally, plasma, sera and DNA specimens were obtained from healthy volunteers from the Anthony Nolan Research Institute, London after obtaining informed consent (Appendix B).

2.2 Molecular Biology

2.2.1 General methods

DNA extraction from whole blood

Patient, donor and healthy control DNA used in this study was extracted using an automated robotic system and Qiagen reagents and performed by the histocompatibility laboratories at Anthony Nolan following guidelines and procedures for a professionally accredited laboratory.

Nucleic acid quantification

Nucleic acids were quantified using a modified form of the Beer-Lambert law on a Shimadzu Mini1240 Spectrophotometer (Shimadzu, Duisburg, Germany) or on a Nanodrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE. USA). One OD unit was assumed to be on average equivalent to 50 μg/ml of double stranded DNA (dsDNA) or 38 μg/ml of single stranded DNA (ssDNA). The Nanodrop software automatically adjusted the absorbance measurements in order to compensate for the 1 μm path length when calculating OD values on this instrument. Purity of the nucleic acid solutions was assessed by the ratio of the absorbance measurements taken at 260 and 280 nm (OD260:OD280) where a pure dsDNA solution has an OD260 absorbance that is exactly twice the value of its OD280 absorbance. Nucleic acid stocks were stored at 4°C or -20°C for longer storage duration.

Quantification of total RNA

To determine the yield of total RNA, the OD was determined using UV spectrophotometry. RNA absorbs light at 260 nm. One OD unit is equivalent to 40 μg/ml of single-stranded RNA.

Polymerase Chain Reaction (PCR)

PCR enables *de novo* synthesis of a target region of genomic or cDNA, flanked by two primers complimentary to the 3' and 5' strands. The reaction comprises three steps, denaturation which separates the double-stranded DNA to form two strands, annealing to allow binding of the primers to their complimentary sequence and extension during which, *Thermus aquaticus* (Taq) DNA polymerase synthesises new copies of the DNA, flanked by the primers. The three steps were repeated a number of times to amplify the DNA region of

interest. The reaction required the addition of deoxynucleotides to provide energy and nucleotides for the synthesis of new DNA, heat stable polymerase, specific 5' and 3' primers, DNA template and buffer containing magnesium. Amplification conditions (temperature and duration) must be optimised based on nucleotide composition of the primers and length of the desired DNA product. Magnesium concentration must be optimised as it is mandatory for activation and fidelity of Taq polymerase and affects primer annealing.

Primer Design

Primers for PCR amplification were custom made and complimentary to the sequences flanking the DNA region of interest. Primers were typically 15-30 nucleotides long and in addition to the sequence complimentary to the DNA template, additional sequence could be added and incorporated into the amplified DNA product. The melting temperature (Tm) is the temperature at which half of the DNA in the reaction is single-stranded and half is double stranded and was calculated for oligonucleotides with the following formula:
Tm (°C) = 2 (nA + nT) + 4 (nG + nC). In this formula, n is the number of A (adenine), T (thymidine), G (guanine) and C (cytosine) nucleotides. The temperature for annealing of primers was 4°C below the calculated Tm for a given primer.

2.2.2 Sequence-based typing for MICA allele assignment

PCR amplification of the MICA gene

Primers used for amplification of exons 2-4 or 2-5 of the MICA gene and for bi-directional sequencing of exon 2-4 (and exon 5 for GCT triplet identification) are given in Table 2.2.1. PCR reactions were performed in an MJ Research PTC-200 thermal cycler (MJ Research Inc, Watertown, MA, USA). Reaction volumes of 25 µl were set up using amplification primers listed in Table 2.2.1 and reaction mixtures described in Table 2.2.2. Reactions were set up on ice and placed in a thermal cycler using the cycling conditions outlined in Table 2.2.3.

Table 2.2.1: Primers used for amplification and sequencing MICA from genomic DNA (SBT) and sequencing of cDNA

Name	Sequence	Description
MICA-Shao[a]	CAC CTG TGA TTT CCT CTT CCC CAG AGC	5' amplification primer intron 1
MICA8999[b]	GAT GCT GCC CCC ATT CCC TTC CCA A	3' amplification primer intron 5
5MICA-1C[c]	GAG CCC CAC AGT CTT CGY TAT	Exon 2 sequencing primer forward
MICA2R[b]	GTG CCG GCT CAC CTC CCC TGC T	Exon 2 sequencing primer reverse
MICA3F[b]	CCC TGG GCT GAG TTC CTC	Exon 3 sequencing primer forward
MICA3R[b]	ATA GCA CAG GGA GGG TTT	Exon 3 sequencing primer reverse
MICA4F[b]	CAG AGT GAG AAC AGT GAA	Exon 4 sequencing primer forward
MICA4R[b]	AGG GAC TTG TTA TAC ACT	Exon 4 sequencing primer reverse
MICA5R[d]	CCT TAC CAT CTC CAG AAA CTG C	Exon 5 sequencing primer reverse

[a](Shao *et al.*, 2004); [b](Zwirner *et al*., 2000); [c](Molinero *et al*., 2002b); [d](Ota *et al*., 1997); [c]

Table 2.2.2: Reagents and volumes required for genomic DNA PCR amplification of exons 2-5 of the MICA gene

PCR Reagent	**Volume (μl)**
TDMH[a]	8
5' primer (25 pmol/μl)	0.25
3' primer (25 pmol/μl)[b]	0.25
TAKARA® Taq polymerase (8 Units/μl)	0.2
DNA template	2.5
Distilled H_2O	13.8
Total Volume	25

[a]TDMH – Ten times PCR buffer, dNTPs, $MgCl_2$, H_2O. (42.25 μl 10X PCR buffer; 12 μl dNTPs; 26 μl 25mM $MgCl_2$; 82 μl distilled H_2O).

[b]Primer MICA 8999 situated in intron 5 allows amplification of exon 5.

Table 2.2.3: PCR cycling conditions for MICA gene amplification

Step	Temp °C	Duration
1	95	5 mins
2	95	30 secs
3	67	1 min 30 secs
4	72	2 mins 30 secs
5	Repeat steps 2-4 for 34 cycles	
6	72	10 mins
7	4	Hold

DNA electrophoresis

To confirm successful PCR amplification of the target gene, agarose gel electrophoresis was performed. In an electric field, the negatively charged DNA migrates towards the anode at a speed relative to the size of the DNA fragment, enabling separation of fragments of different lengths. The 1% agarose gel was prepared by dissolving 1g of electrophoresis grade agarose (Invitrogen, Paisley, UK) in 100 ml Tris-boric acid-EDTA (TBE) buffer (TBE: 0.1 M Tris, 0.09 M boric acid, 1 mM EDTA, Bio Whittaker, Maryland, USA). Ethidium bromide (1 μg/ml final concentration, Sigma, UK) was added to the gel for ultraviolet (UV) light visualisation of the DNA. The gel was poured into a sealed gel tray (Biorad, Hercules, Ca, USA) and allowed to solidify after placing gel combs in the gel to create the wells. The gel was submerged in 1X TBE buffer in an electrophoresis tank before loading the samples. One tenth of the PCR reaction volume (2.5 μl) was mixed with 1 μl 6X blue loading dye solution (Fermantas Inc, Maryland, USA). To enable sizing and/or quantification of the amplified products, a range of molecular weight markers were available in a ready to use format. Hyperladder I (Bioline, London UK) was used with PCR products in the expected range of 1000 bp to 10000 bp and Hyperladder IV (Bioline, London UK) used with fragments <1000 bp. If 5 μl (720 ng) is loaded onto lanes adjacent to the PCR products, the quantity (ng) of DNA as well as the size can be determined by referring to the product insert.

Post-PCR amplification clean up

PCR-amplified DNA was purified using a GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Piscataway, USA) according to manufacturer's instructions. For purification of DNA from agarose gels, an agarose slice containing the DNA of interest was

excised from the gel using a clean scalpel blade. The DNA was eluted by the addition of 75 µl of distilled H_2O (diluting the PCR DNA 1:3). If the DNA was to be used for cloning, only 50 µl of distilled H_2O was used for elution.

Cycle sequencing reaction for direct sequencing and SBT

Sequencing primers, as listed in Table 2.2.1, were used for cycle sequencing. Sequence reactions were performed using Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an MJ Research PTC-200 thermal cycler. Conditions for cycle sequencing were as follows: 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 sec and extension at 60°C for 4 minutes. Products were precipitated in 30 µl 100% ethanol and 2.5 µl 125 mM EDTA for 15 minutes at room temperature and recovered by centrifugation at 1800g for 15 minutes. Pellets were washed with 30 µl of 75% ethanol followed by centrifugation at 1800g for 30 minutes. Products were resuspended in 10 µl of HiDi Formamide (ABI, CA, USA) and sequenced for 50 minutes on a 3730xl ABI Genetic Analyser.

Sequencing Analysis and Heterozygous MICA typing

Sequencing Analysis and MICA typing was performed using AssignSBT™ (Connexio Genomics, Australia) based on exons 2-4 of the MICA gene. Ambiguous types were analysed further if distinguishable by the number of GCT repeats in exon 5 using Genescan (Applied Biosystems, Foster City, CA, USA) or analysis of overlapping sequence patterns as described (Zou *et al.*, 2006a) and is discussed further in Chapter 3.

2.2.3 Short Tandem Repeat (STR) Analysis of MICA Exon 5 using Genescan

PCR Primers and Amplification of Exon 5 of the MICA gene

PCR primers flanking exon 5 (trans-membrane region) were ordered based on the work of Ota (Ota *et al*., 1997). Primer details are as follows, MICA5F: CCTTTTTTTCAGGGAAAGTGC; MICA5R: CCTTACCATCTCCAGAAACTGC. MICA5R was labelled at the 5' end with the fluorescent amidite reagent, 6-FAM (Applied Biosystems, USA). The MICA5F primer corresponds to the boundary region within intron 4 and exon 5 and MICA5R is located in intron 5. PCR was carried out in a 50 µl reaction volume (Amplitaq Gold – ABI, CA, USA) containing 50 ng of template genomic DNA, 200 µM of each primer, 1.25 mM of each dNTP, 2 mM $MgCl_2$, 1.8 U TaqGold and 1X PCR buffer. The reaction mixture was placed in an MJ Research PTC-200 thermal cycler:

denaturation at 94°C for 2 minutes followed by 30 cycles of: 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. A negative control was included to detect contamination. Amplified products were checked on a 1% agarose gel as previously described.

Typing of Trinucleotide Repeat Polymorphism in the TM region of MICA gene

A gel loading cocktail was prepared as follows, per sample: 2.5 µl formamide, 0.5 µl Blue Dextran (50 mM EDTA, 50 mg/ml Blue Dextran), 0.5 µl Genescan Rox 500 size standard (Applied Biosystems). Three volumes of PCR product were tested for optimal results per sample – 1.0 µl, 0.5 µl and 0.3 µl made up to 1.5 µl with water. For electrophoresis an ABI 377 DNA sequencer was used with Genescan software (Applied Biosystems). A 4% denaturing acrylamide gel was prepared using 36 cm well to read plates. Immediately prior to loading samples on the gel, 1.5 µl of sample was mixed with 3.5 µl of loading cocktail in MicroAmp tubes and placed in an MJ Research PTC-200 thermal cycler at 95°C for 5 minutes. Samples were then placed on ice until ready to load. A 36 well comb was used and 1.5 µl of denatured sample mixture was loaded into separate wells. Electrophoresis was performed for 2 hours under conditions appropriate for the plates as recommended by the manufacturer.

Assignment of Alleles

The designation of MICA type was based on the number of repeat units present in the amplified allelic products of MICA exon 5. The alleles have 4, 5, 6, or 9 GCT repetitions (alleles with 7, 8 or 10 GCT repeats were not detected). In addition, alleles with 5 GCT repeats may also have an additional insertion of 'g' and will be referred to as 5.1. The amplified sizes are expected to be as follows: 180 bp (4 GCT), 183 bp (5 GCT), 184 bp (5.1 GCT), 186 bp (6 GCT) and 195 bp (9 GCT).

2.2.4 Production of cDNA from RNA

RNA Extraction and DNase Treatment

Total RNA was isolated from cultured cells using RNeasy® Mini Kit (Qiagen, GmBH, Germany). This allows the purification of up to 100 µg RNA from animal cells and tissues. An aliquot of 5-10 x 10^6 cells were used per extraction following manufacturer's instructions. The mRNA was used immediately as a template for complementary deoxyribonucleic acid (cDNA) synthesis or stored at -70°C until required.

Reverse-Transcriptase (RT) PCR – cDNA synthesis

10 µl of mRNA from the previous step was added to a 1.5 ml Eppendorf tube. 1 µl of 0.5 µg/ml Random Primers (Promega, Wisconsin, USA) and 1µl 10µM dNTPs (Bioline, London, UK) was added to the tube containing RNA and incubated in a water bath at 65°C for 5 minutes. Tubes were then placed on ice and 4 µl 5 x Buffer (Promega), 2 µl 0.1M Dithiothreitol (DTT) (Promega), 1 µl RNase inhibitor (Promega) and 1 µl Maloney-Murine Leukaemia Virus (Promega) was added bringing the total volume to 20 µl. The RT-PCR reactions were placed in a water bath at 37°C for 50 minutes followed by 70°C for 10 minutes and reactions were kept on ice or frozen until needed.

2.2.5 Generation of plasmid constructs for protein expression in Escherichia coli

PCR Amplification of MICA and MICB from cDNA

MICA and MICB exons 2-4 were amplified in a 50 µl reaction volume (ABgene, Surrey, UK) containing 10-50ng template cDNA, 600 µM of MICA/B forward and reverse primers (described below), 1.25 mM of each dNTP, 2.5 mM $MgCl_2$, 5 U RedHot Taq and 1xPCR buffer. PCR: 95°C for 30 seconds followed by 30 cycles of: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes. Amplified products of 800 bp. were checked on a 1% agarose gel. The 5' primer originally used for MICB cDNA amplification by my colleague, Jenny Luxton, contained an Xba1 restriction endonuclease site and the 3' primer contained a restriction endonuclease site for BamH1, however, the Xba1 site was found within the MICA sequence that was not present in MICB and the only alternative was to also use BamH1 on the 5' primer and screen for correctly orientated inserts in the latter stages. MICA/B Forward primer: ggatccaatgccccacagtctt, MICA/B Reverse primer: ggatccccagagggcac. Underlined sequence is the BamH1 restriction site.

Purification of PCR cDNA from a gel slice

The PCR product was run on a 1% agarose gel for gel excision and purification using GFX PCR DNA and gel band purification as described above.

Ligation of MICA cDNA into pCR®4-TOPO® plasmid

This preliminary cloning step using TOPO TA cloning kit for sequencing (Invitrogen, Paisley, UK) was carried out to enable screening of a large number of transfectants as many were expected to have MICB sequences or nucleotide misincorporations. The TOPO TA cloning kit utilises an efficient enzymatic ligation reaction facilitated by topoisomerase I.

Topoisomerase I from the *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phophodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester bond is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The main advantage of this system is that ligation is efficient and can be carried out in five minutes at room temperature using the following method. A volume of 4 μl of purified MICA PCR product was mixed with 1 μl of salt solution and 1 μl of pCR®4-TOPO® vector (both provided with kit) and made up to a final volume of 10 μl with 4 μl of distilled H_2O. Reactions were gently mixed and incubated at room temperature (22-23°C) for five minutes.

Transformation of TOP10 chemically competent cells

The competent cells were thawed on ice and 3 μl ligation reaction was added and mixed with the cells by stirring with a pipette tip. The cells and vector were incubated on ice for 5 minutes then heat-shocked at 42°C in a water bath for exactly 30 seconds and transferred to ice for 2 minutes. 250 μl of SOC medium was added and the tube was placed horizontally in a shaking incubator set at 37 °C for 1 hour. Luria Bertani (LB) agar plates were prepared (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing 50 μg/ml kanamycin as pCR®4-TOPO® contains a kanamycin resistance gene. In order to ensure well-separated colonies, 2 plates were prepared for the transformation reaction – one plated with 10 μl and one with 50 μl of transformation reaction. The agar plates were incubated at 37°C overnight. Individual colonies were then picked and transferred to Falcon 2059 14 ml tubes (Becton Dickinson and Co., New Jersey, USA) containing 2 ml LB medium containing 50 μg per ml kanamycin and incubated in a shaking incubator overnight (about 16 hours).

Miniprep isolation of plasmid DNA

A Strataprep® Plasmid Miniprep Kit (Stratagene, Ca, USA) was used for the extraction of plasmid DNA following manufacturers instructions. Sequencing of plasmids was performed to ensure ligated DNA was MICA specific and contained no nucleotide misincorporations. This required the design of new primers situated in exon 3, allowing sufficient overlap to enable the sequencing of the whole insert with these two primers:

5MICAint: 5'-ATC CAT GAA GAC AAC AGC AC-3'
3MICAint: 5'-AGT CCT CCA GAG CTC AGA CC-3'

DNA endonuclease restriction enzyme digestion

DNA endonulceases and their respective buffers were purchased from Gibco BRL®, (Paisley, UK). The specific DNA sequences recognised and digested by restriction enzymes used in experiments are given in Table 2.2.4. In brief, 1 to 5 µl of DNA (maximum 5 µg) was transferred to a sterile microcentrifuge tube with 1 µl reaction buffer, 1 µl restriction enzyme (when two different restriction enzymes were used, 0.5 µl of each was added) and made up to a final volume of 10 µl with sterile, double distilled water. The digestion was carried out for 1 to 2 hours at 37°C in a water bath.

Specific DNA sequences recognised by restriction enzymes used in this study are listed in Table 2.2.4. The arrows indicate digestion sites and the reactions were carried out in the presence of optimal buffers as indicated. The optimal buffer for Kpn1 is buffer 1, however, buffer 2 has 75% activity and was used to allow dual digestion with Xho1.

Table 2.2.4: DNA endonuclease restriction enzymes, digestion sites and optimum buffers

Name	Recognition site	Reaction conditions
EcoR1	5'-G⇓AATTC-3' 3'-CTTAA⇑G-5'	REACT® buffer 3 50 mM Tris-HCl (pH 8) 10 mM $MgCl_2$ 100 mM NaCl
BamH1	5'-G⇓GATCC-3' 3'-CCTAG⇑G-5'	
Kpn1	5'-GGTAC⇓C-3' 3'-C⇑CATGG-5'	REACT® buffer 2 50 mM Tris-HCl (pH 8) 10 mM $MgCl_2$ 50 mM NaCl
Xho1	5'-C⇓TCGAG-3' 3'-GAGCT⇑C-5'	

Restriction Digestion to release MICA fragments from pCR®4-TOPO®

Restriction digestion was carried out using BamH1 restriction sites encoded by PCR amplification primers at either end of the amplicon to release the verified MICA insert from this plasmid. 20 µl of pCR®4-TOPO® plasmid, containing the desired MICA insert was used in a 30 µl reaction volume containing 1U of BamH1 (1 µl) and 2 µl REACT® buffer 3. The reactions were incubated at 37°C for 2 hours in a water bath before loading the entire

digest on a 1% agarose gel to isolate the MICA fragment using GFX purification columns as described above.

Digestion with BamH1 to Linearise Plasmid pAC-5

Restriction digests were also carried out using 10 µl of pAC-5 plasmid (a total of around 7 µg plasmid DNA) in a 20 µl reaction containing 1U of BamH1 and the REACT® buffer 3 as above.

Ligation of plasmid pAC-5 with purified MICA PCR product

Ligation reactions for each MICA allele were set up in a 20 µl volume containing 10 µl buffer, 2 µl vector and 4 µl purified cloned PCR product and 2 µl T4 DNA ligase (Promega, Maddison, WI, USA). The ligation reaction was carried out at 16°C overnight.

Transfection of AVB101 expression host with pAC-5 – MICA construct

The protein expression system chosen for the production of rMICA was Biotin Avitag™ (Avidity, Colorado, USA), a simple and efficient method for the production of any biotinylated protein. The plasmid pAC-5 was selected because it enabled construction of a sequence in the correct reading frame.

AVB101 *E. coli* cells were grown up and made competent by the following method. A scraping of AVB101 cells was taken from glycerol stocks and plated onto 2TY (2% tryptone, 0.5% yeast extract, 0.1 M NaCl, 0.01 M $MgCl_2$) agar medium containing 10 µg/ml chloramphenicol and placed at 37°C overnight. A single colony was picked from the plate using a pipette tip, which was then ejected into 10 ml of 2TY medium containing 10 µg/ml chloramphenicol and placed in a shaking incubator until an $O.D._{.}^{600}$ of 0.5 was reached. The tube was centrifuged at 5000g for 15 minutes at 4°C. The supernatant was removed on ice before adding 80 ml of TFBI (30 mM KOAC, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$ and 15% glycerol pH to 5.8 using 0.2 M acetic acid and filter sterilised), ensuring the pellet was dissolved. The tubes and contents were left on ice for 30 minutes, then centrifuged at 3000g for 10 minutes at 4°C. The supernatant was removed on ice and 8 ml of TFBII (10 mM MOPS pH 7.0, 75 mM $CaCl_{2,}$ 10 mM KCl, 15% glycerol, pH to 6.5 using KOH/glaciel acetic acid) was added, ensuring pellet was dissolved. 200 µl was aliquoted into Eppendorf tubes and stored at -70°C. Stocks were also replaced by making glycerol stock – 1 ml of cells + 50% glycerol and stored at -70°C.

For the transformation reaction, 2 μl of purified plasmid DNA containing the MICA allele was heat shocked into 200 μl of competent AVB101 cells (using the same method as was used to transfect TOP10 cells) and 10 μl and 50 μl aliquots were spread on LB plates containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol and incubated at 37°C overnight. Individual colonies of transfected AVB101were picked and transferred to a Falcon 2059 tube (Becton Dickinson and Co., New Jersey, USA) containing 2 ml LB medium + 100 μg/ml ampicillin and 10 μg/ml chloramphenicol, and grown for 16 hours at 37°C in a shaking incubator set to 125rpm. Plasmid DNA was extracted using a miniprep kit (Stratagene, CA, USA) as described earlier in this section. MICA cDNA insert orientation and reading frame was verified by sequencing with MICA internal sequencing primers.

Induction of protein expression

Bacterial glycerol stocks, harbouring correctly constructed plasmids, were prepared for future use by taking 500 μl of the overnight culture, placing in a cryotube vial (Nunc, Kamstrupvej, Norway) and adding 75 μl sterile 100% glycerol. The tubes were mixed by vortexing and stored at -70°C. 100 μl of the overnight culture was added to a fresh Falcon 2059 tube containing 1.9 ml of fresh LB medium + 100 μg/ml ampicillin and 10 μg/ml chloramphenicol. The tubes were then placed in a shaking incubator at 37°C for about 1 hour or until the O.D. at 550 nm reached 0.5. For the 0 hour (uninduced) time-course sample, 100 μl of each culture was removed and placed in a microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute to pellet the cells. The supernatant was removed and 20 μl of 2X SDS protein sample buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added to each tube, which was then stored at -20°C. To induce protein expression, 2 μl of 100 mM isopropyl-β-D-thiogalactoside (IPTG) was added to each of the cultures to give a concentration of 1 mM. The tubes were then placed in a shaking incubator at 37°C for 4 hours. Further samples were taken for SDS-PAGE analysis at 1, 2, 3 and 4 hourly intervals.

Inclusion body purification

Proteins synthesised as inclusion bodies in *E. coli* strains such as AVB101 require purification from other bacterial proteins, which can be achieved with the following method. Transformed AVB101 cells were inoculated into 30 ml LB medium (10g tryptone, 5g yeast, 10g NaCl in 1L distilled H_20) containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol and incubated overnight in a shaking incubator at 37°C. 6 ml aliquots of

the 30 ml overnight culture was transferred to six flasks containing 1L of LB medium fortified with antibiotics as above, and placed in a shaking incubator until optical density reached 0.6 at 600 nm. A 1 ml aliquot of culture was removed for later analysis prior to protein induction and stored at -20°C. The remaining culture was induced by adding 1M IPTG to a final concentration of 1 mM, and placed in a shaking incubator for a further 4 hours at 37°C. An aliquot of 0.5 ml post-induction culture was removed and stored at -20°C for later analysis. The culture was transferred to 1L Beckman polypropylene screw top centrifuge bottles and centrifuged in a Beckman Avanti J-20 centrifuge with JLA 8.1000 rotor at 4000 rpm for 15 minutes at 4°C. Bacterial pellets were pooled and resuspended in PBS to a total volume of 60 ml.

The resuspended bacteria was transferred to a 100 ml polypropylene beaker containing a half-inch magnetic stirrer and placed on a stirring plate at half speed. The following were added drop-wise to the stirring mixture: 1.2 ml 50 mg/ml lysozyme (final = 1 mg/ml), 300 µl 1.0M $MgCl_2$ (final = 5mM), 1.5 ml 2 mg/ml DNAse I in 50% glycerol containing 75 mM NaCl, 600 µl Triton-X 100 (final 1%), 600 µl 1M DTT (final = 10 mM).

The 100 ml beaker containing the bacterial lysate was placed into a disposable 500 ml polypropylene beaker containing ice, ensuring the smaller beaker was firmly held in place. The beakers and contents were placed on the lab jack inside a sonicator and the CL4 ultrasonic converter was placed in the 100 ml beaker containing the lysate. The ultrasonic converter was adjusted to its maximum depth without touching the beaker. The solution was sonicated for 90 seconds at 0.5 second alternations on power 4. Lysate was transferred to three 25 x 89 mm Beckman tubes and centrifuged for 10 minutes at maximum speed at 4°C in a Beckman CS-15R centrifuge with FO 630 rotor. The supernatant was discarded and the pellet resuspended using a glass rod in 1-2 ml of wash buffer (100 ml 1M Tris pH 8.0, 40 ml 5M NaCl, 4 ml 5M EDTA, 20 ml 10% Na Azide and 0.5% v/v Triton-X to a final volume of 2L) then topped up to 15 ml with wash buffer. The solution was sonicated on ice for 90 seconds as above before centrifugation as above. The supernatant was discarded and the wash step was repeated 3x until the pellet was white and the supernatant clear. Pellets were then resuspended in wash buffer (as above but without Triton-X) followed by centrifugation as above. After discarding the supernatant, 200-1000 µl distilled H_2O was added to form a white paste, which was then resuspended in 10 ml of 8M urea solution (1.25 ml MES (2-N-morpholino-ethane-sulphonic acid), 24 g urea, 1 ml EDTA made up to 50 ml with distilled H_2O).

The 10 ml of inclusion bodies was centrifuged for 10 minutes as above before transferring the supernatant to a 15 ml polypropylene tube (saving a small aliquot for SDS-PAGE analysis and determination of protein concentration).

Measurement of protein concentration

Protein concentration in solution was measured using the Micro BCA™ assay kit (Pierce, UK). The principle of this assay is based on protein quantification by the Lowry methods. The peptide bond reduces Cu^{+2} in alkaline solution to produce Cu^{+1} that chelates two molecules of bicinchoninic acid (BCA). A purple-coloured reaction was produced that strongly absorbs at 562 nm with intensity linear and proportional to the protein concentration of the sample, determined by comparison with a protein standard curve produced with proteins of known concentration. Bovine serum albumin (BSA) is included in the kit as a protein standard at a concentration of 2 mg/ml and serial dilutions were prepared ranging from 0-32 μg/ml.

2.2.6 Generation of pCMV plasmid constructs for protein expression in 293T mammalian cells

Amplification of the MICA gene from cDNA

PCR primers for amplification of MICA exons 1-4 from cDNA were redesigned according to previously published work (Zou *et al*., 2006a) to incorporate the correct restriction sites for ligation into pBK-CMV vector. The primers included sequence of the restriction site Xho1 on the 5' primer and the 3' primer included sequence for restriction site Kpn1, and a DNA tag encoding the BirA recognition sequence for enzymatic biotinylation. A six-His sequence for protein purification utilising the affinity of histidine with nickel was also included. For mammalian cell protein expression, the leader peptide sequence (exon 1) is required therefore the 5' primer was designed to anneal to the first 18 bases of exon 1 and the 3' primer annealed to the last 21 nucleotides of exon 4.

XhoI

5' forward amplification primer: 5'ACT ATC TAC CTC GAG GCC ATG GGG CTG GGC CCG GTC 3'

3' reverse amplification primer: 5' AGC ACT CAC CCT GTG CCC TCT GGA TCC GGT GGC *GGT CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAA TGG CAC GAA* **CAC CAC CAC CAC CAC CAC** TAG GGT ACC CAT GCG CAT 3'

KpnI

BirA biotinylation enzyme recognition sequence is in italics; six-His tag for up-stream purification is indicated by bold font and restriction enzyme sequences are underlined.

Purification of PCR amplified product and ligation into pGEM-T Easy vector

The resulting fragments were analysed by electrophoresis, cut from the gel and purified as described above. The Taq polymerase used for amplification of the MICA gene did not have proofreading capability therefore there was a high chance that amplicons contained nucleotide mis-incorporations. This first round of cloning enabled screening of multiple clones to find one with the correct sequence of the MICA gene, which was checked by DNA sequencing using MICA internal primers described above. The following procedures were carried out using pGEM®-T Easy vector cloning kit II (Promega Corp., Madison, WI, USA).

The ligation reactions were set up as outlined in Table 2.2.5 and volumes and ratios of DNA and vector were determined by empirical optimisation experiments.

Table 2.2.5: DNA Ligation reaction for T4 DNA ligase and pGEM®-T Easy Vector

Reagent	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	2 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Sterile water to final volume of	**10 µl**	**10 µl**	**10 µl**

The reactions were mixed by pipetting and incubated at room temperature for one hour. Alternatively, to obtain the maximum number of transformants, the incubation was carried out at 4°C overnight.

Transformation of JM109 competent cells using pGEM®-T Easy vector ligation reactions

JM109 high efficiency competent cells were used for transformation reactions. As previous experience with this cloning system showed most colonies contained a DNA insert,

blue/white screening was not necessary and was omitted from the protocol. LB plates were prepared containing 100 μg/ml of ampicillin and two for each ligation reaction prepared, plus two plates for determining the transformation efficiency. LB plates were equilibrated at room temperature before use. Tubes containing the ligation reaction were centrifuged, briefly, to collect the contents in the bottom of the tube. Two microlitres of each ligation reaction was placed in a sterile 1.5 ml microcentrifuge tube on ice. Another tube was set up on ice containing 0.1 ng of uncut plasmid to determine transformation efficiency of the competent cells. Tube(s) of frozen JM109 competent cells were removed from storage and thawed on ice for five minutes and the contents mixed by gently flicking the tube. A volume of 50 μl of JM109 cells was carefully added to each tube prepared above, mixed by gentle flicking and incubated on ice for 20 minutes. To introduce DNA into the competent cells, heat-shocking was carried out at 42°C for 45 seconds before returning the transformation reactions to ice for a further two minutes. At room temperature, 950 μl of SOC medium was added to each transformation, which was then placed in a shaking incubator set at 37°C (150 rpm) for 1.5 hours. Using the two LB plates prepared for each transformation, one was spread with 50 μl and the other 100 μl of the transformation reaction mixture. Plates were placed in an incubator at 37°C for 16-24 hours. When distinct colonies of about 1 mm diameter were present, colonies were picked from the plate and transferred to a 15 ml Falcon tube, and 2 ml of LB medium containing 100 μg/ml was added before loosely capping the tubes and placing in a shaking incubator (220 rpm) at 37°C for 18 hours, or overnight. Plasmid DNA was extracted from the cultures as described above and sequenced to confirm the presence of and integrity of the MICA insert sequence. Plasmid DNA extractions confirmed as having correct inserts were set aside for future work and all others were discarded.

Sub-cloning MICA DNA into pCMV mammalian expression vector

DNA endonuclease restriction enzyme digestion was carried out using Xho1 and Kpn1 enzymes to release the insert from the pGEM®-T Easy vector, which was isolated and purified by gel electrophoresis as described. The pCMV vector was cut with the same restriction enzymes to prepare it for ligation with the MICA DNA insert. Ligation and transformation was carried out exactly as previously described, except that LB plates and medium contained 50 μg/ml of kanamycin instead of ampicillin. Also, as nucleotide mis-incorporation was no longer a problem, only six colonies, per transformation, were picked for sequence analysis.

Endonuclease-free plasmid purification

Once correct pCMV/MICA DNA constructs were identified from plasmid minipreps, plasmid preparation was scaled up using the EndoFree Plasmid Maxi Kit (Qiagen, Crawley, UK) designed for purification of up to 500 μg endotoxin-free plasmid DNA. The procedure was performed following manufacturer's instruction and the DNE pellet was dissolved in 75 μl of endotoxin-free TE buffer. The yield of DNA was then determined by UV spectrophotometry. The purified plasmid DNA was then ready to be used for transfection of 293T cells as detailed in section 2.5.3.

2.2.7 Generation of pIEx-4 plasmid constructs for MICA protein expression in Sf9 insect cell line

MICA amplicons were generated with restriction sites for XhoI and KpnI as with 293T HEK cells, except that the restriction sites were reversed for ligation into pIEx-4 and were re-ordered as follows:

KpnI

5' forward amplification primer: 5'ACT ATC TAC GGT ACC GCC ATG GGG CTG GGC CCG GTC 3'

3' reverse amplification primer: 5' AGC ACT CAC CCT GTG CCC TCT GGA TCC GGT GGC *GGT CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAA TGG CAC GAA* **CAC CAC CAC CAC CAC CAC** TAG CTC GAG CAT GCG CAT 3'

XhoI

The problems of amplification of MICB and MICA pseudogenes are associated with PCR amplification from genomic DNA and were avoided by using a one in ten dilution of purified MICA DNA fragments used to generate pBK-CMV constructs. Purified, amplified MICA DNA and pIEx-4 were digested with Kpn1 and Xho1 restriction enzymes and ligated using T4 DNA ligase as shown in Table 2.2.5. Sequencing was performed to check for successful ligation and correct nucleotide sequence before scaling-up for production of endonuclease-free plasmid DNA, ready for transfection into the cellular host, Sf9, as detailed in section 2.5.3.

2.3 Biochemistry

2.3.1 SDS-PAGE

SDS-PAGE allows the separation of proteins according to their relative size. Using reducing conditions and heat, proteins dissociate into negatively charged polypeptides that bind with SDS and migrate through the gel under an electric current. Polyacrylamide gels have two phases, a stacking gel (high porosity) which concentrates proteins on the surface of a resolving gel that separates proteins. The sample protein size is estimated by comparison with a protein size ladder of known molecular weight.

2.3.2 Setting up, Loading and Running Gels

The apparatus used for protein gel analysis was Bio-Rad Mini-Protean II (Bio-Rad Laboratories, Hercules, Ca, USA) with outer glass plate 10 and inner plate mini PII 10, 1mm spacers and combs. Care was taken to ensure equipment was as clean as possible to avoid possible protein contamination, before assembling the apparatus according to manufacturer's instructions. The gel was made up of two parts: the separating (resolving) gel (30% acrylamide, 1.5M Tris pH 8.8, 10% SDS) made up to 30 ml. 5 ml per gel was used and 50 µl 10% ammonium persulphate (APS) (Amersham Biosciences, Buckinghamshire, UK) and 5 µl tetramethylethylenediamine (TEMED) (Amersham Biosciences, Buckinghamshire, UK) was added immediately prior to casting the gel, as polymerisation begins as soon as these 2 reagents are mixed together. Once the gel was cast it was allowed to polymerise for 30 minutes. Next, the stacking gel was prepared (30% acrylamide, 1M Tris pH 8.0, 10% SDS). A total of 30 ml was prepared and 2 ml was used per gel with 20 µl APS/2 µl TEMED added prior to casting. The comb was inserted and the gel was allowed to polymerise for 30 minutes. The frozen samples from the time-course assay were boiled for 5 minutes to denature the protein, after adding an equal volume of 2X loading buffer (0.5 ml 1 M Tris.HCl pH6.8; 2 ml 10% SDS; 10 mg bromophenol blue; 1 ml 10% glycerol and 6.5 ml distilled H_2O. For reducing gels, 30 µl 1M DTT was added to 270 µl of loading buffer). A molecular weight size ladder (Bio-Rad pre-stained SDS-PAGE standard, low range) was added (5 µl) to the outside lanes. Ten microlitres of each time-course sample, for each clone was added to individual lanes, working quickly to avoid diffusion of sample between lanes. A 1X solution of running buffer (5X running buffer: 15.1 g Tris; 94 g glycine pH 8.3 pure grade and 50 ml 10% SDS made up to 1000 ml) was

added to the tank and electrophoresis was performed at 150V for 1.25 to 1.5 hours or until the dye front had reached the bottom of the gel. The gel was removed from the apparatus and transferred to a tray containing sufficient Coomassie stain to cover (1.25 g Coomassie brilliant blue, 50 ml glacial acetic acid, 225 ml methanol and 225 ml distilled H_2O). The tray, gel and stain were placed on an orbital shaker on a gentle speed for 30 minutes. The stain was poured away (and retained for future use) and the gel was covered with de-stain solution (100 ml glacial acetic acid, 450 ml Methanol, and 450 ml d.H_2O). The tray, gel and de-stain were placed on an orbital shaker on a gentle speed for 4 hours or until bands became visible.

This procedure was also performed for recombinant MICA molecules produced in 293T and Sf9 cells, except the protein collected from cell supernatants was concentrated using Centricon tubes with a 30 kD molecular weight cut-off (Millipore, MA, USA) as described by the manufacturer. An appropriate volume was then mixed with loading buffer and SDS-PAGE carried out as above.

2.3.3 Western Blot Analysis

Proteins were run on polyacrylamide gels, as described above, before being transferred to nitrocellulose membranes using the Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad, Ca, USA) containing transfer buffer (25 mM Tris base, 200 mM glycine, 20% v/v methanol) and applying a current of 200 mA for 2 hours at 4°C. Nitrocellulose membranes were blocked overnight in PBS containing 0.05% v/v Tween 20 and 5% BSA followed by rinsing with PBS containing 0.05% v/v Tween 20. The primary anti-human MICA/B monoclonal antibody stock concentration was 500 μg/ml in sterile PBS which was diluted 1/500 with PBS containing 0.05% v/v Tween 20 and 5% BSA and incubated with the nitrocellulose membranes for 2 hours at room temperature. Membranes were washed five times with PBS containing 0.05% v/v Tween 20 for 5 minutes using gentle agitation. The secondary antibody (peroxidase conjugated goat anti-mouse whole immunoglobulin) was diluted 1/1000 in PBS containing 0.05% v/v Tween 20 and 5% BSA and incubated with the membranes for 30 minutes at room temperature. Membranes were washed as above. The membranes were then treated with a final wash in substrate solution (Fast DAB with colour enhancer (Sigma, Steinhelm, Germany)) for 3 or 4 minutes or until the colour had developed sufficiently. Rinsing with distilled H_2O stopped the reaction. A permanent record of the membrane was made using gel imaging apparatus and software.

2.3.4 Sandwich ELISA for detection of human soluble MICA molecules

The ELISA assay is a widely used laboratory method for the detection of antibodies in serum and is described in Chapter 1, section 1.4.2. The method can be adapted to detect specific antigens or proteins in patient serum by first incubating the plates with a capture antibody specific for the antigen of interest. Following incubation with patient serum, bound antigen is detected with a specific antibody and is why this assay is known as the 'sandwich ELISA'. A commercially available kit was used for detection of soluble MICA in human sera or culture medium, called Human MICA DuoSet ELISA (Cat no. DY1300, R&D Systems, Minneapolis, USA) and was performed according to manufacturer's instructions.

2.3.5 Concentrating recombinant MICA proteins

Recombinant MICA proteins produced in 293T HEK and Sf9 insect cells were secreted into the culture medium, retained for concentrating MICA protein and detected as described above (section 2.3.4). Centricon® Plus-70 centrifugal filter devices with a molecular weight cut-off of 100 kDaltons were used for this procedure (Millipore, CA, USA). These devices were designed for single use but could be re-used several times if used for the same protein (MICA antigen) and could concentrate 70 ml of medium containing protein down to around 300 µl. Prior to concentration, dead cells and debris were removed from solution by centrifugation and filtration using 0.45-micron Minisart syringe filters (Sartorious, Goettingen, Germany). Samples were concentrated following manufacturer's instructions.

2.4 Serological Assays

2.4.1 Luminex Mixed Bead Antibody Detection Screening Assay

LABScreen® LSM12 Mixed Bead assay (LSM12BD, One Lambda, Ca., USA) utilises fluorescent micro-beads (up to 100 different beads) coated with purified HLA class I, class II and MICA antigens and pre-optimised reagents for their detection in human serum. Test serum is first incubated with LABScreen® beads. Any HLA or MICA antibodies present in the test serum bind the antigens, which are then labelled with PE-conjugated goat anti-human IgG (GAH-IgG-PE). The LABScan™ 100 flow analyser detects the fluorescent

emission of PE from each bead (that has bound antibody) and the unique fluorescence of each bead. The reaction pattern of the test serum is compared to the lot-specific worksheet defining the antigen array to assign HLA and/or MICA antibody specificity.

2.4.2 Luminex HLA-Class I and II antibody specificity identification

The specificities of HLA class I antibodies can be determined using LABScreen® LS1PRA micro-beads (LSP1B, One Lambda, Ca., USA). Specificities suggested by this assay can be confirmed using LABScreen® SAg LS1A01 (group 1 – LSP1AB01, group 2 – LSP1AB02, group 3 – LSP1AB03, One Lambda, Ca., USA). The specificities of HLA class II antibodies can be determined using LABScreen® LS2PRA micro-beads (LSP2B, One Lambda, Ca., USA). Specificities suggested by this assay can be confirmed using LABScreen® SAg LS2A01 (group 1 – LSP2AB01, One Lambda, Ca., USA).

2.4.3 Luminex MICA antibody specificity identification

Three independent SAg Luminex assays were used for the detection of MICA-specific antibodies in this analysis.

LABScreen® MICA Single Antigen beads (One Lambda)

The specificities of MICA antibodies detected with LABScreen® LSM12 were determined with the use of LABScreen® LSMICA001, MICA SAg beads (LSPMABD01, One Lambda, Ca., USA). A separate bead was used for the individual detection of antibodies directed against MICA*001, *002, *004, *007, *008, *009, *012, *017, *018 and *019 (also detects MICA*027 which is identical to MICA*008 in the extracellular domains).

Detection of MICA antibodies by Luminex developed by Prof. Peter Stastny (Dallas, USA)

Our sera, which reacted with the MICA specific LSM12 beads, was further tested by our collaborators based at the University of Texas South-Western Medical Center, Dallas, USA, headed by Professor Peter Stastny. Lumavidin® beads were conjugated with biotinylated recombinant MICA (rMICA) produced by expression in insect cellsas detailed (Zou *et al*., 2006b). MICA proteins encoded by MICA*001, *002, *004, *006, *007, *008, *009, *012, *017, *018 and *019 were used in this assay.

Lifecodes LSA™ -MIC Single Antigen beads (Gen-Probe)

The specificities of MICA antibodies detected with LABScreen® LSM12 were determined with the use of Lifecodes LSA™ -MIC SAg beads (LSAMIC, Gen-Probe). A separate bead was used for the individual detection of antibodies directed against 28 MICA antigens, including MICA*001, *002, *004, *006, *007, *008, *009, *012, *017, *018 and *019 (also detects MICA*027 which is identical to MICA*008 in the extracellular domains).

2.4.4 Procedure for antibody detection using Luminex

Specimen collection and preparation

Serum samples were extracted from clotted blood samples by centrifugation at 3000 rpm for 10 minutes and aliquots stored at -70^{0}C. Positions A1 and A2 of a 96-well plate were assigned to negative and positive control sera. Serum samples to be tested were thawed and mixed thoroughly before transferring 100 µl into 0.45 µm filter plates (Cat # MSHVN4510, Millipore, USA). A receiving 96 well microplate was placed under the filter plate and centrifuged at 1300-1500g for 5 minutes. The top filter plate was removed and the receiving plate was re-centrifuged at 4000g for 5 minutes to precipitate any impurities.

General Test procedure

Filters to be used in the assay were pre-wetted and the beads were mixed thoroughly by gently vortexing and pipetting up and down several times. 2.5 µl was placed into filter wells, followed by addition of 10 µl negative control, positive control or test serum. Plates were covered with a foil seal, vortexed to mix and incubated for 30 minutes at room temperature on a plate shaker (250rpm). During the incubation, the GAH-IgG-PE was prepared by diluting 1 µl of 100X stock solution with 99 µl of wash buffer per sample. After incubation, 250 µl of 1x wash buffer was added to each well used to wash the beads. Wash buffer was removed with a vacuum manifold. This was repeated for an additional 4 times.

100 µl of GAH-IgG-PE was added to each well and the pate was covered with a foil seal and vortexed briefly. The tray was placed on a plate shaker set at position 3 and incubated for 30 minutes at room temperature. The plates were washed with buffer 5 times as above and the beads were resuspended in 80 µl of 1X sheath fluid, sealed and placed on plate shaker for 10 minutes. The samples were then processed in the Luminex™ analyser or

stored for up to 24 hours in the dark at 4°C. The data was saved automatically in the data collection computer.

Importing templates for data acquisition in the Luminex™ fluoroanalyser

The template for new batches of beads (determined by Lot No.) was either obtained from the CD provided by the supplier or by downloading from the following URL: http://www.download.onelamda.com/pub/software_update/. The correct template for the new lot of beads (eg. LS1PRA Lot 005 V1.idt) was saved to disc before copying the saved file to the computer.

Amendments to procedure for Gen-Probe MICA antibody Luminex™

Sample filter plates were pre-wetted with distilled water instead of wash buffer. To each of the assigned wells, including negative and positive controls, 20 µl of bead mix was added, followed by 5 µl of patient's serum. Plates were then incubated as described above before washing three times with wash buffer (100 µl wash buffer for the first wash, then 250 µl).

The GAH-IgG-PE (included with the kit and LOT –specific) was diluted by adding 22.5 µl of buffer to 2.5 µl of GAH-IgG-PE concentrate per sample. Beads were resuspended in 25 µl of diluted GAH-IgG-PE, the plates were covered with a foil plate sealer and placed and the plate shaker, set at position 3, for 30 minutes. Prior to data acquisition, a further 65 µl of wash buffer was added to the samples to give a final reading volume of 90 µl.

2.5 Cell Culture

2.5.1 General cell culture procedures

All cell culture techniques were carried out in a laminar-flow tissue-culture cabinet using aseptic technique and sterile lab ware and consumables.

Culture media

Media used for general cell culture was RPMI 1640 medium with L-glutamine supplemented with 10% heat-inactivated (55°C for 1 hour) foetal bovine serum (FBS) and 1% penicillin/streptomycin solution - 1U/ml penicillin and 1 µg/ml streptomycin (all from BioWhittaker, Wokingham, UK). This is referred to as 'basic media'.

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood supplemented with 1% heparin (Monoparin 1000 U/ml, CP Pharmaceuticals, UK) by density gradient centrifugation. Whole blood was layered onto an equal volume of Lympholyte®-H ficoll separation medium (Cedarlane Laboratories, Canada), taking care not to disturb the layer at the interface by slow pipetting. The tubes were centrifuged at 2400 rpm for 30 minutes without braking to ensure no disruption to the layered lymphocytes. The lymphocyte layer was collected using a Pasteur pipette, transferred to a 50 ml Falcon tube, washed with basic media and centrifuged again for 15 minutes at 1800 rpm. The wash was repeated to ensure all of the ficoll medium was removed and cells were resuspended in 10 ml of basic media for enumeration.

Counting cells and checking viability

Lymphocytes were counted using the Trypan Blue dye exclusion method. An aliquot of 10 µl of the cell suspension was mixed with an equal volume of 0.4% Trypan Blue solution (Sigma, UK) and transferred to a haemocytometer counting chamber with a depth of 0.1 mm. Cells were visualised and counted, excluding dead cells dyed blue, using a phase contrast Leica BM LB microscope (Meyer Instruments).

Cryopreservation of cells

Cells requiring storage for later use were cryopreserved in cooled freezing media (heat inactivated FBS and 10% dimethyl sulphoxide (DMSO), BDH, UK). Cells were aliquoted into cryotubes (Nunc, Denmark) at a concentration of 5-10^6 cells/ml and immediately transferred to a polystyrene box and placed in a -80°C freezer for 24 hours (cooling rate is 1°C per minute) before transferring to liquid nitrogen for long-term storage.

2.5.2 Cell lines used to obtain MICA cDNA

The use of commercial cell lines to locate MICA cDNA

Cell line Hela (ECACC, Wiltshire, UK) is known to express MICA*008. Cell line HCT116 (ECACC) is known to express MICA*009:02. Cells were despatched in 1 ml cryotubes containing FCS + 10% DMSO. To resuscitate the cells, the vials were left at room temperature for 1 minute then transferred to a water bath at 37°C, for 1-2 minutes until fully thawed. For Hela cells, the culture medium was Eagles Minimal Essential Medium (Gibco, Invitrogen, The Netherlands) + 2 mM Glutamine (Gibco, Invitrogen, The Netherlands) +

1% non-essential amino acids (Gibco, Invitrogen, The Netherlands) + 10% HI-FCS. For HCT116 cells, the culture medium was McCoy's 5a (Gibco, Invitrogen, The Netherlands) + 2 mM Glutamine + 10% HI-FCS. Cells were slowly pipetted into flasks containing 25 ml of the appropriate pre-warmed medium and incubated at 37°C with 5% CO_2. Both cell types are adherent and half media changes were made 2-3 times per week until confluence was around 80%. The culture medium was decanted and the cell monolayer washed with PBS twice. 2 ml of trypsin/EDTA solution (Gibco, Invitrogen, The Netherlands) was added to the flask, ensuring all cells were covered. The excess trypsin/EDTA solution was decanted. The cells were incubated at 37°C for 2-10 minutes or until cells had become detached. The cells were washed, counted and re-suspended in pre-warmed fresh medium at a density of 1-3 x 10000 cells per cm^2. For freezing of stocks and use in RNA extraction, cells were harvested at 80-90% confluence. Freezing was carried out as for B-LCLs except that only 2-4 x 10^6 cells were frozen per cryovial.

The Use other Cell Lines to Locate MICA cDNA

A cell line expressing MICA*002 and/or *004 was sought to obtain cDNA for two other MICA antigens of high frequency. The European Searchable Tumour Database (ESTDAB: http://www.ebi.ac.uk/cgi-bin/ipd/estdab) and Cell Bank provide a service enabling investigators to search online for HLA typed, immunologically characterised tumour cells as part of the European Commission Fifth Framework Infrastructures Program. With the aid of previous Linkage Disequilibrium (LD) studies of MICA with HLA-B (Gao *et al.*, 2006; Petersdorf *et al*., 1999), a cell was searched for that expressed HLA-B*3501 and/or B*4403 which are in strong LD with MICA*004 and *002 respectively, and several cells were selected to screen for MICA types. A cell line called ESTDAB-172 (or alternatively Ma-Mel-39a) was found and the HLA type was listed on the database as HLA-B*35:01/02/03/04/06, *4102. To confirm whether the cell line also encodes sequence for MICA*002 and *004, MICA-SBT was carried out using genomic DNA as described. That result showed that this cell line was positive for MICA*002 and *004 and FACS characterisation data provided on the ESTDAB database showed this cell did not express HLA but had good cell surface MICA expression.

2.5.3 Cell lines used to produce recombinant MICA

E. coli strain AVB101

Strain AVB101, an *E. coli* B strain (*hsdR, lon11, sulA1*), contains pBirAcm, an IPTG inducible plasmid containing the BirA gene engineered into pACYC184. It is compatible

with most cloning vectors and is maintained with chloramphenicol (10 μg/ml). This strain is recommended for protein expression because of its robust growth and the absence of the OmpT and Lon proteases. Avidity, LLC (Colorado, USA) provides the strain in three forms - glycerol stock (AVB101), chemically competent cells (CVB101), and electrocompetent cells (EVB101). Glycerol stocks were obtained and competent cells were produced with the method described in section 2.2.5.

Human embryonic kidney (HEK) 293T cells

The 293T HEK cell line was a kind gift from Rob Anderson (formerly of Haematology Dept., The Royal Free Hampstead, UK). A frozen vial of $5x10^6$ 293T cells was placed in a water bath at 37°C until it began to thaw and then resuspended in 10 ml of basic media. The tube and contents were centrifuged at 1800rpm for three minutes and resuspended in 10 ml of basic media before centrifuging again to remove traces of freezing mix. A total of $1.2x10^6$ cells were added to 5 ml of CD 293 medium (Invitrogen, Paisley, UK) supplemented with 10% FCS and 1% penicillin/streptomycin in $25cm^2$ vented culture flasks (Sarstedt, NC, USA) and placed in a 37°C incubator with 5% CO_2. HEK 293T cells grow as an adherent monolayer and were grown to a density of 40-80% confluence before splitting into fresh flasks (24-48 hours). After 2-3 passages cells were healthy, fast growing and ready for transfection. Cells that have been passaged for an extended number of times (>50) change their growth behaviour, morphology and transfectability therefore this was avoided.

***Spodoptera frugiperda*-derived insect cells (Sf9)**

After thawing, Sf9 insect cells (Novagen, USA) can be grown as a semi-adherent monolayer in tissue culture flasks at 28°C with no CO_2. The vial of Sf9 cells was revived by placing in a water bath at 28°C for 2 minutes with gentle swirling. The cells were slowly pipetted into a 50 ml Falcon tube and 10 ml of pre-warmed 28°C BacVector® Insect cell Medium (Novagen, USA) without serum or antibiotics was added drop-wise. The solution was gently pipetted 3-5 times and transferred to a $75cm^2$ vented culture flask and gently rocked to disperse the cells before placing in a 28°C incubator for 30-60 minutes (without CO_2). After this time, cells were adherent and medium could be removed with a pipette and replaced with 10 ml fresh pre-warmed BacVector® medium. Incubation was continued until cells were 85-95% confluent, checking viability and confluence every other day using a microscope. Sf9 cells required several passages before becoming healthy and growing exponentially. Healthy Sf9 cells appeared rounded with distinct cell boundaries

and unhealthy cells appeared granular. Sf9 cells were not grown beyond confluency as they detach and float in the medium, although they are still viable and able to divide. Cultures were split until cell numbers doubled every 48 hours before proceeding to transfection.

2.5.4 Transfection procedures

Transient transfection of 293T cells

Optimum conditions for transfection were determined empirically and are described more fully in Chapter 3. The day before transfection, 2.4 x10^6 293T cells from a healthy culture were seeded in a 175cm^2 vented culture flask containing 20 ml of CD 293 medium supplemented with FBS and antibiotics as described. Cells were grown in a 37°C incubator with 5% CO_2 and were about 60% confluent on the day of transfection. A total of 8 μg of endotoxin-free plasmid DNA containing the relevant MICA allele gene was diluted in RPMI 1640 containing no FCS or antibiotics (decreases transfection efficiency) to a final volume of 300 μl. To this, 80 μl of Polyfect® (Qiagen, Crawley, UK) transfection reagent was added and the tube contents were mixed by vortexing for 10 seconds and incubated at room temperature for 10 minutes to allow complex formation. During this time, the medium was aspirated and the cells washed with 10 ml of 1X polybuffered saline (PBS) before adding 17.5 ml of fresh medium (with supplements). A volume of 2 ml of CD 293 medium (supplemented) was added to the transfection complexes, mixed by pipetting up and down twice before transferring the entire volume to the cells in a 175cm^2 vented culture flask followed by gentle rocking to disperse the complexes. The flask was placed in a 37°C incubator with 5% CO_2 for 24 hours to allow transfection to take place. After 24 hours, the medium was collected and stored at -80°C ensuring an aliquot of 500 μl was also collected to test for rMICA protein concentration. The medium was replaced with 20 ml of fresh CD 293 medium with supplements. This procedure was repeated every 72 hours for up to two weeks, or until cell death was noticeable.

Transient transfection of Sf9 cells

Optimum conditions for transfection were determined and are described more fully in Chapter 3. Sf9 cells were passaged until rapidly dividing (doubling every 48 hours) prior to transfection. On the day of transfection, 1x10^7 Sf9 cells were seeded into a 125 ml Erlenmeyer flask containing 8 ml SF900 II serum-free insect cell medium (Invitrogen, Paisley, UK). In a sterile tube, 20 μg of endotoxin-free pIEx-4 plasmid containing the MICA allele of interest was diluted in 1 ml of insect cell medium with no supplements. In a

separate tube, 100 µl of Insect Genejuice transfection Reagent (Novagen, USA) was diluted with 1 ml of insect cell medium with no supplements. The DNA solution was added dropwise to the Genejuice solution and mixed gently with vortexing to prevent precipitation, before incubating for 15 minutes. The transfection mixture was then added to the cells and the flask placed in an orbital shaker set at 150 rpm and incubated for 48 hours at 28°C.

2.6 Data storage and Statistical analysis

A database of renal graft recipient and donor demographic, clinical and genetic data was established by myself and maintained on a password-protected file using Filemaker Pro Software (Filemaker Inc., Santa Clara, CA, USA). Access to this database was restricted to my supervisors and myself.

The prevalence of various risk factors, clinical characteristics and immunological features and the presence or absence of MICA antibodies was compared using Pearson's chi-square test or Fisher's exact test. For odds ratios (OR), 95% confidence intervals (CI) were used. A two-sided P value of less than 0.05 was considered significant. Bonferroni's correction (P_c) for multiple comparisons was applied for analysis of MICA amino acid mismatches between patients and donors. P_c values less than or equal to 0.05 were considered highly significant. Data examining age differences, eGFR and follow-up in study cohorts were expressed as mean ± standard deviation and differences between means were assessed by Student's t-test for independent variables. Serum creatinine values produced a skewed frequency distribution therefore the non-parametric Mann-Whitney U test was used for univariate analysis. Binary logistic and linear regression was used for multivariate analysis. For time-dependent statistics, overall survival (OS) was assessed using the Kaplan Meier method using the log rank statistic and Cox regression was used for multivariate analysis. In binary logistic, linear and Cox regression models, covariates were included where $P \leq 0.100$. SPSS 17.0 was used for analysis (SPSS Inc., Chicago, IL).

MICA and HLA-B haplotype and allele frequencies were generated by Hazael Maldonado-Torres, a former PhD student of the Anthony Nolan Research Institute, using a statistical program he designed himself, called Cactus. Statistical analysis was performed by implementing the Expectation-Maximisation (EM) algorithm (Dempster *et al.*, 1977) for calculating an estimate of haplotype frequencies (Excoffier and Slatkin, 1995). The

population samples represented unrelated individuals requiring calculation of haplotypes based on gene-counting and the EM algorithm, as opposed to family studies. The analysis calculated allele and haplotype frequencies from genotypic data that was demonstrated to comply with the principle of Hardy-Weinberg equilibrium. The reliability of the Cactus program has been verified using a number of other statistical packages, including Arlequin (Excoffier *et al.*, 2005).

CHAPTER 3

Development of techniques for MICA allele typing and MICA antibody detection

3.1 Introduction and aims

In 2005 when this research was begun, commercial methods for both MICA allele typing and MICA antibody detection were unavailable, requiring development of 'in-house' techniques. The method chosen for MICA allele typing of patients and their donors was SBT as this can potentially offer the highest resolution of MICA types and, compared to PCR-SSOP or SSP, did not require large numbers of primers or probes or large volumes of DNA. My previous experience in the field of HLA typing involved establishing SBT methods for HLA class I allele identification, therefore this approach for MICA allele typing seemed the most straightforward to set up. A disadvantage of using SBT over other methods such as PCR-SSOP is that results analysis can create a bottleneck in the throughput, particularly when SBT of three different exons is required, as is the case for MICA typing. In addition, many types can only be unambiguously resolved by characterisation of the number of GCT repeats in exon 5 and this cannot normally be achieved with heterozygous sequencing as differing lengths in exon 5 render the sequences unreadable. This can be overcome by molecular sizing of exon 5 fragments or alternatively identifying the number of GCT repeats carried by each allele by comparison with sequence patterns obtained with all possible combinations of GCT triplet polymorphisms, and both methods are explored in this chapter.

Obtaining a method to detect MICA antibodies was more problematic and required the use of methodologies unfamiliar to me. We decided to attempt to produce an ELISA method using rMICA molecules produced from *Eschericia coli (E. coli)* bacterial cell hosts, using a plasmid DNA vector. Other systems for native rMICA production were also explored, including a mammalian cell host, HEK 293T and an insect cell host *Spodoptera frugiperda* (Sf9), using liposome-based transient transfection protocols.

3.2 Development of MICA sequence based typing

The final, developed MICA-SBT protocol is described in Chapter 2, section 2.2.2.

3.2.1 Amplification of exons 2-5 of the MICA gene

Previous work by other researchers was available to devise a strategy for amplification of the MICA gene. The amplification primers used for targeting exons 2-5 of the MICA gene (Fodil *et al.*, 1996) were also used in the study by Katsuyama (Katsuyama *et al*., 1999) and Zwirner and colleagues (Zwirner *et al*., 2000) for their MICA-SBT strategies. These generic primers are situated in introns 1 and 5 and amplify a 2201-bp MICA gene fragment encompassing exons 2, 3 and 4 (and also the transmembrane domain encoded by exon 5). The 5' (forward) primer was redesigned by Zwirner *et al* to include a polymorphism (C – C/T (Y)) that was previously unknown and presumably was included to avoid non-amplification of one of the alleles (known a 'allele dropout'), although they did not mention the reason in their paper. The MICA amplification primers are given in Table 3.2.1.

Table 3.2.1: MICA PCR amplification primers targeting exons 2-5 of the MICA gene

Primer Name	Primer Sequence (5' – 3')
5MICA[a]	CGT TCT TGT CCC TTT GCC CGT GTG GC
MICA 6823[b]	CGT TCT TGT CCC TTT GCC YGT GTG GC
MICA 8999[a,b]	GAT GCT GCC CCA TTC CCT TCC CAA

[a](Fodil *et al*., 1996); [b](Zwirner *et al*., 2000)

As the redesigned 5' MICA primer reflects a previously unknown polymorphism (intron 1 sequences are generally unavailable for the MICA gene, therefore it is unclear which alleles may be affected), I decided to purchase this version rather than the original. The PCR amplification protocol is given in Chapter 2, section 2.2.2 and is based on my previous work for amplification of full-length HLA-B genes (3.2 Kb) using a total volume of 25 µl. The Taq polymerase used was TaKaRa® (Takara Bio Inc., Japan) and had proven to be reliable for amplification of larger products with better results than other similar products. Figure 3.2.1 shows the amplified region of the MICA gene and amplification results using 100 ng/ml BLCL DNA.

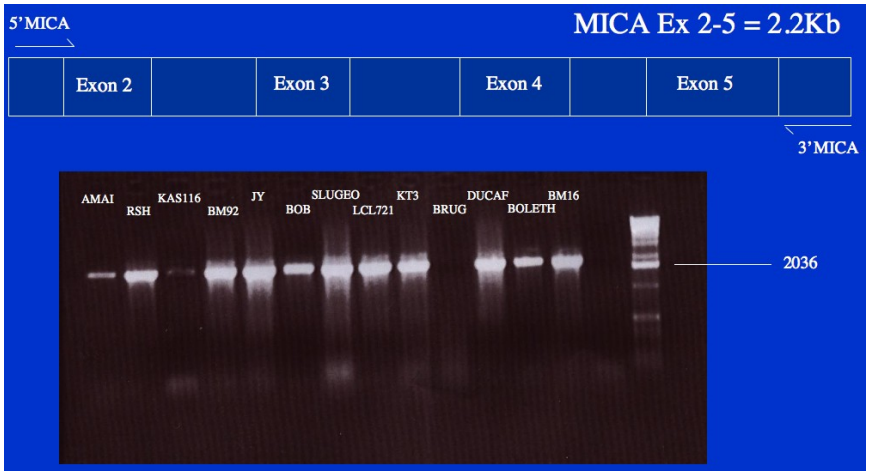


Figure 3.2.1: Amplification of the MICA gene. B-lymphoblastoid cell line DNA (100 ng/ml) was used to test amplification of exons 2-5 of the MICA gene.

The amplification of the MICA fragment was successful for most of the cell line DNA used with weaker amplification of cell line DNA AMAI and KAS116 and no amplification of BRUG. Usually an intense band of the correct size was produced with no other bands present. A different preparation of AMAI, KAS116 and BRUG DNA was used to repeat the amplification and was successful (not shown).

Following visualisation of 2.5 µl (one tenth of the PCR volume) of the amplified DNA on a 1% agarose gel, the reactions were purified to remove excess primer and dNTPs using GFX™ PCR DNA and gel band purification kit (GE Healthcare, UK) as described in Chapter 2, section 2.2.2. Purified DNA was eluted in 75 µl of distilled H_2O ready for cycle sequencing.

3.2.2 Selection of MICA sequencing primers

To ensure that MICA sequencing was carried out for both sense and antisense DNA strands, primers were selected to give bi-directional sequence of exons 2-4 of the MICA gene. The two studies giving comprehensive details of primers for cycle sequencing MICA DNA fragments were from Katsuyama and Zwirner (Katsuyama *et al*., 1999; Zwirner *et al*., 2000). The primer details and sequences from these two studies are given in Tables 3.2.2 and 3.2.3.

Table 3.2.2: MICA sequencing primers from Katsuyama study[a]

Primer Name	Sequence	Exon coverage
MICA51	ATT TCC TGC CCC AGG AAG GTT GG	Exon 2 forward
3AIn2/127	GTG CCG GCT CAC CTC CCC TGC T	Exon 2 reverse
5Ain/107	GTG AGG AAT GGG GTC AGT GGA A	Exon 3 forward
MICA3	CAA CTC TAG CAG AAT TGG AGG GAG	Exon 3 reverse
5An3/516	AAG AGA AAC AGC CCT GTT CCT CTC C	Exon 4 forward
3An4/34	TCC CTG CTG TCC CTA CCC TG	Exon 4 reverse

[a](Katsuyama *et al*., 1999)

Table 3.2.3: MICA sequencing primers from Zwirner study[a]

Primer Name	Sequence	Exon coverage
MICA6838	GCC YGT GTC CAT TTC CTG	Exon 2 forward
MICA7302	TGA GCC AGA TCC AGT GGG	Exon 2 reverse
MICA7403	CCC TGG GCT GAG TTC CCT C	Exon 3 forward
MICA7843	ATA GCA CAG GGA GGG TTT	Exon 3 reverse
MICA8263	CAG AGT GAG AAC AGT GAA	Exon 4 forward
MICA8705	AGG GAC TTG TTA TAC ACT	Exon 4 reverse

[a](Zwirner *et al*., 2000)

The MICA amplified cell line DNA, described in the previous section, was used to test effectiveness of the primers from each study. Cycle sequencing reactions were set up as described in Chapter 2, section 2.2.2. Briefly, 10 µl reaction volumes comprised 2 µl purified, PCR DNA template, 1 µl primer (1.6 pmol/µl) and 7 µl Big Dye Terminator™ V3.1 mixture (Applied Biosystems, CA, USA) (1 µl Big Dye reagent, 0.5 µl 5X sequencing buffer and 5.5 µl distilled H_2O) were placed in a thermal cycler. Conditions for cycle sequencing were: 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 minutes. Products were precipitated in 30 µl 100% ethanol and 2.5 µl 125 mM EDTA (BioWhittaker, UK) for 15 minutes at room temperature and recovered by centrifugation at 1800g for 15 minutes. Pellets were washed with 30 µl of 75% ethanol followed by centrifugation at 1800g for 30 minutes. Products were resuspended in 10 µl of HiDi Formamide (ABI, CA, USA) and sequenced for 50 minutes on a 3730xl ABI Genetic Analyser. Sequences of exons 2-4 were analysed for MICA allele assignment using Assign software (Conexio Genomics, Australia). The sequences obtained using these primers were, in general, very good, however Zwirner's primers usually gave better sequences or were more reliable. There was one problem area, in exon 2, where Katsuyama's exon 2 forward primer (MICA51) consistently failed and Zwirner's exon 2 forward primer (MICA6838) also failed. Following a search of the literature, I found another primer that appeared suitable as an exon 2 forward sequencing primer – MICA-1C

(Molinero *et al*., 2002b). Testing of this primer, which is situated in intron 1 and extending into exon 2, gave acceptable results although the first 50 bases of exon 2 were unusable as shown in Figure 3.2.2.

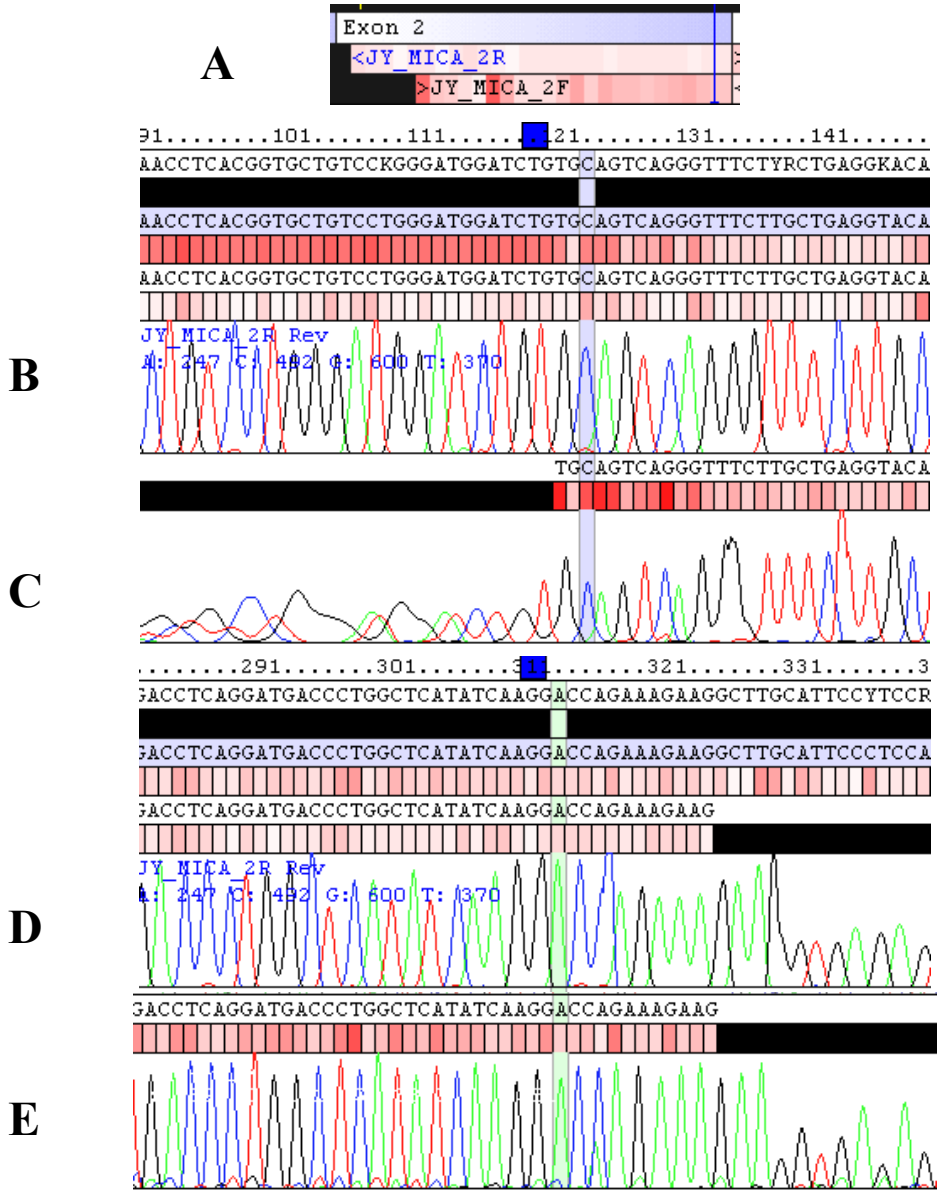


Figure 3.2.2: Exon 2 sequences with Zwirner MICA sequencing primers. A. Coverage of exon 2 – red shading indicates poor sequence. B. Beginning of exon 2 with exon 2 reverse primer (2R). C. Beginning of exon 2 with exon 2 forward primer (2F). D and E - Exon 2 forward and reverse sequences at the end of exon 2.

MICA sequences for exons 3 and 4 were very good with all the cell line DNA tested using Zwirner's primers. Although Katsuyama's primers produced good sequence that was only slightly noisier than with Zwirner's primers, two different primers failed with two of the

cell line DNAs and possibly indicates that these primers are not so robust. An example of exon 3 and 4 sequence obtained with Zwirner's primers using cell line JY is given in Figure 3.2.3 and is representative of all the cell line DNA tested.

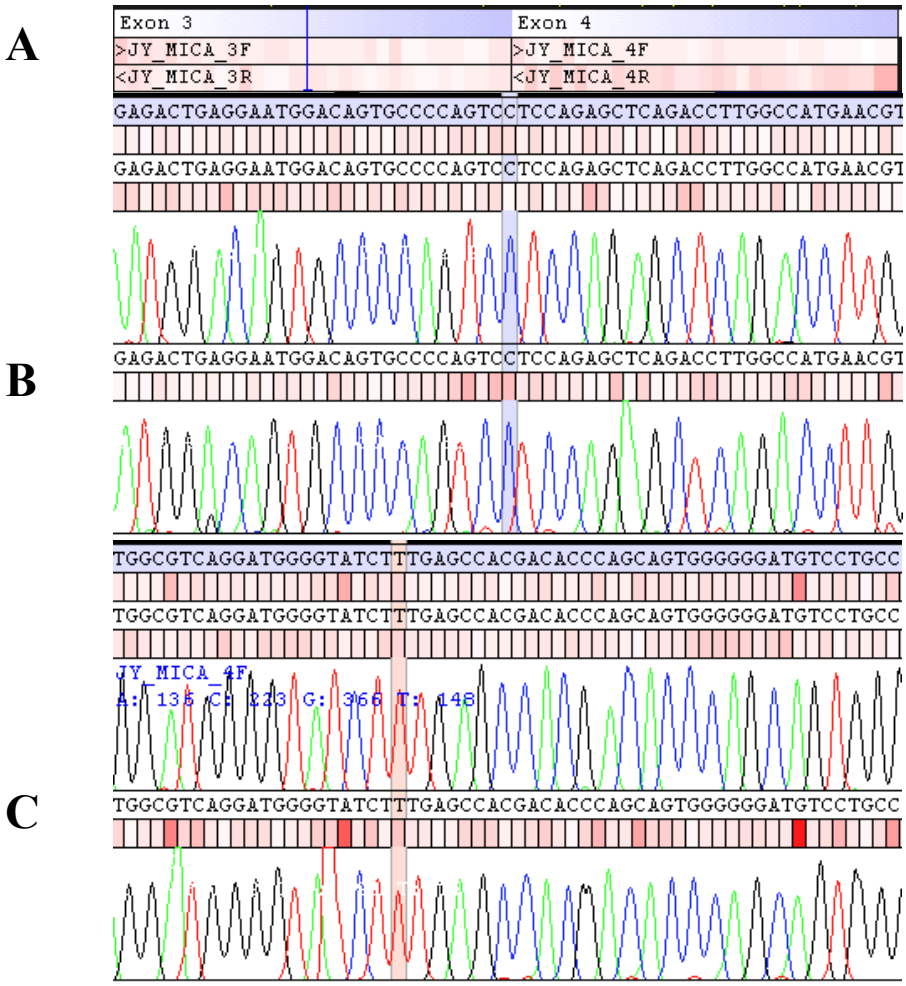


Figure 3.2.3: Exon 3 and 4 sequence obtained using Zwirner's primers and cell line JY DNA. A. Good coverage in both directions was achieved. B. Exon 3 sequence in both directions. C. Exon 4 sequences in both directions.

3.2.3 MICA allele typing results using exons 2-4

MICA typing results of homozygous and heterozygous cell line DNA, selected for alleles with high frequencies in various populations, were generated using Assign™ software and the latest allele libraries obtained from the IMGT/HLA database. The results are given in Table 3.2.4 and are compared to the official MICA typing for the cell obtained from the IMGT/HLA database.

Table 3.2.4: Cell line DNA MICA typing using exons 2-4 compared with IMGT/HLA entry

Cell Name	IMGT/HLA entry	SBT result (exons 2-4)
DUCAF	MICA*001	MICA*001
SLUGEO	MICA*002, *008	MICA*002:01, *008:01/008:04/027/048 or MICA*008:01/04/*027, *020/023/052 or MICA*020/*023, *027/*48
RSH	MICA*004	MICA*004
KAS116	MICA*006	MICA*006
BM92	MICA*007:01	MICA*007:01/*026
JY	MICA *008:01	MICA*008:01/04/*027/*048
LCL721	MICA*008, *009	MICA*008:01/04, 009:01/*049 or MICA*009:01, *027/*048 or MICA*027/*048, *049
BOLETH	MICA*010	MICA*010
KT3	MICA*012:01	MICA*012:01
BM16	MICA*018:01	MICA*018:01

It can be seen from the results that heterozygous MICA allele combinations give very ambiguous typing results. Although most of the homozygous cell lines gave unambiguous MICA allele types, cell line BM92 produced a MICA type of MICA*007:01 that could not be distinguished from MICA*026. The difference between these two alleles is solely based on the exon 5 GCT triplet number and MICA*008 and MICA*027 also only differ in this transmembrane region. These problems affect common and rare MICA alleles therefore identification of the GCT triplet number in exon 5 is crucial for MICA allele level typing.

3.2.4 MICA exon 5 sizing using Genescan

Many of the MICA typing strategies employed to detect polymorphisms associated with diseases and MICA allele frequencies in different populations mainly focussed on determining the number of GCT short tandem repeats in exon 5. As discussed in Chapter 1, GCT repeats encode four to ten alanine residues, referred to as A4, A5, A6, A7, A8, A9 and A10. Another group of alleles, mainly MICA*008 have an insertion of 'g' after the second of five GCT repeats resulting in a premature stop codon and is designated A5.1. As these polymorphisms determine the size of exon 5 DNA fragments, accurate sizing of PCR amplicons is a useful way to identify and discriminate MICA allelic combinations. One

such technique (Ota *et al*., 1997) utilises Genescan software (Applied Biosystems, CA, USA) on an automated sequencing platform, in our case it was an ABI 377 sequencer (Applied Biosystems). Details of the full method are given in Chapter 2 section 2.2.3 and involves PCR amplification of MICA exon 5 with one primer labelled with a fluorescent tag as shown in Figure 3.2.4. The amplicons are loaded onto a denaturing polyacrylamide gel alongside a fluorescent DNA size marker and the software calculates the size of the fragments relative to the size marker.

Figure 3.2.4: PCR amplification of exon 5 of the MICA gene using a 5' 6-Fam fluorescently labelled primer. The size of the products varies at around 200 base pairs when run on a 1% agarose gel.

The purified MICA exon 5 fragments were then run on the ABI 377 with Genescan software to accurately determine the number of nucleotides present in the MICA allele(s). Typical results are shown in Figure 3.2.5.

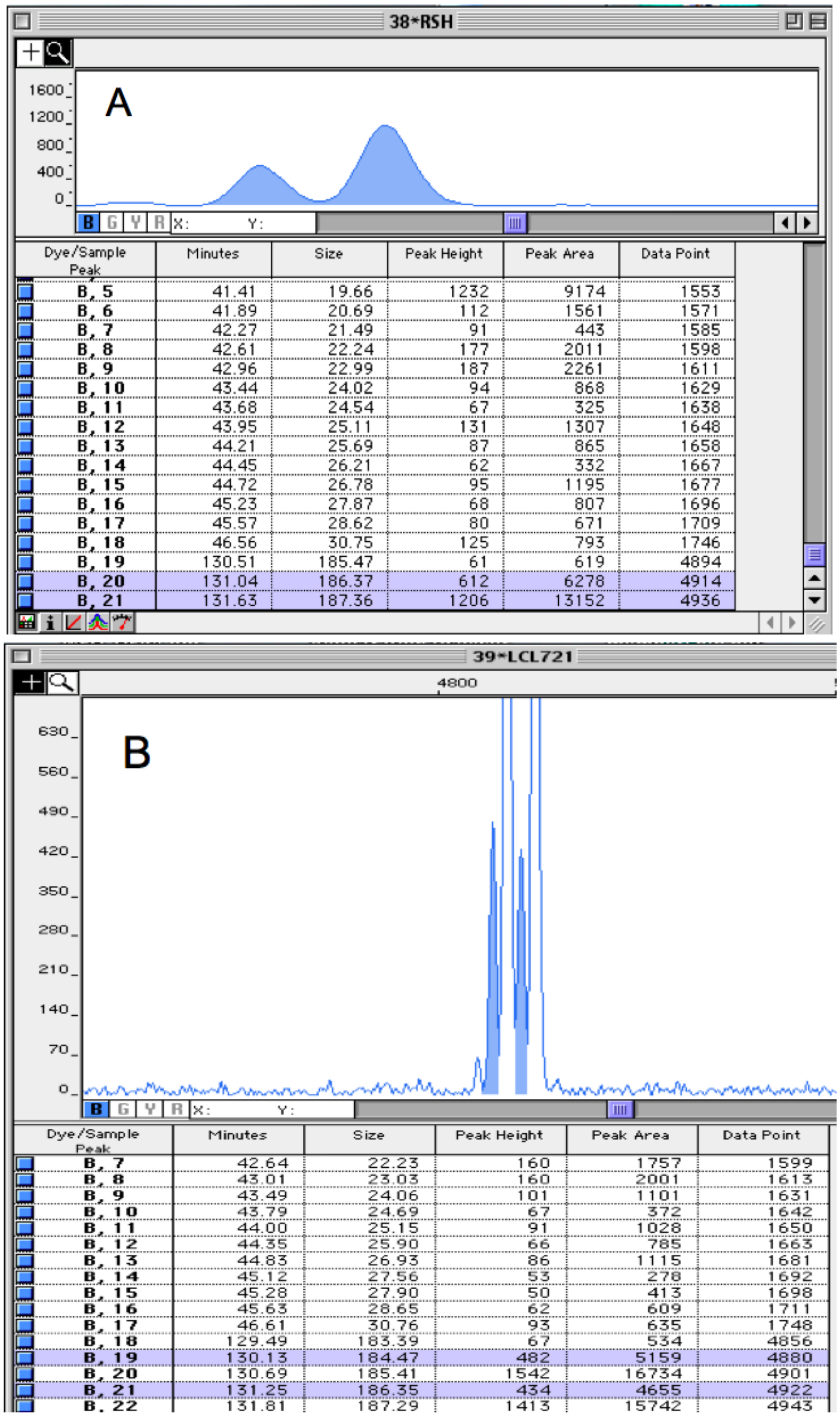


Figure 3.2.5: MICA exon 5 size results generated using Genescan software. A. Homozygous cell-line RSH typed by SBT as MICA*004 produced 2 peaks of 186 and 187 base pairs (bp). The 186 bp fragment corresponds to MICA-A6 and the 187 bp fragment has an additional non-template adenosine base generated by the PCR. A mixture of both types was present with all samples. B. Heterozygous cell line LCL721 generated two peaks specific for MICA-A5.1 (184bp) and MICA-A6 (186bp).

The Genescan assay was successful, although an artefact of the PCR reaction was a fragment equal to the length of the fragment +1 base pair formed by the addition of adenosine to the blunt ended PCR product. As the gel is denaturing, only the fragment with the fluorescent tag is detected hence only one additional nucleotide. The PCR artefact

formed the majority of the products as seen by the peak height in Figure 3.3.5, however a sufficient number of amplicons were the correct size and were easily detected. The results were combined with the SBT results and are shown in Table 3.2.5.

Table 3.2.5: MICA allele typing results of cell line DNA using both SBT and Genescan analysis

Cell Name	IMGT/HLA entry	SBT result (exons 2-4)	Exon 5 size (TM)
DUCAF	MICA*001	MICA*001	180 bp (A4)
SLUGEO	MICA*002, *008	MICA*002:01/*052, *008:01/04	184 bp (A5.1), 195 bp (A9)
RSH	MICA*004	MICA*004	186 bp (A6)
KAS116	MICA*006	MICA*006	186 bp (A6)
BM92	MICA*007:01	MICA*007:01	180 bp (A4)
JY	MICA *008:01	MICA*008:01/04	184 bp (A5.1)
LCL721	MICA*008, *009	MICA*008:01/04, 009:01/*049	184 bp (A5.1), 186 bp (A6)
BOLETH	MICA*010	MICA*010	183 bp (A5)
KT3	MICA*012:01	MICA*012:01	180 bp (A4)
BM16	MICA*018:01	MICA*018:01	180 bp (A4)

TM = transmembrane region

Ambiguity in allele assignment was reduced considerably for heterozygous samples using both SBT and Genescan sizing. MICA*008:01 and *008:04 cannot be distinguished from one another as they only differ in exon 1 and MICA*009:01 and *049 are also identical in exons 2-5, differing by one nucleotide in exon 6. MICA*002:01 and *052 are also identical in exons 2-4 and have the same number of GCT repeats. However, as exon 5 is included in the amplified MICA product used for SBT, scrutiny of exon 5 sequences can resolve this ambiguity.

3.2.5 Determining exon 5 GCT triplet number using sequence analysis

Although sizing of exon 5 MICA fragments could accurately determine the TM-STR present for each allele, it is not well suited to high-throughput MICA typing. Since I planned to MICA type several hundred patients and at least 200 donors, requiring at least six sequences each, the workload including exon 5 sizing would have been immense. However, in 2006 a paper was published describing a method of resolving exon 5 MICA

polymorphism by direct sequence analysis (Zou *et al*., 2006a). As exon 5 was already included in the PCR amplification strategy for the MICA gene, the addition of an extra primer to sequence exon 5 was the only modification required to employ the method of Zou *et al*. Conveniently, the unlabelled exon 5 amplification primer used for Genescan analysis was ideal as a reverse sequencing primer for exon 5. The principle of this method is that sequences of alleles with different lengths will overlap with heterozygous DNA samples, creating unique sequence patterns that can identify the various combinations of TM polymorphisms. The IUB coding system for heterozygous sequence peaks was used and the various patterns observed are reproduced in Table 3.2.6.

The exon 5 TM-STR polymorphisms of individual MICA alleles are shown in Table 3.2.7. Exon 5 sequences are unavailable for all MICA alleles rendering 25 alleles with unknown exon 5 polymorphisms, of which only 10 MICA sequences have been confirmed by a different laboratory or sequencing of a different cell. Most of these alleles were relatively rare in most populations and have polymorphisms in the extracellular domains defining them as a novel allele, and could be detected with regular SBT.

There are three further TM polymorphisms – MICA*050 (A7), MICA*055 (A8) and MICA *020 (A10), but these MICA alleles are rare and associated with ethnic diversity. MICA*050 has a unique polymorphism in exon 3, MICA*020 is mainly associated with American Indian populations and MICA*050 with East Asians, therefore I decided to investigate these for MICA exon 5 polymorphism only if required.

Table 3.2.6: Exon 5 sequences of TM polymorphisms and heterozygous combinations of MICA alleles with differing TM-STRs. Modified from Zou et al., 2009. Permission granted by Springer.

TM	MICA antisense nucleotide sequence
A4	AGC AGC AGC AGC AAC AGC AGA AAC ATG GAA TGT CT
A5	--- --- --- --- -G- -A- --C -GA -AC ATG GAA TG
A5.1	--- --- --- CAG C-G CAA CAG C-G -AA C-T G-A A-
A6	--- --- --- --- -G- --- -AC -G- -GA A-C ATG GA
A9	--- --- --- --- -G- --- --C -G- -GC A-C A-C AG
A4/A5	--- --- --- --- -R- -R- --M -RM -WS RWR KRW YK
A4/5.1	--- --- --- MRS M-S MRM MRR M-S -WR S-W K-W MT
A4/A6	--- --- --- --- -R- --- -RM -R- -KR R-M WKK SW
A4/A9	--- --- --- --- -R- --- --M -R- -KS R-M W-Y MK
A5/A5.1	--- --- --- MRS MRS MAM MRS MRR -AM MWK GRA WK
A5/A6	--- --- --- --- --- -R- -RC -GM -RM AWS RWR KR
A5/A9	--- --- --- --- --- -R- --C -GM -RC AWS RRM WG
A5.1/A6	--- --- --- MRS MRS MRM MAS MRS -RA M-Y RKR RW
A5.1/A9	--- --- --- MRS MRS MRM MRS MRS -RM M-Y R-M AK
A6/A9	--- --- --- --- -G- --- -RC -G- -GM A-C AKS RR

Notes: IUB codes - A+G = R; A+C = M; A+T = W; C+T = Y; C+G = S; G+T = K

Table 3.2.7: MICA TM-STR exon 5 polymorphisms associated with different MICA alleles

MICA Allele				
A4	**A5**	**A5.1**	**A6**	**A9**
001	010	008:01	004	002:01
007:01	016	008:02	006	015
007:03	019	008:04	009:01	017
012:01	027	023	009:02	041
018:01	033	028	011	046
018:02	048	053	026	052
029	054	058	030	
043	056		047	
045			049	
051				
061				

Notes: Other TM polymorphisms: MICA*050 (A7); MICA*055 (A8); MICA*020 (A10)

The sequences obtained using the exon 5 reverse sequencing primer were acceptable and the heterozygous patterns easily recognisable when compared to the predicted patterns. Typical results are shown in Figure 3.2.6 where four different patterns identify MICA-A4/A6 (A), MICA-A6/A9 (B), MICA-A5.1/A9 and MICA-A5.1/A6. Exon 5 sequences were checked when ambiguous allele combinations were present or to confirm homozygosity.

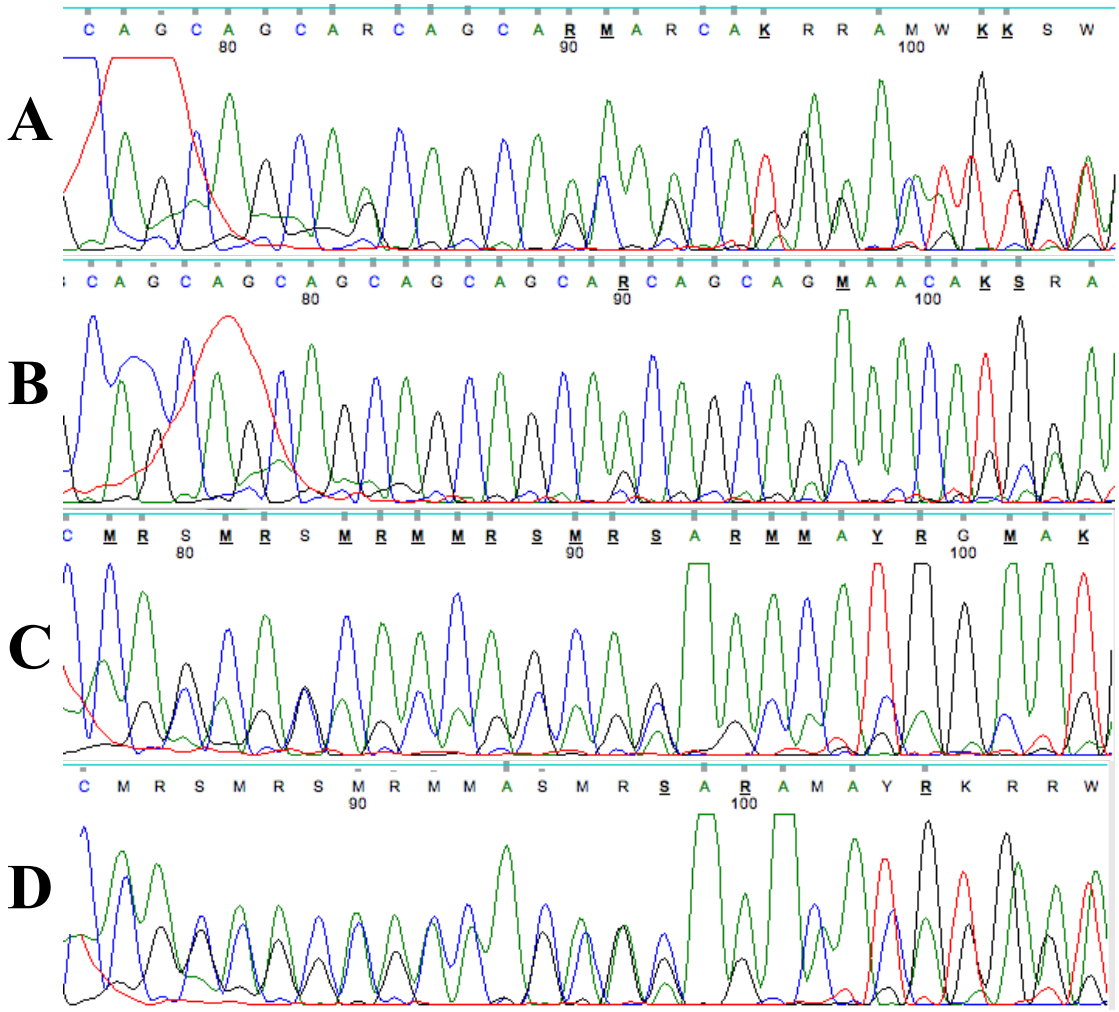


Figure 3.2.6: Heterozygous MICA sequence patterns obtained from sequencing exon 5. Comparisons with Table 3.2.6 reveal - A. MICA-A4/A6. B. MICA-A6/A9. C. MICA-A5.1/A9. D. MICA-A5.1/A6

3.2.6 MICA ambiguous allele combinations not resolved

The only MICA allele heterozygous combination that could not be resolved was MICA*009:01 and *049 which differ in exon 6, outside the region amplified. MICA*008:01 and 008:04 also could not be distinguished as they differ in exon 1.

3.2.7 MICA international DNA exchange scheme

To ensure high quality MICA allele typing and detect any problems that may be inherent in the system, I enrolled on the MICA International DNA Exchange program organised by Marie Lau and colleagues from University College of Los Angeles (UCLA, USA). This program began in May 2007 and involved MICA typing of four cell line DNAs three times a year. I have submitted results for each of the eleven exchanges up until December 2010. The participation in this program has increased from seven centres to 22 centres now currently enrolled. Most centres use the reverse SSO technique (One Lambda, CA, USA) however Peter Stastny's group and myself were the two original submitters using SBT, including exon 5 analysis and currently a further two groups use this method. My results were mostly concordant with the consensus and reports can be downloaded from http://www.hla.ucla.edu/cellDNA/Cell/summaryMica.htm.

Participation in the MICA DNA exchange was extremely useful for not only gaining confidence in the MICA typing results but also for highlighting problems that could not be foreseen. In particular a problem with MICA 'allele dropout', where one of the alleles was not amplified or amplified with a weak signal, is discussed in the next section.

3.2.8 Strategies for resolving MICA amplification 'allele dropout'

A problem of no or low amplification of MICA*001 was initially noticed from the results of the 3rd MICA exchange and confirmed again in the 5th exchange where I noticed that one of the alleles in one sample had a low signal. In both cases the allele affected was MICA*001. I discovered a publication that indicated some MICA primers used for amplification or sequencing, located in intron 1, may be problematic (Shao *et al*., 2004) and decided to investigate this further. However, the problems highlighted by these authors should not have affected my sequencing as the amplification primer was located before the problem area and the sequencing primer was located after as shown in Figure 3.2.7.

```
MICA Intron 1
Pattern 1: TCC CAC CCT CAC AGT TTT CTT TGT ATA TGA AAT CCT CGT TCT
Pattern 2: --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Pattern 3: --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                MICA6823: cgt tct

Pattern 1: TGT CCC TTT GCC CGT GTG CAT TTC CTG CCC C-- -AG GAA GGT
Pattern 2: --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Pattern 3: --- --- --- --- --- --- --- --- --- --- -CT C-- --- ---
           tgt ccc ttt gcc ygt gtg c (amp primer[1])

Pattern 1: TGG GAC AGC AGA CCT GTG TGT TAA ACA TCA ATG TGA AGT TAC
Pattern 2: --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Pattern 3: --- --- --- --- --- --- -A- --- --- --- --- --- --- ---

                                                        Intron 1|
                                                              gag
Pattern 1: TTC CAG GAA GAA GTT TCA CCT GTG ATT TCC TCT TCC CCA GAG
Pattern 2: --- --- --- --- --- --- --- A-- --- --- --- --- --- ---
Pattern 3: --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 MICA new amp primer MICA-Shao: ca cct rtg att tcc tct tcc cca gag[2]

MICA Exon 2
           ccc cac agt ctt cgy tat (modified exon 2 forward primer - MICA-1C[3])
Pattern 1: CCC CAC AGT CTT CGC TAT
Pattern 2: --- --- --- --- --T ---
Pattern 3: --- --- --- --- --T ---
```

Figure 3.2.7: MICA intron 1 region used for amplification of the MICA gene. [1]Amplification primer (Zwirner *et al*., 2000) located before a three-nucleotide insertion present in some MICA alleles. [2]Redesigned primer (Shao *et al*., 2004), located after the three-nucleotide insertion and extending one nucleotide into exon 2 (italics). [3]MICA exon 2 forward sequencing primer (Molinero *et al*., 2002b) was modified to accommodate a polymorphism present in some alleles. Alignment modified from Shao *et al*., 2004 (Permission granted by Nature Publishing Group).

I decided to try the forward amplification primer described by Shao, in place of the MICA 6823 primer and repeated amplification and SBT with a MICA*001 homozygous cell line (DUCAF) and the two heterozygous MICA DNA exchange samples that typed as MICA*001 in previous MICA exchanges. This time there was no problem identifying heterozygous sequence peaks and the homozygous MICA*001 cell line DNA also typed successfully, as previously. Some heterozygous samples were also successfully typed for

MICA*001 previously, indicating that dropout of MICA*001 did not always occur. Although there were no apparent reasons why one primer should be more specific for MICA*001 than the other (MICA 6823 Vs MICA-Shao), there may be MICA intron 1 polymorphisms that are not well defined or known of that could affect the efficiency of MICA 6823 primer. Nevertheless, using the new primer I could avoid allele dropout of MICA*001 and decided to permanently replace the forward amplification primer with that designed by Shao. I tested a range of cell line and patient DNA encoding common MICA alleles to ensure there were no other problems and found the combination of 5'MICA-Shao and 3'MICA 8999 gave consistent results.

As I had already carried out some patient and donor MICA typing, I checked the results for homozygosity that may have been the result of MICA*001 dropout although this allele has low frequency in most populations. This was facilitated by linkage disequilibrium with HLA-B alleles where HLA-B*18:01 is the predominant allele associated with MICA*001 in European-American and African-American populations (Gao *et al*., 2006) with an haplotype frequency of just over 1%. Two further samples were found to be positive for MICA*001 after repeat amplification with MICA-Shao primer, whereas typing using the original MICA amplification primer did not detect this allele.

One other problem that I was unaware of was that the exon 2 forward sequencing primer which is located at the beginning of exon 2, extends into a polymorphic area affecting MICA*002:03, 010, 025, 054 and 062. These alleles encode a 'c' instead of the consensus 't' at nucleotide position 87, therefore I reordered the primer with a 'y' at that position to avoid problems with non-priming that particularly could affect MICA*010 sequencing as this allele had a frequency of 4-5% in our renal patient and donor cohorts. However, the reverse exon 2 primer gave excellent coverage and the polymorphism at position 87 was easily detected.

3.3 Production of recombinant MICA in *E. coli*

3.3.1 Cell Lines for MICA cDNA Amplification and Cloning

To produce a MICA DNA fragment that can be used for recombinant MICA (rMICA) molecule production, it was decided to obtain cells expressing MICA that were used in previous published studies utilising rMICA for the detection of MICA antibodies (Molinero

et al., 2002b; Zwirner *et al*., 2000). HeLa and HCT116 tumor cell lines express MICA*008 and *009:02 respectively in contrast to BLCLs where MICA is not expressed and therefore not transcribed from messenger RNA into cDNA, the essential template for cloning. Another cell line that expressed MICA was obtained from ESTDAB (http://www.ebi.ac.uk/cgi-bin/ipd/estdab). ESTDAB 172 (melanoma) was typed for MICA by SBT, among other available cells, after being selected based on linkage disequilibrium with HLA-B using data shown in Table 3.3.1. The B-locus HLA type for this cell as listed on the ESTDAB database was B*35:01/02/03/04/06, B*41:02 and the MICA type determined by my own typing was MICA*002, 004, the two most frequent MICA alleles after MICA*008. The ESTDAB database also contains FACS characterisation data showing no expression of HLA but good cell surface expression of MICA. This cell was therefore ideal for obtaining cDNA for MICA*002 and 004 in addition to MICA*008 and 009 using the other two cell lines.

Table 3.3.1: Common MICA and HLA-B haplotype frequencies and their linkage disequilibrium

MICA	HLA-B	Haplotype Frequency[1]	Linkage Disequilibrium[1]
*008	*08:01	0.206	0.095
*008	*07:02	0.120	0.054
*008	*44:02	0.106	0.048
*008	*40:01	0.050	0.022
*004	*44:03	0.048	0.044
*010	*44:03	0.015	0.012
*010	*15:01	0.047	0.045
*002	*35:01	0.053	0.046

[1]Frequencies found in North American Caucasoids (Gao *et al*., 2006)

3.3.2 Obtaining cDNA from cell lines and ligation with plasmid vector

RNA extraction and RT-PCR was performed according to methods described in Chapter 2, section 2.2.4. A MICA cDNA fragment was amplified from cDNA produced by RT-PCR using the following primers:

MICA/B Forward: 5'-GGA TCC AAT GCC CCA CAG TCT T-3'
MICA/B Reverse: 3'-GGA TCC CCA GAG GGC AC-5'
Underlined sequence is the BamH1 restriction site.

cDNA obtained from cell lines Hela (MICA*008) and HCT116 (MICA*009:02) were amplified using MIC specific primers as detailed above. For comparison, cDNA extracted from patient and donor BLCLs were also included and results are shown in Figure 3.3.1.

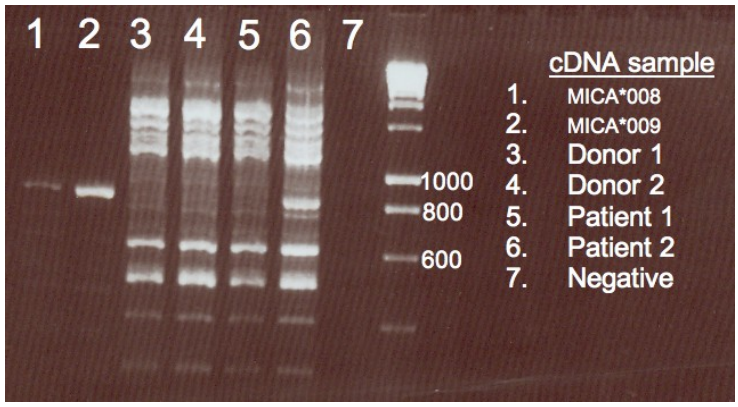


Figure 3.3.1: MIC-specific products (MICA and MICB) amplified using cDNA from cell lines. Hela (lane 1), HCT116 (lane 2) cDNA and cDNA extracted from BLCLs from two potential BMT donors and patients are shown. A band equal to just over 800 base pairs was obtained from tumor cell line DNA and one of the BMT patents BLCL cDNA.

A band equal to the expected size of around 800 base pairs was obtained with cDNA from both tumor cell lines. BLCL cDNA was not expected to produce a MIC-specific band, however one of the patient's cDNA produced a band equal to the size of the MIC amplicon, which was not present in the other BLCL cDNAs. This interesting result may relate to unknown cellular processes occurring in response to bone marrow transplantation or the drug regimen associated with the transplant.

As MICA contained an internal sequence identical to the Xba1 restriction site, this enzyme could not be used. The only solution was to use BamH1 at both ends of the insert and screen carefully to ensure the product was ligated in the correct orientation in the expression plasmid (pAC-5), requiring analysis of the plasmid sequence preceding and following the MICA insert using the MICA internal primers as follows: 5MICAint: 5'-ATC CAT GAA GAC AAC AGC AC-3', 3MICAint: 3'-GGT CTG AGC TCT GGA GGA CT-5'.

3.3.3 Cloning procedures for bacterial expression of MICA protein

As MIC amplified products from cDNA could be MICA, MICB or MIC pseudogenes and because nucleotide misincorporations could also be present in cloned DNA fragments, a

first round of cloning in pCR®4-TOPO® vector and TOP10 chemically competent *E. coli* cells using TA cloning, detailed in Chapter 2, section 2.2.6, was used as a simple procedure to screen several clones for the desired insert. Successful ligation of MIC inserts with pCR®4-TOPO® plasmid was verified using restriction enzyme digestion with BamH1 and results are shown in Figure 3.3.2. Sequences were verified by direct sequencing of plasmid DNA using the MICA bi-directional internal sequencing primers detailed above.

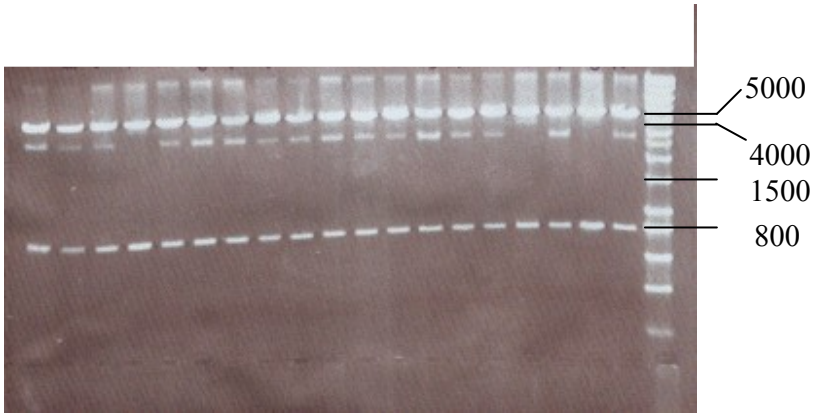


Figure 3.3.2: Restriction digestion of pCR®4-TOPO® ligated with MICA*008 cDNA. Digestion with BamH1 cut the DNA either side of the insert at BamH1 restriction sequences incorporated into the MIC amplification primers. The two main bands observed correspond to the pCR®4-TOPO® vector (3957 bp) and the MICA fragment (836 bp). The remaining bands are partially digested and supercoiled plasmids with different mobilities.

Several of the clones contained inserts that had many differences compared to MICA*008, that were more related to MICB or possibly pseudogenes, which can also be amplified with the amplification primers used. Some clones had nucleotide misincorporations but several were identical to the sequence of MICA*008 and selected for sub-cloning into the pAC-5 protein expression plasmid.

A larger volume of 20 µl pCR®4-TOPO® vector DNA containing verified MICA*008 cDNA sequence was used to prepare MICA fragments for subcloning in pAC-5 expression plasmid. Restriction digestion with BamH1 of the pAC-5 plasmid was carried out to prepare it for ligation with MICA fragments, which were released from pCR®4-TOPO® by restriction digestion with BamH1 and isolated by gel excision. The linearised pAC-5 plasmid DNA and MICA*008 DNA fragments were ligated and then sequenced using MICA internal sequencing primers. All methods are described in Chapter 2, section 2.2.6.

A total of six ligation reactions were performed as it was predicted that some MICA fragments could have a reverse orientation rendering them unsuitable for protein expression. pAC-5 plasmid DNA containing MICA*008 in the correct orientation and reading frame was identified as shown in Figure 3.3.3.

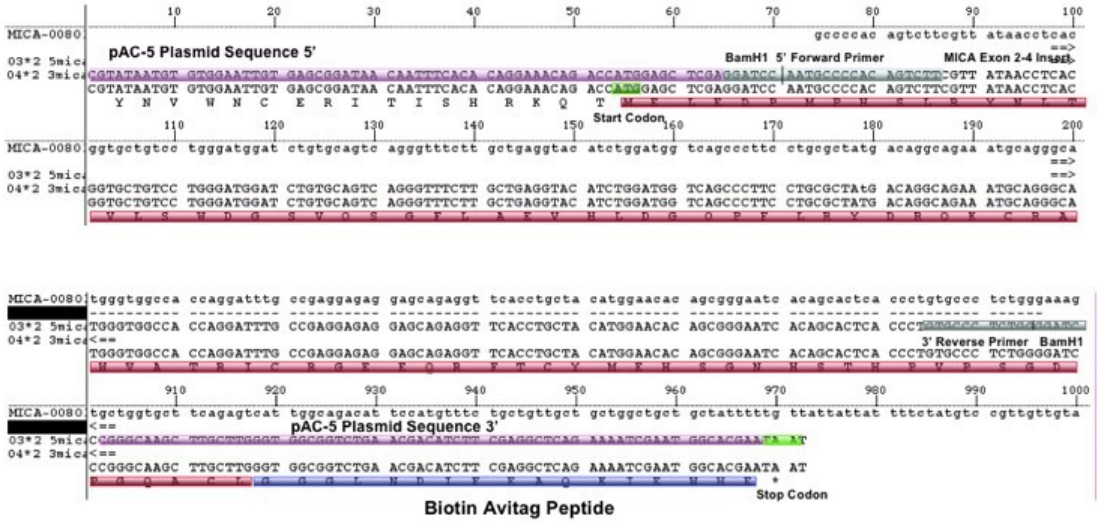


Figure 3.3.3: Sequence alignment of MICA*008 coding sequence with sequence of pAC-5 plasmid containing a MICA DNA insert. The 5' plasmid sequence shows that the promoter region is in frame and continuous with the MICA sequence. The MICA coding sequence, highlighted in red is also in frame with the biotin Avitag peptide sequence (blue) at the 3' end of the MICA sequence, which includes a stop codon to terminate transcription.

A protein coding sequence of 305 amino acids, including the biotin tag sequence was produced and the full protein sequence was as follows:

MFLEDPMPHSLRYNLTVLSWDGSVQSGFLAEVHLDGQPFLRYDRQKCRAKPQGQWAEDVLGNKTWDRETR
DLTGNGKDLRMTLAHIKDQKEGLHSLQEIRVCEIHEDNSTRSSQHFYYDGELFLSQNLETEEWTVPQSSR
AQTLAMNVRNFLKEDAMKTKTHYHAMHADCLQELRRYLESGVVLRRTVPPMVNVTRSEASEGNITVTCRA
SSFYPRNIILTWRQDGVSLSHDTQQWGDVLPDGNGTYQTWVATRICRGEEQRFTCYMEHSGNHSTHPVPS
GDPGQACLGGGLNDIEFAQKIEWHE

The predicted molecular weight of the molecule produced by the protein sequence above was estimated to be 35.0 kilo Daltons (kDa) by the bioinformatics tool available on the website: http://bioinformatics.org/sms/prot_mw.html. AVB101 transformants containing pAC-5 with MICA*008 inserts were induced for protein expression and samples taken at 0, 1, 2, 3 and 4 hour intervals as detailed in Chapter 2, section 2.2.5. SDS-PAGE analysis results are shown in Figure 3.3.4.

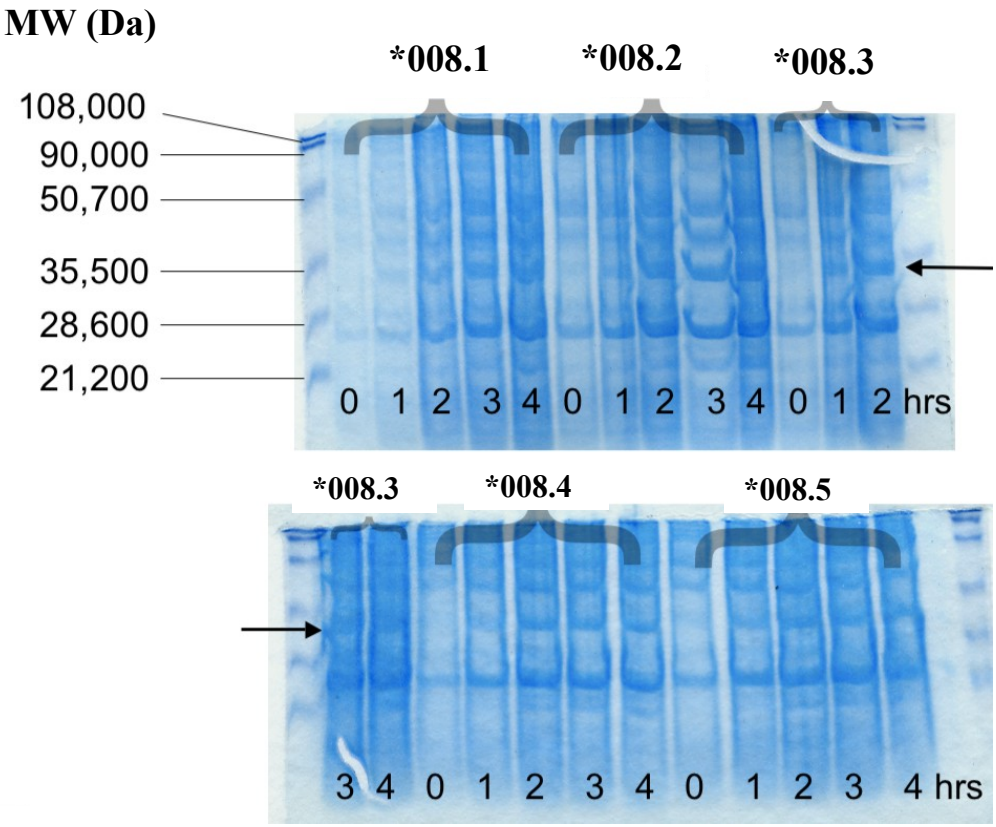


Figure 3.3.4: SDS-PAGE analysis of MICA protein with Coomassie Blue staining. The gel images show protein induction by IPTG of MICA*008 at 0, 1, 2, 3 and 4 hours for 5 different samples. Arrows indicate likely position of induced MICA protein band, predicted to be 35.0 kDa by the internet tool: http://bioinformatics.org/sms/prot_mw.html

SDS-PAGE analysis revealed the presence of a band corresponding to around 30 kDa that was present after one hour of protein induction but since other bacterial proteins were also present the result by SDS-PAGE alone was inconclusive. To verify with more certainty that a band was present that was specifically MICA-derived, Western blotting was used with detection of MICA protein using a mouse anti-human MICA/B specific antibody as detailed in Chapter 2, section 2.3.3. Six samples were tested by Western blot using 0 hour and 4-hour post-induction samples and the results are shown in Figure 3.3.5.

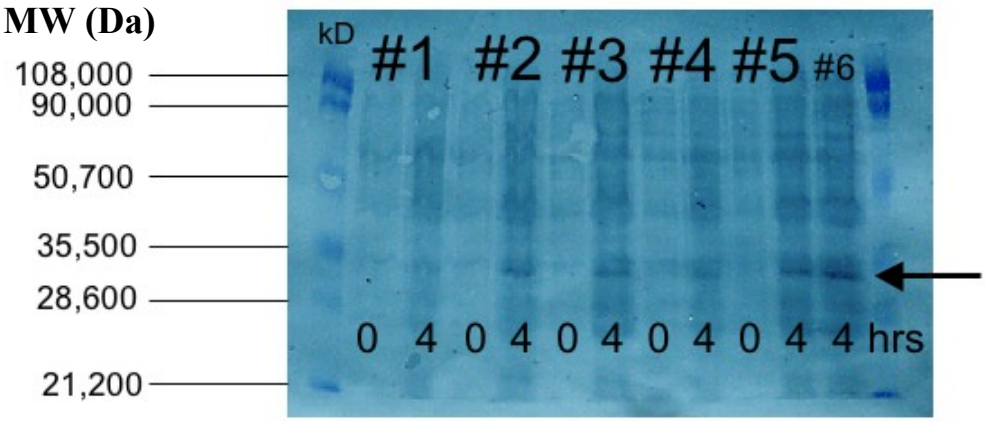


Figure 3.3.5: SDS-PAGE gel Western Blot Analysis. Six different samples comparing uninduced (0 hours) with 4 hours post-induction with IPTG are shown. A stronger band of around 33 kDa is seen in the post-induction samples indicated by the arrow. Sample 6 has 0 hours omitted due to lack of space on the gel. These results confirm those shown in Figure 3.3.4 and, in addition, show the induced band is MICA specific.

As the miniprep testing confirmed the production of a MICA specific induced protein band, cultures were scaled up for inclusion body purification, to remove contaminating bacterial proteins and produce milligram quantities of MICA protein as detailed in Chapter 2 section 2.2.5. The SDS-PAGE results of pre and post-induction and purified inclusion bodies are shown in Figure 3.3.6.

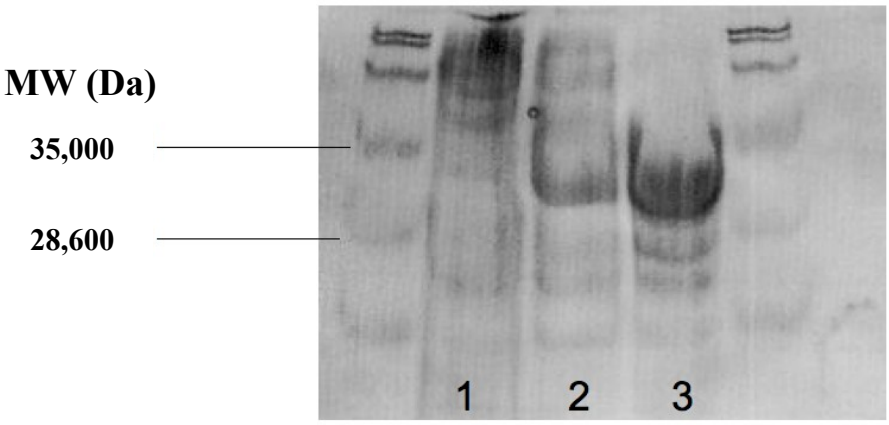
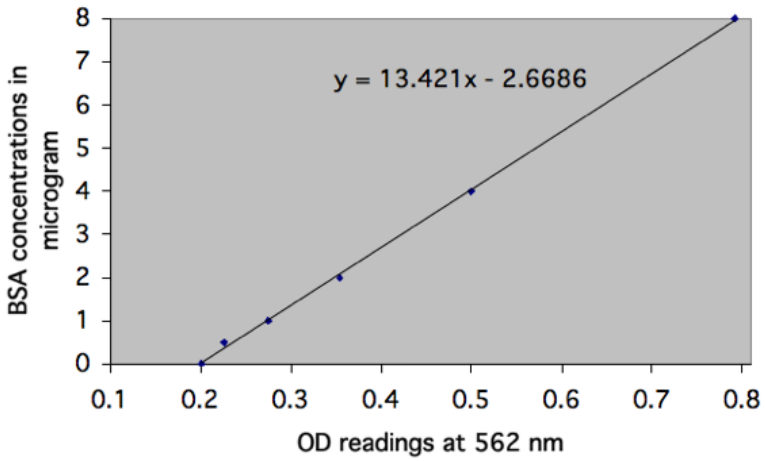


Figure 3.3.6: SDS-PAGE analysis of scaled-up MICA protein production. 1. Pre-induction. 2. Post-induction. 3. Purified inclusion bodies.

The concentration of MICA protein obtained from inclusion body purification was determined using the Micro BCA™ assay (Pierce Scientific, UK) as detailed in Chapter 2,

section 2.2.5. The results are given in Figure 3.3.7 and show a mean concentration of purified protein of 4.6 mg/ml.



sample	OD at 562 nm	protein in µg	mg/ml
MICA*008 0.5 µl	0.369	2.3	4.6
MICA*008 1 µl	0.541	4.6	4.6
MICA*008 2 µl	0.898	9.4	4.7
MICA*008 3 µl	1.206	13.5	4.5

Figure 3.3.7: Measurement of protein concentration from MICA inclusion body purification. Test samples were compared to a BSA standard curve of known concentration and the OD for the blank (no protein) was subtracted from the test ODs. The mean concentration of protein in test samples was 4.6 mg/ml.

This work was carried out, in part, with a post-doctoral colleague Jenny Luxton, who wanted to produce recombinant MICA molecules to construct MICA tetramers for a separate study. While I was preparing MICA*008 protein, my colleague worked on producing MICA*009 from the cDNA that I produced from HCT116 cells. My colleague encountered a problem at the protein re-folding stage, which was the next step to follow on from the work described above. The re-folding method was based on that of Steinle (Steinle *et al*., 2001), who also produced MICA molecules in *E. coli*. The principle was to dialyse from 8M urea, in which the inclusion bodies were resuspended, through 4M, 2M 1M and no urea solutions, gently allowing the proteins to fold into their native conformation. Following this procedure folded proteins can be purified by fast protein liquid chromatography (FPLC) and the fractions obtained tested for purity. Once pure refolded protein is obtained the final stage is to use the BirA enzyme for enzymatic biotinylation utilising the Avitag peptide incorporated into the cloned molecule.

My colleague, who encountered problems with protein coming out of solution and aggregating, could not successfully demonstrate the re-folding of the MICA molecule despite spending a considerable amount of time trying to perfect this technique. As correctly folded MICA molecules were essential to produce an ELISA assay to detect clinically relevant MICA antibodies it was not possible to continue this line of investigation.

At around the same time, during 2006, One Lambda released commercial products to detect MICA antibodies for the first time. This solid-phase assay utilised Luminex™ technology as described in Chapter 1, with MICA screening and SAg beads. The MICA screening assay comprised two beads, each coated with a mixture of MICA antigens, that were included free of charge with the HLA antibody Luminex screening assay. The MICA SAg assay, although not free, was available to confirm the MICA specificities detected by screening, covering a range of 11 frequent MICA antigens. As our renal histocompatibility laboratories were already testing patients with the HLA antibody Luminex screening beads that included the MICA screening beads, it was decided to continue the project using Luminex to detect MICA antibodies in renal transplant patients.

3.4 Production of rMICA in 293T HEK mammalian cells

Although I carried out this study using commercial Luminex assays to detect MICA antibodies, I was still interested in how rMICA molecules produced in different systems interacted with MICA antibodies. This interest arose during testing of renal patients for the presence of MICA antibodies using three different Luminex assays, where differences in reactivity were observed between the different tests as discussed at length in Chapter 4. The variable MICA antibody reactivity with rMICA antigens using the same serum sample indicates that there are differences in the rMICA molecules attached to beads used in the assays. These differences may be structural (native or denatured), conformational, related to differing degrees of glycosylation or due to fusion tags or molecules used to attach recombinant MICA proteins to the beads.

The aim of this part of the study was to produce two or three different recombinant MICA molecules as secreted, native products, produced by either mammalian HEK 293T cells or Sf9 insect cells. Once enough recombinant protein had been generated, reactivity with

MICA positive human sera would be compared with Luminex beads coated in 293T-cell derived or Sf9 cell derived MICA antigens to investigate any differences.

In this section I discuss the production of rMICA molecules using 293T HEK cells. These cells were originally generated in Alex Van der Eb's laboratory in Leiden in the early 1970s by using sheared adenovirus type 5 DNA to transform normal human embryonic kidney cells obtained from a healthy, aborted foetus. Frank Graham, who published his findings in the late 1970s (Graham *et al.*, 1977), carried out the transformation experiments and the number 293 relates to his practice of numbering his experiments. Today, HEK 293T cells are widely used in molecular biology for mammalian recombinant protein production because they are readily transfected and easy to culture.

3.4.1 Generation of plasmid constructs for MICA expression

For molecular cloning in eukaryotic systems, it is necessary to include the leader sequence encoded by exon 1, in contrast to cloning in prokaryotes where it is not required. The PCR amplification is detailed in Chapter 2, section 2.2.5. PCR primers for amplification of MICA exons 1-4 from cDNA were redesigned according to previously published work (Zou *et al*., 2006b) to incorporate the correct restriction sites for ligation into pBK-CMV vector. The primers included sequence of restriction site Xho1 on the 5' primer and Kpn1 on the 3' primer. A DNA tag encoding the BirA recognition sequence for enzymatic biotinylation and a six-His sequence for protein purification utilising the affinity of histidine with nickel was also included on the 3' primer. The 5' forward primer was designed to anneal to the first 18 bases of exon 1. The 3' reverse primer annealed to the last 21 nucleotides of exon 4. The size of the fragment generated was 1082 base pairs.

XhoI

5' forward amplification primer: 5'ACT ATC TAC CTC GAG GCC ATG GGG CTG GGC CCG GTC 3'

3' reverse amplification primer: 5' AGC ACT CAC CCT GTG CCC TCT GGA TCC GGT GGC *GGT CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAA TGG CAC GAA* **CAC CAC CAC CAC CAC CAC** TAG GGT ACC CAT GCG CAT 3'

KpnI

The gel image shown in Figure 3.4.1 is the result of using the above primers to amplify cDNA obtained from Hela (MICA*008), HCT116 (MICA*009) and ESTDAB-172

(MICA*002 and 004) cDNA. A single band around 1000 bp is clearly visible compared to the negative control lane.

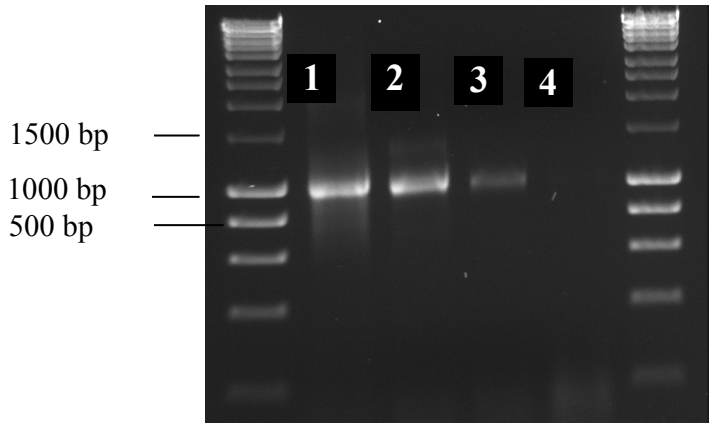


Figure 3.4.1: PCR amplification of cell line cDNA. MICA*008 (lane 1), MICA*009 (lane 2) and MICA*002/004 (lane 3). Lane 4 is the negative control (water instead of DNA).

The remaining PCR amplification products were run on another agarose gel for excision and purification of the correctly sized DNA band as detailed in Chapter 2, section 2.2.2. The Taq polymerase used for amplification of the MICA gene did not have proofreading capability, therefore amplicons could have nucleotide misincorporations. A first round of cloning enabled screening of multiple clones to find one with the correct sequence of the MICA gene, which was checked by DNA sequencing using MICA internal primers described above. As I previously encountered some problems using the TOPO-TA cloning kit from Invitrogen, where ligation was often unsuccessful, I used the pGEM-T Easy vector cloning kit II (Promega Corp., Madison, WI, USA) instead with very good results. The method is described in detail in Chapter 2, section 2.2.6. Following identification of pGEM clones with MICA inserts without misincorporations, restriction digestion using Xho1 and Kpn1 enzymes was carried out as detailed in Chapter 2, section 2.2.5. The results are shown in Figure 3.4.2.

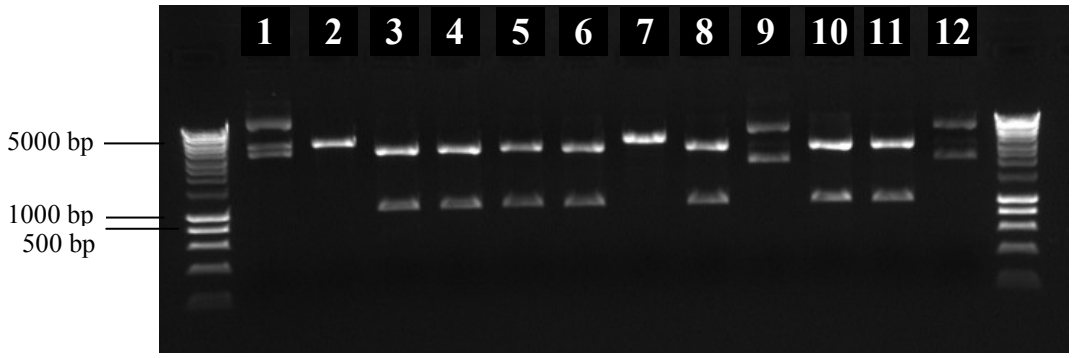


Figure 3.4.2: Restriction enzyme digestion with Xho1 and Kpn1 of pGEM plasmid ligated with MICA*008 cDNA amplicons. Seven of the 12 clones (lanes 3,4,5,6,8,10,11) contained a MICA insert shown by the band corresponding to 1000 bp.

The clones yielding a correctly sized band after restriction digestion were sequenced with MICA internal sequencing primers to verify the integrity of the cloned sequences. One clone with correct sequence for each of the alleles was selected for ligation with pBK-CMV mammalian expression vector. 20 µl pGEM plasmid DNA was digested with Kpn1 and Xho1 restriction enzymes to release the insert, which was recovered by agarose gel electrophoresis and gel excision and purification as previously described. The pBK-CMV vector was also digested with the same enzymes. The prepared MICA DNA inserts and pBK-CMV plasmid are shown in Figures 3.4.3 and 3.4.4.

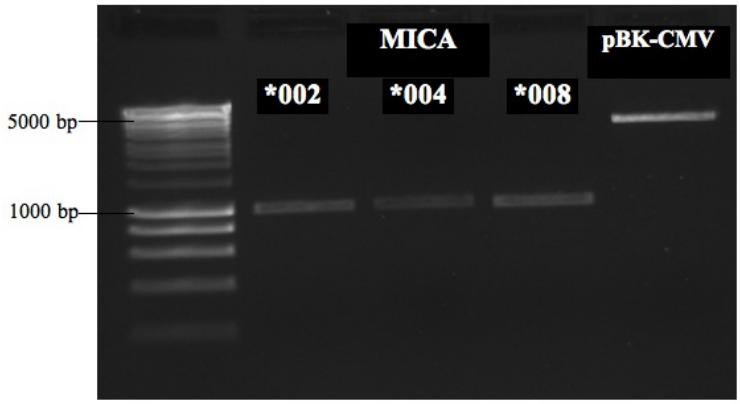


Figure 3.4.3: Cloned MICA inserts and pBK-CMV plasmid vector digested with Xho1 and Kpn1 restriction enzymes and purified by gel excision. The MICA inserts are 1082 bp and pBK-CMV vector is 4518 bp.

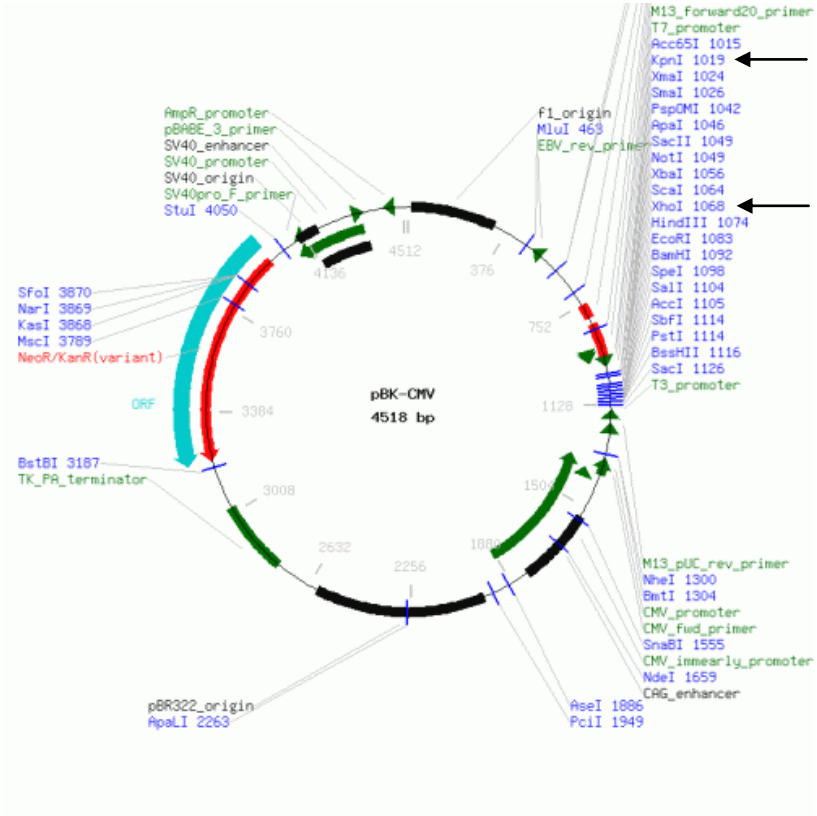


Figure 3.4.4: Plasmid map of pBK-CMV. This plasmid has multiple cloning sites and a strong CMV promoter for improving protein expression. The Kpn1 and Xho1 restriction enzyme sites used to clone MICA fragments are indicated by the black arrows. Direction of transcription indicated by large green arrows. Source: http://www.lablife.org compiled from data collected by Addgene.

MICA*002, 004 and 008 inserts were ligated with pBK-CMV using the same protocol used for ligation with pGEM as described in Chapter 2, section 2.2.6. The ligation reactions were used to transform JM109 cells and selected using kanamycin resistance encoded by the pBK-CMV plasmid. Six colonies for each MICA allele were selected and cultured overnight at 37°C in LB medium containing kanamycin. Plasmid minipreps were prepared as described and sequenced using MICA internal sequencing primers to check integrity of sequence and reading frame. A map of pBK-CMV indicating restriction enzyme sites used for ligation of the MICA insert is shown in Figure 3.4.4. One correctly constructed plasmid for each MICA allele (*002, 004, 008 and 009) was selected for scaling-up endonuclease-free plasmid preparations as described in Chapter 2, section 2.2.6. The results after extraction of MICA*004 and 008 plasmid DNA using the Endofree Plasmid Maxi Kit (Qiagen, UK) are shown in Figure 3.4.5

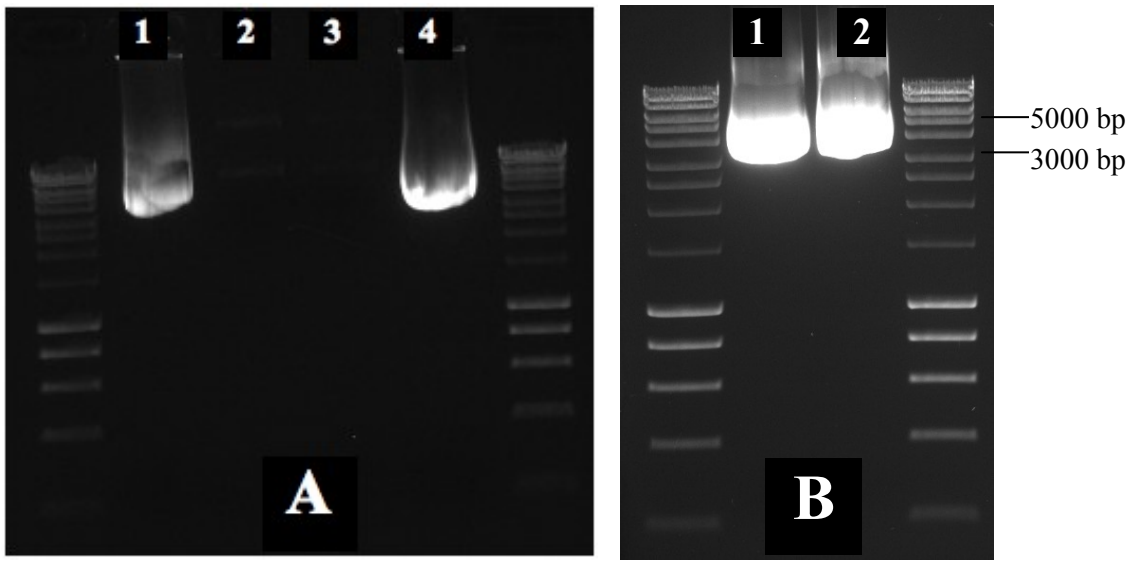


Figure 3.4.5: Endonuclease-free plasmid extraction using the Qiagen maxi kit. Test samples were taken during the extraction procedure to monitor efficiency. A. Lane 1: lysate; lane 2: lysate after DNA binding on columns; lane 3: column wash solution; lane 4: Eluted DNA. B. Endonuclease-free plasmid DNA for MICA*004 (lane 1) and MICA*008 (lane 2) constructs.

The plasmid constructs of pBK-CMV with MICA*002, 004, 008 and 009 are shown in Figure 3.4.6 and the position of the CMV promoter, primers, restriction sites, coding sequence, BirA biotinylation tag and six-His tag are indicated. Plasmid constructs were prepared for MICA*002, 004, 008 and 009 and the concentration of endonuclease-free plasmid DNA was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop, DE, USA) as described in Chapter 2, section 2.2.1. The results are shown in Table 3.4.1.

Table 3.4.1: Concentration and purity of endonuclease-free plasmid DNA

MICA allele	Concentration (μg/μl)	260nm/280nm ratio
*002	4.0	1.8
*004	3.1	1.8
*008	4.7	1.7
*009	1.8	1.8

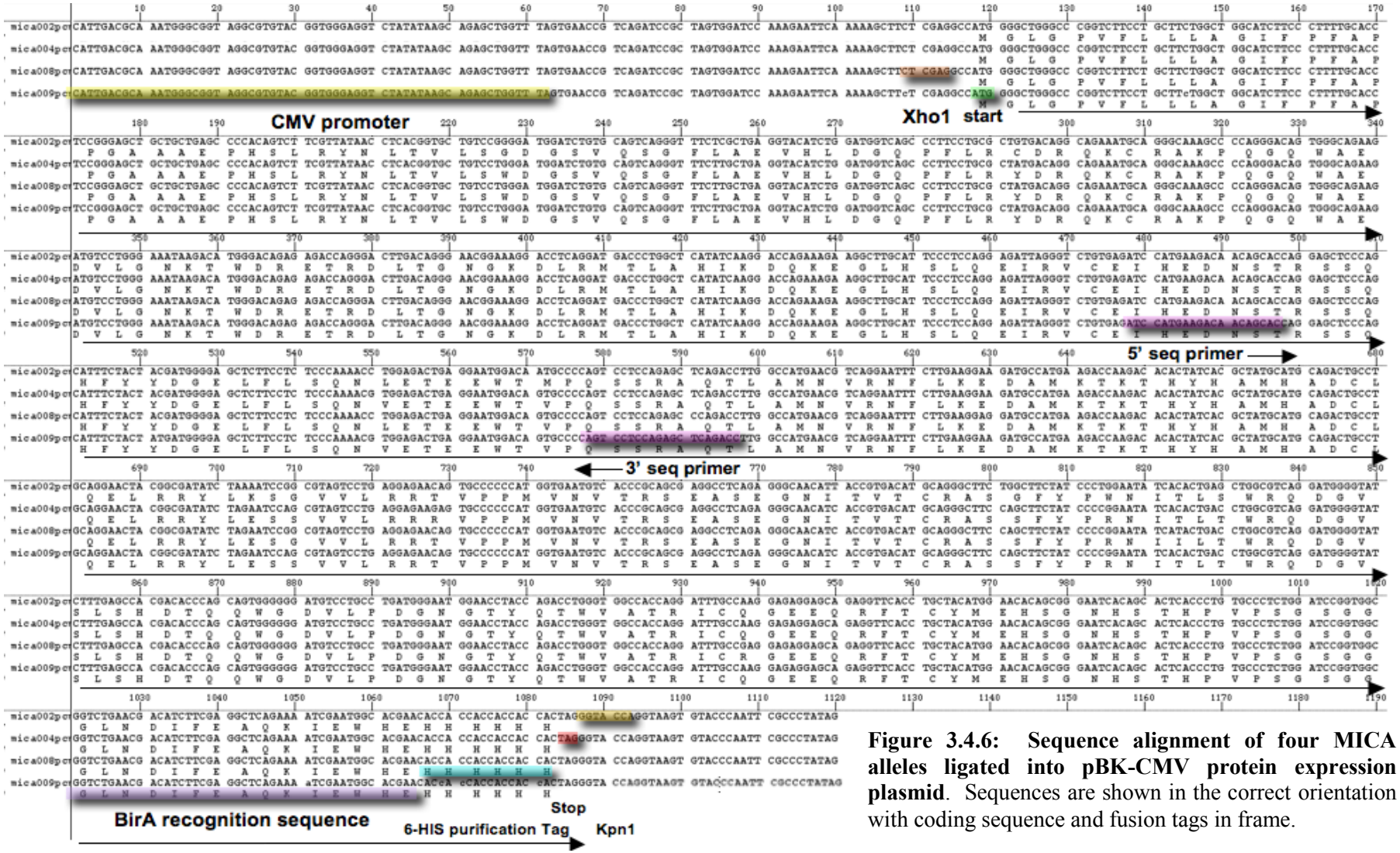


Figure 3.4.6: Sequence alignment of four MICA alleles ligated into pBK-CMV protein expression plasmid. Sequences are shown in the correct orientation with coding sequence and fusion tags in frame.

3.4.2 Liposome-mediated transient transfection of 293T HEK cells

The cells used for transfection had a low passage number and were maintained in cell culture using CD 293 protein-free media (Invitrogen, Paisley, UK) supplemented with antibiotics as described in Chapter 2, section 2.5.3. Cells were grown at 37°C with 5% CO_2 as an adherent monolayer, split to new flasks when the cell density reached 40-80% confluence and after 2-3 passages cells were healthy and ready for transfection.

The transfection reagent chosen was Polyfect® (Qiagen, UK) consisting of liposomes possessing a defined spherical architecture, with branches radiating from a central core and terminating at charged amino groups. Polyfect® reagent assembles DNA into compact structures to optimise entry into the cell. The Polyfect®-DNA complexes have a net positive charge allowing them to bind negatively charged receptors on the surface of eukaryotic cells. Once inside the cell, Polyfect® reagent buffers the lysosome after it has fused with the endosome, leading to pH inhibition of lysosomal nucleases ensuring stability of Polyfect®-DNA complexes and the transport of intact DNA into the nucleus (Polyfect® transfection handbook, 09/2000).

To find optimal requirements for transfection and protein production efficiency, testing was carried out in 6-well tissue culture plates testing parameters including DNA concentration, Polyfect® (Qiagen, UK) liposome reagent concentration and time post-transfection. The first test was with varying amounts of MICA*008 plasmid DNA either 2, 4 or 6 μg and the recommended 20 μl of Polyfect® reagent. The day before transfection (24 hours), $6x10^5$ 293T-cells were seeded into each of 5 wells of a tissue culture plate and transfected as follows:

Well 1: No DNA
Well 2: No Polyfect® reagent
Well 3: 2 μg plasmid DNA
Well 4: 4 μg plasmid DNA
Well 5: 6 μg plasmid DNA

Aliquots of medium were removed to analyse MICA-specific protein concentration after 16, 24 and 48 hours using the soluble MICA sandwich ELISA as described in Chapter 2, section 2.3.4. Concentration of MICA protein was determined using a MICA protein

standard curve of 4000, 2000, 1000, 500, 250, 125 and 62.5 pg/ml. Results are shown in Figure 3.4.7. It can clearly be seen from the chart in Figure 3.4.7 (B) that 2 µg of plasmid DNA achieved the highest concentration of rMICA*008 produced by 293T cells, where 3 ng/ml was produced after 48 hours.

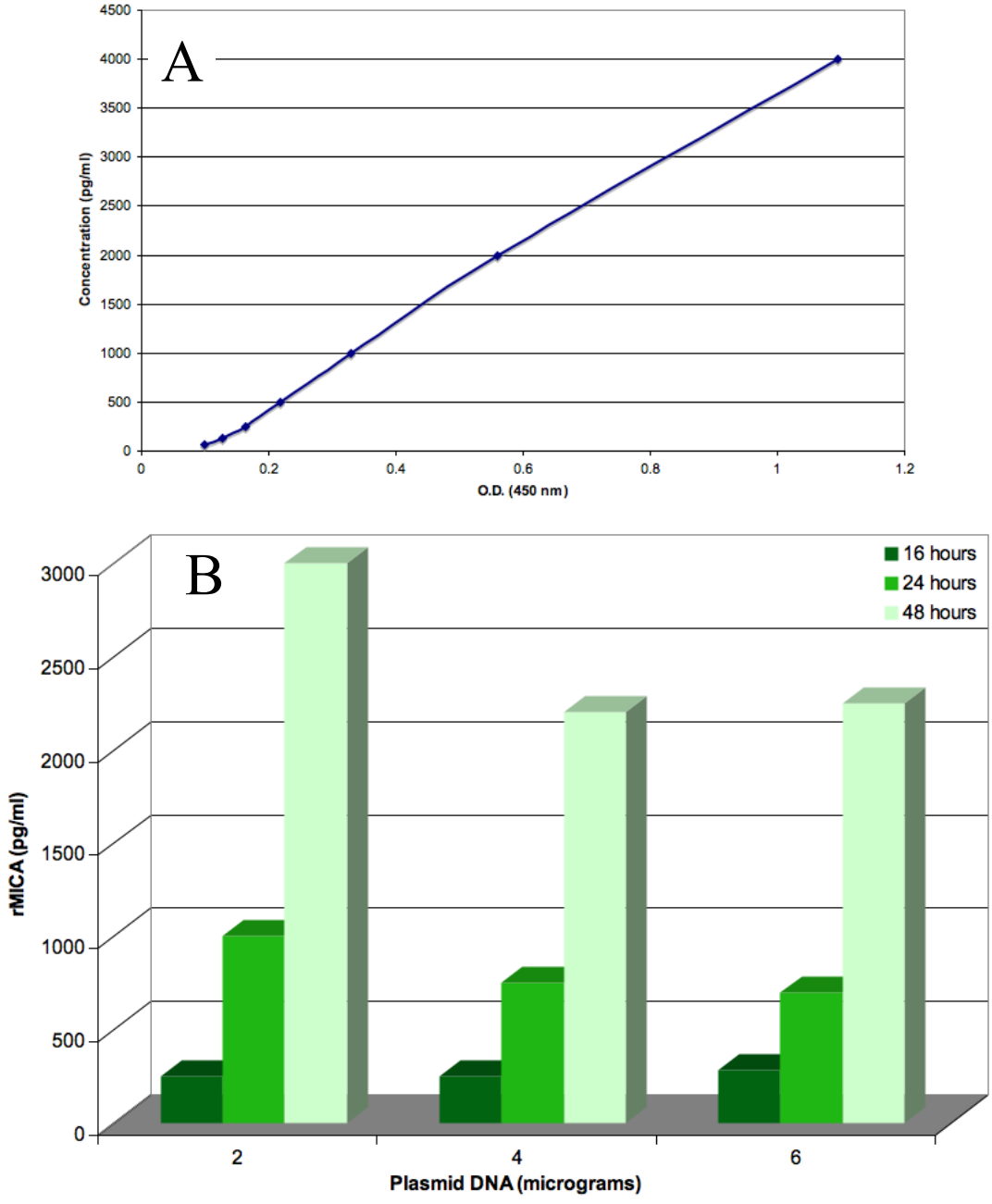


Figure 3.4.7: Amount of rMICA*008 produced with varying quantities of plasmid DNA. A. Protein standard curve produced from known concentrations of rMICA using an ELISA sandwich technique. B. Amount of rMICA*008 produced 16, 24 and 48 hours post-transfection using 2, 4 and 6 µg of MICA*008 plasmid DNA. The cells transfected with 2 µg of produced more rMICA after 24 and 48 hours.

The Polyfect® reagent was the most expensive part of the procedure, therefore it was important to determine the correct amount to use, not only to produce the most recombinant protein but also to reduce costs by using only what was necessary. The experiment was again carried out in 6-well tissue culture plates as above, using 2 µg MICA*008 plasmid DNA with 5, 10, 15, 20 or 30 µl of Polyfect® reagent. Testing for rMICA production was performed 48 hours post-transfection. The results are shown in figure 3.4.8.

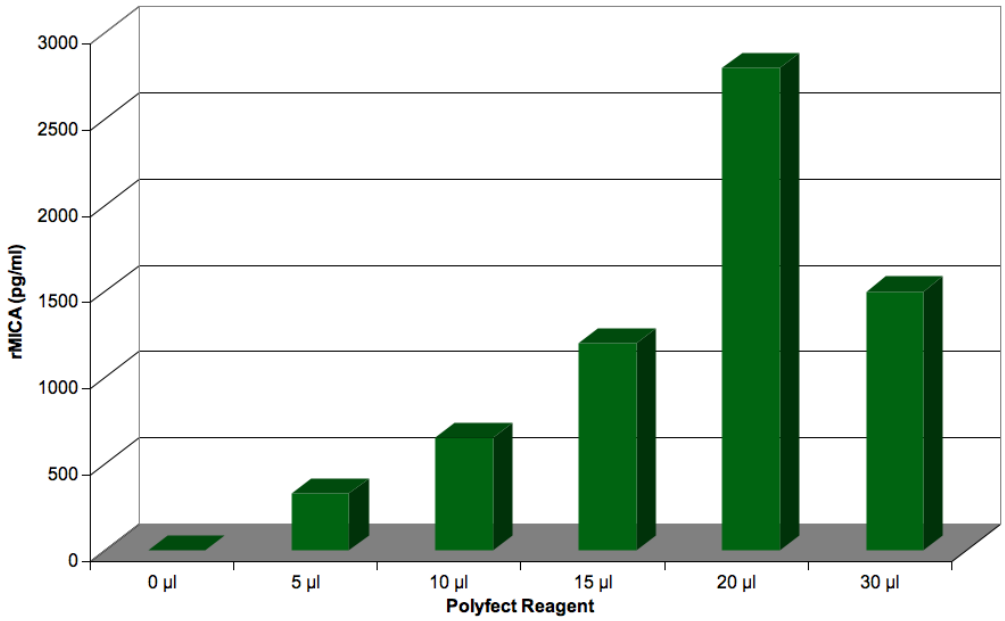


Figure 3.4.8: Effect of varying amount of 0-30 µl of Polyfect® reagent on rMICA*008 production. The cells transfected using 20 µl of Polyfect® reagent produced the most rMICA*008 protein at 2.8 ng/ml.

The results of optimisation experiments carried out at this point determined that 2 µg of plasmid DNA and 20 µl of Polyfect® reagent in a 6-well format produced optimal amounts of rMICA molecules, as recommended by Qiagen. However, due to an ambiguous part of the Polyfect® reagent transfection handbook, I had made a mistake in the transfection procedure. This related to the time cells were incubated with Polyfect® reagent and DNA complexes, as I believed the complexes were toxic to the cells and should not be left on too long. I had been using a 2 hour incubation of cells with DNA complexes before removing the media containing the complexes and replacing with fresh protein-free media. As I was unsure I was carrying out the protocol correctly I contacted technical support at Qiagen who confirmed that the Polyfect® reagent and DNA complexes can be incubated with the cells

for 24-48 hours. They recommended an incubation time of 24 hours as 48 hours could be too long and result in some cell death.

I repeated the transfection using the previously determined optimal amounts of MICA*008 plasmid DNA and Polyfect® reagent which were incubated with the cells at 37°C with 5% CO_2 for 24 hours. Four transfections were carried out in a 6-well plate with a negative control (no DNA). An aliquot of medium was retained from each well before removing the entire medium with the complexes and replacing with fresh protein-free media. The ELISA results for rMICA production showed that after 24 hours there was an average of 5000 pg/ml rMICA protein determined from one in ten dilutions of the retained media. The results showed a five-fold increase in rMICA production compared to the results shown in Figure 3.4.7 where only 1000 pg/ml was produced using the same DNA and Polyfect® conditions.

This experiment was continued with media changes every 48 hours until the 293T cells began to die, storing all the harvested media at -80°C for later recovery of rMICA. The results of this time-course experiment of rMICA production are shown in Figure 3.4.9.

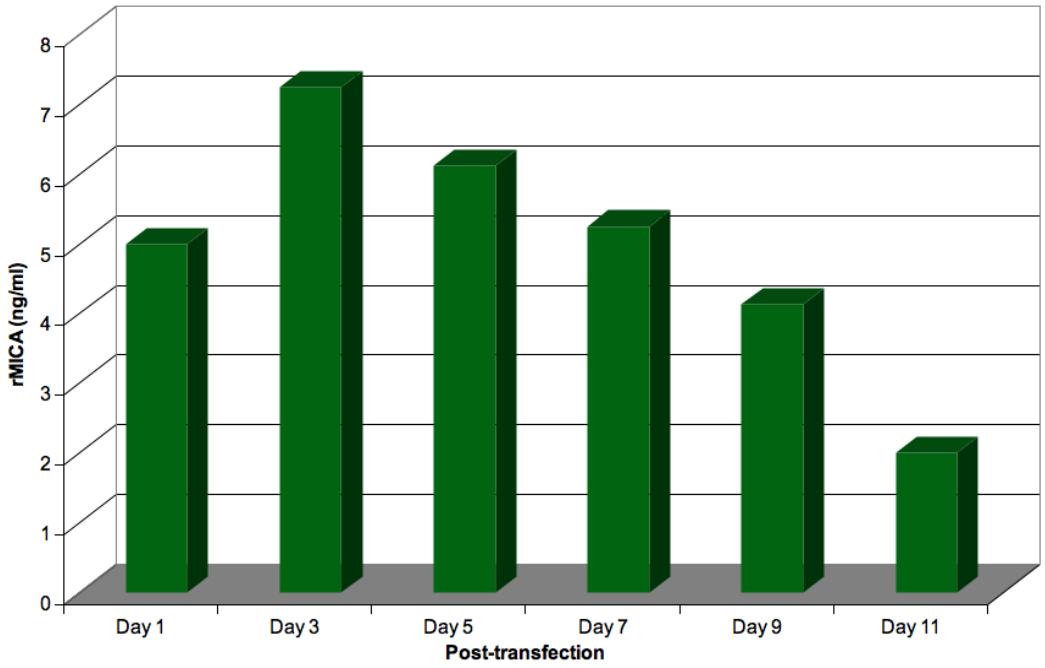


Figure 3.4.9: Production of rMICA*008 by transfected 293T cells over eleven days. DNA complexes were removed after 24 hours and changes of protein-free media made every 48 hours until day 11 post-transfection. Media was collected and stored and aliquots tested by ELISA for rMICA production.

The time-course experiment showed that rMICA could be produced for up to eleven days after transfection. The amount of rMICA produced declined gradually after day 3, which is most likely related to cell death as detached cells became noticeable when harvesting the media. However, the mean amount of protein produced was 5 ng/ml for almost two weeks.

The low level of protein expression compared with prokaryotic cloning and expression became a concern as the amount of rMICA necessary for conjugation with Luminex™ beads was quoted to be 500 µl of 5 µg/ml (Zou *et al*., 2006b). This amount would be sufficient for conjugation with $2.5x10^6$ Luminex beads, equivalent to 1 vial of beads as supplied, therefore could be scaled down slightly. However, this amount of recombinant protein is post-purification and biotinylation and it would be likely that a certain amount would be lost during these steps. To obtain this amount of unpurified rMICA it would be necessary to harvest around one litre of media containing rMICA. I calculated that this could be achieved by scaling-up production using large 175 cm^2 tissue culture flasks (Sarstedt, NC, USA) seeded with $2.4x10^6$ 293T cells in 20 ml of protein-free media 24 hours before transfection. Using ten 175 cm^2 tissue culture flasks with six media harvests at 48 hour intervals (first harvest after 24 hours) it was possible to obtain 10 x 20 x 6 = 1200 ml of medium, equal to about 6 µg of rMICA. It was therefore possible to obtain enough rMICA to create MICA molecule-coated Luminex beads, but would require a lot of time and effort.

As I could only produce a maximum of two different rMICA antigens with sufficient quantity for conjugation with Luminex beads using 293T cells, I was interested in producing a construct of pBK-CMV with MICA*002 cDNA obtained with cell line ESTDAB-172 in addition to rMICA*008. This would enable the production of beads to detect antibodies directed against the two main MICA ancestral lineages as discussed in Chapter 1. However, despite several attempts at re-cloning and endonuclease-free DNA purification, where the yield was 2.2 µg/µl and 260:280 nm ratio was 1.87, all attempts with these constructs to transfect 293T cells were unsuccessful. I decided to try a small-scale transfection test, with four MICA plasmid constructs (MICA*002, 004, 008 and 009) in a six-well plate format using the defined optimal conditions and testing for rMICA production after 24 hours. The results are shown in Figure 3.4.10

There was no obvious explanation why the MICA*002 construct was the only one where protein expression could not be achieved. The construction of the plasmids was the same

for all constructs and there were no sequence mutations in the CMV promoter region inhibiting transcription or MICA coding region causing a premature stop codon.

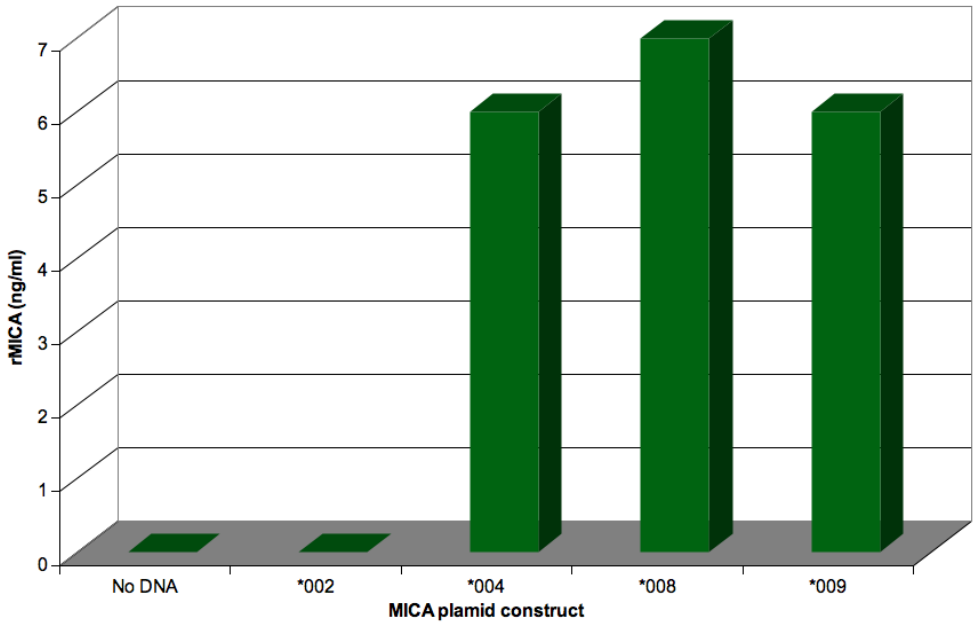


Figure 3.4.10: Expression of rMICA using four different MICA pBK-CMV constructs. Using the same conditions for the different constructs, no expression was obtained with MICA*002 compared with MICA*004, 008 and 009 where expression was at the expected levels.

I tried releasing the MICA*008 insert from its plasmid and ligating MICA*002 with this plasmid as I knew it worked successfully previously, but this was also unsuccessful with MICA*002 DNA. The successful transfection using MICA*004, 008 and 009 alleles may be related to their MICA-L2 lineage, whereas MICA*002 is MICA-L1 lineage which may have affected the transcription and expression, although this is unlikely.

Although not having rMICA molecules representative of the MICA-L1 lineage was a problem, time constraints did not permit further investigation and I decided to focus my efforts on producing adequate quantities of rMICA*004 and 008 molecules. Several rounds of transfection, using ten 175 cm^2 tissue culture flasks each time, were performed. The large volumes of medium containing rMICA molecules were concentrated using Centricon® Plus-70 (Millipore, USA) as described in Chapter 2 section 2.3.5. Using these protein concentrators it was possible to reduce 70 ml of medium to 200-500 µl. Concentrated rMICA proteins were quantified using the soluble MICA ELISA technique with a 1:10,000 dilution of rMICA protein, revealing a concentration of 1-2 µg/ml. A total of 22.8 µg rMICA*004 and 44.3 µg rMICA*008 was produced. This higher productivity

compared to earlier tests and the projected forecast may have been related to personal improvements in carrying out the tissue culture and transfection procedures, resulting in better expression. Alternatively, the high dilution used with concentrated MICA protein may have allowed more accurate quantification by the soluble MICA ELISA assay.

Concentrated rMICA proteins were analysed by SDS-PAGE to visualise and estimate their size and purity. The MICA protein standard from the ELISA kit (R&D systems, USA) was used to compare with recombinants produced in 293T cells. A 0.5 µl volume of MICA standard and 2 µl of rMICA*004 (1 µg/ml) and *008 (2 µg/ml) was loaded on the gel and results are shown in Figure 3.4.11.

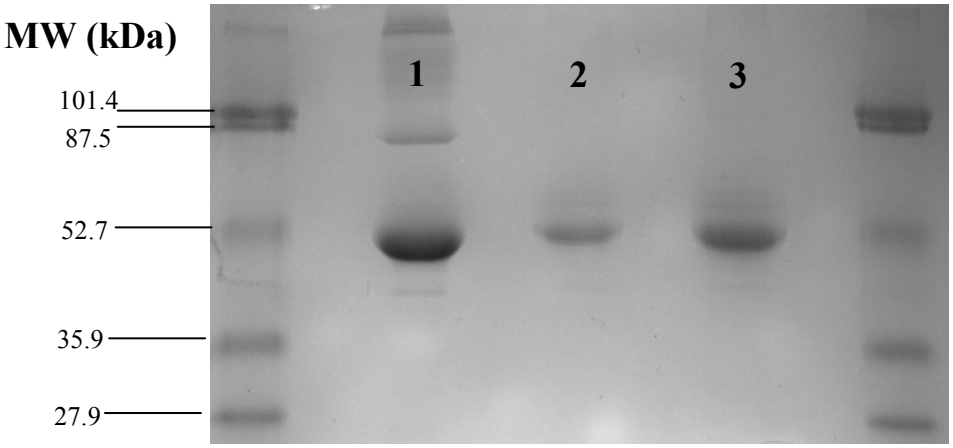


Figure 3.4.11: SDS-PAGE results after electrophoresis of a commercial rMICA (lane 1), rMICA*004 (lane 2) and rMICA*008 (lane 3) produced by 293T cells. The protein produced in 293T cells was purer than the commercial rMICA and both types were estimated to be 45-50 kDa.

3.5 Production of rMICA in Spodoptera frugiperda (Sf9) insect cells

Mammalian cells such as 293T HEK are an attractive means of obtaining recombinant proteins if the purpose is to use the protein in assays designed to detect molecules, for example antibodies, in other mammalian subjects such as humans. However, low expression of protein, evident in experiments described above, is a common problem. An alternative that has become very popular is to use insect cells for transfection as native protein very similar to the mammalian counterpart can be obtained in much higher quantities using similar methods. *Spodoptera frugiperda* or 'Fall Armyworm' is the

caterpillar stage of a moth and cells derived from this organism, called Sf9, originate from cell line OPLB Sf21-AE. This cell line has been tested for optimal growth and use in transfection, plaque assays, virus production and protein expression (Novagen User Protocol TB329 Rev C0108). As with 293T HEK cells, Sf9 cells can be transfected for protein expression with liposome-mediated reagents for transient or stable expression.

In this section I describe the production of rMICA*002 and rMICA*008 by transient transfection of Sf9 cells with liposome-based reagents.

3.5.1 Generation of plasmid constructs for MICA expression

The vector chosen for transfection of Sf9 cells was pIEx-4 (Novagen, Cat. No. 71235-3) and was designed for cloning and high-level expression of proteins in transiently transfected Sf9 insect cells. Transcription is driven by the AcNPV-derived hr5 enhancer and intermediate early promoter, IE1. Conveniently, the same restriction enzymes used to construct pBK-CMV-MICA plasmid were used with pIEx-4 but were situated at opposite ends of the MICA insert. Therefore primers were obtained with restriction sites for Kpn1 and Xho1 at the 5' and 3' ends respectively as follows:

Kpn1

5' forward amplification primer: 5'ACT ATC TAC GGT ACC GCC ATG GGG CTG GGC CCG GTC 3'

3' reverse amplification primer: 5' AGC ACT CAC CCT GTG CCC TCT GGA TCC GGT GGC *GGT CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAA TGG CAC GAA* **CAC CAC CAC CAC CAC CAC** TAG CTC GAG CAT GCG CAT 3'

Xho1

BirA biotinylation enzyme recognition sequence is in italics; six-His tag for up-stream purification is indicated by bold font and restriction enzyme sequences are underlined.

The plasmid map for pIEx-4 showing the available restriction sites is shown in Figure 3.5.1.

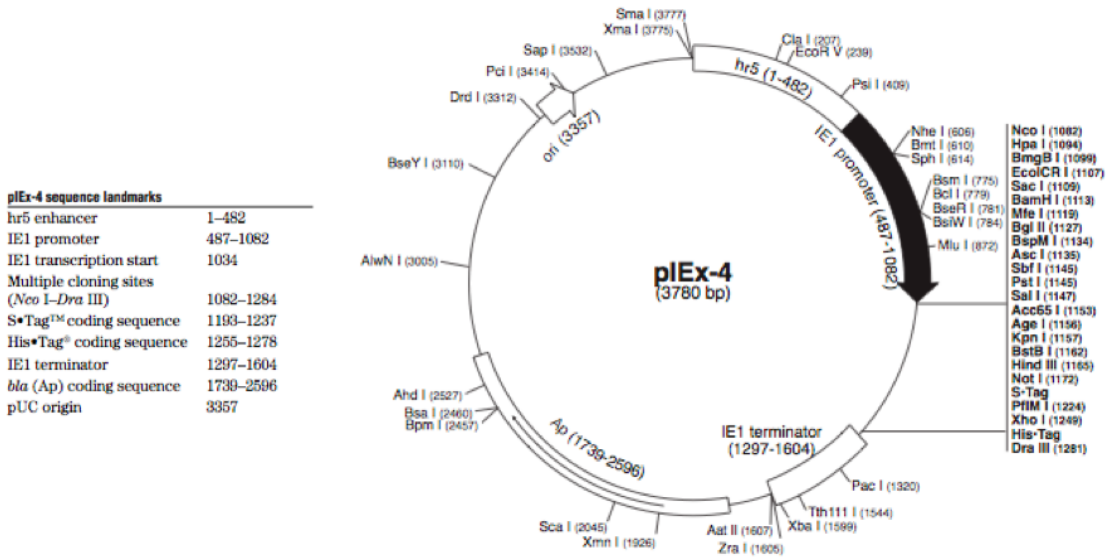


Figure 3.5.1: Map of plasmid pIEx-4 showing restriction enzyme sites and features. A MICA amplicon was generated with Kpn1 and Xho1 restriction sites for ligation between the IE1 promoter and terminator.

To avoid amplification of MICB, MICA pseudogenes and misincorporation of nucleotides, gel-excision purified MICA inserts, used in 293T HEK cell transfection, were used for amplification using the new primers. A one in ten dilution of the MICA DNA was made and utilised for PCR amplification as described in Chapter 2, section 2.2.5. Cloning in pGEM was not necessary therefore new MICA amplicons and pIEx-4 were digested with Kpn1 and Xho1 restriction enzymes and ligated using T4 DNA ligase as described in Chapter 2, Table 2.2.5. Sequencing was carried out using the MICA internal sequencing primers and the correctly assembled pIEx-4-MICA constructs for MICA alleles MICA*002 and *008 are shown in Figure 3.5.2.

3.5.2 Liposome-mediated transient transfection of Sf9 insect cells

The protocol for culture and maintenance of *Sf*9 insect cell cultures is detailed in Chapter 2, section 2.5.3. A vial of *Sf9* cells, as supplied by the manufacturer (Novagen, USA) and stored in liquid nitrogen, was used as a starting culture. Cells were cultured as a semi-adherent monolayer in tissue culture flasks and passaged until healthy and rapidly dividing which took 3-4 weeks of culture after revival from liquid nitrogen. Cells were initially split two times a week and more often as they became healthier and were passaged 7 or 8 times prior to transfection. The maximum manufacturer recommended number of passages was 20-25 in order to maintain experimental consistency.

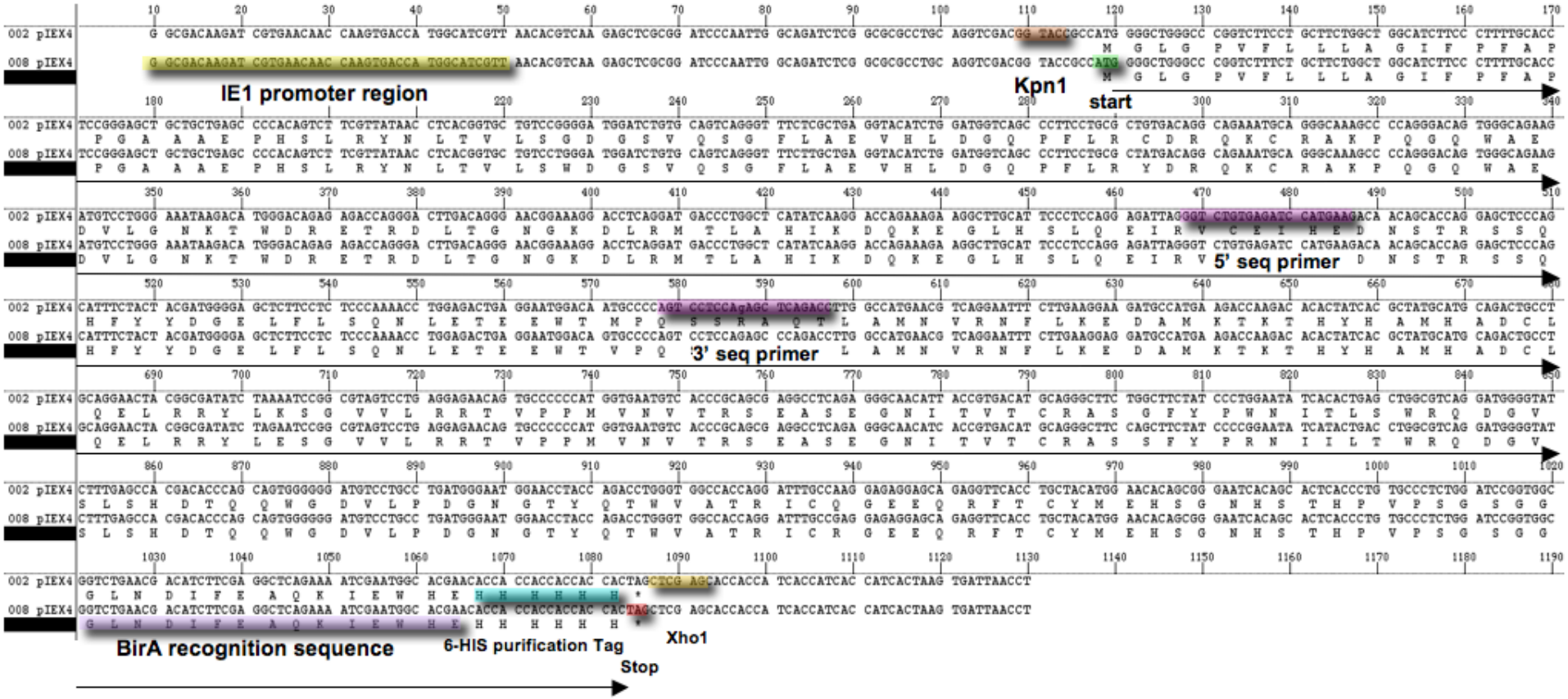


Figure 3.5.2: Sequence alignment of two MICA alleles ligated into pIEx-4 protein expression plasmid. Sequences are shown in the correct orientation with coding sequence and fusion tags in frame.

As I was limited by time, I chose to use liposome-mediated transient transfection using plasmid DNA to produce rMICA proteins, as baculovirus transfection was a much longer and more complicated process. It was expected that protein expression with insect cells would be much higher than with 293T cells.

There was a vial of two different insect cell liposome transfection reagents in the laboratory, and I decided to compare them for transfection and protein expression efficiency. One was called Insect GeneJuice® (Novagen USA), a proprietary liposome formulation optimised for transfection of *Sf9* cells. This reagent has extremely low cytotoxicity, can be used in serum-containing or serum-free media and is ideal for large-scale protein expression in *Sf9* suspension cultures when using pIEx™ plasmid vectors. Cellfectin® II Reagent is a cationic-lipid formulation designed for optimal transfection of insect cells and is an improved version of the original Cellfectin®. Cellfectin® II Reagent is suitable for transient transfection and consistent and efficient transfection of *Sf9* cells. Healthy, rapidly dividing cells were used in these experiments. The concentration of endonuclease-free pIEx-4:MICA plasmid DNA was 4.8 μg/μl for MICA*002 and 5.0 μg/μl for MICA*008 and both had a 260:280 nm ratio of 1.7. The conditions for transfection in 24-well tissue culture plates using both reagents with manufacturer's recommended volumes are shown in Table 3.5.1.

Table 3.5.1: Conditions for transient transfection of *Sf9* cells in 24-well plates using either Cellfectin® or Genjuice® transfection reagents and pIEx-4 with MICA DNA inserts

Transfection of Plasmid DNA	**Cellfectin II®**	**GeneJuice®**
Number of cells	$2x10^5$	$2x10^5$
Culture volume before transfection	500 μl	500 μl
Amount of plasmid DNA	0.4 μg	0.4 μg
Volume of transfection reagent	2 0 μl	2 0 μl
Total volume of media and transfection complexes	40 μl	50 μl
Total volume serum-free medium[1] added to transfection complexes after incubation[2].	160 μl	200 μl
Incubation time of DNA complexes with cells	4 hours	4 hours

[1]Grace's unsupplemented medium was used with Cellfectin® and SF900 II serum-free medium was used with GeneJuice®. [2]incubation time was 15 minutes.

The day before transfection, wells of a 24-well tissue culture plate were seeded with $2x10^5$ healthy *Sf9* cells grown in serum free medium (SFM). On the day of transfection, wells were 60-80% confluent (as an adherent monolayer). For each well to be transfected, 0.4 µg of DNA was diluted with 20 µl SFM (25 µl for Cellfectin II) and 2 µl of transfection reagent was diluted with 20 µl SFM (30 µl for Cellfectin II). The DNA was added slowly, drop-wise to the diluted transfection reagent and immediately vortexed gently to avoid precipitation. The DNA and transfection reagent mixture was incubated at room temperature for 15 minutes before aspirating medium from cells and replacing with the transfection complex mixture. Covers were placed on the plates and left to incubate for 4 hours at 28°C to allow transfection to take place. The transfection complexes were then removed from the cells, replaced with SFM and tested 48 hours later for recombinant protein production. The results given in Figure 3.5.3 show transfection with Cellfectin II® reagent produced a concentration of 100 ng/ml rMICA*002 and 500 ng/ml rMICA*008. Transfection with GeneJuice® reagent produced a concentration of 200 ng/ml rMICA*002 and 700 ng/ml rMICA*008.

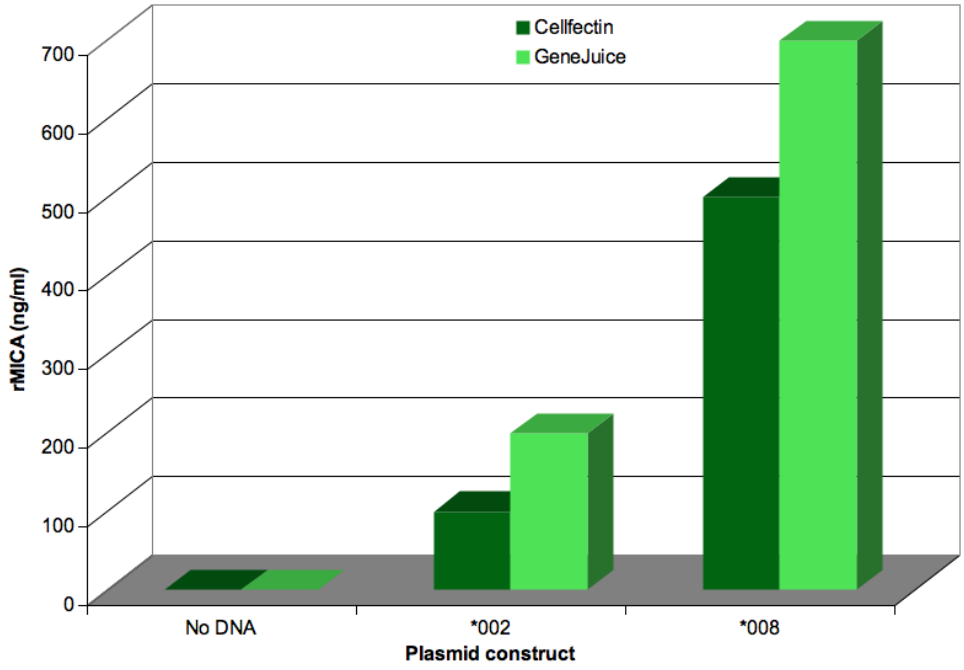


Figure 3.5.3: Concentration of rMICA protein produced using MICA*002 and MICA*008 plasmid DNA and either Cellfectin® reagent or GeneJuice® reagent.

The results show that rMICA produced using GeneJuice® reagent was more successful and therefore, was used in all subsequent experiments. These results also show that more

protein was produced with the MICA*008 plasmid than with MICA*002, even though both had the same concentration and purity.

The rMICA produced was secreted into the surrounding medium as soluble molecules but rMICA may also be inside the cells. To test whether it was worthwhile lysing the *Sf9* cells to extract more recombinant protein, I separated the medium from the cells (using 6-well culture format), replaced with an equal volume of fresh SFM and lysed the cells using Insect PopCulture reagent (Novagen, USA). Briefly, a 200 µl volume of Insect PopCulture reagent and 0.4 µl (10U) Benzonase Nuclease (Novagen, USA) per 1 ml of medium was added to the wells and allowed to incubate for 15-30 minutes. I then analysed the amount of rMICA present in the culture medium and cells separately, after culturing for four days post-transfection. The results are given in Figure 3.5.4 and show that most of the rMICA is secreted into the surrounding medium with only a small proportion remaining inside the cells. There was also much more MICA*008 produced than MICA*002, confirming the previous experiments.

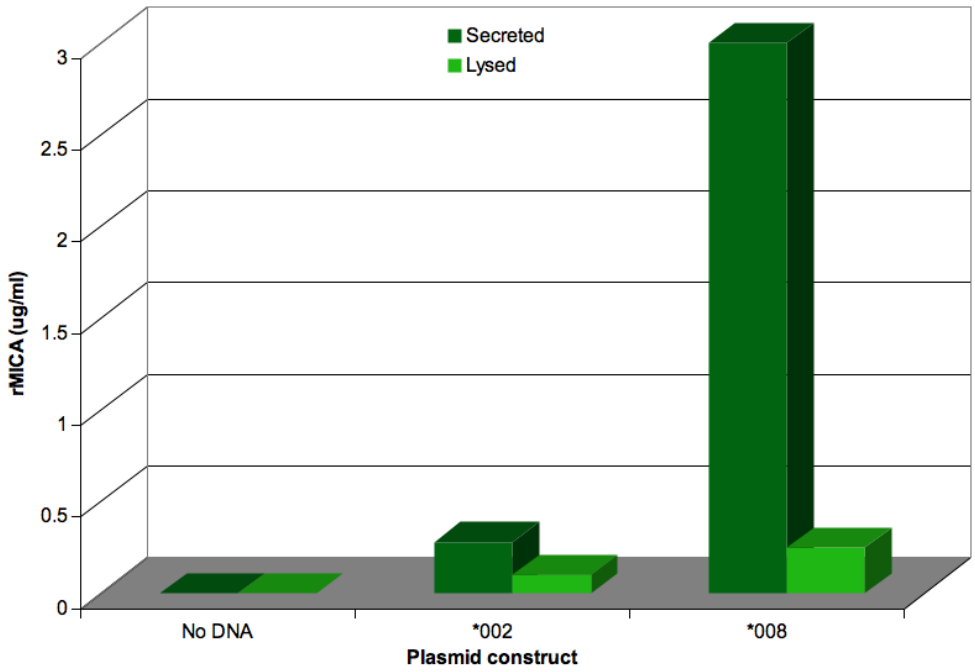


Figure 3.5.4: **Concentration of rMICA secreted into culture medium and lysed from cells.**

As only a small amount of rMICA was present inside cells and because protein from cell lysates would contain a lot of other contaminating proteins, I decided not to use lysis as a method of recovering rMICA protein

The results also showed an improvement in yield of rMICA, especially with MICA*008 transfected cells, and this may be related to improvements in carrying out the procedures and the *Sf9* cells becoming more healthy. Therefore I decided to try scaling up to transfection of 10 ml suspension cultures in 125 ml Erlenmeyer conical flasks. Briefly, three transfections were carried out for each of the two MICA plasmids (MICA*002 and *008) using 20 µg of plasmid DNA and 100 µl of GeneJuice® reagent. A total of $1x10^7$ cells from an exponentially growing culture were seeded into the flasks, DNA and GeneJuice® transfection complexes were added to the cells after incubation for 15 minutes. Flasks were then placed in an orbital shaker (150 rpm) at 28°C for 48 hours, to allow transfection and production of rMICA to take place, and tested for rMICA production. The results are given in Figure 3.5.5 and show that again, more rMICA*008 is produced than MICA*002 where a mean concentration of 6.3 µg/ml was produced by MICA*008 transfectants and 1.5 µg/ml by MICA*002 transfected cells.

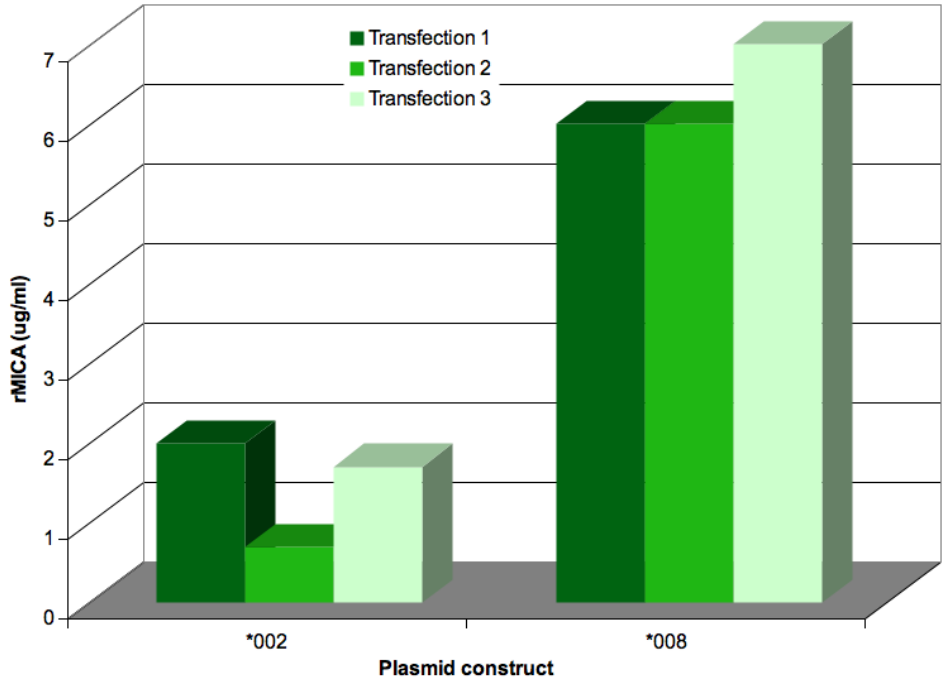


Figure 3.5.5: Concentration of rMICA produced by transfection of 10 ml suspension cultures. The test was carried out in triplicate and assayed for rMICA after 48 hours post-transfection.

This method produced the best rMICA production in terms of yield but a lower amount of rMICA*002. In total, from this experiment 44 µg of rMICA*002 and 190 µg of rMICA*008 was produced. SDS-PAGE was performed using 0.5 µl of the rMICA standard from the MICA ELISA assay and 6 µl of rMICA*002 (1.5 µg/ml) and *008 (2.5 µg/ml) produced by the above experiment and results are shown in Figure 3.5.6.

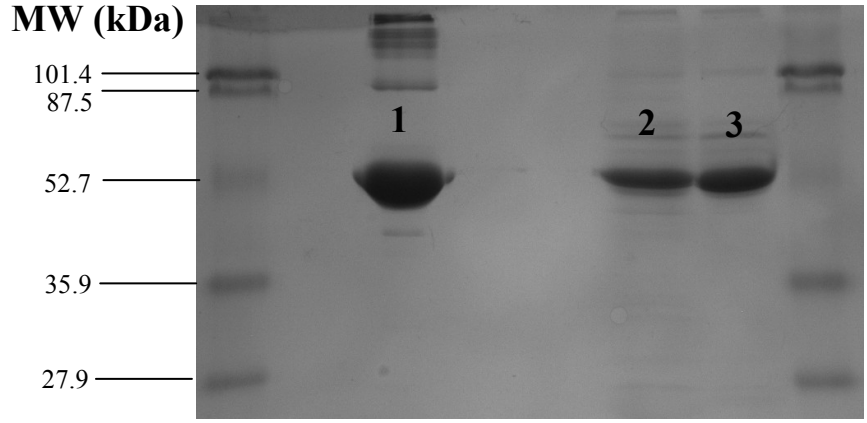


Figure 3.5.6: SDS-PAGE results after electrophoresis of rMICA. Proteins loaded were a commercial rMICA (lane 1), rMICA*002 (lane 2) and rMICA*008 (lane 3) produced by *Sf9* insect cells. The protein produced in *Sf9* insect cells was purer than the commercial rMICA and both types were estimated to be 45-50 kDa.

3.6 General Discussion

The MICA-SBT worked well in conjunction with analysis of exon 5 sequence patterns, consequently I was able to carry out in excess of 600 individual MICA typings including the identification of two novel MICA alleles (discussed in Chapter 5). No further problems were encountered with allele dropout and the method generally proved to be reliable and robust. The application of SBT for MICA typing in a routine laboratory is feasible although currently there is no easy solution to the problem of characterising exon 5 polymorphism. However, with sufficient demand heterozygous sequence typing algorithms, such as Assign™, could be adapted by the manufacturer to automatically identify exon 5 sequence patterns, enabling implementation, and commercial MICA-SBT typing kits would soon follow.

The production of recombinant MICA to identify MICA antibodies in patient serum proved to be problematic and ultimately was unsuccessful. Fortunately, commercial methods became available enabling me to concentrate on obtaining results and characterising the renal patients for MICA antibody status. As discussed at length in the next chapter, three different MICA Luminex assays were used to detect MICA antibodies and there were differences in the results obtained. It is probable that these differences were related to the various methodologies used to produce the rMICA molecules attached to the beads, leading to differences in antibody specificity. The aim of my experiments to generate rMICA

molecules from 293T mammalian and *Sf9* insect cells was to produce Luminex beads with homologous rMICA molecules from each cell line and compare their reactivity with patient serum that was well-characterised for MICA antibodies. I did not have time to complete this aim but did produce MICA proteins from 293T cells and *Sf9* cells in addition to rMICA produced using *E. coli*.

The use of *E. coli* to produce recombinant molecules is the first system that should be attempted as it is relatively straightforward, cheap and milligram quantities can be obtained. The final preparation of rMICA*008 isolated from inclusion bodies contained a concentration of 4.6 mg/ml and would have been more than sufficient to prepare an assay if the proteins had refolded correctly. The reason why it would not refold is unclear but could be related to the technique as it was achieved in other laboratories. Alternatively it is possible that rMICA molecules are particularly difficult to fold into their native structure because, unlike HLA class I molecules, they do not bind peptides or associate with β-2 microglobulin. This renders the MICA molecule more flexible but also less rigid and is evident from x-ray crystallographic analysis (Bahram *et al*., 1994). This could indicate that MICA protein produced in *E. coli* may not refold stably and become unfolded again, leading to linear proteins being used in assays to detect MICA antibodies that could react with clinically irrelevant patient antibodies.

The second method used to produce rMICA using 293T HEK cells did produce folded MICA that was determined using a MICA sandwich ELISA technique utilising antibodies that recognise MICA epitopes of native molecules. The problem was that 293T cells did not produce very large quantities of native MICA molecules. In contrast, *Sf9* insect cells produced much greater quantities of folded MICA proteins and proved to be an efficient method. In hindsight it would have been more productive to use insect cell transfection first and develop the techniques for HIS-tag purification, biotinylation and conjugation to beads, while collecting the rMICA produced by 293T cells.

It is puzzling why I was unable to produce rMICA*002 with 293T cells and consistent relatively low amounts of MICA*002 was produced with *Sf9* cells. A reason, in addition to those discussed above, could be that 293T cells were transcribing and producing MICA*002 protein but it was all in a linear form and not detected using the ELISA. Similarly *Sf9* cells may have produced the same amounts of rMICA*002 compared with rMICA*008 but less was refolded, perhaps relating to differing amino acid composition. Unfortunately I did not save any supernatant from MICA*002 transfected 293T-cell

cultures for analysis on an SDS-PAGE gel to identify a protein band equal to the size of MICA molecules to prove or disprove this theory. However, SDS-PAGE analysis of MICA*002 and MICA*008 produced in *Sf9* cells shows that, relative to the concentration of protein loaded on the gel, there were equal amounts of protein. Therefore, at least as far as *Sf9* cells are concerned, there was simply less protein produced by MICA*002 transfected cells than with MICA*008 transfected *Sf9* cells.

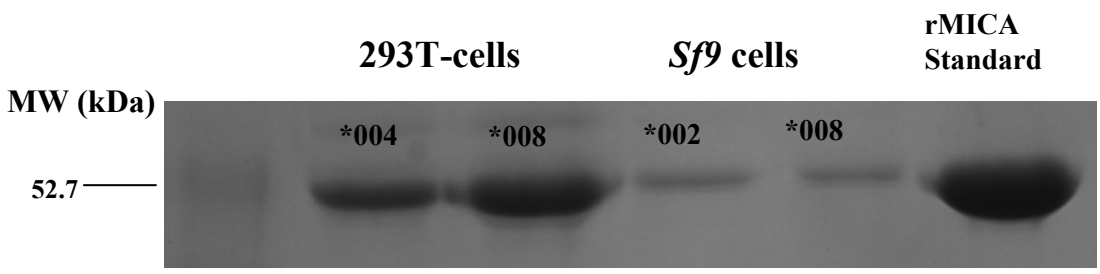
Another observation relating to native and linear rMICA was made by comparison of SDS-PAGE analysis of MICA proteins from all three methods. I was initially concerned that in contrast to rMICA derived from *E. coli*, the low amounts of protein detected by ELISA would not be visible with regular Coomassie Blue staining. The protein detection limit using Coomassie Blue is 0.3-1 μg per lane. Therefore if I used 10 μl of rMICA with a concentration of 2 μg/ml about 20 ng of MICA would be loaded onto the gel, below the detection limit. Before investigating more sensitive techniques, I decided to analyse MICA*008 produced by 293T cells with Coomassie Blue and was very surprised that the protein band of around 50 kDa had overloaded the gel. I repeated the SDS-PAGE analysis with the same amounts (2.5 ng) of rMICA produced in my experiments and also included the protein standard from the MICA ELISA (70 pg). A summary is given in Table 3.6.1.

Table 3.6.1: Characteristics of MICA proteins loaded onto SDS-PAGE gels

Source	MICA allele	rMICA conc.	Vol. loaded	Quant. loaded
293T cells	004	2.5 μg/ml	1 μl	2.5 ng
	008	2.5 μg/ml	1 μl	2.5 ng
Sf9 cells	002	1.25 μg/ml	2 μl	2.5 ng
	008	2.5 μg/ml	1 μl	2.5 ng
MICA standard[1]	-	140 ng/ml	0.5 μl	70 pg

[1]MICA sandwich ELISA (R&D systems) reconstituted as per manufacturer's instructions giving a final concentration of 140 ng/ml.

Result:



Firstly, only 70 pg of rMICA standard was loaded on the gel but this is the most intense band compared with rMICA produced from 293T or *Sf9* cells. Coomassie blue is unable to

detect this amount of protein therefore what is seen on the gel must represent linear MICA protein. Similarly, the amounts of native protein produced by 293T and *Sf9* and loaded on the gel would be barely detectable, if at all. These results show that most of the recombinant protein produced in these systems is linear, however less linear MICA products are observed with Sf9 cell rMICA production.

Another observation relates to the size of the proteins, estimated to be 45-50 kDaltons in the results shown above. This is larger than the protein isolated from inclusion bodies with *E. coli* transfection, which was approximately 33 kDaltons. This corresponds to a MICA protein produced using *E. coli* (Zwirner *et al*., 2000), estimated to be 33 kDaltons. The larger size of MICA protein produced by eukaryotic cells is most likely due to heavy N-linked glycosylation that has been reported previously (Zou *et al*., 2006b) and this is known to increase the apparent molecular weight of recombinant proteins (Lu *et al.*, 1997). It is interesting that rMICA produced by 293T cells (mammalian) is the same size as that produced by *Sf9* cells, indicating they are equally glycosylated.

In conclusion, the presence of linear MICA protein, and heavy N-linked glycosylation have implications regarding the use of these molecules in assays to detect MICA antibodies. Co-purified linear forms of the MICA protein will ultimately become conjugated with Luminex beads (or other solid-phase support) and may cross-react with irrelevant antibodies leading to false positive results. Conversely, a heavily glycosylated MICA molecule may mask epitopes important for MICA antibody recognition, and a false negative result may be obtained. The results of these experiments show that rMICA production in *Sf*9 insect cells is more efficient compared with 293T HEK cells, more protein is re-folded and products are of equal size. The testing of renal transplant recipients for the presence of MICA antibodies using three independent MICA Luminex, assays was carried out and is discussed in the next chapter. One of these assays used rMICA produced in insect cells, developed in Professor Peter Stastny's laboratories (University of Texas, USA).

CHAPTER 4

Evaluation of MICA antibody detection in renal patients and healthy controls using fluorescent bead-based assays

4.1 Introduction and aims

It is now approaching 50 years since the advent of CDC testing for HLA antibodies, first described in 1964 (Terasaki and McClelland, 1964). This was followed, five years later, by the introduction of CDC as a pre-transplant crossmatch test (Patel and Terasaki, 1969), which had a dramatic effect by reducing hyperacute and AR associated with a positive crossmatch in renal transplantation. This technique remained the mainstay of HLA antibody testing for over 40 years, providing a reliable estimate of risk associated with potential donors in terms of pre-sensitisation of the patient to HLA antigens. During the last decade there has been a revolution in this field enabling increased sensitivity and specificity of HLA antigens, namely the solid-phase assay.

The solid-phase assay for HLA antibody detection utilises recombinant HLA molecules or HLA antigens cleaved from the surface of cells, immobilised on a solid surface such as wells of a plastic plate (ELISA) or a polystyrene fluorescent micro-bead (Luminex). The latter has emerged as the current 'gold standard' (arguably) because of its superior sensitivity and multiplexing allowing extremely high throughput in a relatively short period of time using automation and computerised interpretation of results. The term 'Luminex' has been adopted by the H&I community to describe this technology after the company that developed the polystyrene fluorescent beads and instrumentation used in most assays of this type (Luminex Corp., Austin, TX, USA). It is now possible to test for HLA antibodies that were difficult or impossible to detect with the CDC assay for example antibodies to HLA-C and HLA-DP can now be tested for routinely. Also available are beads to detect HLA-DQα, -DQβ and MICA antibodies, allowing their clinical relevance in transplantation to be assessed.

There are three levels of HLA antibody detection using Luminex technology based on the number of different beads and the array of antigens on those beads. The first level utilises beads with a large number of different HLA class I or class II antigens on the surface,

which essentially provides the investigator with a screening method to obtain a positive or negative result. The second level employs a panel of 32 beads coated with different purified Class I or Class II antigens. The 32 different antigen beads in each test are divided into four HLA Groups with eight antigen beads in each group. This test can reveal antibody specificities at an antigenic level and is a cheaper alternative to the next level. The third level employs beads, which each have a different antigen, for a wide range of HLA antigens, attached to individual beads and is known as the single antigen (SAg) Luminex assay. This assay is very useful because it identifies the precise HLA antibody specificities present in patient serum, enabling avoidance of potential donor organs expressing those antigens.

As already discussed in the introduction, patients with antibodies to MICA pre-transplant, or who develop MICA antibodies post-transplant, may be at increased risk of acute and/or chronic graft rejection and loss. One Lambda was the first company to include MICA Luminex beads in their first level mixed HLA antigen bead assay and also a SAg MICA antibody detection assay (third level) to identify individual MICA antibody specificities that could be purchased separately. This was chosen as the method to identify MICA antibodies and the specificities of those antibodies in a cohort of renal transplant patients from our centre.

In 2008, I attended the IHIWC in Brazil, where I met a number of leading scientists and pioneers in this field, including Professor Peter Stastny whose group had developed their own bead-based Luminex assay for MICA antibody detection. Realising our group faced the same problems as many others who were interested in MICA antibody testing, Professor Stastny offered to test our sera with their technique at their laboratories. In addition, another commercial method for MICA antibody detection using Luminex technology became available late 2008 (Gen-Probe, previously Tepnel Lifecodes) and was also used to test the same patient cohort. This chapter deals with the evaluation of these assays, the elucidation of MICA antibody reactivity with MICA antigens and establishing criteria for assigning test subjects as either MICA antibody positive or MICA antibody negative.

4.2 Identification of MICA antibodies by One Lambda Luminex screening beads

The first test used for identification of individuals who may have MICA antibodies was the Labscreen™ Mixed LSM12 (One Lambda, CA, USA). This Luminex antibody screening kit incorporates beads conjugated with mixed antigens to detect HLA class I (12 beads), HLA class II (5 beads) and MICA (2 beads). The specificities identified were limited to the range of antigens conjugated to the bead with a positive reaction, as this assay does not identify single antibody specificities. The two MICA beads, bead 16 and bead 17, each identify five MICA antibodies based on antigens encoded by the most frequent MICA alleles. Bead 16 was conjugated with MICA*001, 004, 008/027, 012, 018 and bead 17 was conjugated with MICA*002, 007, 009, 017 and 019 antigens.

4.2.1 Calculations used for assignment of positivity with the One Lambda mixed bead assay

The following calculation was applied to the data obtained by the Luminex instrument to obtain a normalised background ratio (NBR) using negative control (NC) beads and NC serum:

NBR =

$$\frac{\text{Sample specific fluorescent value for bead N - Sample specific fluorescent value for NC bead}}{\text{Background NC serum fluorescent value for bead N - Background NC serum fluorescent value for NC bead}}$$

At this point in time, there were no internationally recognised sera standards for MICA antibodies and, therefore it was not possible to accurately determine the cut-off value as detailed in the product literature. Therefore, the manufacturer's recommended cut-off value of NBR equal to or greater than 2.2 was used to assign MICA antibody positivity, to be re-assessed later, following confirmatory testing with MICA SAg beads. Calculations were performed with the dedicated software supplied with the product (HLA Visual™ 2.2.0, One Lambda).

A total of 758 individuals consisting of 442 transplant recipients, 200 ESRD patients who were awaiting transplant and 116 healthy controls were screened for HLA and MICA antibodies. This assay was performed by the renal team from the Anthony Nolan H&I laboratories, Royal Free Hampstead for patient samples and by myself for healthy controls and selected patients.

4.2.2 MICA antibody frequencies - kidney transplant recipients

In total, sera from 150 of the 442 renal graft recipients (34%) were positive for MICA antibodies. Twenty-two individuals had positive reactions with bead 16 only (5%) and 55 were positive for bead 17 (12%). Both beads together reacted with the sera from 73 of the 442 recipients (16.5%).

4.2.3 MICA antibody frequencies – ESRD patients waiting for transplant

A MICA antibody frequency of 23% was detected in the serum of 46 out of 200 patients awaiting transplant (Untransplanted). Bead 16 was positive with 7 out of 200 serum samples (3.5%) and bead 17 was positive with 23 individuals (11.5%). Both beads 16 and 17 were positive with sixteen sera (8%).

4.2.4 MICA antibody frequencies - healthy controls

Among the 116 healthy control sera, there were a total of 33 positive reactions (28%). Eighteen sera (15.5%) reacted with bead 16 and 26 (22%) reacted with bead 17. Both beads 16 and 17 were positive with eleven of the 116 sera (9.5%).

Comparisons of frequencies are detailed in Table 4.2.1 and Figure 4.2.1.

Table 4.2.1: MICA antibody frequencies in 442 renal graft recipients, 200 untransplanted patients and 116 healthy controls using One Lambda screening beads

	Bead 16 n (%)	**Bead 17 n (%)**	**Bead 16 + 17 n (%)**	**Total positive n (%)**
Transplanted	22 (5)	55 (12)	73 (16.5)	150 (34)
Untransplanted	7 (3.5)	23 (11.5)	16 (8)	46 (23)
Healthy Control	7 (6)	15 (12)	11(9.5)	33 (29)

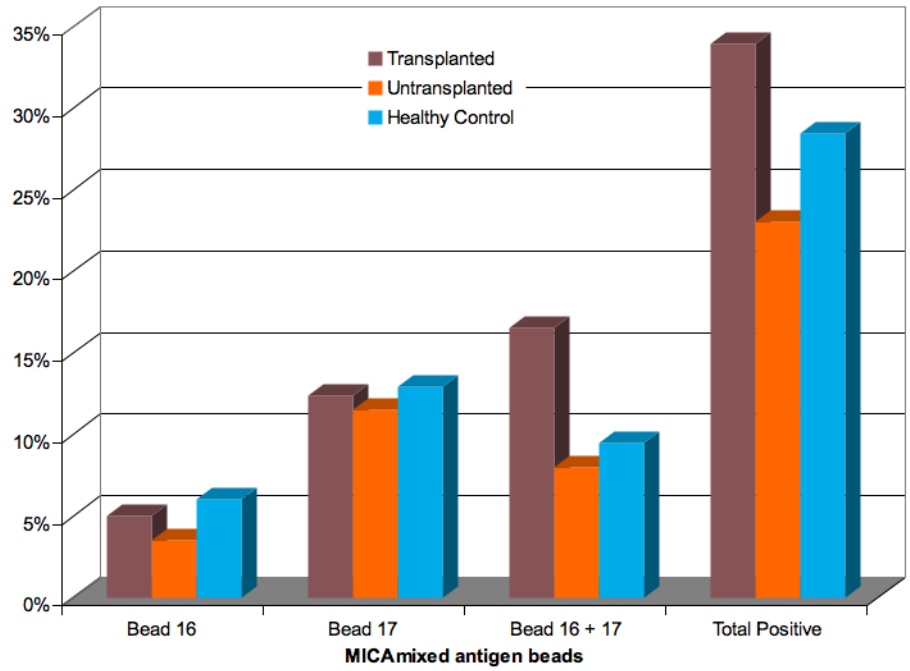


Figure 4.2.1: Frequencies of MICA antibodies by Luminex screening assay. Frequencies of MICA antibodies detected by beads 16, 17 or both, and overall positive cases in renal graft recipients (n=442), untransplanted renal patients (n=200) and healthy controls (n=116).

4.3 Identification of MICA antibodies by One Lambda Luminex single antigen beads

The product used for identification of MICA antibodies using SAg beads was the LABScreen® MICA SAg antibody detection test - LSMICA001 (One Lambda Inc., CA, USA). This kit includes SAg beads covering the same range of MICA antigens as the LABScreen® Mixed Class I and II test (One Lambda Inc., CA, USA). The MICA specificities detected were MICA*001, 002, 004, 007, 008/027, 009, 012, 017, 018 and 019. As MICA*008 and MICA*027 are identical in the extracellular regions, this bead reacts with antibodies against both specificities.

4.3.1 Calculation of results for baseline Mean Fluorescence Intensity

The following calculation was applied to the raw data obtained by the Luminex instrument to obtain baseline MFI using negative control (NC) beads and NC serum:

Baseline MFI =

$$\frac{\text{Sample specific MFI value for bead N - Sample specific MFI value for NC bead}}{\text{Background NC serum MFI value for bead N - NC serum MFI value for NC bead}}$$

Again, it was not possible to accurately determine the cut-off value as detailed in the product literature due to no MICA antibody reference sera. Therefore, the manufacturer's recommended cut-off – baseline MFI> 1000 was used. Calculations were performed with the dedicated software supplied with the product (HLA Visual™ 2.2.0) using lot-specific values.

4.3.2 MICA antibody frequencies - kidney transplant recipients

Screening for MICA antibodies using the One Lambda mixed bead assay revealed 34% of renal graft recipients (150 of 442 patients) were possibly positive. Testing for MICA antibodies using the One Lambda SAg assays showed that, of the 150 who were positive for MICA antibodies by screening beads, only 76 (51%) were positive. Full results are given in Table 4.3.1 and Figure 4.3.1. The most frequent MICA antibody detected in this group of 76 with One Lambda MICA SAg beads was MICA*019 (71%). The next most frequent MICA antibody was MICA*001, detected in 49% followed by MICA*018 with a frequency of 43%. The least frequent antibody detected was MICA*009 and tested positive in 9/76 recipients (11.8%).

4.3.3 MICA antibody frequencies - patients ESRD, awaiting transplant

A total of 46 of the 200 untransplanted patients (23%) tested positive for MICA antibodies with the screening method described above. Of these, 21 were confirmed positive by One Lambda SAg beads (45.6%). The most frequent antibody detected in this group of 21 patients was MICA*019 (48%). None of the patients had MICA*004 antibodies in this group.

4.3.4 MICA antibody frequencies - healthy controls

Sera from 116 healthy controls were tested with the One Lambda mixed Luminex assay and 33 were positive for MICA antibodies with a frequency of 28.4%. Confirmatory testing using One Lambda SAg beads revealed 14 had MICA antibodies (42.4%). The most frequently detected MICA antibody was MICA*001 (71%), MICA*019 had a frequency of 43%, MICA*018 was 29% and MICA*002 and MICA*008 antibodies were detected with a frequency of 14%. One of the healthy controls (7.1%) had antibodies detected against MICA*017.

Table 4.3.1: MICA antibody frequencies among 76 renal graft recipients, 21 untransplanted patients and 14 healthy controls testing positive using One Lambda MICA single antigen beads

MICA antibody	Transplanted n (%)	Untransplanted n (%)	Healthy Control n (%)
001	37 (49)	8 (38)	10 (71)
002	23 (30)	4 (19)	2 (14)
004	12 (16)	0 (0)	0 (0)
007	12 (16)	2 (9.5)	0 (0)
008/027	18 (24)	4 (19)	2 (14)
009	9 (12)	1 (5)	0 (0)
012	17 (22)	1 (5)	0 (0)
017	15 (20)	1 (5)	1 (7)
018	33 (43)	3 (14)	4 (29)
019	54 (71)	10 (48)	6 (43)

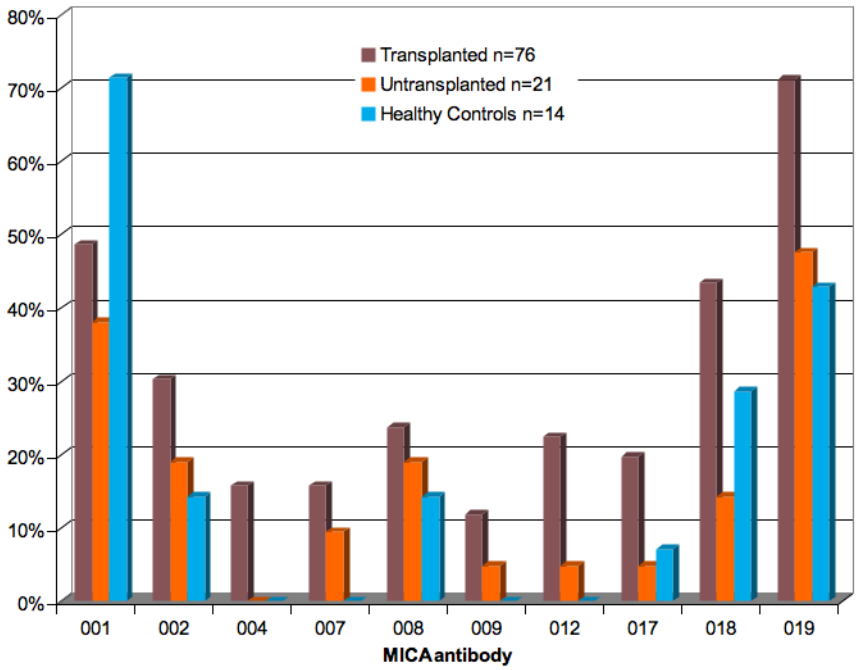


Figure 4.3.1: Frequencies of MICA antibodies by single antigen Luminex assay. MICA antibody frequencies detected by One Lambda SAg Luminex testing of renal transplant recipients, untransplanted patients and healthy controls.

4.4 Identification of MICA antibodies using a flow cytometry-based technique with recombinant MICA molecules produced from insect cells

This assay was developed at the laboratories headed by Professor Peter Stastny in Dallas, USA (Zou *et al*., 2006b) and will be referred to as the 'Stastny assay'. The recombinant MICA (rMICA) molecules used for conjugation with Luminex beads were produced using an insect cell (Sf9) transfection system. This assay includes SAg beads covering the same range of antibodies detected by the LABScreen® Mixed Class I and II test (One Lambda Inc., CA, USA). The MICA specificities detected were MICA*001, 002, 004, 007, 008/027, 009, 012, 017, 018 and 019. Testing was carried out to confirm the positive reactions with MICA antibodies observed using the One Lambda MICA SAg beads – 76 graft recipients, 21 untransplanted patients and 14 healthy controls. Full results are given in Table 4.4.1 and Figure 4.4.1

4.4.1 Calculation of Mean Fluorescence Intensity and interpretation of results

Yizhou Zou from Professor Stastny's laboratories in Dallas, USA, carried out this assay and analysis of results. Data files were generated with the bead numbers and their median fluorescence intensities (MFI) in Excel format. Standard curves were plotted to calculate the relative amount of MICA antibody for each sample, using aliquoted serial dilutions of a positive control serum. The raw data MFI obtained by Luminex FC were normalized to the relative amount of MICA antibodies according to a standard curve using a computer program developed in their laboratory. The means of the relative amount plus threefold SD of IgG binding in serum from normal subjects were used to set the threshold for this assay. Based on the relative amount of each MICA allele-specific antibody and the threshold for each antigen bead, scores were assigned as follows: negative, $\leq$ threshold; doubtful, > threshold and $\leq$ threshold + 200; weak positive, > threshold + 200 and $\leq$ threshold + 600; positive, > threshold + 600.

4.4.2 MICA antibody frequencies - kidney transplant recipients

Testing for MICA antibodies with this assay revealed that 29 of the 76 graft recipients, who were positive with the One Lambda SAg beads, had MICA antibodies detected (38%). The most frequent MICA antibody detected was MICA*007 with a frequency of 26%. The next

most frequent antibody was MICA*002 detected in 24% followed by MICA*017 (18%). MICA*001 and MICA*018 had a frequency of 17%. The two least frequent MICA antibodies detected in transplant recipients with this assay were MICA*004 and MICA*009 which were each detected with a frequency of 8%.

4.4.3 MICA antibody frequencies - patients with ESRD, awaiting transplant

Three patients from the untransplanted group of 21 were positive for MICA antibodies with this method (14%). Two patients had antibodies directed against MICA*002 (9.5%) and MICA*001, *004, *009, *009 and *019 antibodies were each detected in one patient (5%). No patients in this group had MICA*007, *012, *017 or *018 antibodies.

4.4.4 MICA antibody frequencies - healthy controls

Only one of fourteen healthy controls tested for MICA antibodies showed a positive result, with two specificities detected – MICA*002 and MICA*017, accounting for a frequency of 7%.

4.5 Identification of MICA antibodies using Gen-Probe MICA single antigen Luminex assay

Towards the end of 2008, another commercial method became available for the detection of MICA antibodies using Luminex technology. This assay identifies a wider range of MICA antibodies compared to One Lambda SAg or the Stastny SAg beads, which identify ten MICA antigens. However, MICA specificities MICA*001, 002, 004, 007, 008, 009, 012, 017, 018 and 019 only were considered, to allow comparison with the other techniques. Testing was carried out to confirm the positive reactions with MICA antibodies observed using the One Lambda MICA SAg beads – 76 graft recipients, 21 untransplanted patients and 14 healthy controls and full results are given in Table 4.5.1 and Figure 4.5.1.

Table 4.4.1: MICA antibody frequencies among 76 renal graft recipients, 21 untransplanted patients and 14 healthy controls using insect cell rMICA single antigen beads (Stastny assay)

MICA antibody	Transplanted n (%)	Untransplanted n (%)	Healthy Control n (%)
001	13 (17)	1 (5)	0 (0)
002	18 (24)	2 (9.5)	1 (7)
004	6 (8)	1 (5)	0 (0)
007	20 (26)	0 (0)	0 (0)
008/027	9 (12)	1 (5)	0 (0)
009	6 (8)	1 (5)	0 (0)
012	12 (16)	0 (0)	0 (0)
017	14 (18)	0 (0)	1 (7)
018	13 (17)	0 (0)	0 (0)
019	11 (14.5)	1 (5)	0 (0)

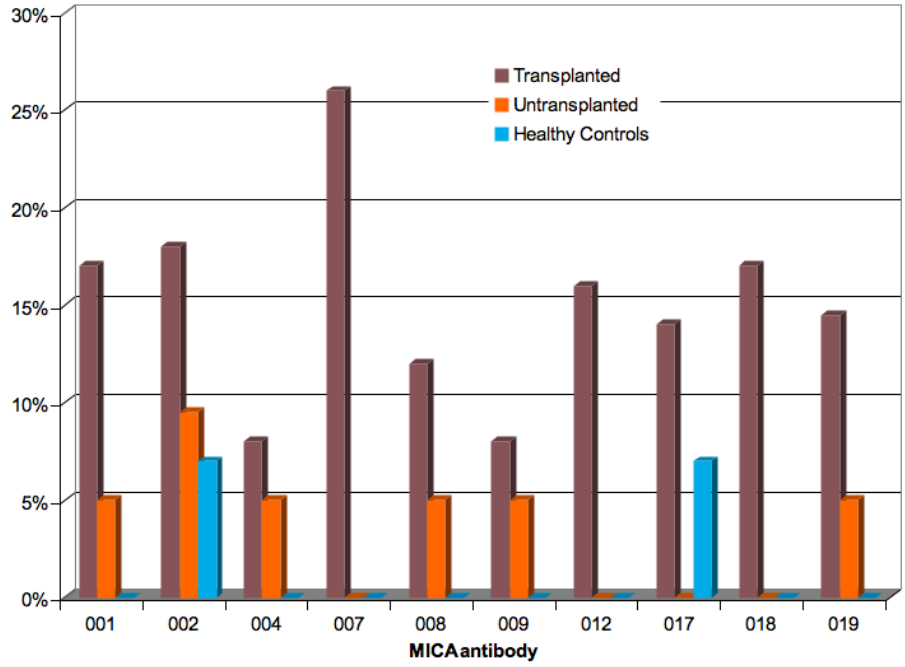


Figure 4.4.1: Frequencies of MICA antibodies using the Stastny Luminex assay. MICA antibody frequencies detected in transplant recipients, untransplanted patients and healthy controls who previously tested positive using the One Lambda Luminex assay.

4.5.1 Calculation of adjusted Median Fluorescence Intensity

To determine if a bead was positive, the background MFI was subtracted from the raw MFI, for each bead, to give the Adjusted Value 1. The background MFI is the background noise due to bead variation and was included on the lot-specific recording sheet provided with the kit. The Adjusted Value 1 was divided by the MFI of the calculated control (CalcCON) to generate the Adjusted value 2. The CalcCON is the raw MFI value of the lowest ranked antigen bead.

Adjusted Value 2 for antigen'x' =

$$\frac{\text{Individual Bead Raw MFI} - \text{Background MFI}}{\text{CalcCON MFI}}$$

Adjusted Value 3 was generated by dividing Adjusted Value 2 by the relative amount of antigen on each bead as detailed in the lot-specific recording sheet which also lists the antigens present on each bead and the suggested cut-offs for estimating a positive or negative result. Higher or lower sensitivities can be achieved by adjusting the cut-off. The calculations were performed using the analysis software (Quicktype for Lifematch version 2.5) using the recommended cut-off MFI of 3000.

4.5.2 MICA antibody frequencies - kidney transplant recipients

Overall, 31 of the 76 graft recipients' sera tested for MICA antibodies had reactions against at least one MICA specificity, representing 41% of recipients who originally tested positive using the One Lambda SAg assay. The most frequently detected MICA antibody was MICA*001 (26%). MICA*018 was detected with a frequency of 25% and MICA*007 was detected in 22%. The MICA antibodies detected with the lowest frequencies were MICA*004 and MICA*009 detected in 13% and 10.5% respectively.

4.5.3 MICA antibody frequencies - patients with ESRD, awaiting transplant

A total of five patients in this group tested positive for MICA antibodies out of 21 who were positive using One Lambda SAg beads, accounting for 24%. The most frequently detected MICA antibody was MICA*019 in three patients (14%) followed by MICA*001, *004, *008 and *018 each with a frequency of 9.5%. MICA*009 and *012 antibodies were detected in one patient (5%). None of the patients in this group had MICA*007 or MICA*017 antibodies detected.

4.5.4 MICA antibody frequencies - healthy controls – Not performed due to insufficient sera.

Results are shown in Table 4.5.1 and Figure 4.5.1.

Table 4.5.1: MICA antibody frequencies among 76 renal graft recipients and 21 untransplanted patients using Gen-Probe single antigen Luminex beads

MICA antibody	Transplanted n (%)	Untransplanted n (%)
001	20 (26)	2 (9.5)
002	13 (17)	0 (0)
004	10 (13)	2 (9.5)
007	17 (22)	0 (0)
008/027	14 (18)	2 (9.5)
009	8 (10.5)	1 (5)
012	15 (20)	1 (5)
017	13 (17)	0 (0)
018	19 (25)	2 (9.5)
019	12 (16)	3 (14)

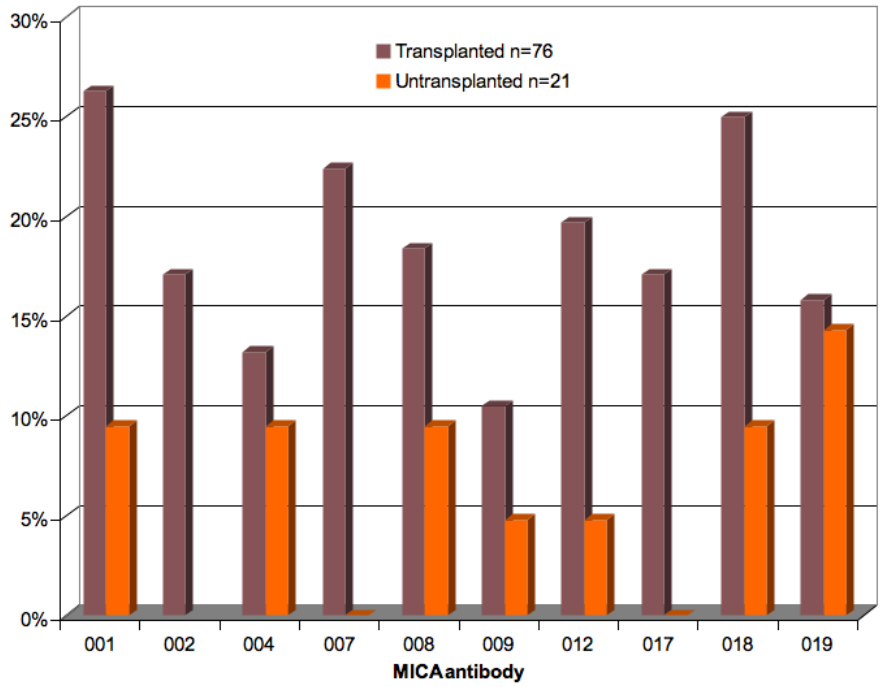


Figure 4.5.1: Frequencies of MICA antibodies detected using Gen-Probe single antigen Luminex assay. MICA antibody frequencies in transplant recipients and renal patients awaiting transplant

4.6 Comparison of MICA antibody testing results obtained with three methods

Whilst analysing the data obtained from MICA antibody testing using the three different assays it became apparent that many of the individuals who were assigned as positive for MICA antibodies using the One Lambda SAg Luminex assay were negative for MICA antibodies using the other two assays. Figure 4.6.1 compares frequencies of MICA

antibodies detected in the graft recipient cohort as a percentage of the 76 recipients who tested positive with the One Lambda SAg assay. The chart reveals that many of the positive cases defined using One Lambda's assay, in particular those with antibodies detected against MICA*001, *018 and *019, were not so frequently detected using the other two assays. MICA*001 had a frequency of 49% using One Lambda's assay compared to 17% using the Stastny assay and 26% with Gen-Probe's assay. Similarly, MICA*018 was detected in 43% of recipients with One Lambda's assay, 17% with the Stastny assay and 25% with the Gen-Probe assay. The largest discrepancy was with detection of antibodies specific for MICA*019 and detected in 71% of recipients using the One Lambda assay compared to 14.5% with the Stastny assay and 16% with the Gen-Probe assay.

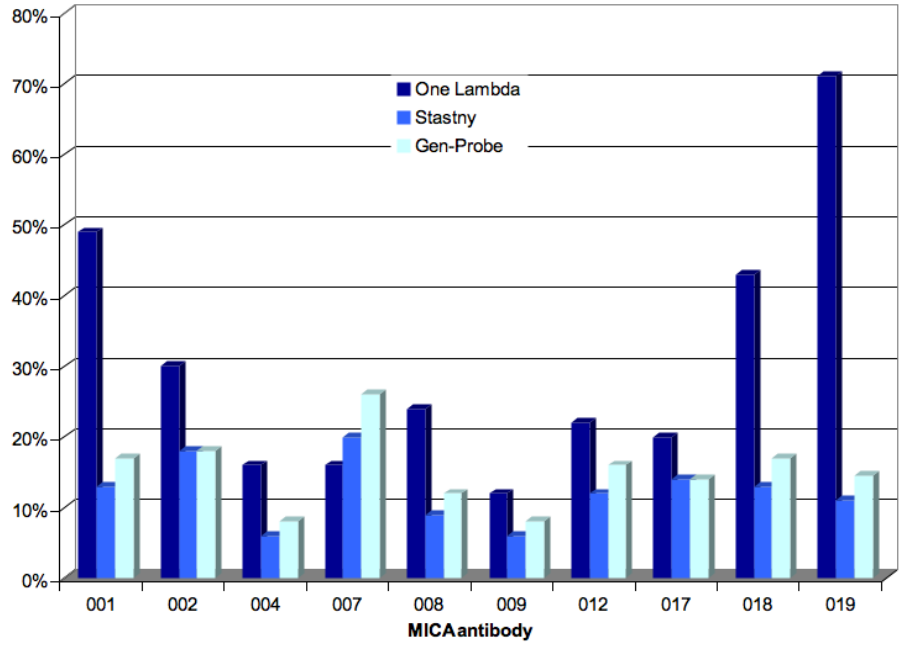


Figure 4.6.1: Comparison of MICA antibodies detected in renal graft recipients using three independent MICA single antigen Luminex assays. Percentages relate to the number of patients testing positive out of the cohort of 76 individuals positive by the One Lambda MICA SAg assay.

The overall frequencies of MICA antibodies detected in the graft recipients (n=442), untransplanted patients (n=200) and healthy controls (n=116) are shown in Figure 4.6.2. There is a clear difference between the frequencies detected using One Lambda's assay compared to the other two methods and these differences are statistically significant. In the graft recipient group, MICA antibodies were detected with a frequency of 17.2% with the One Lambda assay, 6.6% with the Stastny assay ($P<0.0001$) and 7% with the Gen-Probe assay ($P<0.0001$). In the untransplanted cohort, MICA antibodies were detected with a

frequency of 10.5% using One Lambda's assay, 1.5% with the Stastny assay (P=0.0002) and 2.5% with the Gen-Probe assay (P=0.001). MICA antibodies were detected with a frequency of 12% in healthy controls using One Lambda's assay compared to 1% with the Stastny assay (P=0.0005).

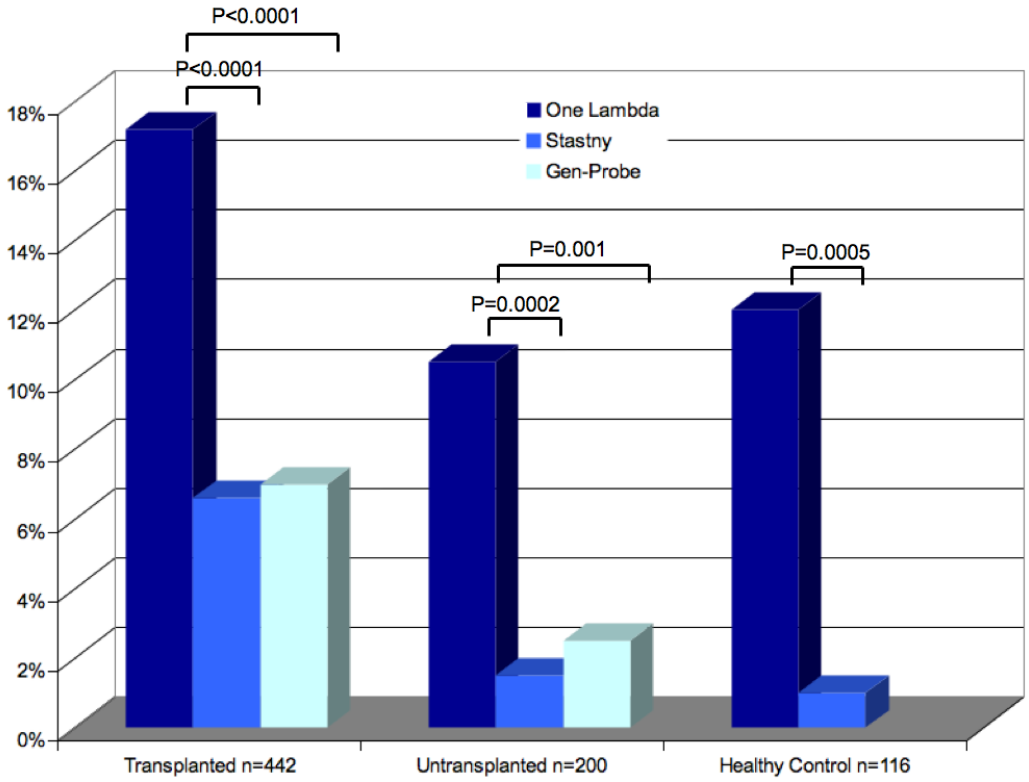


Figure 4.6.2: Comparison of MICA antibody frequencies detected with three single antigen Luminex methods. Overall frequencies of MICA antibody positive individuals among transplant recipients, untransplanted renal patients and healthy controls.

The MICA antibody reactions causing the higher frequency of MICA antibody detection using the One Lambda assay were easily identified by alignment of MFI values for each SAg bead and each assay. The main patterns of reactivity seen using the One Lambda assay but not the Stastny assay or the Gen-Probe assay (Figure 4.6.3) were with MICA*001 only, MICA*001 and *018, MICA*001 and *019, MICA*002 and *019, MICA*008 only or MICA*019 only. MICA*001 antibody only was detected in four of 76 (5%) graft recipients, one of the 21 untransplanted patients (5%) and four of fourteen healthy controls (29%). Reactions for both MICA*001 and *008 were positive for 5% of graft recipients, 14% of untransplanted patients 21% of healthy controls. MICA*001 and *019 were positive for 4% of transplanted recipients, 5% of untransplanted patients and 21% of healthy controls. MICA*002 and *019 were positive with 5% transplant recipients, 14%

untransplanted patients and one healthy control (7%). MICA*008 antibodies only were detected in 2.5% graft recipients, 14% untransplanted patients and 14% healthy controls. MICA*019 only was detected in 17% graft recipients, 29% untransplanted patients and none of the healthy controls. There was also a difference in the antibody specificities detected between the different groups. In the healthy control group, frequencies above 20% were seen with reactions involving antibodies to MICA*001. In the recipient and untransplanted patient groups, higher frequencies were observed for MICA*019 antibodies.

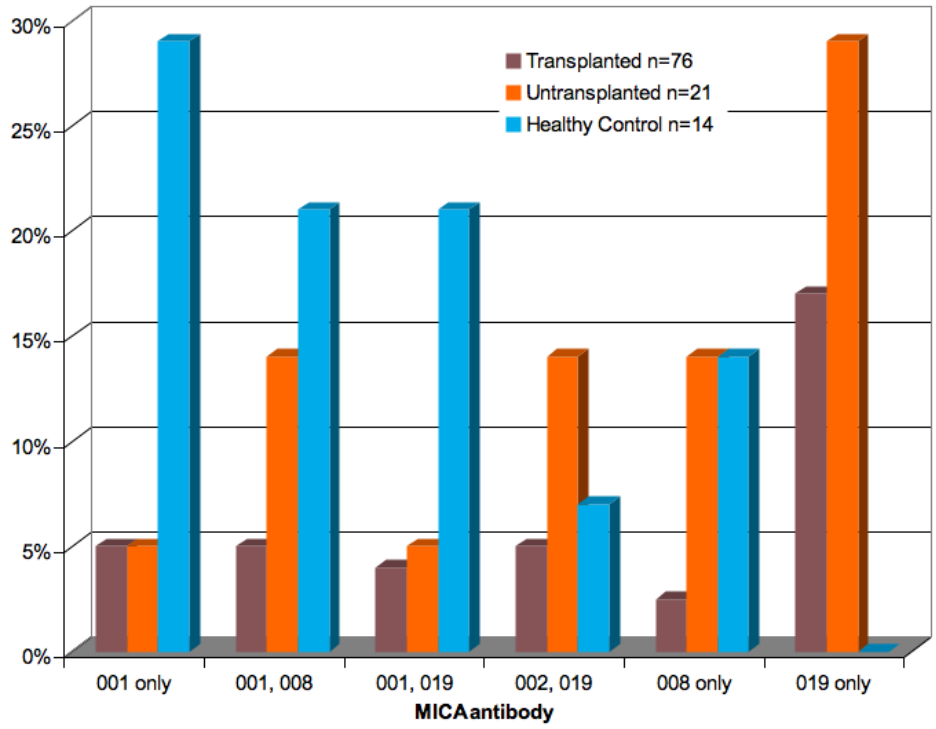


Figure 4.6.3: MICA antibody reaction patterns detected with One Lambda Luminex but not confirmed with the Stastny and Gen-Probe assays. These reactions were mainly limited to identification of MICA*001 only, MICA*001 and *018, MICA*001 and *019, MICA*002 and *019, MICA*008 only or MICA*019 only.

As there are differences between the methodologies used to detect MICA antibodies and there are no reference standards of sera with known MICA specificities, I decided to use a consensus of the three methods to assign positive or negative cases. MICA antibody positivity was assigned if at least two methods were positive for a given MICA specificity in the same serum. Using this approach the final frequencies of MICA antibodies in the three groups were as follows. In the graft recipient group, 33 individuals were positive for MICA antibodies (7.5%), in the untransplanted patient group six of 200 patients had MICA antibodies (3%) and one of the healthy control subjects had MICA antibodies (1%).

4.7 Identifying patterns of MICA antibody allorecognition of MICA antigenic determinants

Once a strategy for assigning MICA antibody status was decided and extra, possibly non-specific reactions were removed, it became easier to identify polymorphic MICA residues that may be antigenic determinants recognised by MICA antibodies. Table 4.7.1 provides an alignment of the polymorphic amino acids across the α-1 to α-3 domains of the MICA molecule. Unlike HLA molecules, where amino acid residues can be highly polymorphic, most of the polymorphic MICA residues are dimorphic. MICA polymorphism is mainly generated by inter-allelic recombination of polymorphic nucleotide motifs in exons 2, 3 and 4 encoding the extracellular α-1 to α-3 domains. Therefore, MICA antibodies could be cross-reactive and not specific for MICA allelic variants. In addition, more than one MICA antibody specificity may be present, further confounding interpretation.

After further analysis of MICA polymorphism and patterns of MICA antibody reactivity, it was possible to identify shared polymorphisms among the specificities in the sera of some patients. For example MICA*001, *012 and MICA*018 often produced positive reactions together and was reproduced by sera from different individuals. Figure 4.7.1 shows MFI values produced by the three assays with serum from one individual, using cut-off MFI values as follows: One Lambda 1000, Stastny 700, Gen-Probe 3000.

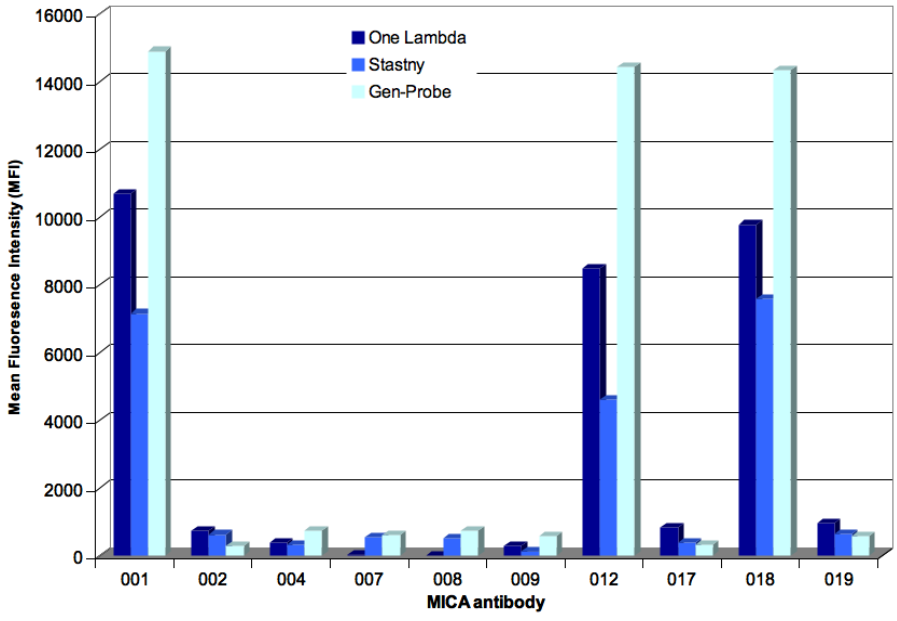


Figure 4.7.1: **Typical example of serum reactive with Thr at residue 24.** MICA antibodies reactive with MICA*001, 012, 018 antigens may have specificity for an epitope where 24T is expressed rather than 24A.

Table 4.7.1: Alignment of MICA polymorphic amino acid residues across the α-1 to α-3 extracellular regions of the MICA molecule. A '-' denotes identity with MICA*001.

MICA	6	14	24	26	36	90	91	105	114	122	124	125	129	142	151	156	173	175	176	181	206	208	210	213	215	221	251	255	268	271
001	R	W	T	V	C	L	Q	R	G	L	T	K	M	V	M	H	K	G	V	T	G	Y	W	T	S	V	Q	Q	S	P
002	-	G	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
004	-	-	A	-	Y	-	-	-	-	V	-	E	V	-	-	-	E	S	-	R	S	-	R	-	T	-	-	-	-	-
005	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	-	-	-	-	S	-	R	-	T	-	R	-	-	-
006	-	-	A	-	Y	-	-	-	-	V	-	E	V	-	-	-	E	S	I	-	S	-	R	-	T	-	-	-	-	-
007	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
008	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	-	-	-	S	-	R	I	T	-	R	-	-	-
009	-	-	A	-	Y	-	-	-	-	V	-	E	V	-	-	-	E	S	-	-	S	-	R	-	T	-	-	-	-	-
010	P	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	-	-
011	-	G	A	-	-	-	-	-	-	-	-	E	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A
012	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
013	-	G	A	-	-	-	-	-	-	-	-	E	V	-	-	-	E	-	-	-	-	-	-	-	-	-	R	-	-	-
014	-	G	A	-	-	-	-	R	-	-	-	E	-	-	-	-	E	-	-	R	-	-	-	-	-	-	-	-	-	-
015	-	G	A	-	-	-	-	R	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
016	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	L	R	-	-	-
017	-	G	A	-	-	-	R	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
018	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
019	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	-	-
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021	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
022	-	G	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	-	-
023	-	G	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
024	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	-	-	-	S	-	R	-	T	-	-	-	-	-
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026	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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033	-	-	A	-	Y	-	-	-	-	-	S	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	-	-

Table 4.7.1: Continued.

MICA	6	14	24	26	36	90	91	105	114	122	124	125	129	142	151	156	173	175	176	181	206	208	210	213	215	221	251	255	268	271
001	R	W	T	V	C	L	Q	R	G	L	T	K	M	V	M	H	K	G	V	T	G	Y	W	T	S	V	Q	Q	S	P
034	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	S	-	R	-	T	-	-	-	-	-
035	-	G	A	-	Y	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	S	-	R	I	T	-	R	-	-	-
036	-	G	A	-	-	-	-	K	-	-	-	E	-	-	-	-	E	S	-	-	-	-	-	-	-	-	-	-	-	-
037	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	S	-	R	I	T	-	R	-	-	-
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040	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
041	-	G	A	G	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
042	-	-	A	-	Y	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	S	-	R	I	T	-	R	-	-	-
043	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
044	-	G	A	-	Y	-	-	-	-	V	-	E	V	-	-	-	E	S	-	R	S	-	R	-	T	-	-	-	-	-
045	-	-	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
046	-	G	A	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-
047	-	G	A	G	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
048	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	-	-	-	S	-	R	I	T	-	-	-	-	-
049	-	-	A	-	Y	-	-	-	-	V	-	E	V	-	-	-	E	S	-	-	S	-	R	-	T	-	-	-	-	-
050	-	G	A	-	-	F	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
051	-	-	A	-	Y	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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054	P	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	G	-
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056	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	S	-	-
057	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	-	-	-	-
058	-	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	-	-	-	S	-	R	I	T	-	R	-	-	-
059	-	-	A	G	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-
060	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	L	R	-	-	-
061	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-
062	P	-	A	-	Y	-	-	-	-	-	-	E	V	-	-	-	E	S	-	-	S	-	R	I	T	-	R	-	G	-

Analysis of protein alignments (Table 4.7.1) revealed these three MICA antigens share threonine (T) at residue 24 in the α-1 domain and all other MICA antigens (used in the MICA antibody detection assays) expressed alanine (A).

Other patterns correlated with a dimorphic motif comprising a long epitope across the α-1 to α-3 domains. One motif, termed MICA-G1 is characterised by residue 36 cysteine (C), 129 methionine (M), 173 lysine (K), 206 glycine (G), 210 tryptophan (W) and 215 serine (S). Alternatively MICA-G2 epitopes share residue 36 tyrosine (Y), 129 valine (V), 173 glutamic acid (E), 206 serine (S), 210 arginine (R) and 215 threonine (T). For example MICA*001, 002, 007, 012, 017 and 018 were found to react together in different sera, correlating with MICA-G1 as shown in Figure 4.7.2.

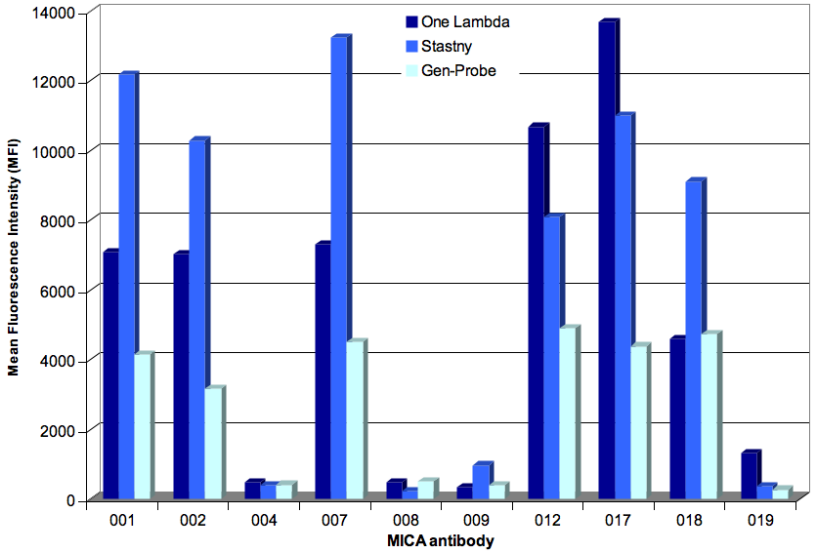

Figure 4.7.2: Typical example of patient serum reactive with MICA-G1 epitopes. MICA antibodies reacting with MICA*001, 002, 007, 012, 017 and 018 may be recognising 36C, 129M, 173K, 206G, 210W and 215S (MICA-G1) across the α1 to α-3 domains of the MICA molecule.

Conversely, MICA*004, 008, 009 and 019 would react together and correlate with the MICA-G2 motif as shown in Figure 4.7.3. The patterns could not be deduced in some samples, presumably because more than one MICA antibody specificity was present in the sera.

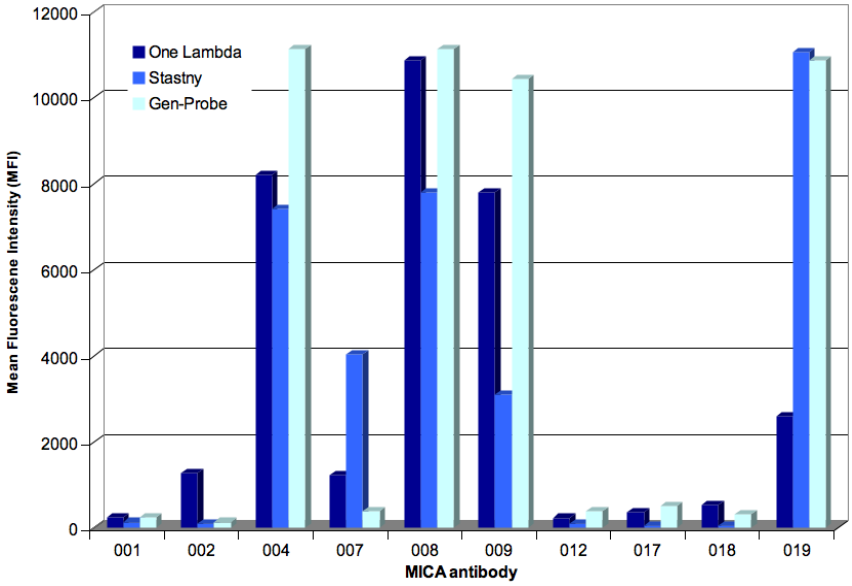

Figure 4.7.3: Typical example of patient serum reactive with MICA-G2 epitopes. MICA antibodies reacting with MICA*004, 008, 009 and 019 may recognise 36Y, 129V, 173E, 206S, 210R and 215T (MICA-G2).

The most common patterns of antibody recognition found by this analysis are shown in Figure 4.7.4. In some cases the reaction patterns belonged to single MICA specificities but often the patterns were mixed and reflected two or more specificities in the same serum.

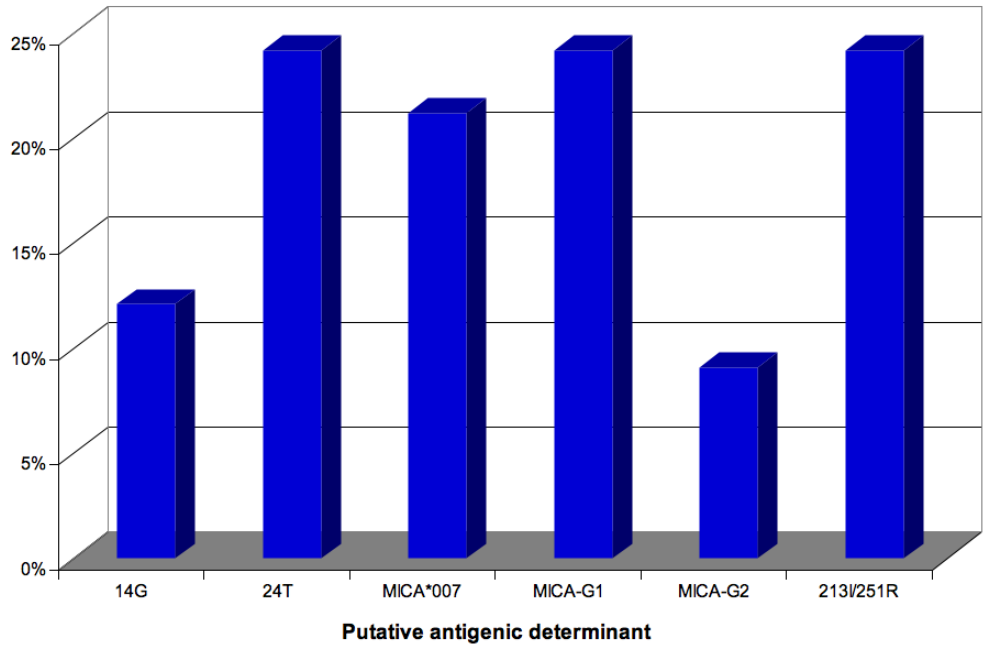

Figure 4.7.4: Frequencies of different MICA antibody recognition patterns. Shared polymorphisms giving rise to putative antigenic determinants in 33 renal graft recipients confirmed as positive for MICA antibodies by three assays.

Starting from the α-1 domain and moving towards the α-3 domain, they were G at residue 14, T at residue 24, MICA*007 with unknown specificity, MICA-G1, MICA-G2 and a specificity unique to MICA*008 and *019 with isoleucine (I) at residue 213 and R at residue 251. The 14G specificity was identified in the sera of 12% of graft recipients positive for MICA antibodies and MICA antibody recognition of 24T was observed in 24%. MICA antibodies reacting with the MICA-G1 epitope were detected with a frequency of 24% and the MICA-G2 specificity was present in 9%. The 213I/251R specificity reacted with MICA*008 and *019 in 24%. Antibodies possibly having more than one MICA specificity were more difficult to characterise. For example, as shown in Figure 4.7.5, this patient's serum had reactions with all MICA beads except MICA*002 and MICA*017 (patient is MICA*002 homozygous) which appears to be a reaction to W at residue 14, however analysis of the differential MFI values indicates that at least two MICA antibodies may be present, one specific for 24T and the other for MICA-G2 epitope.

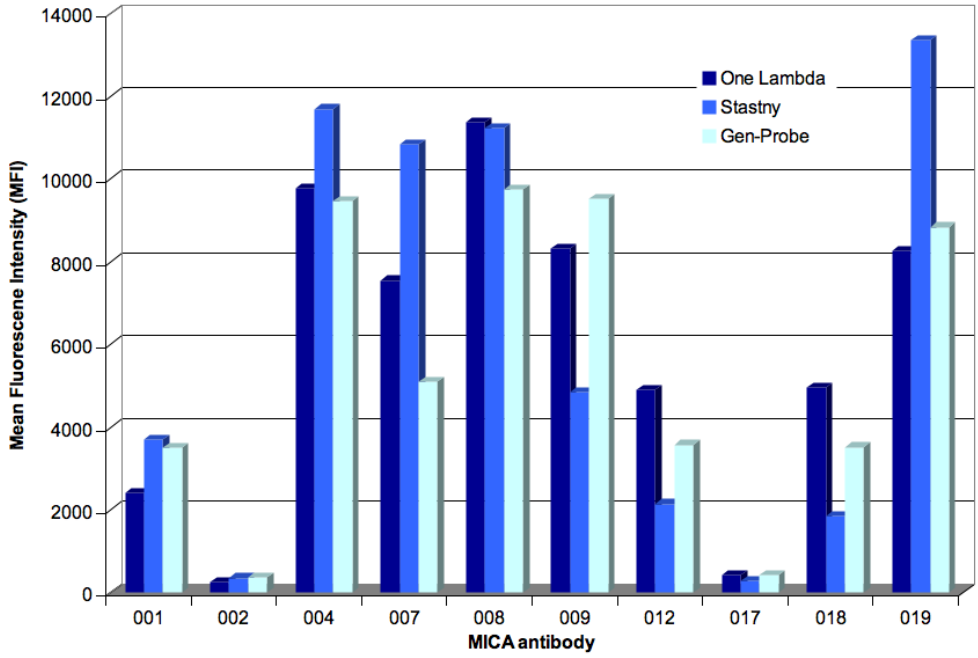

Figure 4.7.5: Typical example of a patient serum with possible mixed MICA antibody specificity 1. This serum appears to react with all MICA single antigen beads except MICA*002 and MICA*017. However, it can be seen that there are two MICA specificities with different strengths (titres). One specificity relates to Thr at residue 24 (MICA*001, 012 and 018). The other specificity relates to alleles with MICA-G2 epitopes (MICA*004, 008, 009, 019). MICA*007 may be an extra reaction.

MICA*007 is also positive and, although not seen in isolation, reactivity with this antigen was fairly common (21% of MICA antibody positive cases) and could accompany any of the other specificities with any of the Luminex assays used.

There were some reactions showing MICA*001 specificity, in addition to other MICA specificities, for example MICA-G2, as shown in Figure 4.7.6. These MICA antibodies could be reacting with epitopes defining MICA-G2 and also L at residue 125, a polymorphism unique to MICA*001 with the assays used but is also seen with MICA*031 and *040 alleles, although rarely. There are three other MICA antigens that may be detected by allele-specific MICA antibodies. MICA*012 has L at residue 156, MICA*017 has R at residue 91 and MICA*004 has R at position 181. Allele-specific reactions for MICA*012 or MICA*017 were not observed, unless masked by other specificities, however there was one clear example showing only reactivity with MICA*004 beads and, could be recognising R at residue 181 and is shown in Figure 4.7.7. Also seen in this serum are extra, non-specific reactions only seen with the One Lambda assay, as described earlier.

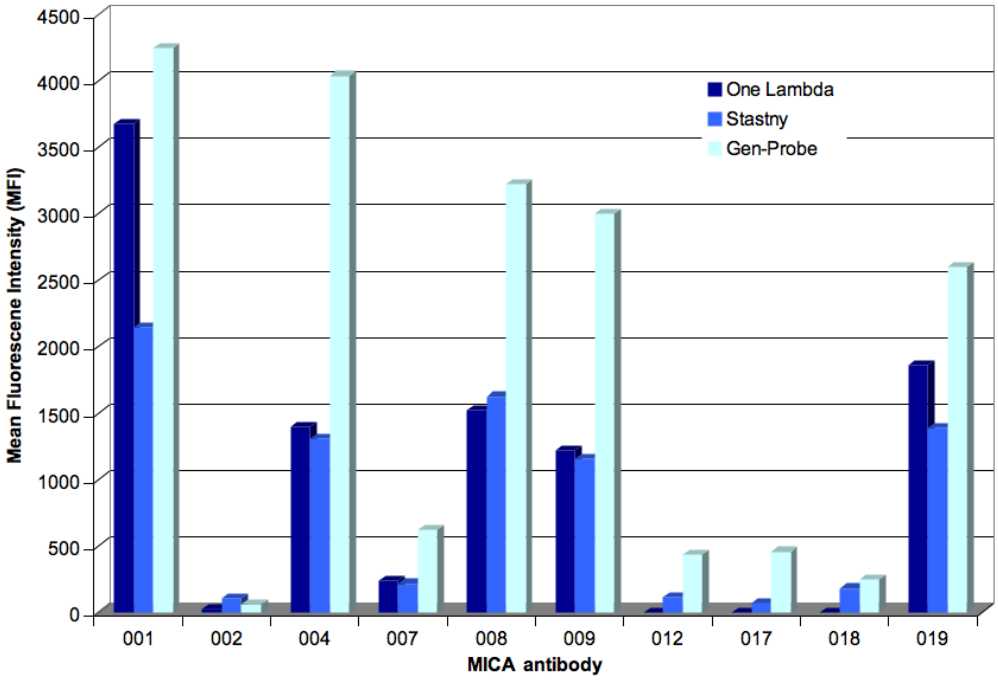

Figure 4.7.6: Typical example of a patient serum with possible mixed MICA antibody specificity 2. Mixed MICA antibody specificities, that may be recognising Lys at residue 125 (unique to MICA*001) and MICA-G2 alleles (MICA*004, 008, 009 and 019).

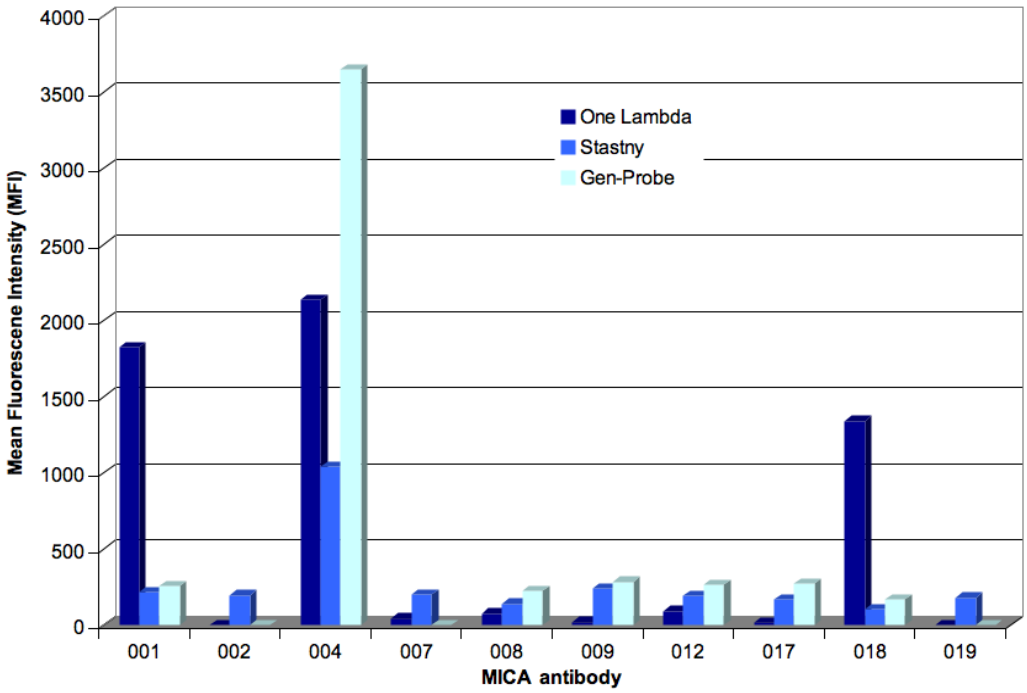

Figure 4.7.7: Example of a patient serum reactive with only one MICA antigen. All three Luminex assays show positive reactions with MICA*004 single antigen beads and patient serum, possibly reactive with Arg at residue 181. One Lambda single antigen beads also react with MICA*001 and MICA*018 in this serum and was a frequent observation with this assay only.

4.8 Discussion

When this study began in 2005, little was known of the frequency of MICA antibodies in healthy individuals, patients with ESRD or renal graft recipients, although it was generally agreed that many solid organ transplant recipients who rejected their grafts had MICA antibodies detected in their serum. Studies up to that point had used either CDC with fresh 721.221 or HMY2.C1R cells transfected with cDNA from MICA alleles (Mizutani *et al*., 2005; Sumitran-Holgersson *et al*., 2002) or recombinant MICA proteins produced in *E coli*, immobilised to wells of a plastic plate to detect MICA antibodies using ELISA (Zwirner *et al*., 2000). My own attempts to produce recombinant MICA molecules from *E. coli* for use in an ELISA test were unsuccessful but, with the release in 2006 of a commercial MICA antibody detection Luminex assay from leaders in the field One Lambda, MICA antibody detection became possible. I could now reliably detect MICA antibodies in our patient and healthy control cohorts and, together with MICA typing of patients and their donors, gain novel insights into the relationship of MICA allele mismatching with the production of MICA antibodies.

4.8.1 MICA antibody frequencies using One Lambda screening and SAg beads

It became apparent, whilst using the One Lambda mixed bead screening and SAg Luminex beads, that the frequency of MICA alleles detected by screening beads was much higher than was being confirmed by the SAg beads. For example, 34% of all graft recipients had positive reactions using screening beads but only 17% using One Lambda's SAg beads, which is approximately half of those originally positive for MICA antibodies. This could have been an indication that the cut-off threshold for the normalised background ratio, based on manufacturer's recommendations, was too low. Upon further analysis, the cut-off ratio was raised to 4.2 from 2.2 since there were no confirmed positive reactions with MICA antibodies below a ratio of 4.2. However, this only accounted for a small percentage of those identified as MICA antibody positive by the screening assay and by raising the threshold further, true positives could be excluded.

The MICA antibody profiles identified by One Lambda SAg beads did not correlate with expected frequencies based on MICA allele frequencies. Why was the MICA*001 antibody detected with high frequency in healthy controls and why was MICA*019 so frequent among the MICA positive renal graft recipients? MICA*001 has an allele frequency of 3% in Caucasoid and non-Caucasoid populations (Petersdorf *et al*., 1999), 2.5% in Caucasoids (Y. Zhang *et al*., 2002) and 15% in Northeastern Thais (Romphruk *et al*., 2001). Since there is unlikely to be a high proportion of Northeastern Thais among UK organ donors, the low frequency of these alleles is not concordant with the high frequency of MICA antibodies directed against their antigenic products. Additionally there were also several instances where graft recipients had MICA antibodies detected against their own MICA antigens, which should not happen unless the patient's primary disease was autoimmune, for example systemic lupus erythematosus. One Lambda's UK representatives were unable to answer my questions regarding these anomalies however, they did say that other groups using the SAg Luminex assay had also found high frequencies of MICA*019 antibodies.

Nevertheless, I continued with analysis of MICA mismatching and other transplant variables and correlating association with MICA antibodies, and submitted an abstract for the 15th IHIWC that was to be held in Brazil in September, 2008. My abstract was accepted for an oral presentation in a session chaired by Professor Peter Stastny. I attended the MICA component workshop discussion groups, comprised of well-known individuals from the field of H&I, and organised by Peter Stastny. After showing my results, the unanimous verdict from the group was that the MICA*019 antigen on One Lambda's SAg Luminex

beads was very over-reactive and, not only MICA*019, but other antigens as well. Suggestions for possible reasons for these 'false positives' were natural antibodies in the serum that could recognise exposed epitopes on denatured or partially folded MICA molecules. As I observed with my experiments to produce soluble MICA molecules in different systems, most of the MICA protein that was isolated from mammalian and insect cells was denatured and not in the native form. Alternatively, MICA molecules produced by cleavage from MICA transfected cells may partially unfold during the manufacturing process and expose epitopes that cross-react with natural antibodies. It was suggested at the workshop that I should analyse my data again and exclude the data obtained by the MICA*019 bead.

During the general discussion following my presentation, Professor Stastny, who chaired the session, commented that my MICA frequencies were high and that patients should not have antibodies towards their own MICA antigens, and this was the result of using the One Lambda Luminex assays. In a private discussion between Professor Stastny, my PhD supervisor and myself, Professor Stastny offered to test all of our positive sera with their beads at their laboratories in the US. The bead assay he referred to was that used to detect MICA antibodies in patient's serum for the MICA component of the workshop, constructed using recombinant MICA molecules produced in Sf9 insect cells.

4.8.2 MICA antibody frequencies using Stastny's MICA Luminex single antigen assay

I was initially very surprised that when I received the results from Professor Stastny's lab, as many of those positive by One Lambda's SAg assay, including all that were positive for MICA*019 only, were negative with their assay. Overall frequencies of MICA antibodies in the transplanted, untransplanted and healthy control groups were considerably lower than previously determined with the One Lambda assay. For example, in the graft recipient group, there was a MICA antibody positive frequency of 7% with the Stastny MICA SAg assay compared to 34% with One Lambda SAg beads. There were also noticeable differences in individual bead frequencies between the two assays. Detection of MICA*001 in positive serum (n=76) using Stastny's assay was down from 49% to 17%, MICA*018 was positive for 17% recipients with Stastny's assay compared to 43% with One Lambda and MICA*019 was detected in 14.5% of recipients using Stastny's SAg assay compared to the very high frequency of 71% with One Lambda's SAg assay. Interestingly, detection of MICA*007 was increased with Stastny's assay from 16% to 26%. This testing confirmed my original concerns, that One Lambda's mixed antigen and

SAg MICA antibody detection assays may be producing false positive reactions. Frequencies of individuals with MICA antibodies were also considerably reduced in the untransplanted group and healthy controls. Of the 200 untransplanted individuals, 23% had MICA antibodies detected with the One Lambda SAg assay but was only 1.5% when tested with Stastny's assay. Similarly, 28% of healthy controls were positive for MICA antibodies with One Lambda's SAg assay compared to 1% (only one individual) with Stastny's assay and none of the healthy control sera with reactions against MICA*001 were confirmed.

4.8.3 MICA antibody frequencies using Gen-Probe MICA Luminex single antigen assay

Another commercially available MICA SAg Luminex assay became available towards the end of 2008, manufactured by Tepnel Lifecodes (now Gen-Probe), however a screening assay was not released. This assay included a further eighteen MICA specificities on SAg beads for approximately the same cost as One Lambda's SAg assay. It was decided to also re-test all those originally positive with One Lambda SAg beads using this assay. Results were, in general, similar to those obtained using Stastny's SAg bead assay although there were differences in individual beads frequencies. MICA*001 was detected with a frequency of 26% in transplant recipients using Gen-Probes's assay but was 17% with Stastny's assay and MICA*018 was detected in 25% graft recipients with Gen-Probe's SAg beads compared to 17% with the Stastny assay. Again, none of those with antibodies to MICA*019 alone detected using One Lambda's assay were confirmed as positive with this method. The overall frequency of MICA antibodies detected in graft recipients using Gen-Probe SAg beads was 7%, the same frequency found by Stastny's SAg beads.

4.8.4 Comparison of three MICA single antigen Luminex assays

By comparing the results obtained by each of the MICA SAg bead assays, it became apparent that there were differences, some subtle and others obvious. Figure 4.6.1 shows frequencies for each of the MICA SAg beads detected with each of the three assays. The most obvious observation is that MICA*001, 018 and *019 are all much higher when One Lambda's SAg assay is used compared to the other two methods. Comparing overall frequencies between the three assays for the three different groups of subjects, the difference between One Lambda frequencies and those obtained by testing with Stastny's or Gen-Probe's MICA SAg beads was statistically significant. Some reaction patterns were only seen using the One Lambda assay and Figure 4.6.3 compares their frequencies in transplanted, untransplanted renal patients and healthy control subjects. This showed a

difference in the specificity of these 'MICA' antibodies between the different groups of patients. Although I have confirmed that these types of reactions are not real MICA specificities, what could be causing this reactivity?

4.8.5 Possible reasons for false positive reactions using One Lambda Luminex assays

As I mentioned already, it was suggested at the IHIWC MICA component discussion group that denatured or partially folded MICA molecules could be the cause of some non-specific reactions. Another explanation could be that antibodies induced by epitopes found in microorganisms, ingested proteins or allergens cross react with antigenic MICA epitopes, and there is plenty of evidence for this with HLA antigenic determinants. It was first reported 40 years ago that the streptococcal M1 protein inhibits cytotoxic reactions with seven different HLA specificities, inferring that the M1 protein has a structure common to HLA antigens (Hirata and Terasaki, 1970). Also, some de-esterified lipopolysaccharides from *E. coli* and *Salmonella* have been shown to inhibit seventeen different HLA specificities (Hirata *et al.*, 1973). The crystal structure of staphylococcal entertoxin that forms a complex with HLA class II antigens has been identified (Fernandez *et al.*, 2006) and it has been reported that *Klebsiella pneumoniae* antibodies can cross react with HLA-B27 (Archer, 1981). Luminex technology now makes it possible to study these types of cross-reacting antibodies and a study published in 2008 (and presented by Paul Terasaki at the 15th IHIWC) investigated the frequency of 'natural' HLA antibodies in non-alloimmunised healthy male blood donors (Morales-Buenrostro *et al.*, 2008). The possibility of these reactions being a consequence of artefacts on the SAg beads was eliminated by repeated testing and observation that the same specificity and the same antibody 'strength' was produced each time. Also, to eliminate the possibility the reactions were particular to the use of One Lambda SAg beads, they also used beads from Gen-Probe, with the same outcomes. It is interesting that the HLA specificities found in these blood donors are rare such as A*3002, A*8001 and B*1512 and match those found in pre-transplant patients who were not expected to be sensitised (Idica *et al.*, 2006). The high frequencies of MICA*001 and MICA*019 found in healthy controls and renal patients in my study are also unexpected because these alleles are relatively rare. Their detection could also be a consequence of cross reactivity with micro-organism-derived antibodies in a similar manner. However, this argument does not hold because the reactions with MICA*001 and MICA*019 were not reproduced using other independent Luminex assays.

It is, therefore, more likely that the unexpected reactivity seen when using the One Lambda assay is caused by antibodies (which could be generated as described above) recognising linear epitopes exposed by partially folded or denatured MICA molecules. The high frequency of reactivity against the MICA*019 SAg bead in the One Lambda assays has now been reported by other groups facing the same problem as myself. Smith *et al* postulate cross-reactivity with bacterial or endemic entities (Smith *et al*., 2009) and exposed epitopes on denatured MICA*019 antigens in the One Lambda assay may be responsible not only for the high frequency of detection but also for the relatively large incidence of autoantibodies. These autoantibodies were against MICA*008 and MICA*019 and in their study, MICA*019 was also more frequently detected using the One Lambda SAg assay. In another study aimed at identifying epitopes and immunodominant regions on the MICA molecule, MICA*019 was also found at unexpectedly high frequencies using the One Lambda SAg assay for MICA antibodies (Suarez-Alvarez *et al*., 2009a). Using their method of epitope mapping, they state that antibodies reacting with MICA*019, often in isolation as with my study, may be recognising a linear epitope exposed by misfolding of the MICA*019 molecule.

It seems logical, therefore, that these 'extra' reactions seen when using the One Lambda SAg Luminex assay are indeed caused by natural antibodies reacting with epitopes exposed by protein misfolding. But why do different groups of patients and healthy controls have differential recognition patterns? The false MICA*019 only reaction was very frequent in renal patients, either wait-listed (some of these would have had a previous transplant) or transplanted but absent in healthy controls. Conversely, MICA*001 only reactions were much more frequent in healthy controls. It is possible that reactions involving MICA*019 arise from antibodies generated by events associated with transplantation such as other mismatched polymorphic antigens or perhaps from immunosuppression therapy or transfusions. It is unlikely to be specific to renal disease as the same observations with MICA*019 were also made with heart transplant patients (Suarez-Alvarez *et al*., 2007). Perhaps the higher frequency of false reactivity with MICA*001 seen in healthy controls reflects antibodies generated against allergens. The mean age of the control group was 33.8 ± 9.9 compared to 42.2 ± 16.0 in recipients or 51.9 ± 13.8 in untransplanted patients. Younger people are more likely to have allergies that may be due to an increase in prevalence of allergy to ingested proteins (Sicherer *et al., 2010*) and other environmental factors. The healthy control subjects were potential bone marrow donors and, incidentally, many of them mentioned allergy in medical questionnaires. Whatever the reason for natural antibody production, cross-reactivity with exposed epitopes on denatured or partially folded

MICA molecules, observed when using the One Lambda beads, seems to be the most likely cause of non-specific reactivity.

4.8.6 Identifying patterns of MICA antibody specificity

Cracking the code of MICA epitope recognition by MICA antibodies seemed a daunting task when faced with the results from One Lambda Luminex mixed and SAg beads. Even assuming that MICA antibodies recognise their antigenic determinants in the same way as HLA antibodies, by cross-reactive recognition of shared polymorphism and recognition of unique epitopes on the surface of the molecules, there were often no clear-cut patterns that made sense. The main problem, as it turned out, were the extra reactions or false positives, which confounded this analysis. Removing these reactions by confirmatory testing with two other methods revealed a much clearer picture and the patterns of MICA antibody reactivity could be related to the protein structure of the MICA molecules. Even so, this analysis would have been only hypothetical and anecdotal in isolation. However, there are currently three separate publications directly addressing the issue of MICA epitope specificity, which corroborate with and give credence to the findings of this study.

The first paper to emerge and shed light on how MICA antibodies recognise MICA epitopes was published by Rene Duquesnoy in collaboration with Tepnel Lifecodes Inc. (Duquesnoy *et al*., 2008). Rene Duquesnoy is well-known in the field of H&I for his work with the HLAMatchmaker computer algorithm that predicts HLA mismatching based on structurally defined HLA mismatches. The algorithm utilises the concept that an HLA (or MICA) epitope is represented by a configuration of polymorphic surface residues with other residues within a radius of around 3 Ångströms. The term 'eplet' describes a potentially functional epitope consisting of polymorphic and conserved residues. This analysis shows that most MICA eplets are single residues and in five cases, two polymorphic MICA residues are close to each other, constituting a single eplet.

The second paper was published a few months later (Zou *et al*., 2009) and provided proof of MICA antibody specificity. This well designed study utilised HMY2.C1R cells transfected with MICA*001, 002, 008 and 009 in absorption and elution experiments using well characterised patient sera testing positive for MICA antibodies. Specificities were determined by testing with their MICA SAg Luminex assay, described above. To define key epitopes involved in recognition of MICA antigens recognising the two broad groups of antigens, MICA-G1 and MICA-G2, hybrid recombinant MICA proteins were constructed

having part of MICA*002 (MICA-G1) and part of MICA*008 (MICA-G2). Two other hybrid MICA molecules were constructed one was MICA*002 with a change of K to E at residue 173 and MICA*008 with residue 129 changed from V to M. The patterns of reactivity after absorption and elution of MICA antibodies in these experiments provide evidence of MICA antibody specificities.

Six months later, a study was published which analysed the sera of MICA antibody positive renal transplant patients and mapped MICA epitopes by screening with a synthetic library of overlapping peptides from the extracellular domains of the MICA molecule (Suarez-Alvarez *et al*., 2009a). In this study, One Lambda mixed antigen and SAg beads were used to identify patients with MICA antibodies. They found nine antigenic regions were reactive with MICA antibodies and four of those epitopes mapped to polymorphic MICA residues.

To enable a more thorough understanding of the results obtained from this analysis, I will discuss them in the context of the findings from the published work. Table 4.8.1 is based on a table published by Zou and shows, with the use of different colours, the various specificities of MICA antibodies that can be present in serum as confirmed by MICA antibody absorption and elution experiments (Zou *et al*., 2009). Figure 4.8.1 shows the polymorphic MICA residues on the 3D space-filling MICA molecule shown in 3 views. Table 4.8.1 and Figure 4.8.1 only show the ten MICA antigens used in the assays but, other antibody specificities with the same polymorphisms may also be detected.

MICA antibodies reacting with 14G were identified by this analysis and were characterised by positive reactions for MICA*002 and MICA*017 bearing beads. This residue is prominent on the surface of the MICA molecule, located on the β-strand in the α-1 domain and forms the start of a coil loop, which is highly exposed. The study by Zou and colleagues showed reactivity to MICA*002 and MICA*017 with patient sera was removed by adsorption with MICA*002 transfected cells, however the authors did not confirm allorecognition of the alternate 14W as no examples were found. The study by Suarez-Alvarez *et al*, however, did observe reactivity in MICA positive patient serum with synthetic peptides that included W at residue 14, although these were linear peptides and the conformational epitope may be different.

Another amino acid in the α-1 domain of the MICA molecule that frequently appeared to be reactive with MICA antibodies was 24T, characterised by reactivity with MICA*001, 012, and 018 SAg beads. Analysis using the MICA Matchmaker algorithm (Duquesnoy *et al*.,

2008) revealed residues 24 and 36, both polymorphic, come together and comprise the same MICA eplet. Although both residues 24 and 36 are only partially visible on the surface of the MICA molecule, the substitution of T for A is non-conservative with polar differences while C and Y differ structurally, therefore MICA antibodies may be reactive with both substitutions. The study by Zou and colleagues found that sera reacting with MICA*001, 012 and 018 beads lost reactivity with all MICA single SAg in their assay when incubated with cells transfected with MICA*001, confirming that the polyspecificity was linked to a shared epitope.

Several sera were identified with reactivity against MICA*008 and MICA*019 antigens on SAg beads and share polymorphic residues at positions 213 (I) and 251 (R). This was confirmed in the study by Zou as it was demonstrated that reactivity to both antigens could be removed by adsorption with MICA*008 transfected cells. Both amino acid substitutions are non-conservative, result in polarity differences and are prominent on the surface of the MICA molecule.

Table 4.8.1 shows MICA alleles arranged according to sequence similarity forming long epitopes differentially recognised by MICA antibodies, dividing the MICA alleles into two broad immunodominant groups.

Table 4.8.1: Protein alignment of polymorphic MICA residues shown experimentally to be involved in allorecognition by MICA antibodies. Each colour represents a different antigenic determinant that can be recognised by MICA antibodies. Modified from Zou *et al.*, 2009 (Permission granted by Springer).

Domain:	α-1			α-2								α-3				
Residue:	014	024	036	091	122	125	129	156	173	175	181	202	210	211	215	251
Antigen																
MICA*001	W	T	C	Q	L	K	M	H	K	G	T	G	W	T	S	Q
MICA*012	-	-	-	-	-	E	-	L	-	-	-	-	-	-	-	-
MICA*018	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*007	-	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*002	G	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*017	G	A	-	R	-	E	-	-	-	-	-	-	-	-	-	-
MICA*004	-	A	Y	-	V	E	V	-	E	S	R	S	R	-	T	-
MICA*009	-	A	Y	-	V	E	V	-	E	S	-	S	R	-	T	-
MICA*008	-	A	Y	-	-	E	V	-	E	-	-	S	R	I	T	R
MICA*019	-	A	Y	-	-	E	V	-	E	S	-	S	R	I	T	R

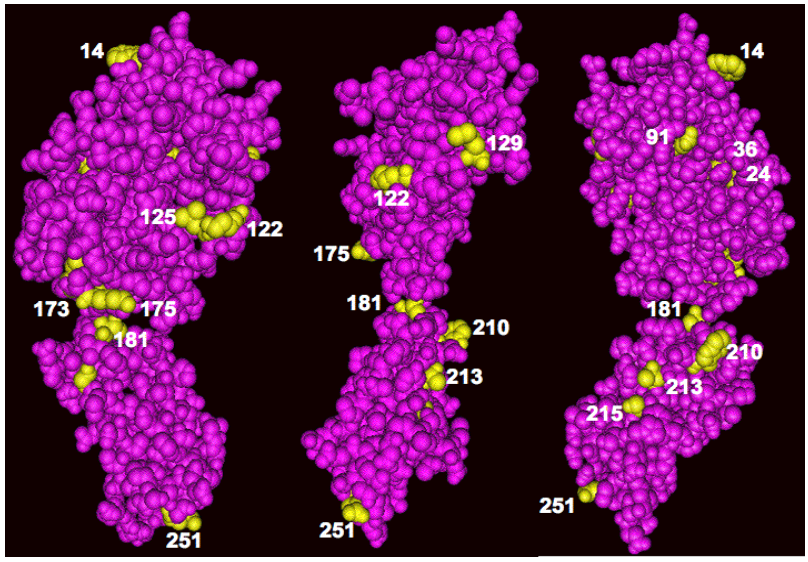

Figure 4.8.1: Polymorphic MICA residues on the MICA molecule shown in 3 views. Based on polymorphic residues of MICA*001, *002, *004, *007, *008, *009, *012, *017, *018 and *019. Stereochemical modelling of MICA*001 using CN3D (PDB code 1B3J).

It could be postulated that the differences in the α-3 domain could be responsible as they are in close proximity but this is not certain. In the study by Zou (Zou *et al*., 2009), reactivity by two different types of MICA antibody was confirmed by removing reactivity with antigens expressing 36C, 129M, 173K, 206G, 210W and 215S (MICA-G1) by adsorption with cells transfected with MICA*001 and MICA*002. The reactivity with

MICA antigens expressing 36Y, 129V, 173E, 206S, 210R and 215T (MICA-G2) could be removed by adsorption with cells transfected with MICA*008 and MICA*009. Mutant MICA proteins were produced, having part of MICA*002 and part of MICA*008. Other MICA mutants had position 173E changed from 173K on a MICA*002 backbone and 129M changed from 129V on a MICA*008 backbone. These constructs were used to try and eliminate some of the reactivity to the long epitopes. It was concluded from these experiments that positions 129 and 173 were not essential for MICA antibody recognition but MICA-G1 antibodies recognised polymorphisms in the α-1 region (36C) and/or the α-3 region (206G, 210W, 215S) and MICA-G2 antibodies had specificity for polymorphisms in the α-3 region (206S, 210R, 215T). It was found by Duquesnoy that position 206 and 210 form part of the same eplet (Duquesnoy *et al*., 2008) and although residue 206 is only barely visible, residue 210 is highly prominent on the cell surface, whereas residue 215 is some distance away and would not be accessible to the same antibody (Duquesnoy *et al*., 2008).

Both analyses described above were performed before more informative MICA beads were included in the MICA SAg array provided by the Gen-Probe assay (formerly Tepnel). When I started using this kit the SAg array had been increased to 28 and included beads for antigens that have interesting polymorphisms. Notably, MICA*028 and and MICA*051 are essentially MICA-G1 type molecules but have 36Y as opposed to 36C. Analysis of MICA-G1 and MICA-G2 antibody reactivity detected using the Gen-Probe assay revealed that all sera with MICA-G1 antibodies reacted with all MICA-G1 SAg beads, except MICA*028 and 051. By contrast, all sera with MICA-G2 reactivity also included positive reactions for MICA*028 and 051. Therefore these antibodies must be specific for either 36C or 36Y. This is contrary to the absorption and elution experiments performed by Zou and colleagues, where mutant transfectants with 36Y, that must have resembled MICA*028 and 051, failed to react in this way (Zou *et al*., 2009). Possibly the mutant transfectants were not performing correctly and the specificity of MICA-G1 and MICA-G2 antibodies requires clarification.

Only a few MICA alleles (in this analysis) differ by a single amino acid detectable by allele specific MICA antibodies. Examples were found for allorecognition of 125K which is unique to MICA*001 and also examples of MICA antibodies directed against 181R, unique to MICA*004. Antibodies to MICA*012 or MICA*017 reacting with 156L or 91R respectively, were not found or were present in combination with other antibodies and could not be determined. There were also no examples of allele-specific MICA antibodies against

MICA*012 or MICA*017 found by Zou and colleagues (Zou *et al*., 2009) even though many thousands of different sera that were tested, leading to the conclusion that if they exist they are extremely rare.

4.8.7 Differences of MICA antibody specificity using different MICA Luminex assays

Comparisons of MFI values between the different tests revealed some possible false negativity with the Stastny and the Gen-Probe MICA SAg assays. There were several examples where detection of antibodies specific for 14G was weak or negative using the Gen-Probe assay, but clearly positive with the other two assays. Other examples showed weaker reactivity with MICA-G1 specific antibodies using the Gen-Probe assay, where reactions with all or some of the MICA-G1 antigens were weak and below the cut-off MFI threshold, compared to the other two tests. Similarly, unexpected negative results were also observed by analysis using the Stastny MICA SAg Luminex. In three of the eight sera where MICA antibodies recognised 24T, the results using the Stastny assay were completely negative but clearly positive by the other two methods. Also, in one serum where antibodies reacted with MICA-G2 with the One Lambda and Gen-Probe assay, the result was negative with the Stastny assay. I also observed that when positive results had MFI values that were very high, for example, over three times the recommended MFI cut-off value for MICA positivity, then all three assays produced concordant results that correlated extremely well with MICA antibody epitope recognition.

The reactivity with the MICA*007 antigen was very frequent but unexpected. This bead was often positive with all three assays when accompanied by other specific reactions but never occurred in isolation. Reactions where MICA antibody epitope recognition of MICA-G2, 24T and 213I/251R were identified usually included reactions with the MICA*007 bead, although there are no shared epitopes that could be cross-reactive. The only explanation, based on the evidence so far, is that there is a conformational epitope unique to MICA*007 that can cross-react with other MICA antibody specificities.

To understand MICA sensitising events in renal transplant recipients, it is necessary to have information regarding the MICA allele types of the recipient and the donor of the graft. There are currently no studies of more than a few patients regarding MICA types of both the patient and donor, therefore I have determined the MICA allelic and antigenic compatibility in 227 recipient and donor pairs and the results are presented in the next chapter.

CHAPTER 5

MICA allele mismatching, production of MICA antibodies and acute rejection in renal transplantation

5.1 Introduction and aims

Despite numerous studies aimed at identifying the presence of MICA antibodies in renal graft recipients, to date there has been very little MICA genotyping of the recipients and even less data is available of donor MICA genotypes. Studies published so far have focused on the relationship of pre-transplant or post-transplant MICA antibodies and associations with acute or chronic graft rejection, but lack information regarding the MICA types of the donors. The MICA gene is polymorphic and as with HLA, this polymorphism leads to allelic and antigenic mismatches between patient and donor. It is known that HLA antigen mismatches can lead to development of DSA against mismatched epitopes expressed on donor organs. However, due to the paucity of information available, it has not been proven that renal graft recipients produce antibodies specific for mismatched epitopes of MICA antigens or that mismatching MICA alleles leads to development of MICA antibodies.

HLA antibodies have been shown by many studies to associate with AR in the early post-transplant period. Both T-cell mediated (ACR) and humoral graft rejection (aAMR) have been implicated. ACR occurs in 10-25% of renal graft recipients, commonly within the first three months post-transplant and more than 90% of AR episodes develop in the first six months. ACR is unusual within the first five days of transplantation as the cellular immune response requires at least this long to develop, however aAMR can occur within the first week, particularly if pre-formed donor-reactive antibodies are present and the risk associated with HLA antibodies is avoided by pre-transplant cross-matching (Torpey et al., 2010, p. 126). AR can be avoided with modern immunosuppression in most cases but is highly associated with reduced graft function and survival.

The presence of MICA antibodies has been implicated with AR but whether or not this associates with MICA-DSA has not been established. Knowledge of MICA types of the donor, together with information regarding the specificities of MICA antibodies produced by the recipient is essential in assessing the relationship between MICA allele mismatches

and the production of antibodies to mismatched MICA epitopes. In the 50 years since the emergence of renal transplantation, much has been learned of the nature of HLA antibody recognition of mismatched HLA antigens. HLA antigens can be categorised into groups based on the sharing of epitopes that are known to induce the production of HLA antibodies. Thus, HLA antibodies can cross-react with different HLA antigens, usually encoded by the same locus, but not always, on the basis of having a shared antigenic epitope. This knowledge enables prediction of mismatches that may be antigenic, and therefore dangerous, and also those that can be tolerated. This is essential for preventing HAR in solid organ transplantation as discussed in Chapter 1. Because HAR has been known to occur in the absence of HLA-DSA, pre-existing antibodies to MICA, which are donor-specific, may also have the potential to initiate this type of rejection, particularly if the antibodies are present in high-titre. There is also the potential for pre-formed MICA antibodies to initiate aAMR in the early post-transplant period. The development of DSA post transplant can associate with AR episodes during the first year of engraftment and, over a period of time, can associate with CAD impairing function and eventually leading to a loss of adequate renal function and return to haemodialysis.

Therefore, to fully understand the role of MICA antibodies in solid organ transplantation, MICA allele typing of both recipients and their donors is paramount. So too is the reliable assignment of MICA antibody status and specificity. To this end, the aims of this chapter are to determine the MICA alleles present in renal transplant recipients and donors for as many transplant pairs as possible. To enable high-resolution detection of MICA antibodies in the sera of renal graft recipients, a sensitive flow-cytometry based technique was used to screen patients utilising mixed MICA antigens attached to micro-sphere beads and to identify the specificities of the MICA antibodies, beads with single MICA antigens attached were used in three independent assays (as discussed in the previous chapter). The information gained from MICA allele typing and MICA antibody identification was then used to correlate the presence of MICA antibodies and MICA-DSA with various transplantation variables including MICA mismatching and AR. Some of the results presented in this chapter have been published in a peer-reviewed journal (Cox *et al.,* 2011).

5.2 Study cohorts

The patient cohort included 442 renal graft recipients (386 primary and 56 re-transplants) and 200 end-stage renal failure patients awaiting transplant. General immunosuppression included calcineurin inhibitors (cyclosporine A or tacrolimus), an antiproliferative agent (mycophenolic acid or azathioprine) and corticosteroids (prednisolone). MICA typing was performed for 227 recipient-donor pairs transplanted between January 2003 and October 2008. A further 74 recipient/donor pairs transplanted between November 2008 and August 2009 were also MICA typed but details of biopsy-proven rejection was not available for these patients. Additionally I wanted to ensure enough time had passed after the transplant to allow for development of antibodies and for these reasons only the 227 MICA typed pairs were used for statistical analysis of transplantation variables. All patients and donors were typed by the H&I department for HLA-A, B, C, DRB1, 3, 4, 5 and DQB1 by PCR-SSP (One Lambda, CA) or PCR-SSO (Luminex™, One Lambda, CA). Sera from 116 healthy controls, 58 male and 58 female (with no known pregnancies) were also screened by the same methods. Biopsies investigating suspected AR were performed on 391 of the larger cohort of 442 recipients and mean follow-up time was 5.9 years.

Pre- and post-transplant screening of patients was performed for HLA and MICA antibodies using LABScreen® Mixed assay (LSM12 – One Lambda inc., CA). Mean time of testing for antibodies was 7 months post-transplant. MICA antibodies were confirmed using three different methods: MICA LABScreen® MICA SAg kit (One Lambda inc., CA), Gen-Probe LSA™ MICA kit, and an 'in-house' bead-based MICA antibody detection assay, as previously described (Zou *et al*., 2006b). All three methods identify MICA IgG antibodies directed against MICA*001, *002, *004, *007, *008, *009, *012, *017, *018, *019 and *027. Recommended MFI values for each method were used as the threshold for antibody positivity. MICA antibody specificities were considered positive if confirmed by at least two methods. DSA against MICA were identified if any of the antibodies were directed against the known MICA mismatches of the donor.

HLA antibody specificity was confirmed after positive screening among the 227-patient cohort, with the same serum sample used to detect MICA antibodies. SAg bead testing for HLA antibodies was performed using LABScreen® HLA Class I - Combi and HLA Class II – Group 1 (LS1A04 and LS2A01 - One Lambda inc., CA). These beads detect antibody specificities against HLA-A, -B, -C, -DRB1, -DQB1 and –DPB1. DSA was defined as a

positive reaction above the empirically determined MFI cut-off of 2000, with antigens expressed by the donor.

5.3 MICA allele typing, frequency and haplotype analysis in a renal transplant cohort

In total, 301 renal graft recipients and their donors were MICA allele typed by MICA-SBT methodology using an in-house protocol developed for this project and described in Chapter 3. Hazael Maldonado-Torres, a former PhD student at the Anthony Nolan Research Institute, performed analysis of MICA allele frequencies, HLA-B antigen frequencies and haplotype estimation. The computer program used to perform the analysis was called Cactus and developed by Hazael. Further details of this statistical method are provided in Chapter 2, section 2.6.

Alleles and haplotypes, linkage equilibrium and disequilibrium

An allele is defined as a variant of a gene with the same function but differing by genetic polymorphism in the coding (exon) or non-coding (intron) regions. Traditionally, a haplotype is defined as the genetic constitution of a single chromosome, referring to the combinations of genes or alleles at particular genetic loci (Ceppellini *et al.*, 1955).

Linkage equilibrium and disequilibrium are parameters to determine the nature of recombination of multiple genes or alleles within a haplotype. Linkage equilibrium is observed when the frequency of a haplotype and its genetic elements is equal to what would be expected by random chance. Linkage disequilibrium (LD) is defined as a non-random combination of genes or alleles, and their haplotype frequencies can be either higher or lower than expected by random chance.

*5.3.1 Identification of two novel MICA alleles: MICA*054 and MICA*056*

During SBT analysis of MICA alleles of recipients and donors, two novel polymorphisms were detected that did not agree with heterozygous combinations of MICA alleles (Figure 5.3.1). Once it was determined that these polymorphisms represented the presence of novel alleles, DNA cloning was performed to separate the alleles and sequencing of exons 2-5 was performed as described in Chapter 2.

MICA*054 allele was identified in a Caucasoid kidney donor (501DW) who was originally typed by SBT as MICA*008, *010 with one novel nucleotide substitution. Cloning revealed an allele identical to MICA*010 except for a novel non-synonymous polymorphism of nucleotide 871 (codon 268) of A to G, which encodes a novel amino acid change of serine to glycine in the α-3 domain.

MICA*056 allele was identified in an African-Caribbean kidney donor (208RP) SBT typed as MICA*008, *019 with one novel nucleotide belonging to the allele most similar to MICA*019. MICA*056 has a non-synonymous mutation at nucleotide position 758 (codon 230) of G to C encoding a novel amino acid change of tryptophan to serine in the α-3 domain. Serine is a small, polar amino acid (MW=105.09) whereas tryptophan is larger and non-polar (MW=204.23). An alignment of MICA sequences comparing MICA*054 and MICA*056 with the reference MICA*001 is provided in Figure 5.3.1.

The names MICA*054 (EMBL accession number AM899996) and MICA*056 (AM944063) were officially assigned by the WHO nomenclature committee in March 2008 and a report was published in Tissue Antigens (Cox *et al.*, 2009). The EMBL flatfile summaries for these submissions are provided in Appendix D.

5.3.2 Comparison of HLA-B antigen and MICA allele frequencies in UK renal transplant recipients and donors

Despite the less polymorphic nature of MICA compared to HLA and the strong linkage disequilibrium between MICA and HLA-B, most recipient and donor pairs were mismatched. Overall 30% were fully MICA matched, 49% had one allele mismatch and 21% had 2 allele mismatches. Twenty-seven different MICA alleles were identified at least once among recipients and donors. MICA*002 allele showed statistically significant frequency differences between patients and donors (P=0.02) and the frequencies of MICA*007 were also significantly different (P=0.01). Full details of MICA allele frequencies are given in Table 5.3.1. The four most frequent MICA alleles (highlighted) were as follows: MICA*008 (36.9% recipients versus 39.4% donors) MICA*002 (17.4% versus 12.6%), MICA*004 (11.6% versus 9.3%) and MICA*009:01 (8% versus 7.6%). A full list of recipient and donor MICA types is provided in Appendix C.

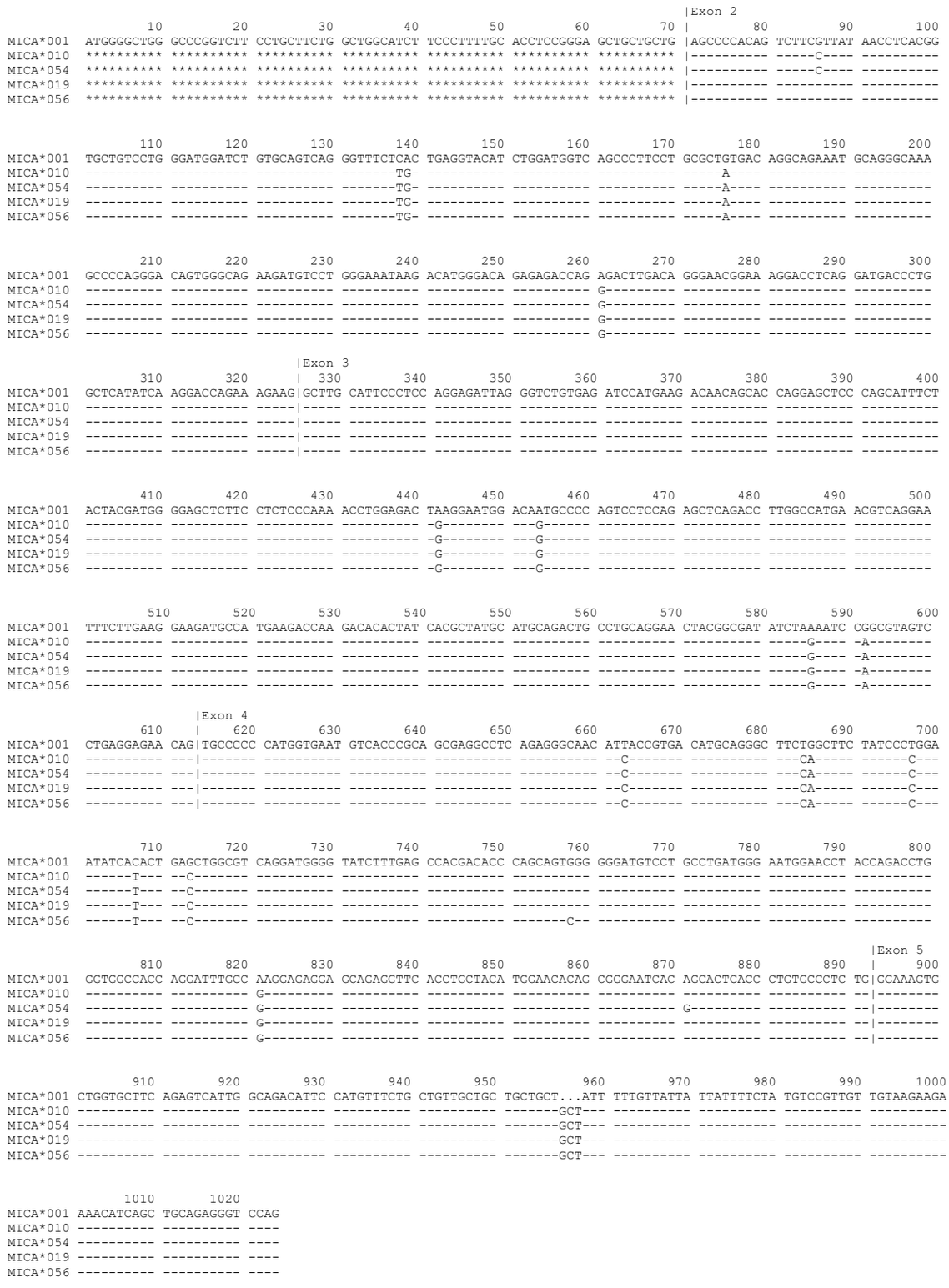

Figure 5.3.1: Nucleotide sequence alignment of exons 1-5 of the MICA gene. MICA*054 nucleotide sequence is compared with MICA*010 and MICA*056 nucleotide sequence is compared with MICA*019 and all sequences are compared with the reference MICA*001 sequence. Asterisk denotes no available sequence, dash denotes homology to reference sequence and full-stop denotes nucleotide deletion (for differentiation of GCT triplet repeats in exon 5). Cox *et al*, 2009 (Reproduced with permission from John Wiley and Sons).

Most recipients were also mismatched for HLA-B, with 15% fully matched, 66.5% with one mismatch and 18.5% with two antigen mismatches. As shown in Table 5.3.1, the HLA-B antigenic repertoire was higher among recipients than donors. Five HLA-B antigens had significantly different frequencies between recipient and donor as indicated in Table 5.3.1. The highest HLA-B antigen frequencies (recipients) were as follows: B44 (12.0% recipients versus 15.6% donors) B35 (11.5% versus 10.5%), B7 (8.3% versus 11.8%) and B8 (6.6% versus 7.6%).

Table 5.3.1: MICA allele and HLA-B antigen frequencies in 301 renal graft recipients and their donors

	Recipient (N=301)		Donor (N=301)			Recipient (N=301)		Donor (N=301)	
MICA*	**n**	**Freq.**	**n**	**Freq.**	**HLA-B$^{\Psi}$**	**n**	**Freq.**	**n**	**Freq.**
001	5	0.0083	7	0.0116	7	50	0.0831[c]	71	0.1179[c]
002:01	105	0.1744[a]	76	0.1262[a]	8	40	0.0664	46	0.0764
004	70	0.1163	56	0.0930	13	13	0.0216	12	0.0199
006	1	0.0017	-	-	18	20	0.0332	30	0.0498
007	16	0.0266[b]	33	0.0548[b]	27	13	0.0216[d]	26	0.0432[d]
008	222	0.3688	237	0.3937	35	69	0.1146	63	0.1047
009:01	48	0.0797	46	0.0764	37	6	0.0100	6	0.0100
009:02	8	0.0133	15	0.0249	38	20	0.0332[e]	8	0.0133[e]
010	13	0.0216	16	0.0266	39	10	0.0166	9	0.0150
011	13	0.0216	19	0.0316	41	7	0.0116	-	-
012:01	17	0.0282	14	0.0216	42	11	0.0183[f]	-	-
012:02	-	-	1	0.0017	44	72	0.1196	94	0.1561
013	1	0.0017	-	-	45	6	0.0100	9	0.0150
015	1	0.0017	2	0.0033	49	12	0.0199	8	0.0133
016	18	0.0299	11	0.0183	50	7	0.0116	8	0.0133
017	8	0.0133	11	0.0183	51	29	0.0482	20	0.0332
018:01	20	0.0332	27	0.0448	52	13	0.0216	13	0.0216
018:02	-	-	1	0.0017	53	12	0.0199	15	0.0249
019	22	0.0365	15	0.0249	55	15	0.0249	12	0.0199
027	7	0.0116	9	0.0150	57	25	0.0415	21	0.0349
029	2	0.0033	2	0.0033	58	19	0.0316	14	0.0233
030	3	0.0050	-	-	60	23	0.0382	17	0.0282
035	2	0.0033	-	-	61	12	0.0199	10	0.0166
038	-	-	1	0.0017	62	22	0.0365	32	0.0532
041	-	-	2	0.0033	63	7	0.0116	-	-
054	-	-	1	0.0017	64	7	0.0116	7	0.0116
056	-	-	1	0.0017	65	16	0.0266	16	0.0266
					71	11	0.0183[f]	-	-
					72	7	0.0116	-	-
					75	7	0.0116	-	-

$^{\Psi}$ Only frequencies above 1% are shown

[a]P=0.02; χ^2=5.5. [b]P=0.01; χ^2=6.1. [c]P=0.04; χ^2=4.0. [d]P=0.03; χ^2=4.5. [e]P=0.02; χ^2=5.3. [f]P=0.001; χ^2=11.1

5.3.3 HLA-B - MICA haplotype frequencies in UK graft recipients and donors

Table 5.3.2 lists HLA-B – MICA haplotype frequencies 301 UK renal transplant recipients. Highest frequencies are highlighted and were found for B7 – MICA*008 (7.9%), B35 – MICA*002 (7.0%), B44 – MICA*008 (6.8%) and B8 – MICA*008 (6.3%).

Table 5.3.2: HLA-B – MICA haplotype linkage disequilibrium frequencies in UK renal transplant recipients

Haplotype		UK Renal Graft Recipients (N=301)			
HLA-B	**MICA**	**[1]Frequency**	**[2]D'**	**χ^2**	**P-value**
7	008	0.079275	0.9279	46.51	< 0.001
35	002:01	0.069684	0.5251	74.36	< 0.001
44	008	0.067579	0.3109	7.52	0.006
8	008	0.062836	0.9139	36.10	< 0.001
44	004	0.045343	0.3071	42.78	< 0.001
51	009:01	0.041524	0.8478	184.34	< 0.001
60	008	0.033213	0.7930	15.62	< 0.001
35	016	0.026535	0.8729	93.80	< 0.001
38	002:01	0.025321	0.7119	39.61	< 0.001
18	018:01	0.024917	0.7414	309.29	< 0.001
58	002:01	0.024487	0.7285	39.40	< 0.001
55	012:01	0.021595	0.8628	373.40	< 0.001
13	008	0.021595	1.0000	14.05	< 0.001
49	004	0.019934	1.0000	80.59	< 0.001
71	008	0.018272	1.0000	11.89	< 0.001
27	007	0.018272	0.8420	328.55	< 0.001
65	011	0.016611	0.7629	269.77	< 0.001
42	004	0.016595	0.8961	59.33	< 0.001
62	010	0.014950	0.6806	152.97	< 0.001
57	017	0.013289	1.0000	176.97	< 0.001
52	009:01	0.013289	0.5759	38.13	< 0.001
39	002:01	0.013289	0.7577	22.44	< 0.001
53	002:01	0.012407	0.5426	13.81	< 0.001
50	009:02	0.011628	1.0000	61.90	< 0.001

Only significant haplotype frequencies above 1% are shown
[1]Maximisation Likelihood Estimate (MLE) – estimated frequency of observed haplotypes
[2]D' (Delta') indicates degree of LD. Values are between -1 and 1 and LD is indicated by values closest to -1 or 1.

Table 5.3.3 lists HLA-B – MICA haplotype frequencies 301 UK renal transplant donors. Highest frequencies are highlighted and were found for B7 – MICA*008 (11.3%), B44 – MICA*008 (8.7%), B8 – MICA*008 (7.3%) and B44 – MICA*004 (6.0%).

Table 5.3.3: HLA-B – MICA haplotype linkage disequilibrium frequencies in UK renal transplant donors

Haplotype		UK Renal Graft Donors (N=301)			
HLA-B	**MICA**	**[1]Frequency**	**[2]Delta'**	**χ^2**	**P-value**
7	008	0.112875	0.9292	57.24	< 0.001
44	008	0.087054	0.2702	6.41	0.011
8	008	0.073082	0.9281	37.00	< 0.001
44	004	0.060482	0.5854	87.53	< 0.001
35	002:01	0.052096	0.4253	68.90	< 0.001
27	007	0.038110	0.8756	324.85	< 0.001
18	018:01	0.034849	0.7361	274.86	< 0.001
60	008	0.026569	0.9024	12.93	< 0.001
51	009:01	0.024800	0.7179	82.15	< 0.001
62	010	0.023256	0.8680	203.30	< 0.001
65	011	0.019934	0.7419	261.66	< 0.001
52	009:01	0.019753	0.9051	84.88	< 0.001
35	016	0.018272	1.0000	84.26	< 0.001
13	008	0.018179	0.8548	8.19	0.004
55	012:01	0.016611	0.8294	338.61	< 0.001
53	002:01	0.016549	0.6157	34.38	< 0.001
58	002:01	0.013731	0.5312	23.89	< 0.001
57	017	0.013289	0.7174	151.17	< 0.001
39	002:01	0.013289	0.8728	41.46	< 0.001
38	002:01	0.013289	1.0000	48.38	< 0.001
50	009:02	0.011628	0.8609	47.26	< 0.001
18	001	0.011628	1.0000	126.82	< 0.001
45	009:02	0.011628	0.7527	40.64	< 0.001
35	008	0.010208	-0.7522	14.03	< 0.001

Only significant haplotype frequencies above 1% are shown
[1]Maximisation Likelihood Estimate (MLE) – estimated frequency of observed haplotypes
[2]D' (Delta') indicates degree of LD. Values are between -1 and 1 and LD is indicated by values closest to -1 or 1.

5.3.4 Analysis of MICA allele mismatching at the amino acid level

To enable a more thorough understanding of MICA mismatching in renal transplantation, it was necessary to compare MICA residues expressed by patient MICA antigens with those expressed on the donated organ. This was achieved by aligning the possible expression patterns of polymorphic MICA residues encoded by both alleles (if heterozygous) of the recipient with those of the donor, using an excel spreadsheet. Mismatches were counted if a residue expressed on the donor organ was different to either residue encoded by the recipient MICA alleles, for each polymorphic residue. This was carried out for 210 recipient and donor pairs where the recipient was negative for MICA antibodies and seventeen recipient and donor pairs where the recipient was confirmed to be MICA antibody positive. The frequencies of mismatched MICA residues for the MICA negative group and MICA positive group were then compared using chi-squared analysis.

Figure 5.3.2 shows fourteen MICA residues significantly mismatched between recipients with MICA antibodies and those without. Bonferroni's correction was used, by multiplying the P value by the number of mismatched residues to give P_c. Mismatches were considered highly statistically significant if P_c was ≤ 0.05.

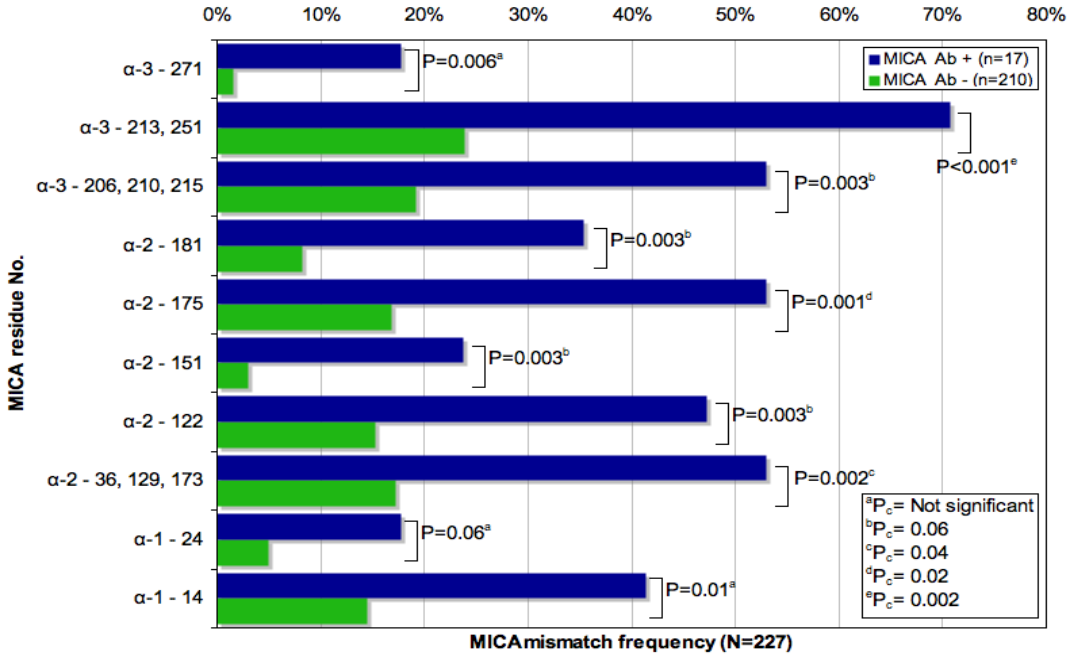

Figure 5.3.2: Histogram comparing the frequency of mismatched MICA amino acid residues between MICA antibody positive (n=17) and negative (n=210) renal graft recipients and their donors. There were nineteen MICA residues mismatched across the α1-α3 regions (exons 2-4) and fourteen of these mismatched residues were significantly correlated with MICA antibody production. The corrected P values for 19 comparisons are indicated. (Cox *et al.,* 2011). Reproduced with permission from Elsevier.

Figure 5.3.3 shows a MICA amino acid alignment of polymorphic residues across the α1-α3 domains of the MICA molecule with alleles listed according to their amino acid similarity. The boxed areas indicate highly significant mismatched amino acid residues in a cohort of 227 transplant pairs.

	MICA polymorphic amino acid residues in extracellular domains																	
	α-1			α-2										α-3				
Allele																		
MICA-G1	14	24	36	91	122	125	129	151	156	173	175	176	181	206	210	213	215	251	271
MICA*001	W	T	C	Q	L	K	M	M	H	K	G	V	T	G	W	T	S	Q	P
MICA*012	-	-	-	-	-	E	-	-	L	-	-	-	-	-	-	-	-	-	-
MICA*018	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA*002	G	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA*017	G	A	-	R	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA*011	G	A	-	-	-	E	-	V	-	-	-	-	-	-	-	-	-	-	A
MICA*007	-	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA-G2																			
MICA*004	-	A	Y	-	V	E	V	-	-	E	S	-	R	S	R	-	T	-	-
MICA*006	-	A	Y	-	V	E	V	-	-	E	S	I	-	S	R	-	T	-	-
MICA*009	-	A	Y	-	V	E	V	-	-	E	S	-	-	S	R	-	T	-	-
MICA*008	-	A	Y	-	-	E	V	-	-	E	-	-	-	S	R	I	T	R	-
MICA*019	-	A	Y	-	-	E	V	-	-	E	S	-	-	S	R	I	T	R	-

Figure 5.3.3. MICA amino acid alignment of polymorphic MICA residues across the α1-α3 domains of the MICA molecule. Alleles are listed according to similarity of protein sequence, dividing them into two broad groups, MICA-G1 and MICA-G2 that are differentially recognized by MICA antibodies (Zou *et al*., 2009). Mismatched MICA residues found to be significant between patients and donors, (or a trend P_c=0.06) after P correction are indicated by the boxed areas. Modified from Zou *et al.,* 2009 (Permission granted by Springer).

The most frequently mismatched MICA residues were 213 and 251 in the α3-domain. In the MICA antibody negative group, 23.8% of recipient and donor pairs were mismatched compared to 70.6% of those who produced MICA antibodies (P<0.001; P_c=0.002). The next most frequently mismatched residue in the MICA antibody positive group was position 175 with a frequency of 52.9% compared to 16.7% when no

MICA antibodies were detected (P=0.001; P_c=0.02). Residues 36, 129 and 173 were highly significantly mismatched with equal frequency between the MICA antibody negative and positive groups. A frequency 17.1% of recipient and donor pairs were mismatched when the recipient did not have MICA antibodies, compared to 52.9% with mismatches where the recipient was positive for MICA antibodies (P=0.002; P_c=0.04). Residue 122 was mismatched in 15.2% of recipient and donor pairs without MICA antibodies, compared to 47.1% in the MICA antibody positive group and was significant (P=0.003). Residue 151 was mismatched in 2.9% of the MICA antibody negative group compared to 23.6% for those with MICA antibodies (P=0.003). Residue 181 was mismatched in 8.1% of transplant pairs without MICA antibodies, compared to 35.3% for those with MICA antibodies (P=0.003). Residues 206, 210 and 215 were mismatched equally in 19% of recipients with no MICA antibodies, compared to 52.9% of recipients with MICA antibodies (P=0.003) Residue 271 was mismatched with a frequency of 1.4% in the MICA antibody negative group and 17.6% of the MICA antibody positive group (P=0.006). Finally, residue 14 was mismatched in 14.3% when negative for MICA antibodies (14.3%) and 41.2% when the recipient was positive for MICA antibodies (P=0.01).

Figure 5.3.3 shows amino acids expressed at mismatched residues. The two residues with most mismatches were expressed by MICA*008 and MICA*019 corresponding to I at residue 213 and R at residue 251 as opposed to 213T and 251Q encoded by most other alleles. A frequent type of mismatching occurred between alleles of the MICA-G1 and MICA-G2 groups and involved mismatches in the α-1 to α-3 domains (Zou *et al*., 2009) (residues 36, 206, 210 and 215), occurring in 53% of those with MICA antibodies and 19% without MICA antibodies. Residues 151 and 271 were more frequently mismatched in MICA antibody positive recipients, and although not highly significant, associates with a polymorphism in the MICA*011 allele encoding 151V and 271A. MICA*011 was to found to have an allele frequency of 2% in recipients and 3% of donors and is therefore a relatively frequently occurring MICA allele in this transplant cohort.

Figure 5.3.4 shows the relative positions of the MICA amino acid residues found to be significantly mismatched from this analysis using computerised models based on x-ray crystallographic analysis (P. Li *et al*., 1999). Space-filling models are used in A and C and ribbon models in B and D. Arrows indicate amino acid residues significantly mismatched between recipient and donor (before correction). The diagrams reveal that mismatched residues are exposed and visible to the extracellular environment. Many of these residues are on highly exposed loops between β-strands, across the α1-α3 domains.

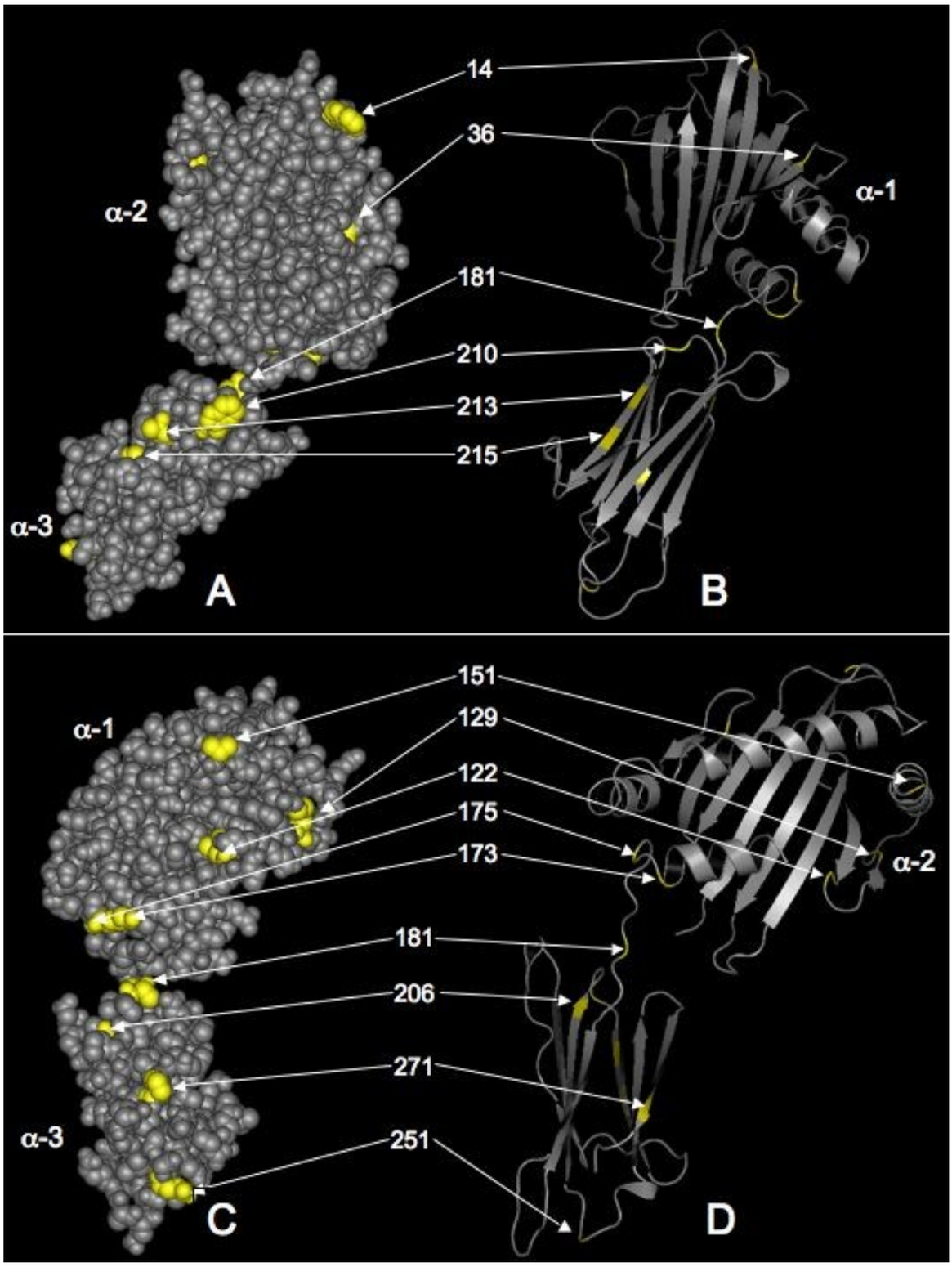

Figure 5.3.4. α-1, α-2 and α-3 extracellular domains of the MICA molecule. Space-filling models are used in A and C and ribbon models in B and D. The models are shown in two orientations, AB (from underneath/behind) and CD (from in front/above). Arrows indicate amino acid residues significantly mismatched between recipient and donor. The diagrams reveal that mismatched residues are exposed and visible to the extracellular environment and across the α1-α3 domains. Images from Molecular Modelling Database (MMDB), The National Centre for Biotechnology Information (NCBI) and visualized using Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) on World Wide Web http://www.pymol.org, and Cn3D http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml. Cox *et al.,* 2011. Reproduced with permission from Elsevier.

5.4 Clinical characteristics and transplantation variables associated with the presence or absence of MICA antibodies

5.4.1 Significance of clinical characteristics with the production of MICA antibodies

The association of MICA antibodies with general clinical characteristics of 442 graft recipients is analysed in Table 5.4.1. Variables considered were recipient and donor gender and age, time of follow-up and whether the transplant was performed using grafts from living or deceased donors. Overall 59% of graft recipients were male and 41% were female.

Table 5.4.1: Clinical characteristics of 442 renal transplant recipients

Recipients	MICA Ab negative (n=409)	MICA Ab positive (n=33)	MICA Ab. (%)	P Value	χ^2	Odds Ratio (95% CI)
Recipient - no. (%)				0.33	0.96	1.5 (0.7 – 3.1)
Male	237 (58)	22 (67)	8.5			
Female	172 (42)	11 (33)	6			
Donor - no. (%)						
Male	141/263	8/19	2.8	0.35	0.94	0.6 (0.2 – 1.6
Female	122/263	11/19	3.9			
Recipient age - yrs	42.2 ± 16.0	43.0 ± 16.2		0.18		
Donor age - yrs	45.5 ± 16.4	49.0 ± 14.2		0.51		
Follow-up - yrs	6.7 ± 5.8	7.1 ± 5.8		0.17		
Donor - no. (%)				0.4	0.80	1.5 (0.6 – 3.8)
Living	107/387 (28)	6/30 (20)	5			
Deceased	280/387 (72)	24/30 (80)	7.9			

Data for ages and time are means ± standard deviation

There was a slightly higher frequency of MICA antibodies detected in males (8.5%) compared to females (6%) but this was not statistically significant. There was also no significant association of donor or patient gender or age with production of MICA antibodies. Mean time of follow-up was 6.7 ± 5.8 years for recipients without MICA antibodies and 7.1 ± 5.8 years for recipients with MICA antibodies. Kidney transplants from living donors were performed for 28% of recipients and 72% received a graft from a deceased donor. There was a slightly higher frequency of MICA antibodies (7.9%)

detected in those who received grafts from deceased donors compared to 5% who received a graft from a living donor but this was not statistically significant.

Gender and age of 200 end-stage renal patients awaiting transplant and 116 healthy controls were analysed for association with the presence of MICA antibodies and results are shown in Table 6.4.2. There was no significant association with any variable with the presence of MICA antibodies. Only one of the 116 healthy controls had MICA antibodies detected and this individual was male.

Table 5.4.2: Clinical characteristics of 200 renal patients awaiting transplant and 116 healthy controls

Untransplanted Patients	**MICA Ab negative (n=194)**	**MICA Ab positive (n=6)**	**MICA Ab (%)**	**P Value**	**Odds Ratio (95% CI)**
Gender - no. (%)				1.0	0.9 (0.2 – 4.6)
Male	107 (55)	3 (50)	2.7		
Female	87 (45)	3 (50)	3.3		
Age	51.9±13.8	50.3±14.9		0.47	
Healthy Controls	**(n=115)**	**(n=1)**			
Gender - no. (%)				1.0	2.1 (0.2 – 24.4)
Male	57 (48)	1 (100)	1.7		
Female	58 (52)	0 (0)	0		
Age	33.8±9.9	45		0.35	

5.4.2 Significance of MICA allele and antigen mismatching with the production of MICA antibodies

Table 5.4.3 shows analysis of MICA allele and amino acid mismatching and association with the production of MICA antibodies. The number of MICA allele mismatches significantly correlated with the presence of MICA antibodies (P=0.006). None of the patients matched for MICA alleles developed antibodies compared to 10% with one MICA allele mismatch and 15% with two MICA allele mismatches. Mismatching of MICA alleles at the amino-acid level was more significantly associated with the presence of MICA antibodies (P=0.001). One of 103 graft recipients without a MICA amino acid

mismatch (1%) developed MICA antibodies compared to 13% of graft recipients with MICA amino acid mismatches.

Table 5.4.3: MICA allele and amino acid residue matching and association with production of MICA antibodies in 442 renal graft recipients

Variable	MICA Ab negative (n=210)	MICA Ab positive (n=17)	MIC A Ab (%)	P Value	χ^2	Odds Ratio (95% CI)
MICA allele mm - no. (%)				0.006	10.2	
0	75 (36)	0 (0)	0			
1	102 (49)	10 (65)	10			
2	33 (15)	7 (35)	15			
MICA allele mm no (%)						
No	75 (36)	0 (0)	0	0.003	9.1	
Yes	135 (64)	17 (100)	7.5			
MICA residue mm no. (%)				0.001	10.6	14 (1.8 - 106)
No	102 (49)	1 (6)	1			
Yes	108 (51)	16 (94)	13			

5.4.3 Association of HLA antigen mismatches with production of MICA antibodies

To assess whether mismatching of HLA antigens also associates with the production of MICA antibodies, HLA-A, B, C, DR and DQ antigen mismatches were compared in the MICA antibody positive group and the MICA antibody negative group (Table 5.4.4). There was no significant difference with HLA class I or class II mismatches and MICA antibodies. There was also no significant difference when individual HLA loci were compared (not shown). However, none of the recipients who were matched for class I loci developed MICA antibodies compared to 8% with 1-3 class I mismatches and 6% of those with 4-6 HLA class I mismatches. The opposite was true for HLA class II mismatching as 7.5% had MICA antibodies with no mismatches and none of the recipients with 3-4 class II mismatches developed MICA antibodies.

Table 5.4.4: Association of HLA class I (HLA-A, B, C) and class II (HLA-DR, DQ) antigen mismatches with the production of MICA antibodies in renal transplantation

Variable	MICA Ab negative (n=409)	MICA Ab positive (n=33)	MICA Ab (%)	P Value	χ^2
HLA-A, B, C mm - no./total no. (%)				0.29	2.5
0	25/303 (8)	0/22 (0)	0		
1-3	143/303 (47)	13/22 (59)	8		
4-6	135/303 (45)	9/22 (41)	6		
HLA-DR, DQ mm - no./total no. (%)				0.39	1.9
0	99/303 (33)	8/22 (36)	7.5		
1-2	180/303 (59)	14/22 (64)	7		
3-4	24/303 (8)	0/22 (0)	0		

5.4.4 Significance of HLA class I and/or HLA class II antibodies and re-transplantation with the production of MICA antibodies

Table 5.4.5 analyses the relationship between the presence of HLA class I or HLA class II antibodies and the effect of re-transplantation with the production of MICA antibodies. In total 120 recipients developed antibodies against HLA class I antigens representing 27% of the 442 graft recipients. Sixteen of those with HLA class I antibodies also had MICA antibodies (13%) compared to a total of seventeen patients with MICA antibodies among 320 patients without HLA class I antibodies (5%). Thus, HLA class I antibodies were significantly associated with the presence of MICA antibodies in renal graft recipients (P=0.004). HLA class II antibodies were detected in 21% of recipients. Thirteen of the 93 recipients with HLA class II antibodies were also positive for MICA antibodies (14%) and of the 349 recipients with no HLA class II antibodies, 20 had MICA antibodies (6%). These findings were statistically significant (P=0.007).

The effect of re-transplantation on the production of MICA antibodies was also evaluated. Among the 442 graft recipients there were 386 (87%) primary transplants and 56 (13%) re-transplants. Five percent of primary graft recipients developed MICA antibodies compared to 20% of re-transplanted patients. Re-transplantation was strongly associated with the production of MICA antibodies (P<0.001).

Table 5.4.5: Association of the production of HLA class I and HLA class II antibodies and re-transplantation with the presence of MICA antibodies in 442 renal graft recipients

Variable	MICA Ab negative (n=409)	MICA Ab positive (n=33)	MICA Ab (%)	P Value	χ^2	Odds Ratio (95% CI)
HLA Class I sensitisation - no. (%)				0.004	8.4	2.8 (1.4 – 5.7)
Class I antibodies	104 (25)	16 (48)	13			
No class I antibodies	305 (75)	17 (52)	5			
HLA Class II sensitisation - no. (%)				0.007	7.2	2.7 (1.3 – 5.6)
Class II antibodies	80 (20)	13 (40)	14			
No class II antibodies	329 (80)	20 (60)	6			
Transplantation number - no. (%)				<0.001	13.8	4.0 (1.8 – 8.9)
Successive transplant(s)	45 (11)	11 (33)	20			
First transplant	364 (89)	22 (67)	6			

5.4.5 Association of MICA antibodies with biopsy-proven acute graft rejection

AR was diagnosed histologically by renal pathologists, following guidelines of the Banff Conference Report, as updated in 2007 (Solez *et al.*, 2008) and designated type I and type II T-cell-mediated rejection (for simplicity called acute cellular rejection, ACR) or acute antibody-mediate rejection (aAMR). Acute AMR with complement deposition was investigated by immunohistological investigation of C4d following Banff guidelines (Racusen *et al*., 2003; Solez *et al*., 2007) using a rabbit antibody to C4d (Biomedica, Vienna) in an indirect immunoperoxidase method on paraffin sections following antigen retrieval by heating to 100°C in EDTA buffer pH 8. As in the Banff guidelines, significant C4d deposition was considered to be when immunostaining was present in the endothelium of at least half of intertubular capillaries.

Protocol biopsies and biopsies investigating suspected rejection, were performed on 391 of the larger cohort of 442 recipients and mean follow-up was 5.9 years. Patients diagnosed with ACR or aAMR and those without rejection episodes were analysed for association with the production of MICA antibodies. The results are shown in Table 5.4.6a and reveal 70/391 recipients with ACR (18%) and 31/391 recipients with aAMR (8%). Nine of the 70

patients diagnosed with ACR produced MICA antibodies (13%), compared to 17 of 321 patients with no ACR (5%) and was statistically significant (P=0.021). Only one of the 31 recipients with biopsy proven aAMR had MICA antibodies (3%) but showed no evidence of C4d deposition, compared to 25 of 360 recipients (7%) with no aAMR who were positive for MICA antibodies (not significant).

Table 5.4.6a: ACR and aAMR and association with the production of MICA antibodies among 391 renal graft recipients

Recipients	MICA Ab negative (n=365)	MICA Ab positive (n=26)	MICA Ab (%)	P Value	χ^2	Odds Ratio (95% CI)
ACR - no. (%)				0.021	5.3	2.6 (1.1 – 6.2)
Yes	61 (17)	9 (35)	13			
No	304 (83)	17 (65)	5			
aAMR – no. (%)				0.4	0.6	0.4 (0.1 –3.3)
Yes	30 (8)	1 (4)	3			
No	335 (92)	25 (96)	7			

It became apparent whilst performing the MICA antibody testing that some patients produced results with much higher MFI values than others and it is generally agreed among the H&I community that high MFI values by Luminex™ antibody testing, correlate with high-titre antibodies. Since I have not performed titration experiments I will refer to this type of antibody as 'strong' and was categorised as such if the MFI value was equal to or greater than three times the recommended, or defined, cut-off value for MICA antibody positivity. Table 5.4.6b compares recipients with strong MICA antibodies or moderate/no MICA antibodies and ACR or aAMR. Strong MICA antibodies were detected in nine of the recipients with ACR (13%) compared to three of 321 patients (1%) with strong MICA antibodies and no ACR (P<0.001). There was no significant correlation of strong MICA antibodies with aAMR.

Table 5.4.6b: ACR and aAMR and association with the production of 'strong' MICA antibodies in 391 renal graft recipients

Recipients	'Strong' MICA Ab negative (n=379)	'Strong' MICA Ab positive (n=12)	MICA Ab (%)	P Value	χ^2	Odds Ratio (95% CI)
ACR - no. (%)				<0.001	27.5	15.6 (4.2 – 6.2)
Yes	61 (16)	9 (75)	13			
No	318 (84)	3 (25)	1			
aAMR – no.(%)				1.0		
Yes	30 (8)	1 (4)	3			
No	349 (92)	11 (96)	7			

5.4.6 Statistically significant associations of 'strong' MICA antibodies with other transplantation variables

Overall, 33 of the 442 patients had MICA antibodies and using the criteria above for defining those with strong MICA antibodies, fourteen were eligible for inclusion in this category. A re-analysis of association with transplantation variables, as carried out for MICA antibodies, was performed to enable a separate multivariate analysis. In addition to ACR, described above, there were statistically significant correlations with strong MICA antibodies and HLA class I antibodies, HLA class II antibodies and re-transplantation.

Of the 56 re-transplanted patients, eight had strong MICA antibodies (14%) compared to six of the 386 patients receiving a primary graft (1.6%) and this difference was highly significant (P<0.001). Of the recipients sensitised to HLA class I antigens, 8% had MICA antibodies that were strong compared to 1.5% of recipients with no HLA class I antibodies. This difference was also statistically significant (P=0.001). The presence of HLA class II antibodies was also associated with strong MICA antibody production in seven of 94 recipients (7.5%) compared to seven (2%) of 349 recipients who did not have class II antibodies (P=0.007).

Table 5.4.7: Transplantation variables significantly associated with the presence of 'strong' MICA antibodies among 442 renal transplant recipients

Variable	Strong MICA Ab negative (n=428)	Strong MICA Ab positive (n=14)	MICA Ab (%)	P Value	χ^2	Odds Ratio (95% CI)
Transplant number no. (%)				<0.001	25.8	10.6 (3.5 – 31.7)
Re-graft	48 (11)	8 (57)	14			
Primary graft	380 (89)	6 (43)	1.6			
HLA Class I Abs no. (%)				0.001	10.2	5.2 (1.7 – 15.8)
Positive	110 (26)	9 (64)	8			
Negative	318 (74)	5 (36)	1.5			
HLA Class II Abs no. (%)				0.007	7.3	4.0 (1.4 – 11.6)
Positive	86 (20)	7 (50)	7.5			
Negative	342 (80)	7 (50)	2			

5.4.7 Multivariate analysis of variables significantly associated with the production of MICA antibodies

Multivariate analysis was performed using binary logistic regression for association of MICA antibodies or strong MICA antibodies including covariates with a P value of less than or equal to 0.100 and the results are shown in Table 5.4.8. Covariates included in the binary logistic regression model for MICA antibodies were HLA class I antibodies, class II antibodies, ACR, re-transplantation and MICA residue mismatching. Significant independent variables were re-transplantation (P=0.020) and MICA residue mismatching (P=0.008). Variables included in the model for strong MICA antibodies were HLA class I antibodies, class II antibodies, ACR and re-transplantation. Two variables were independently significant after regression analysis, ACR (P=0.001) and re-transplantation (P=0.008).

Table 5.4.8: Univariate and multivariate analysis of renal transplantation variables associated with MICA antibody status and MICA antibody strength in 442 renal graft recipients

Antibodies	Variable	Univariate		Multivariate	
		P value	OR	CI (95%)	P value
MICA	Class I antibodies	0.004	0.5	0.1 – 3.3	0.485
	Class II antibodies	0.007	1.5	0.2 – 10.3	0.663
	ACR	0.021	1.1	0.3 – 4.2	0.827
	Re-transplantation	<0.001	5.8	1.3 – 25.6	0.020
	MICA residue mm	0.001	16	2.0 – 127	0.009
Strong MICA	Class I antibodies	0.001	2.5	0.5 – 12.5	0.249
	Class II antibodies	0.007	1.1	0.2 – 5.7	0.880
	ACR	<0.001	9.9	2.4–40.2	0.001
	Re-transplantation	<0.001	6.9	1.6 – 28.8	0.008

5.4.8 Correlation of HLA and/or MICA antibodies with ACR and aAMR

So far I have only considered association of MICA antibodies with ACR or aAMR, but it was important to also consider what effects the presence of HLA antibodies or HLA and MICA antibodies together might have in relation to AR. In total 391 patients out of the cohort of 447 had biopsy information regarding diagnosis of ACR or aAMR and the results of the full analysis are presented in Table 5.4.9. A separate analysis was also performed using the smaller cohort of 227 MICA-typed transplant pairs as they were confirmed by SAg testing for both MICA and HLA antibodies and allowed categorisation of donor-specific HLA antibodies as shown in Table 5.4.10.

From the larger cohort, statistically significant associations were found with ACR and HLA antibodies overall (P=0.042), MICA antibodies overall (P=0.021) HLA and MICA antibodies together (P<0.001), HLA class I (P=0.012) and strong MICA antibodies (P<0.001). Significant associations with aAMR were HLA antibodies overall (P=0.001), HLA class I (P<0.001), HLA class II (P=0.024) and co-production of HLA class I and II (P=0.001).

Table 5.4.9: Association of different categories of HLA and MICA antibodies with acute cellular and acute antibody-mediated rejection

Antibodies	ACR[1] n (%)					aAMR[1] n (%)				
	Neg n=321	Pos n=70	P value	χ^2	Odds Ratio (95% CI)	Neg n=360	Pos n=31	P value	χ^2	Odds Ratio (95% CI)
HLA Overall	93 (29)	29 (41)	0.042	4.2	1.7 (1.0 – 3.0)	104 (29)	18 (58)	0.001	11.3	3.4 (1.6 – 7.2)
MICA overall	17 (5)	9 (13)	0.021	5.3	2.6 (1.1 – 6.2)	25 (7)	1 (3)	NS	-	
HLA and MICA	5 (2)	7 (10)	<0.001	13.8	7.0 (2.2 – 22.8)	11 (3)	1 (3)	NS	-	
HLA Class I	73 (23)	26 (37)	0.012	6.3	2.0 (1.2 – 7.5)	83 (23)	16 (52)	<0.001	12.3	3.6 (1.7 – 7.5)
HLA Class II	62 (19)	16 (23)	NS	-		67 (21)	11 (35)	0.024	5.1	2.4 (1.1 – 5.3)
HLA Class I + II	44 (10)	14 (20)	NS	-		47 (13)	11 (35)	0.001	11.4	3.7 (1.6– 8.2)
Strong MICA	3 (1)	9 (13)	<0.001	27.5	15.7 (4.1 – 59)	11 (3)	1 (3)	NS	-	

[1]N=391 (51 patients had no biopsy details from the 447 cohort).

Table 5.4.10: Association of different categories of HLA and MICA antibodies with acute cellular and acute antibody-mediated rejection

Antibodies	ACR[1] n (%)					aAMR[2] n (%)				
	Neg n=184	Pos n=41	P value	χ^2	Odds Ratio (95% CI)	Neg n=207	Pos n=17	P value	χ^2	Odds Ratio (95% CI)
HLA Overall	51 (28)	16 (39)	NS	-		54 (26)	12 (70)	<0.001	15.0	6.8 (2.3 – 20.2)
MICA overall	11 (6)	5 (12)	NS	-		15 (7)	1 (6)	NS	-	
MICA-DSA	8 (4)	5 (12)	0.051	3.8	3.1 (0.9 – 9.9)	12 (6)	1 (6)	NS		
HLA and MICA	4 (2)	4 (10)	0.018	5.6	4.9 (1.2 – 20.3)	7 (3)	1 (6)	NS	-	
HLA Class I	40 (22)	15 (37)	0.045	4.0	2.1 (1.0 – 4.3)	43 (21)	11 (65)	<0.001	16.6	7.0 (2.5 – 20.0)
HLA Class II	35 (19)	12 (29)	NS	-		36 (17)	11 (65)	<0.001	21.2	8.7 (3.0 – 25.0)
HLA Class I + II	24 (13)	11 (27)	0.028	4.8	2.4 (1.1 – 5.5)	25 (12)	10 (59)	<0.001	26.0	10.4 (3.6– 29.8)
HLA Class I-DSA	11 (6)	4 (10)	NS	-		8 (4)	7 (41)	<0.001	35.0	17.4 (5.3 – 57.6)
HLA Class II-DSA	10 (5)	5 (12)	NS	-		7 (4)	8 (47)	<0.001	48.0	25.4 (7.5– 85.5)
Class I + II-DSA	6 (3)	2 (5)	NS	-		2 (1)	6 (35)	<0.001	53.8	56 (10.1 – 310)
Strong MICA	0 (0)	5 (12)	<0.001	22.9	1.1 (1.0 – 1.3)	1 (0.5)	4 (23)	NS	-	

[1]N=225 (two patients from the 227 cohort had no biopsy details); [2]N=224 (three patients had no biopsy details)

Among the MICA-typed transplant pair cohort, 225 recipients had biopsies available for ACR diagnosis and 224 for aAMR. Significant associations with ACR were HLA and MICA antibodies together (P=0.018), HLA class I antibodies (P=0.045) both HLA class I and II (P=0.028), and strong MICA antibodies (P<0.001). There was a trend of association with MICA-DSA and ACR (P=0.051). Significant associations with aAMR were HLA antibodies overall (P<0.001) and all other categories with HLA antibodies were also highly significant (P<0.001). There was no significant association of aAMR with MICA antibodies.

5.4.9 Identification of donor-specific MICA antibodies

Seventeen graft recipients from the cohort of 227 with DNA available for both patient and donor were confirmed as positive for MICA antibodies. Those who were found to have MICA antibodies were also tested with pre-transplant sera to establish whether MICA sensitisation was '*de novo'* meaning a new alloresponse to the transplanted organ, and results are shown in Table 5.4.11. The table also shows whether or not the patient had a re-transplant and whether they have HLA class I or class II DSA or NDSA (non-donor-specific antibody).

Analysis revealed four patients with the same antibody profile before and after transplantation, three of them having had more than one transplant. Thirteen patients (76%) who had no antibodies to MICA before transplantation, developed *de novo* MICA antibodies post-transplant and eleven of these recipients (92%) had antibodies against MICA-DSA. In total, thirteen patients of the seventeen with MICA antibodies had MICA-DSA.

Eight patients out of the seventeen developed MICA antibodies but no HLA antibodies and seven developed MICA-DSA with HLA antibodies. Six patients had HLA-NDSA and four of them had a previous transplant. Two of the three patients with HLA-DSA also had MICA-DSA.

Table 5.4.11: Details of individual MICA antibody positive renal graft recipients indicating recipient and donor MICA types and MICA antibodies detected pre- and post-transplantation

Pat. No.	Sex	Donor	Year Txp	Patient MICA type	Donor MICA type	MICA Abs pre-transplant[ζ]	MICA Abs post-transplant	Re-Tx	HLA Class I[1]	HLA Class II[1]
1	F	DD	2006	002, 004	**008**, 00901/049	Negative	**008**, 019	Yes	No	DSA
2	M	DD	2006	004	**001**, 00901/049	Negative	**001**, 007, 012, 018	No	No	No
3	M	1. DD 2. LD	1. 2004 2. 2006	008, 00902	1. 002 2. 004, 00902	Negative	001, 002, 007, 012, 017, 018	Yes	NDSA	No
4	M	DD	2006	002	002, **008**	001, 004, 007, **008,** 009, 012, 018, 019	001, 004, 007, **008,** 009, 012, 018, 019	Yes	NDSA	NDSA
5	M	DD	2007	002, 01801	011, **016**	004, 008, 009, **016[2],** 019	004, 008, 009, **016[2],** 019	Yes	NDSA	NDSA
6	M	DD	2004	008	**004**, 008	Negative	**004**	No	No	No
7	M	DD	2005	011, 016	**002**, 008	Negative	**002**, 017	No	No	No
8	F	LD	2005	004, 008	008, 00901/049	001, 002, 007, 017	001, 002, 007, 017	Yes	DSA	No
9	M	LD	2006	010, 011	008, 010	Negative	001, 012, 018	Yes	No	No
10	M	DD	2007	002, 008	008, **00901**/049	Negative	004, **009**	Yes	DSA	DSA
11	M	DD	2007	008	**002**, 041	Negative	**002**, 017	No	No	No
12	M	LD	2007	008	011, **01801**	Negative	**018**, 019	No	NDSA	No
13	M	LD	2008	002	002, **00901**/049	Negative	004, 008/027, **009**, 019	No	No	No
14	M	DD	2008	004, 010	008	Negative	001, 012, 018	No	No	No
15	F	DD	2008	008	**004**, 008	Negative	001, **004**, 008, 009, 019	No	No	No
16	M	DD	2008	00902	**002**, 008	Negative	**002**, 017	No	No	NDSA
17	F	DD	2008	00901/049, 017	008, **01201**	001, **012**, 018	001, **012**, 018	No	No	No

[1]Confirmed with single antigen Luminex™ testing for HLA class I/II. [3] MICA*016 antibody was detected using the Gen-Probe Luminex assay but not confirmed.. MICA-DSA and corresponding donor alleles are indicated in bold typeface. [ζ]Where patients had more than one transplant, testing for pre-transplant MICA antibodies was assessed prior to their latest graft.

5.5 Discussion

5.5.1 MICA allele polymorphism and mismatching in UK renal graft recipients and donors

There are currently 76 MICA alleles listed on the IMGT/HLA database, release 3.4.0, April 2011 (Robinson and Marsh, 2007). This number has increased steadily since the discovery of the MICA gene and the initial flood of new MICA allele reports in the 1990's and is likely to continue to rise as more ethnically diverse populations are investigated for MICA polymorphism. My own investigation of MICA polymorphism in UK renal transplant recipients and donors, involving MICA sequencing of around 600 individuals, yielded two new MICA allelic variants (Cox *et al*., 2009). MICA*054 and MICA*056 were identified in Caucasoid and African-Caribbean kidney donors respectively, both with non-synonymous amino acid substitutions in the α-3 domain. Interestingly MICA polymorphism can occur equally in any of the three α-1 to α-3 domains in contrast to HLA polymorphism, which is mostly restricted to the α-1 and α-2 regions around the peptide-binding site of the TCR.

There have been several population studies carried out to determine MICA allele frequencies and linkage disequilibrium with HLA-B to date. These include American-Indians (Y. Zhang *et al*., 2002), African-Americans and European-Americans (Gao *et al*., 2006), Japanese (Nishiyama *et al.*, 2004), Koreans (Mok *et al.*, 2003), Chinese (Tian *et al.*, 2006), Brazilians (Tian *et al*., 2006) and Moroccans (Piancatelli *et al*., 2005). These studies were often carried out to establish disease association or susceptibility to certain epithelial carcinomas with MICA alleles. However, this data is limited and not as comprehensive as that for HLA polymorphism, which has been carried out extensively. Nevertheless, enough data is available to gain an insight into how frequent certain alleles are in different regions of the world and the conclusion can be made that as with HLA, MICA polymorphism is driven by evolution under pressures that differ in ethnic groups, for example disease and environment.

When considering how MICA polymorphism may influence mismatching in renal transplantation, information regarding the frequencies of MICA alleles for both patient and donor cohorts is essential. There are reasons to suppose that based on ethnicity there may be differences between a very ethnically heterogeneous patient population from the London

region of the UK and the mainly Caucasoid population of donors from the UK as a whole. However, the frequencies of MICA alleles in 301 patients and donors were, in fact, very similar overall. The only MICA alleles with a significant difference in frequency between recipients and donors were MICA*002 (P=0.02) and MICA*007 (P=0.01). While MICA*007 is an infrequent allele, MICA*002 is very common and the frequency was highest in recipients compared to the donors (17.4% and 12.6% respectively). The reason MICA*002 was more frequent in our cohort of patients may be that there are many of South Asian (Indian and Pakistani) ethnic origin due partly to UK demographics and the fact that CKD is more prevalent, affecting 10-15% (Bhowmik *et al.*, 2008). Information of MICA allele frequencies in South Asian populations would be useful but is not available. It is possible that the higher frequency of MICA*002 in this cohort of renal graft recipients may reflect differences in ethnicity, compared with UK donors. There was also significant variation of HLA-B antigens between recipients and donors. B7 and B27 had a higher frequency among donors (P=0.04 and P=0.03, respectively), and B35 and B42 had higher frequencies in recipients (P=0.02 and P=0.001, respectively).

The MICA allele frequencies overall were similar between patients and donors but analysis revealed that most donor and recipient pairs were mismatched for MICA alleles, despite strong linkage disequilibrium between HLA-B and MICA genes. However, from a total of 301 renal transplants, only 15% were fully matched for HLA-B antigens, 66.5% had one mismatch and 18.5% had two mismatches, indicating MICA mismatching was also very likely. Surprisingly, MICA matching was better than HLA-B with 30% fully matched, 49% with one allele mismatch and 21% with 2 allele mismatches. These observations indicate that knowledge of MICA allele frequencies and HLA-B – MICA haplotype frequencies and linkage disequilibrium is necessary.

There were 27 different MICA alleles identified in this study, although frequencies of 13 alleles were below 1%. The highest frequencies of MICA alleles in both recipient and donor groups were associated with MICA*002:01, 004, 008 and 009:01 and accounted for 75% and 69% of allele frequencies in the two groups, respectively. These data are remarkably similar to published work (Gao *et al*., 2006) where MICA allele and HLA-B – MICA haplotype frequencies in European-American (N=1238) and African-American (N=587) populations were compared. The results of my study show similarities with results obtained from European-Americans, although there are obvious differences. The frequency of MICA*008 was lower in renal graft recipients (37%) than in UK donors (39%) or European-Americans (43%). In contrast, the frequency of MICA*004 was higher in UK

renal graft recipients (12%) than in UK donors (9%) or European-Americans (7.5%). Comparison with data for African-Americans (Gao *et al*., 2006) reveals similarities with UK kidney recipients as MICA*008 frequency is lower (26%) and MICA*004 is very high (19%). Again, these data highlight the heterogeneity of UK renal transplant recipient ethnicity, particularly in the London area.

HLA-B and MICA haplotype and LD analysis provided further information important for this study. Highest LD frequencies were found for B7 – MICA*008 in both groups but was higher in donors. The results indicate that different HLA-B antigens can associate with the same MICA allele, for example HLA-B7 and B8 are in strong LD with MICA*008 and B35, 38, 39, 53 and 58, associate with MICA*002. The sequence homology of B35, 53 and 58 and that of B38 and 39 indicate they have originated from a common progenitor allele and association with MICA*002 existed before HLA-B allelic divergence. MICA sub-types such as MICA*009:01 and 009:02 were also found to associate with different HLA-B antigenic groups. MICA*009:01 was found to be in strong LD with HLA-B5 subtypes, B51 and B52 and the variant MICA*009:02 correlated with B45 and B50, which are known to be closely related (Hildebrand *et al.*, 1992). For this analysis it was only possible to provide serological types for HLA-B (this was not ideal, however I could not obtain high-resolution data in time for the analysis). Nevertheless, as noted by Gao and colleagues (Gao *et al*., 2006), in general HLA-B serological specificities associate with individual MICA alleles, with two important exceptions. HLA-B44 showed frequent association in both recipients and donors (highest in donors) with two different MICA alleles, MICA*008 and MICA*004. The analysis by Gao, using high-resolution HLA-B typing, revealed MICA*008 was in LD with B*44:02 and MICA*004 was in LD with B*44:03. Similarly, B*35:01 associated with MICA*002, B*35:02 with MICA*016 and B*35:03 with MICA*009:01.

Improving HLA-B matching in renal transplantation may help matching for MICA alleles, as HLA-B antigens frequently associate with individual MICA alleles. Even if matching is only considered for HLA-B antigen cross-reactive groups, the common lineage of these antigens would be likely to result in matching for MICA. However, MICA mismatching can commonly occur with allelic subtypes of the same HLA-B antigen (B44 or B35) and reliable matching could only result from high resolution MICA genotyping of recipients and donors.

MICA amino acid mismatching

To enable an assessment of MICA allele mismatches that can be tolerated and those that may be responsible for the production of MICA antibodies in renal graft recipients, analysis of mismatching at the amino acid level was performed. This analysis showed nineteen residues across the α1-α3 domains were mismatched at least once between renal transplant recipients and donors and frequencies were always highest in the group who were positive for MICA antibodies. Fourteen MICA residues were significantly mismatched and highly significant corrected probabilities were achieved at twelve residues. Residues 213 and 251 were mismatched in 71% of MICA antibody producers, some of them having MICA-DSA for MICA*008, compared to 24% with no detectable MICA antibodies. These residues differ in both MICA*008 and MICA*019 alleles and this polymorphism has been demonstrated as responsible for MICA antibody recognition (Zou *et al*., 2009). Since MICA*008 is very frequent in both recipients and donors (around 50%) there is an equal chance of being matched or mismatched but when mismatched could be a very immunogenic difference leading to MICA sensitisation. Mismatching of residue 175 (expressing G or S) was also very frequent as 53% of MICA antibody positive recipients were mismatched compared to 17% who were mismatched without MICA antibodies. MICA antibodies have been found to react specifically with S at residue 175. Serine is expressed at this residue by MICA*004, *006, *009 and *019 antigens and could be considered an unacceptable MICA mismatch for recipients who do not have any of these alleles. The most striking observation involved mismatching residues that comprise the long epitope discriminating MICA-G1 alleles from MICA-G2 alleles (described in Chapter 4) and includes six residues mismatched in 53% of MICA antibody producers and around 18% of those recipients with no MICA antibodies. The epitope responsible for allorecognition of this mismatch was mapped to the α-3 domain (Zou *et al*., 2009). In simple terms, matching for MICA-G1 and MICA-G2 groups would avoid many of the mismatches that have been observed and would be an important consideration if matching strategies were to be implemented to reduce *de novo* MICA sensitisation. Mismatching of individual MICA alleles also associated with the production of MICA antibodies. MICA*011 had a frequency of 4-5% in recipients and donors and mismatches at residues specific for this allele were significantly more frequent in MICA antibody positive recipients. Similarly, MICA*004 encodes R at residue 181, a highly exposed loop region, and antibodies can react specifically with this residue (Zou *et al*., 2009). Mismatching for residue 181 was significantly more frequent in the group producing MICA antibodies.

An analysis of amino acid mismatches and association with the production of MICA antibodies was also carried out. The results show that mismatching MICA residues was more significantly associated with a positive MICA antibody status compared to MICA allele mismatches. Multivariate analysis by logistic regression revealed MICA residue mismatching as a significant independent factor associated with the presence of MICA antibodies. This novel finding indicates mismatching of group-specific MICA epitopes, as well as polymorphisms defined by individual MICA alleles, are important considerations if the aim were to minimise sensitisation to MICA antigens in renal transplantation.

5.5.2 Association of MICA antibody production with general renal transplant variables

The observation of increased frequencies of MICA antibodies in solid organ transplant recipients, compared to patients awaiting transplant or healthy controls, infers that an event specific to transplantation is responsible. As described above, MICA allele and amino acid mismatching may be one factor, and significantly associated with MICA antibody production in this study. However, other factors such as recipient and donor age and gender, whether the donor was deceased or living, previous transplantation, HLA mismatching, HLA antibodies and rejection may also influence MICA sensitisation. These particular patient demographics have not been examined in most published studies investigating the significance of MICA antibodies in organ transplantation.

A study of MICA antibodies and heart transplantation (Suarez-Alvarez *et al*., 2007) in a fairly small cohort of 44 heart transplant recipients found no significant association with donor or patient age or gender. In a large study, using sera obtained from the Collaborative Transplant Study organised by Gerhard Opelz, 1910 kidney transplant recipients were tested for MICA antibodies using pre-transplant sera (Zou *et al*., 2007). No significant association of donor or patient age with MICA antibody production was found. The gender of the donor was not considered in this study, however, a statistical trend was observed with recipient gender (P=0.07) and the highest frequency of MICA antibody producers was in the male group where 14% were positive for MICA antibodies compared to 11% of female recipients. Another study of 425 renal graft recipients (Lemy *et al*., 2010) found, as with the study by Zou, that more male recipients had MICA antibodies than female recipients (15% versus 11%). My study of 442 renal graft recipients found no significant association of MICA antibodies with recipient or donor age or gender, although this study also found a higher number of males with MICA antibodies (8.5%) compared to female recipients (6%).

The fact that more male graft recipients develop MICA antibodies than females seems to be a paradox as studies have been published suggesting pregnancy is a sensitisation event for MICA antibodies. The study by Mizutani and colleagues was not large and their pregnancy cohort of fourteen (only three with MICA antibodies) did not achieve statistical significance when compared to sera from individuals with no pregnancies (Mizutani *et al*., 2006b). From the evidence available it appears that there is no influence on the production of MICA antibodies by the age or gender of the donor. The finding from three separate, reasonably large studies that more male graft recipients have MICA antibodies than females seems to suggest that, unlike HLA antibody production, MICA sensitisation is not induced by pregnancy and further studies are warranted.

It is known that long-term GS is improved with renal transplants performed from live donors compared to deceased donors (Fuggle *et al*., 2010.). This is thought to be because of increased ischaemia time after removal of the organ and some studies investigating MICA antibodies in transplantation make comparisons with the length of cold or warm ischaemia time and the presence of MICA antibodies. Details of ischaemic time were not available to me for this study but the large study of pre-transplant MICA antibodies in 1910 renal graft recipients (Zou *et al*., 2007) found significant association (P=0.007) with cold-ischaemia time and the production of MICA antibodies. However, the MICA positive group had a lower ischaemic time of 18.1 hours compared to 19.7 hours for MICA negative recipients. As only pre-transplant serum was tested in this group of patients, the reason for this comparison is unclear as the ischaemia event occurs after testing for MICA antibodies. The renal transplant study of post-transplant sera (Suarez-Alvarez *et al*., 2009a) found no significance with the production of MICA antibodies and cold-ischaemia time although patients with MICA antibodies had a 1.3 hour longer cold-ischaemia time compared to MICA negative recipients. In another study (Lemy *et al*., 2010), there was also no significant association with the production MICA antibodies post-transplant. The MICA antibody positive group had a cold-ischaemia time of 16.6 hours compared to 15.7 hours for recipients with no MICA antibodies. Increased cold-ischaemia time could have an influence on the production of MICA antibodies as it could induce expression of allogeneic MICA antigen within the graft vasculature and increase the possibility of sensitisation. This remains to be proven as the results of these studies, although not significant, show a longer cold-ischaemia time in MICA positive recipients.

Previous exposure to allogeneic transplants is a known factor significantly associated with the production of HLA class I and II antibodies but it is unclear whether this is also an event

associated with the production of MICA antibodies. Only a few studies have investigated re-transplantation in relation to MICA antibodies in transplantation. Zou and colleagues found no significant association although recipients who had previous transplants had a higher frequency of MICA antibodies compared to recipients of their first transplant (13.4% versus 11%) (Zou *et al*., 2007). The smaller study by Suarez-Alvarez (Suarez-Alvarez *et al*., 2009a) also did not find significant association with re-transplantation and MICA antibody production. Moreover, in this group of 131 patients, 16% had MICA antibodies and a first transplant compared to 11% with MICA antibodies and a re-transplant. However, the larger study of 425 renal graft recipients by Lemy (Lemy *et al*., 2010) found that re-transplantation was highly significantly associated with MICA antibodies ($P<0.001$). They found that 27% of re-transplanted patients had MICA antibodies compared to 11% who had their first graft and was significant after multivariate analysis ($P=0.016$). It is also worth remarking that the authors of this study used the LSA-MIC assay from Gen-Probe to identify MICA antibodies and, as discussed in Chapter 4, this assay is probably more reliable than the other commercially available product from One Lambda. The results were very similar to those obtained from my study of 442 renal graft recipients. Among the re-transplanted patients, 20% had MICA antibodies compared to 6% of recipients of first transplants ($P<0.001$). After logistic regression multivariate analysis, re-transplantation was an independent significant factor associated with MICA antibodies ($P=0.020$). Therefore it is highly likely that re-transplantation is an event associated with the production of MICA antibodies in renal graft recipients.

I also included the presence or absence of HLA class I/II antibodies to investigate correlation of their presence with MICA antibody status. Zou and colleagues also carried out this analysis in their study and found that 17% of recipients with class I antibodies also had MICA antibodies compared to 11% with class I antibodies and no MICA antibodies (Zou *et al*., 2007). Likewise 17% of recipients with HLA class II antibodies had MICA antibodies compared to 11% with class II antibodies and no MICA antibodies ($P=0.007$ respectively). This was very similar to the outcome from my own analysis where 16% of recipients with either class I or II antibodies had MICA antibodies compared to 5% of recipients without either HLA class I or II antibodies ($P<0.004$ and $P=0.007$ respectively). This could mean that cellular events post-transplantation that result in the generation of HLA antibodies can also result in the production of MICA antibodies. Alternatively, it could be that the statistical association of HLA and MICA antibodies arises because both are more frequently detected in re-transplanted patients as discussed. There was also a significant association of HLA class I or II antibodies with re-transplantation ($P<0.001$) and

if patients with both HLA and MICA antibodies were analysed (P<0.001). Additionally, HLA class I or II antibodies were not independent significant factors associated with MICA antibodies after multivariate analysis by logistic regression. It is therefore probable that events leading to HLA sensitisation do not necessarily generate the production of MICA antibodies and *vice versa*. Separate immunological events involving MICA antigens and antibodies may be additional events involved in the aetiology of ACR or aAMR and will be discussed next.

5.5.3 Association of MICA allele mismatching and MICA antibody production with acute renal graft rejection

The observation that many transplant recipients with rejection have antibodies to MICA was made over ten years ago (Zwirner *et al*., 1999; Zwirner *et al*., 2000) and numerous studies have since investigated MICA antibodies and graft rejection. Around a quarter of kidney transplant recipients experience ACR or aAMR, often in the first year post-transplant. Improvements in immunosuppressive therapy, aimed at limiting the effects of T-cell mediated immune responses to the graft, have increased overall GS (Meier-Kriesche *et al*., 2004) and reduced AR. However rejection due to antibody-mediated graft damage arising from B-cell responses to mismatched HLA antigens remains a problem. The production of post-transplant, *de novo* DSA to HLA antigens is associated with acute and chronic allograft rejection (P. C. Lee *et al*., 2002; Trpkov *et al*., 1996). Although many renal transplant recipients with rejection have developed HLA antibodies, previous studies suggest that 11-20% of patients without HLA antibodies may develop CAD (P. C. Lee *et al*., 2002; Worthington *et al*., 2001). Additionally, HAR can occur in the absence of HLA antibodies, implicating other alloantigens (Brasile *et al*., 1986; Sumitran-Karuppan *et al.*, 1997; Zou *et al*., 2006b). Possible candidates are the polymorphic proteins encoded by the MICA and MICB genes.

For this study, diagnosis of rejection from biopsy analysis was available for 391 of 442 graft recipients. Patients were categorised as having no rejection, ACR or aAMR. The results show that ACR was significantly associated with the presence of MICA antibodies in graft recipients but no association was found with MICA antibodies and aAMR. This is in contrast to the highly significant association of HLA antibodies with aAMR episodes. For example, 70% of patients with both HLA class I-DSA and class II-DSA had aAMR compared to 26% without both types of HLA antibody (P<0.001). However, in a recent study of heart transplant recipients, 72 patients and donors were typed for MICA alleles and

the authors found significant association (P=0.01) of MICA-DSA with aAMR (Q. Zhang *et al.*, 2011).

The detection of C4d in the endothelium of intertubular capillaries is generally accepted as the hallmark of antibody-mediated immune responses within the graft (Feucht, 2003). Only one patient confirmed as positive for MICA antibodies experienced aAMR and immunoperoxidase analysis for C4d deposition was negative (Racusen *et al*., 2003). By contrast 50% of the six recipients with co-production of HLA class I+II-DSA had significant C4d staining. One study specifically investigated the association of MICA antibodies with C4d staining (Alvarez-Marquez *et al*., 2009). MICA allele typing was also performed for both patients and donors (n=58) to investigate MICA donor specificity but no details of MICA types were provided. The authors found that of the 19 patients who were C4d+, four patients had MICA antibodies (21%) compared to three patients (7.7%) with MICA antibodies and no C4d staining, however this was not significant. It remains unclear whether MICA antibody-mediated graft damage can be detected with C4d staining and may implicate involvement of other, non-complement mediated, mechanisms of MICA antibody allorecognition.

Most studies investigating the presence of MICA antibodies with graft rejection classify rejection as either acute or chronic. Significant association has been observed with both types of rejection and I will focus on AR in this section. Unless the purpose was to identify antibody-mediated rejection involving MICA antibodies, no study has stated whether the rejection was ACR or aAMR. In this study ACR was found to be significantly associated with the presence of MICA antibodies and indicates cellular rejection processes are either a trigger for MICA antibody production or the involvement of MICA antibodies exacerbates cellular rejection to pathological levels. A study of 44 heart transplant recipients (Suarez-Alvarez *et al*., 2007), using One Lambda's MICA SAg Luminex assay, revealed 55.5% of recipients with MICA antibodies had AR compared to 44% without MICA antibodies (P=0.002) and was confirmed by CDC using MICA transfected cells. All patients in this study were tested within the first year post-transplant. This compares to ACR occurring in 35% of renal graft recipients with MICA antibodies in my study with significant association (P=0.021). The higher frequency of MICA antibodies detected within the first year of heart transplantation by Suarez-Alvarez and colleagues correlates with the high degree of AR in the early post-transplant period. In a study of 139 kidney transplants (Mizutani *et al*., 2006b), 37% of recipients with MICA antibodies (detected by MICA transfected CDC) and

no HLA antibodies had graft rejection compared to 19% with functioning grafts and MICA antibodies but no HLA antibodies (P<0.01).

Another finding from my study was that some patients with ACR have very high MFI values when tested with MICA Luminex assays. Using an arbitrary cut-off, patients were categorised as having 'strong' MICA antibodies and compared to patients who did not have strong MICA antibodies. In this analysis there was a highly significant association between strong MICA antibodies and ACR (P<0.001) and was confirmed by logistic regression multivariate analysis (P=0.001). It was also observed that all of the recipients with MICA antibodies who experienced ACR had strong MICA antibodies.

The association of ACR across different categories of HLA and/or MICA antibodies was also examined and revealed association with both HLA and MICA antibodies independently and together. The combination of both MICA and HLA antibodies has been shown previously to have detrimental effects leading to rejection or decreased GS (Mizutani *et al*., 2006a; Panigrahi *et al.*, 2007b). In my study there was also association with MICA-DSA and ACR, although only approaching significance (P=0.051).

I was able to determine the association of donor-specific MICA antibodies with transplant rejection and outcomes for seventeen donor and recipient pairs where the recipient was positive for MICA antibodies. This analysis showed that 71% of the seventeen MICA positive individuals developed *de novo* MICA antibodies post-transplant and within this group 92% developed antibodies reactive with MICA antigens expressed by the donor organ. Five of the seventeen MICA positive individuals had MICA antibodies defined as strong and all five had ACR rejection. Among them, three patients had strong MICA-DSA and ACR but no HLA donor-specific antibodies, confirmed by HLA SAg Luminex. One of the patients currently has very poor function (eGFR<15 ml/min/1.73m^2) and a biopsy examined within nine months of the AR episode showed a chronic damage (CD) index of 59% and a CD index >40% is considered highly predictive of renal graft failure (Howie *et al.*, 2004). This patient first tested positive for MICA antibodies one-year post transplant (two days after biopsy was taken) with moderate strength. Eight months later these MICA antibodies, with the same specificities, increased to strong MFI and at this time HLA-DSA DQ9 was detected but was weak and below cut-off for assigning positivity. Serial serum sample analysis of the five patients with AR and MICA antibodies revealed that all developed MICA antibodies after the AR episode. This is in contrast to previous studies where it was stated that 'most' had MICA antibodies detected before the rejection episode

(Panigrahi *et al*., 2007b; Suarez-Alvarez *et al*., 2007). It is very clear from my study however that the MICA antibodies, which were strong, were detected following the ACR episode. Alternatively, MICA antibodies could be involved in the pathogenesis of ACR but were not detected prior to rejection because they were adsorbed within the graft where they were causing complement-mediated damage via interaction with mismatched allogeneic MICA antigens. Consequently the MICA antibodies could be depleted and undetectable in the periphery as is known to occur with HLA antibodies (Terasaki and Cai, 2005). In this case, once the AR episode subsides, the MICA antibodies may re-enter the periphery where they are detected with strong MFI.

From these results, it is likely that T-cell allorecognition of mismatched MICA epitopes can give rise not only to T-cell help for the production of IgG antibodies against MICA, but also cell-mediated immunity resulting in cellular rejection. By comparison, a study investigating MICA matching and graft versus host disease (GvHD) in hematopoietic stem cell transplantation found a significantly higher rate of grade II-IV acute GvHD in MICA mismatched patients, indicating a T-cell response to mismatched MICA antigens (Parmar *et al.*, 2009). This type of immune response to solid organ transplants is the hallmark of indirect allorecognition and may occur where allogeneic, MICA peptides from necrotic or apoptotic graft cells are taken up by recipient APCs such as DCs or B-cells and presented to recipients naïve T-cells in the lymph nodes. The end result is the production of effector cells and a cellular and antibody-specific response against mismatched allogeneic MICA molecules leading to graft destruction.

The association of AR with MICA antibodies remains unclear and the finding that ACR is associated needs to be clarified. A large study of patients with rejection, several hundred if not more, together with MICA antibody testing and MICA allele typing of both patient and donor would be required to draw conclusions of any certainty. Collaborative studies are the easiest way of achieving this and currently Peter Stastny is organising a MICA component for the next IHIWC to be held in the UK in 2012.

In Chapter 7, I investigate association of MICA antibodies with five-year GS and decreased graft function, as determined by measurement of eGFR post-transplant, in an attempt to correlate the presence of MICA antibodies with the longer-term pathogenesis of CAD. In the next chapter (Chapter 6) I carry out a time-course investigation of several renal graft recipients with MICA antibody production to determine any association of events post-transplant with changes in MICA antibody MFI as detected by Luminex.

CHAPTER 6

MICA antibody production and evolution in renal transplant recipients: a longitudinal analysis

6.1 Introduction and aims

Undoubtedly, the most important advance leading to a reduction in T-cell mediated rejection in renal transplantation was the introduction of cyclosporine as an immunosuppressive agent (Calne and Wood, 1985). Twenty-six years later, little has changed in the prevention of late graft rejection due to chronic rejection associated with both immune and non-immune factors (Thorogood *et al.*, 1992). CAD and death with a functioning graft remain the largest cause of graft loss after the first year of transplantation with a loss rate of 3-5% each year (Pascual *et al*., 2002). One of the factors associated with CAD is the presence of HLA antibodies (Terasaki and Cai, 2005; Vasilescu *et al.*, 2006) and evidence is also accumulating that implicates MICA antibodies with chronic dysfunction (Mizutani *et al*., 2005; Terasaki *et al*., 2007). HLA and/or MICA antibodies are frequently found before graft failure and can occur many years after the transplant. It is thought that HLA antibodies initiate a slow process of damage and repair to the graft endothelium leading to luminal occlusion and loss of function (Terasaki and Cai, 2005). MICA antibodies may also be involved in the same processes and particularly target the glomerulus in renal transplantation, as MICA expression is high in this compartment (L. Li *et al*., 2010; L. Li *et al.*, 2009).

The development of fluorescent micro-bead assays (Luminex) for the detection of HLA and MICA antibodies has enabled a more precise evaluation of their role in renal transplantation and whether or not antibody specificities are directed towards the donor. It is thought that up to 60% of CAD in organ transplants is caused by HLA antibodies (Piazza *et al.*, 2001) and HLA-DSA are highly associated. Lee and colleagues found that all patients with graft loss due to chronic rejection had developed *de novo* HLA antibodies (P. C. Lee *et al*., 2002). In a five-year longitudinal study of 54 renal graft recipients, serial serum analysis revealed extremely high association with HLA-DSA and graft failure in thirteen of fifteen cases (Mao *et al.*, 2007). Additionally the appearance of HLA antibodies always came before an elevation of SCr creating a window of opportunity for therapeutic intervention. Rise in SCr also preceded the appearance of MICA antibodies in recipients with no HLA

antibodies in a similar study (Kinukawa *et al.*, 2006) but in one case, levels of SCr began to rise before MICA antibodies were detected. In another longitudinal study of 266 patients for HLA and MICA antibody production, 11.6% were found to have MICA antibodies and 21% of recipients with graft failure had MICA antibodies compared to only 7.2% of patients with successful grafts (Ozawa *et al.*, 2006). However in the study by Ozawa and colleagues, all but one of the MICA antibody positive recipients also had HLA antibodies and no MICA typing information of the donors was available in their study or that of Kinukawa.

There are currently no detailed published studies investigating the long-term course of MICA antibodies in renal transplant recipients and many questions remain unanswered. If MICA antibodies have a pathological role in transplantation it is important to know whether the appearance of antibodies identified in renal graft recipients for this study represents a permanent MICA antibody status or a transient phenomenon in the post-transplant period. It is known that HLA antibodies can be detected within twelve months but often disappear completely and have no effect on transplant outcome (Ozawa *et al*., 2006) and this could also be the case with MICA antibodies. The results presented in Chapter 5 show that most recipients with *de novo* MICA antibodies have specificities directed towards antigens or group-specific epitopes expressed on the donor organ but how long do they take to appear and do they persist? MICA antibodies having high MFI values associated with ACR in this study, but does the strength of MICA antibodies remain high or decline? Most importantly, does the continued presence of MICA antibodies affect graft function or lead to failure?

The logical way to begin to answer the above questions is by serial analysis of serum samples from graft recipients, and this has been carried out extensively with regard to HLA antibodies. Current Luminex technology now allows sensitive detection of antibodies and it is possible to detect very low levels. Thus, it is possible to detect sub-clinical appearance of antibodies that may predict the emergence of higher-titre antibodies that could be implicated with acute or chronic dysfunction. It is also possible to define DSA pre and post-transplantation. Pre-transplant HLA and MICA antibodies can be implicated with aAMR (Lefaucheur *et al.*; Zou *et al*., 2007) and crossmatch negative HLA-DSA are highly associated with AR. Development of *de novo* HLA antibodies may also be a mechanism involved in CAD (Lachmann *et al.*, 2006). Therefore, Luminex is an excellent tool to investigate the longitudinal development of MICA antibodies in renal transplant recipients.

In this chapter I analyse a group of MICA antibody positive renal graft recipients by longitudinal serum sample testing using Luminex antibody detection technology. With this approach, I was able to determine the time-period between transplantation and the *de novo* appearance of MICA antibodies. The relative MFI values of MICA-DSA and NDSA, the association with ACR and decreased function as measured by eGFR, was also assessed. In patients who were transplanted too long ago to determine the MICA types of the donor, serial serum analysis enabled a 'snapshot' of MICA antibody production many years post-transplant. Taken together these analyses reveal novel insights into MICA antibody production and evolution in renal graft recipients.

6.2 Detection of MICA antibodies

MICA antibodies were originally detected using three independent fluorescent bead assays as described in Chapter 4. For longitudinal analysis, the primary method for MICA antibody detection was the Gen-Probe MICA SAg Luminex assay, carried out as described in Chapter 2. Most of the serum samples were also confirmed for specificity and MFI strength by the Stastny MICA SAg Luminex assay in their laboratories in Dallas, USA.

6.3 Patients and sera

Among the 33 recipients with MICA antibodies only sixteen were selected for longitudinal analysis, mainly due to the high cost of commercial Luminex assays and the high number of sera to be tested. Patients with MICA typing details of their donors were of primary importance, especially those with ACR and were selected on the basis of serum availability. Other patients with older transplants were also selected to evaluate antibody production in long-term recipients. The time-points of serum samples depended on availability but ideally included a sample at one month pre-transplant followed by all available serum in the first three months post-transplant and every three or six months after that for two or three years of follow-up. In total, 108 serial serum samples from the sixteen recipients were analysed for MICA antibodies.

6.4 Assessment of graft function

To investigate association of MICA antibodies with graft function, eGFR was calculated from serum creatinine values and expressed as ml/min/1.73m^2 according to the 4-point

Modification of Diet in Renal Disease method (MDRD) as described (A.S. Levey *et al.*, 2006), (Burden and Tomson, 2005). Measurements of eGFR were taken at one, two and three years post-transplant and were available for the MICA-typed pair cohort only. An eGFR <30 is considered poor function and indicates chronic renal damage. Assessment of chronic renal damage in confirmed MICA antibody positive recipients was made by renal pathologists on renal biopsies using a morphometric index of chronic damage (CD), which expresses the cross-sectional area of chronically damaged cortex as a percentage of the cross-sectional area of the renal cortex. An index >40% was strongly predictive of renal failure (Howie *et al*., 2004).

6.5 Longitudinal testing of individual patients for MICA antibodies

The data obtained from MICA antibody testing were used to plot charts of MFI against time (months) for each of the sixteen patients included in this part of the study. The first time-point was the earliest sample to be tested, in most cases around one month before the transplant. Also included in the charts are the eGFR measurements for up to three years of follow-up and their values are indicated on the secondary axis. MICA specificities and major events such as transplantation or ACR are indicated by arrows on the charts. Where appropriate, the MICA types of the patient and donor are displayed and the MICA-DSA and NDSA are indicated. Some MICA antibody specificities, not related to the donor, may also be detected due to cross-reaction with specific shared epitopes as described in Chapter 4 and are indicated by bold typeface on the charts (epitope-specific).

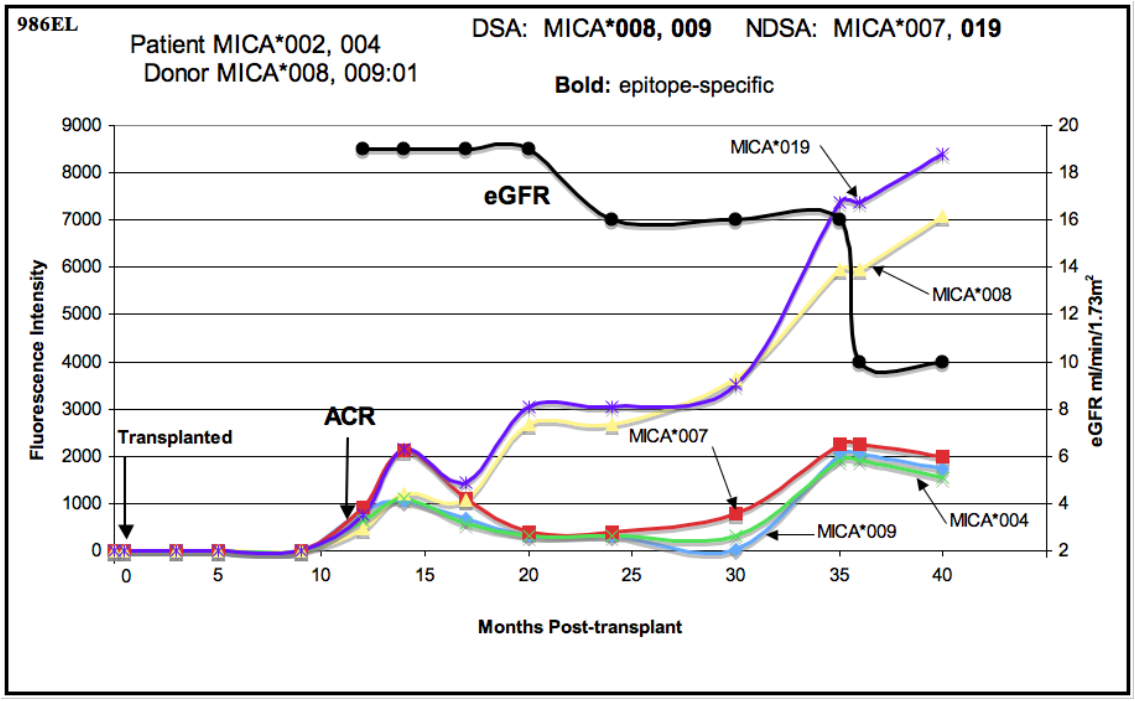

Figure 6.5.1: Longitudinal MICA antibody analysis of patient 986EL. MICA antibody production occurred between nine and twelve months post-transplant when MICA*004, 007, 008, 009 and 019 antibodies were detected with low MFI. At the same time MICA antibodies were detected, biopsy evidence showed ACR. The fluorescence intensity of MICA*008 (DSA) and *019 then increased to high levels while in the same period eGFR decreased. In addition, this patient has CAD with an index of 59% chronic damage in the renal cortex.

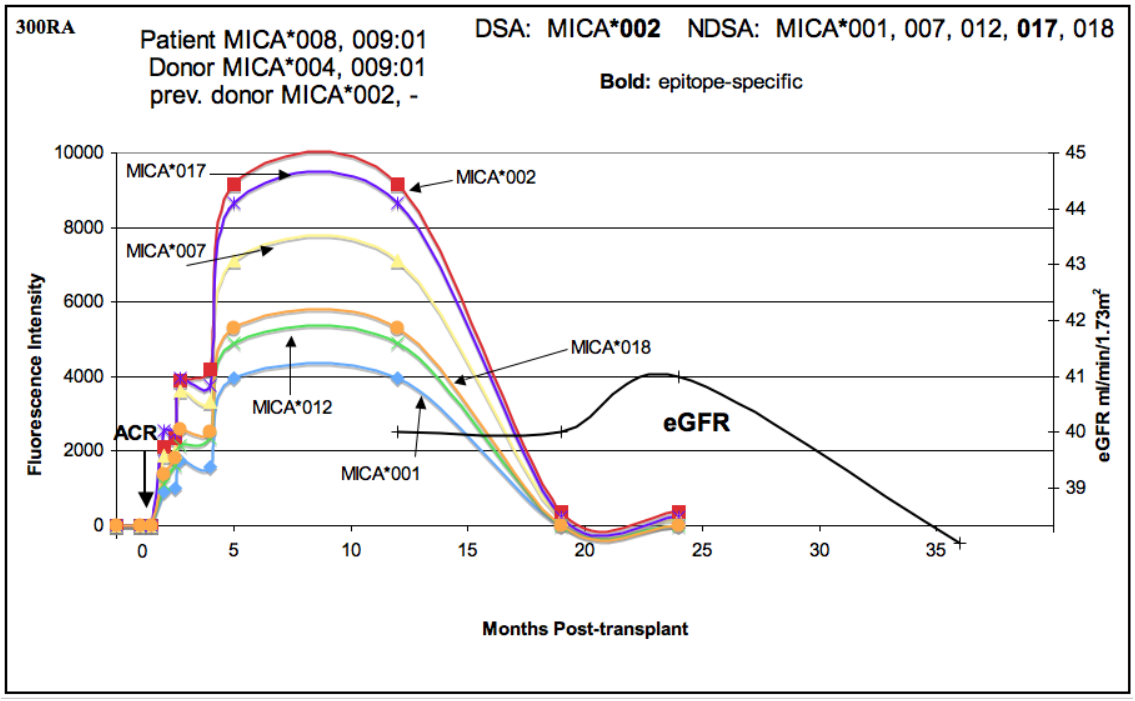

Figure 6.5.2: Longitudinal MICA antibody analysis of patient 300RA. Only HLA antibodies with weak specificity were found and the patient developed MICA antibodies by 6 weeks post-transplant. MFI then increased steadily over the following months with highest values achieved by previous donor-specific MICA*002 and epitope-specific (14G) MICA*017. No MICA antibodies directed towards the current graft were detected. ACR was diagnosed before the appearance of MICA antibodies. By nineteen months post-transplant, MICA antibodies were almost undetectable. After an initial improvement in eGFR during the second year of transplant, a decline was evident after three years.

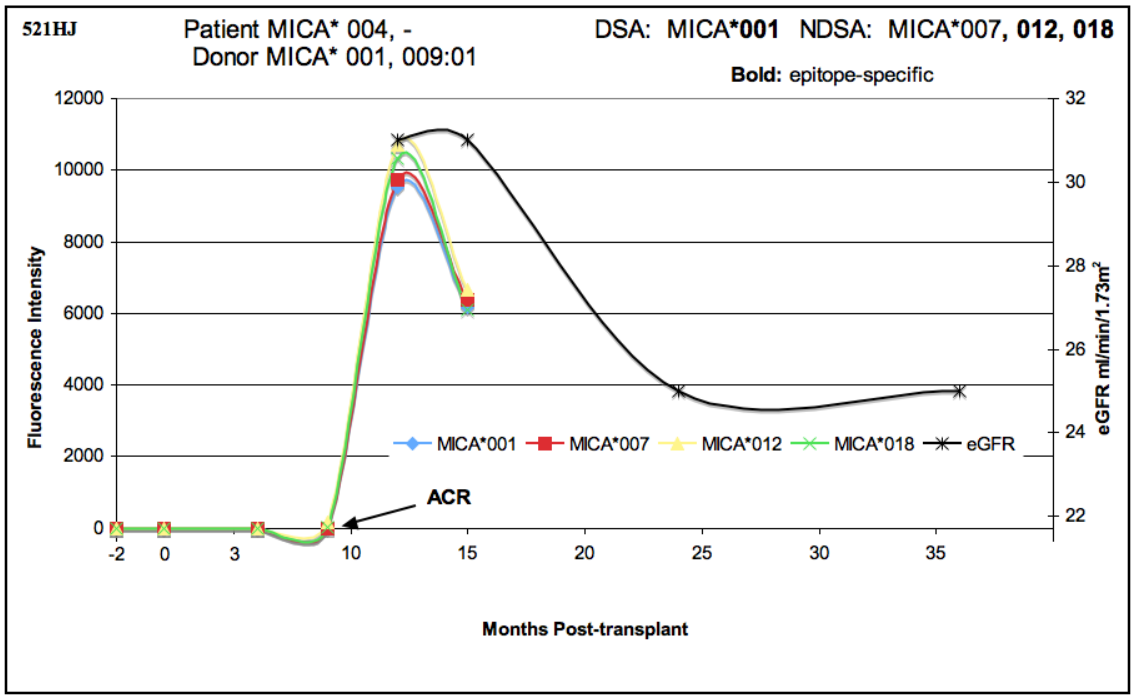

Figure 6.5.3: Longitudinal MICA antibody analysis of patient 521HJ. No HLA antibodies were detected in this patient. MICA antibodies were not detected until ten months post-transplant but sensitisation could have occurred between eight and ten months. Sera collected four days before diagnosis of ACR showed a weak positive signal for MICA*001, *012 and *018 which rose to high levels two months later in addition to MICA*007. MFI for all MICA antibodies fell to 6000 over the next five months. The rise and fall of MICA antibodies was followed by a decline in eGFR after 24 months.

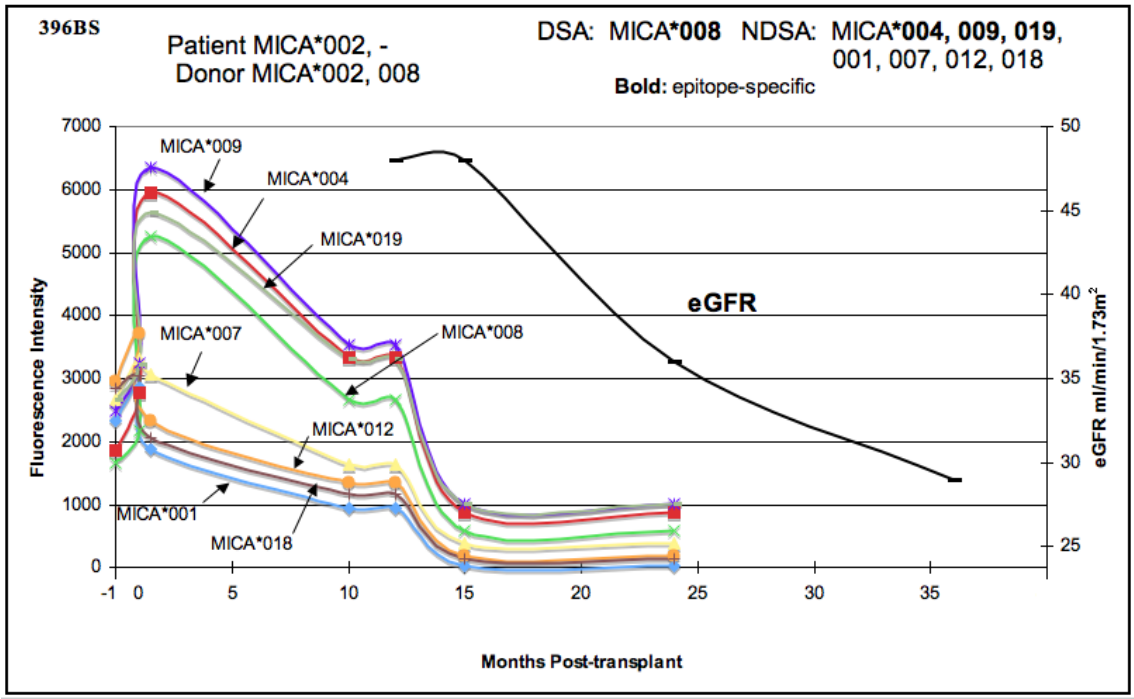

Figure 6.5.4: Longitudinal MICA antibody analysis of patient 396BS. This patient produced MICA-DSA and NDSA pre-transplant and had a previous graft failure in 2003. No HLA-DSA was detected. Following re-transplantation in 2006 there was a rise in MFI for MICA-DSA and epitope-specific antibodies and a decline with NDSA. All antibodies declined to low levels by 24 months post-transplant when a decline in eGFR was also recorded.

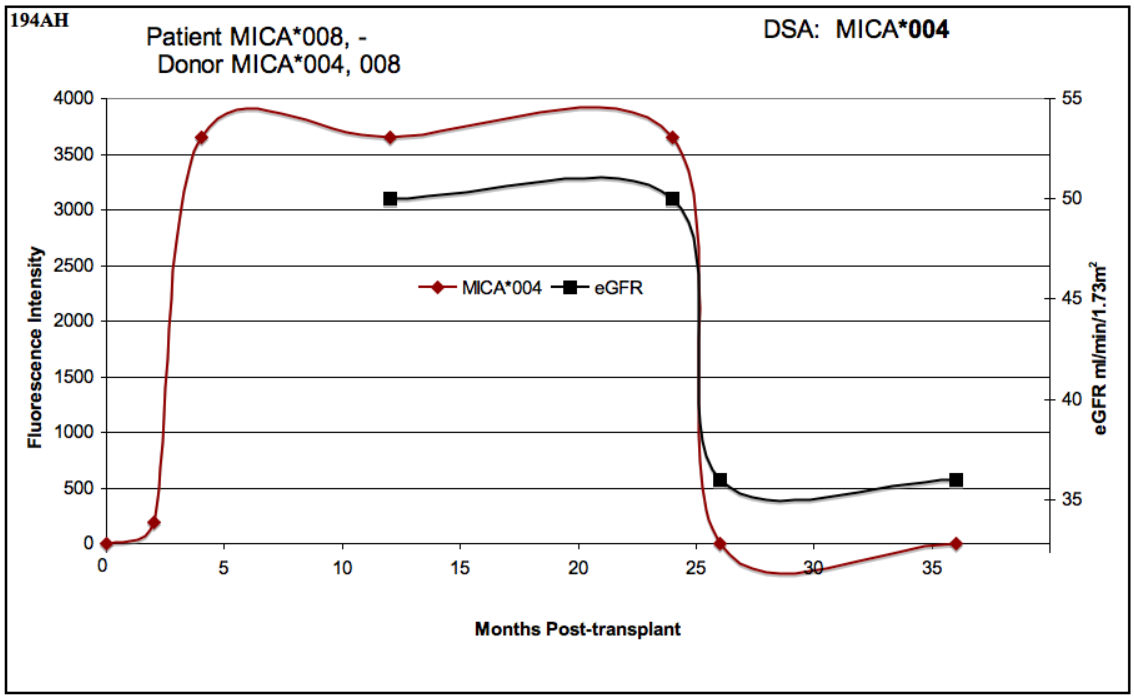

Figure 6.5.5: Longitudinal MICA antibody analysis of patient 194AH. No HLA antibodies were detected in this patient who was negative for MICA antibodies prior to transplant, developing low levels of DSA MICA*004 after two months post-transplant. MICA*004 MFI then increased to positive levels and remained steady for up to two years before disappearing completely and still undetectable by three years post-transplant. The presence of MICA*004 DSA coincided with a steep decline in eGFR between the second and third year of transplantation.

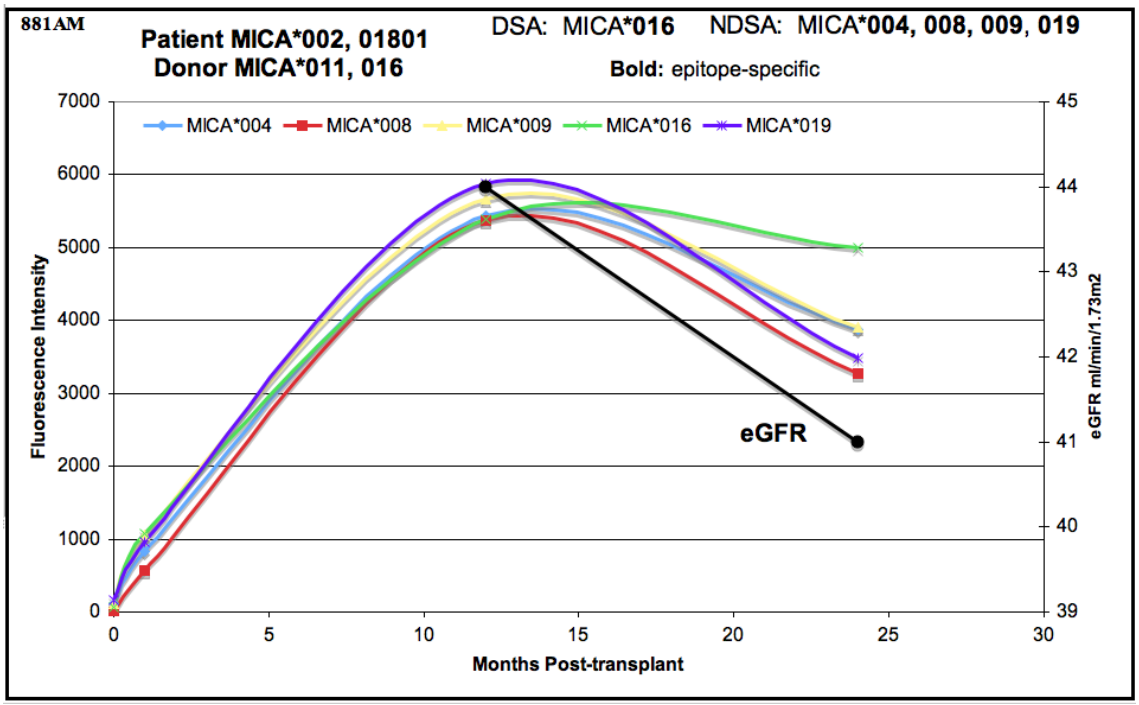

Figure 6.5.6: Longitudinal MICA antibody analysis of patient 881AM. This patient had a failed transplant in 2001 following ACR and was re-transplanted in 2007. Pre-transplant sera was negative for MICA antibodies but detectable MICA-DSA and epitope-specific antibodies were present three weeks after transplantation and by twelve months had increased to high positive levels. After 24 months MICA antibody MFI-levels declined slightly but were still high. There was also a modest decline in eGFR 12-24 months post-transplant.

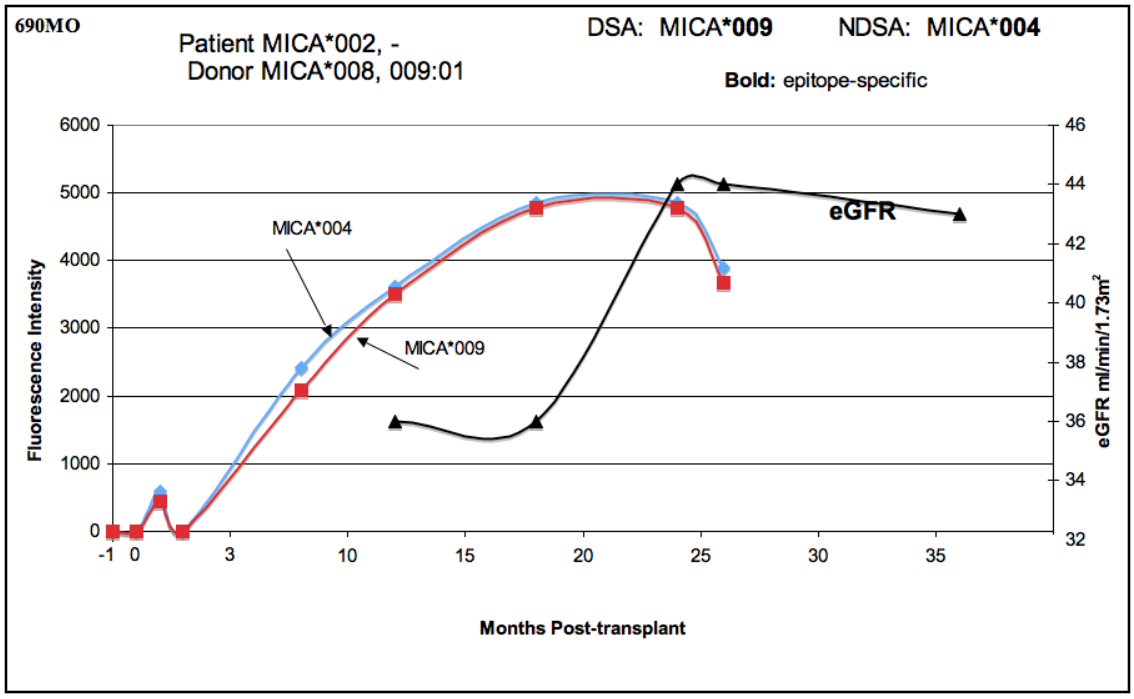

Figure 6.5.7: Longitudinal MICA antibody analysis of patient 690MO. This patient, transplanted in 2007, was negative for MICA antibodies pre-transplant and had received a previous graft. At one month post-transplant there were detectable MICA*004 and *009 DSA but these disappeared the next month. At six months post-transplant MICA antibodies were positive and increased steadily over the next ten months before declining slightly at around 24 months post-transplant. There was an improvement in eGFR, which was initially very poor but shows signs of further decline after three years post-transplant.

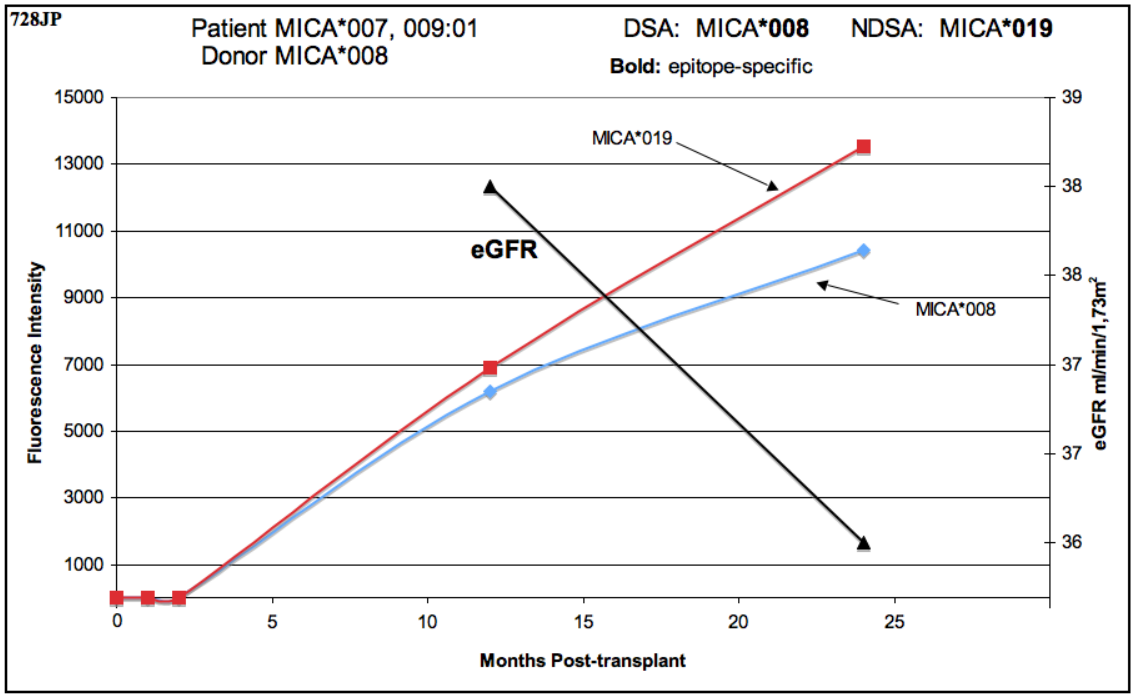

Figure 6.5.8: Longitudinal MICA antibody analysis of patient 728JP. This patient did not test positive for MICA antibodies until twelve months post-transplant when MICA*008 DSA and MICA*019 (shared epitope) were detected with high MFI, rising higher over the next twelve months. Between one and two years post-transplant there was also a modest decline in eGFR.

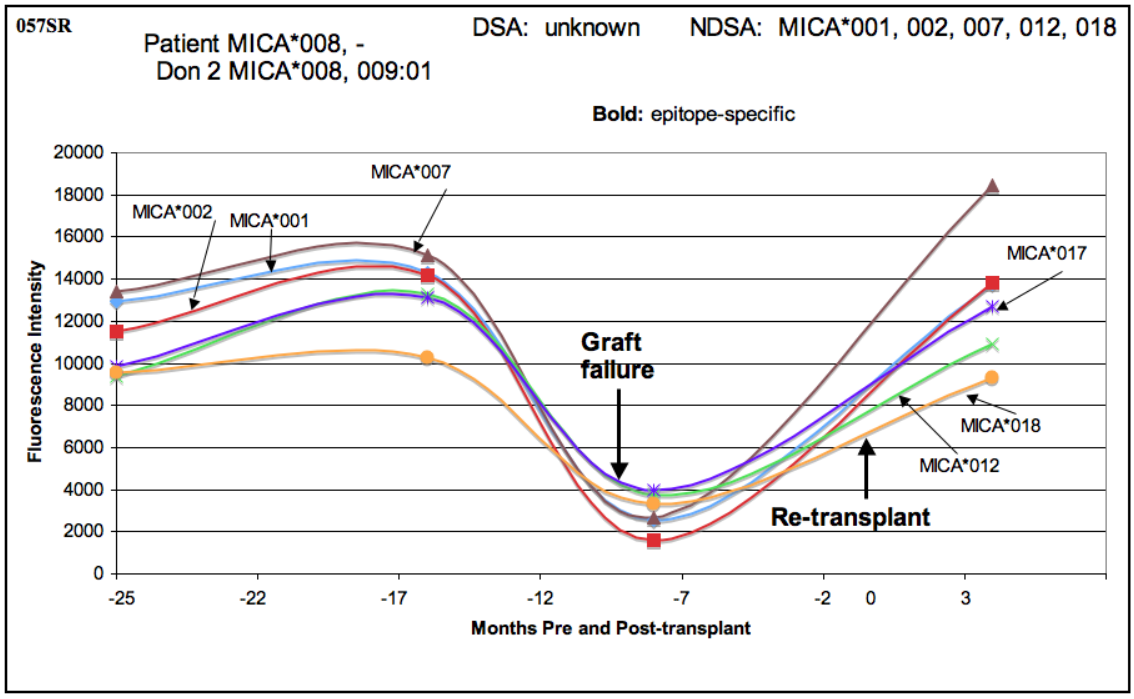

Figure 6.5.9: Longitudinal MICA antibody analysis of patient 057SR. This patient had no HLA antibodies, was originally transplanted in 1990 and recently lost the graft due to chronic dysfunction. High MFI MICA antibodies were present and detected ten months prior to failure. After graft failure MICA antibodies had a much lower MFI but were still positive. A few months after re-transplantation, MICA antibody MFI values for the same specificities became high again.

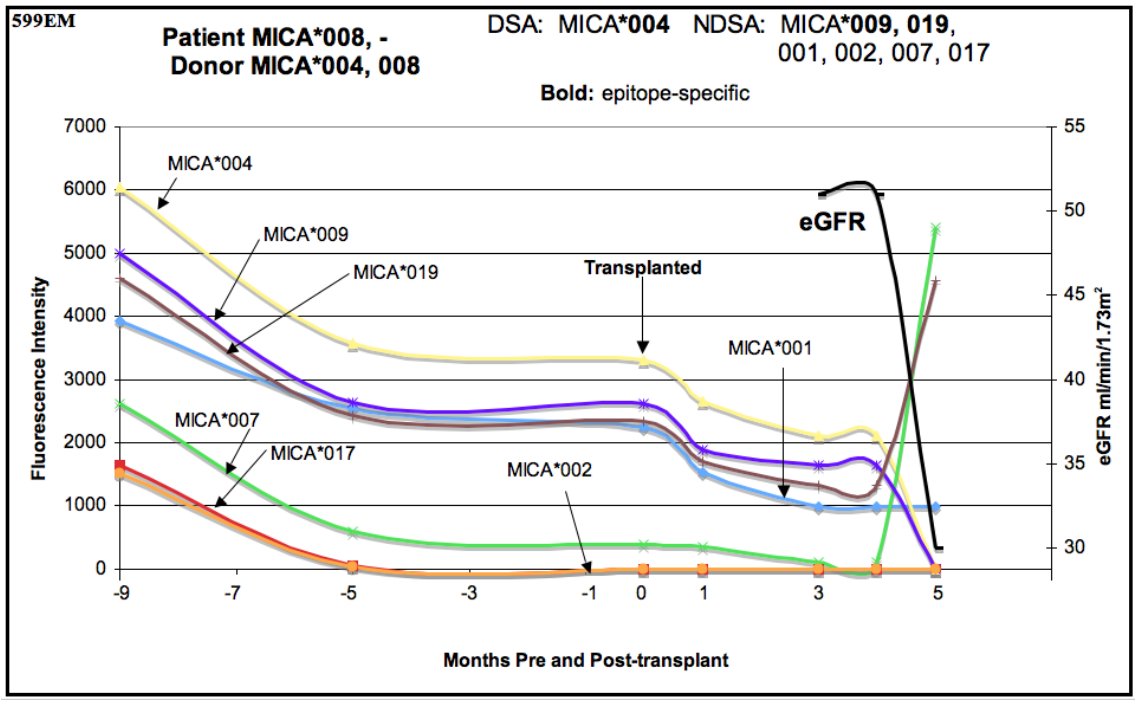

Figure 6.5.10: Longitudinal MICA antibody analysis of patient 599EM. This patient was positive for MICA antibodies prior to transplantation in 2008. MICA*004 DSA were present pre-transplant with the highest MFI. Following transplantation there was an initial decline in MICA antibodies. MICA*002, 004 and 017 became undetectable whereas MICA*007 and 019 antibodies increased to high levels five months after transplantation. There was also a large decline in eGFR during the first year of transplantation. This patient did not develop HLA antibodies and died with a functioning graft of unrelated cause fifteen months after transplantation.

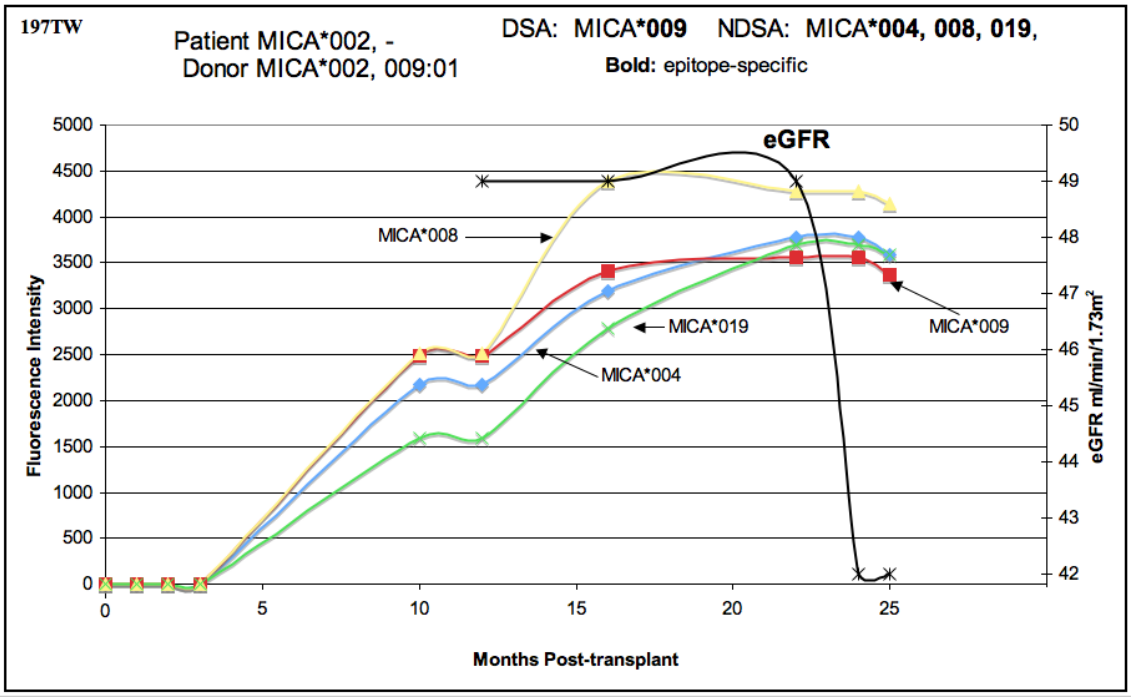

Figure 6.5.11: Longitudinal MICA antibody analysis of patient 197TW. This patient had no HLA antibodies and no MICA antibodies before transplantation, developing MICA-G2 antibodies (MICA*004, 008, 009, 019) 3-9 months post-transplant. The MICA antibodies then persisted for >24 months and eGFR declined in the second year of transplant. There are signs of CAD with 47% chronic damage in the renal cortex.

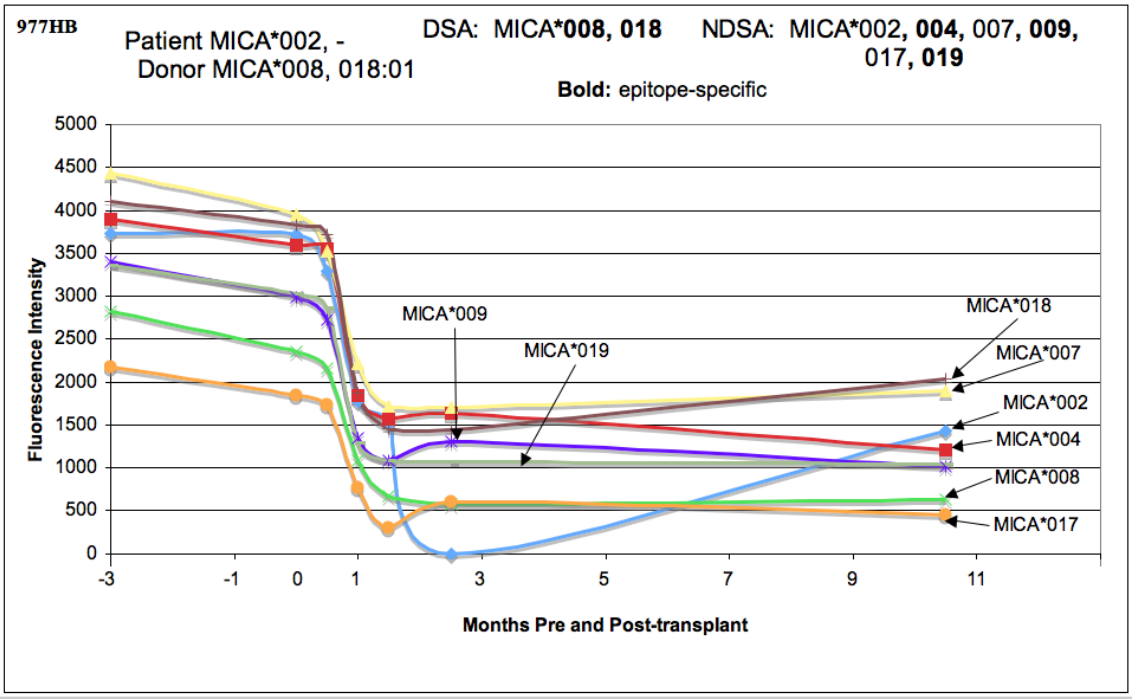

Figure 6.5.12: Longitudinal MICA antibody analysis of patient 977HB. This patient was re-transplanted in 2009 with pre-existing MICA antibodies. There is an autoantibody MICA*002 detected in this patient and primary disease is lupus erythematosus. There are also MICA antibodies specific for donor MICA antigens comprising MICA-G2 (MICA*004, 008, 009, 019) and MICA*018. MFI for all antibodies declined sharply just after transplantation then remained steady, at a lower level than pre-transplant, for ten months..

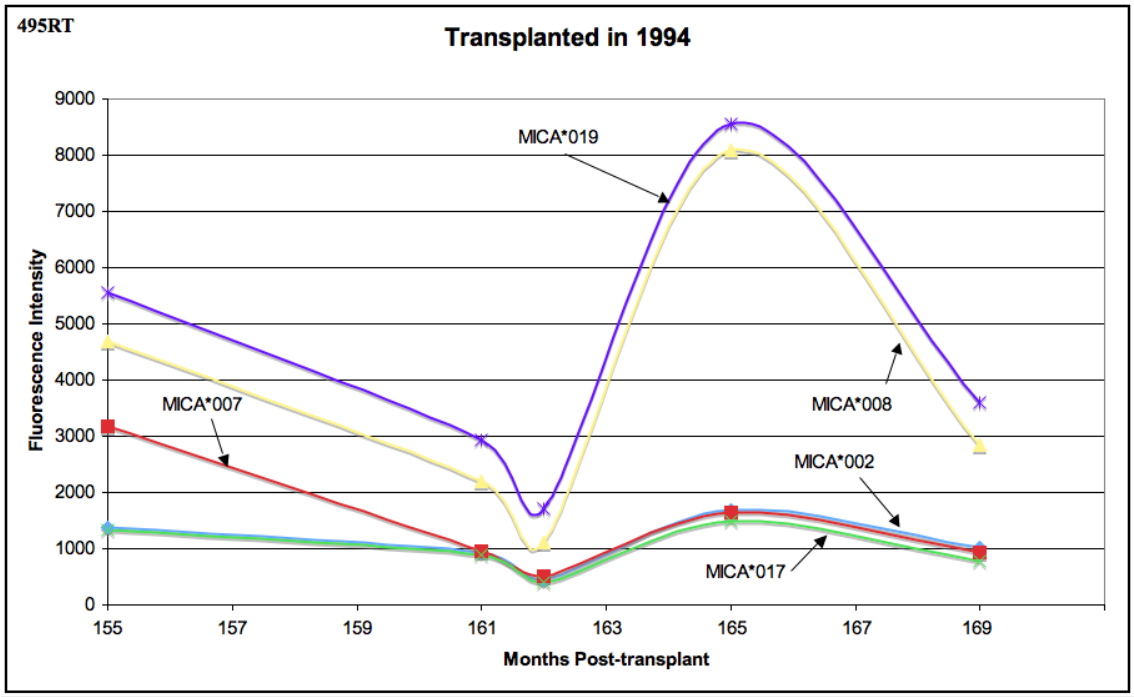

Figure 6.5.13: Longitudinal MICA antibody analysis of patient 495RT. This patient has a functioning graft transplanted in 1994, with an early AR episode. There appears to be two groups of antibodies with different MFI values. MICA*008 and 019 share polymorphisms at residues 213 and 251 and have similar fluorescence intensity values. MICA*002 and 017 both have G at residue 14 and are below the threshold of MICA antibody positivity.

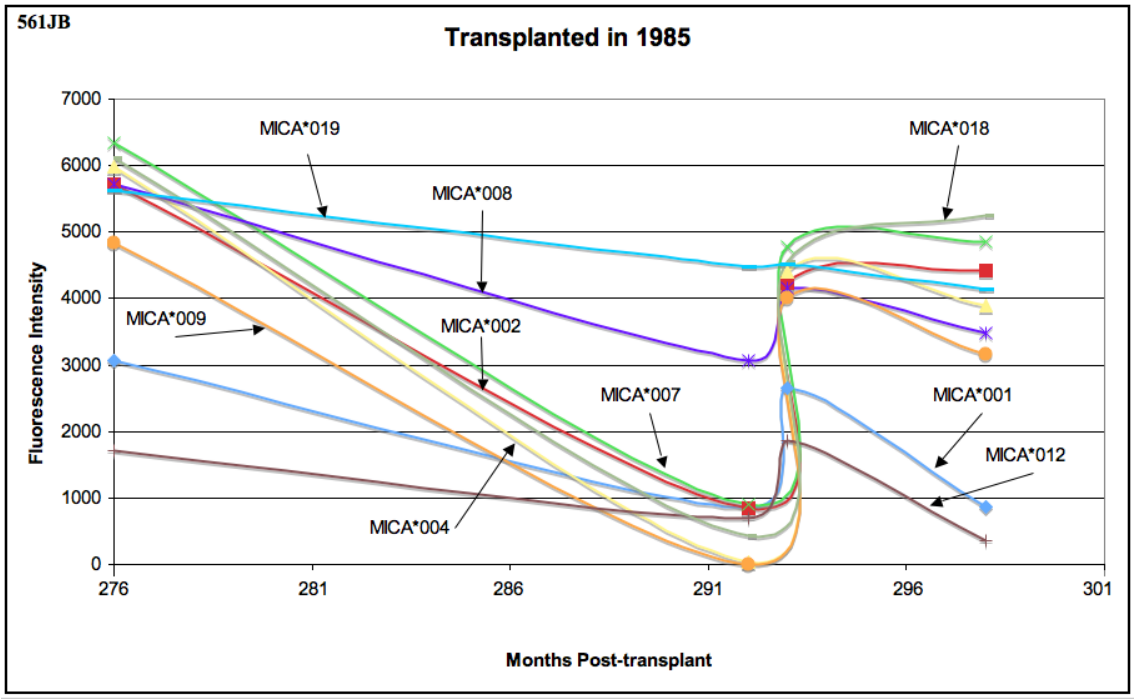

Figure 6.5.14: Longitudinal MICA antibody analysis of patient 561JB. This patient was transplanted in 1985 with an unknown rejection history during the early stages of transplantation. The MICA antibody profile shows two types of antibody may be present. MICA-G2 antibodies are all present (MICA*004, 008, 009 and 019) and MICA*001, 012 and 018 which each express T at residue 24. The presence of MICA*007, as with many of these patients, remains unexplained. Despite slight fluctuations, the level of antibodies appears to be steady.

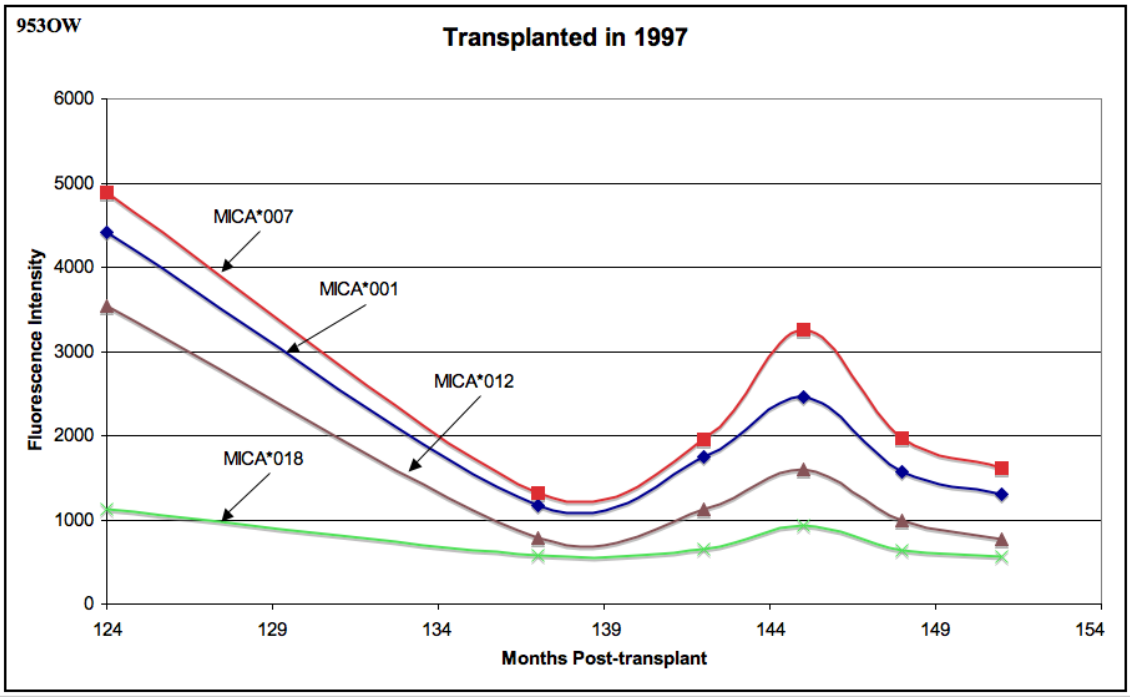

Figure 6.5.15: Longitudinal MICA antibody analysis of patient 953OW. This patient did not experience AR and appears to have developed MICA antibodies against 24T shared by MICA*001, 012 and 018. MICA*007 is also present and has the highest fluorescence intensity.

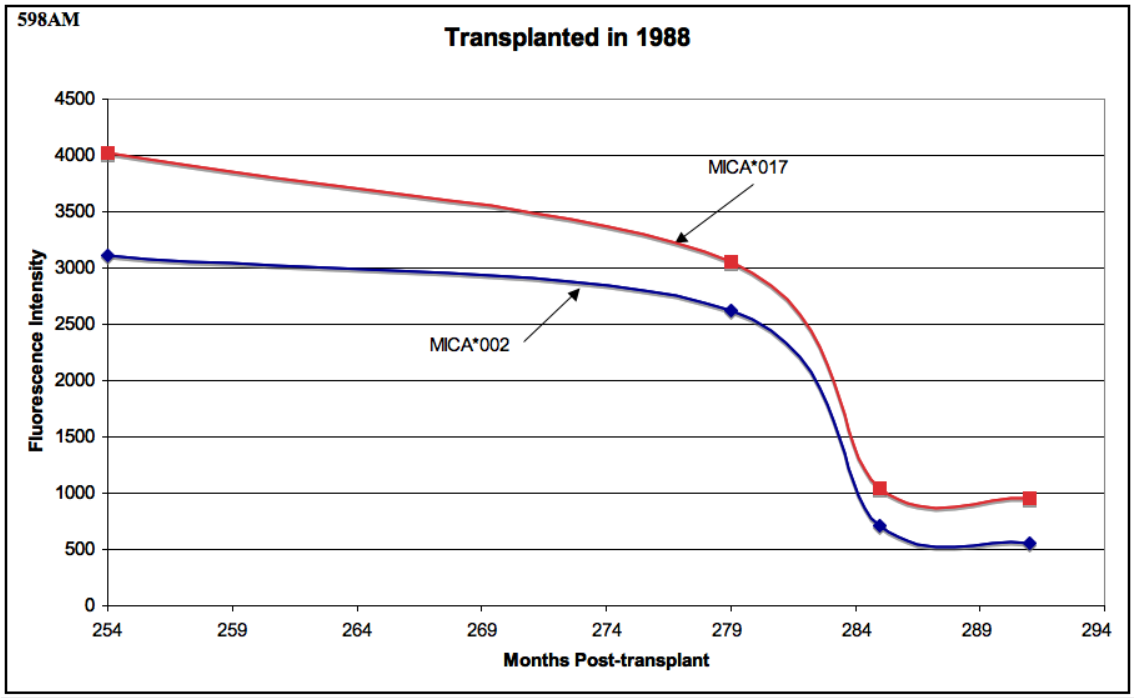

Figure 6.5.16: Longitudinal MICA antibody analysis of patient 598AM. MICA*002 and MICA*017 antibodies were detected in this patient possibly via allorecognition of 14G although no typing was available for the donor, the patient typed as MICA*008, -. The level of fluorescence intensity decreased gradually over 3 years and the last sera tested in the series have low MFI.

Table 6.5.1: Transplant details of patients enrolled in the longitudinal study

ID	Fig. No.	Gender	Age at Txp	Donor	Re-txp	Date of txp	Latest eGFR[1]	%CD[2]
986EL	6.5.1	F	28	DD	Yes	12/01/2006	<15	59
300RA	6.5.2	M	53	LD	Yes	22/03/2006	38	25
521HJ	6.5.3	M	59	DD	No	09/02/2006	25	28
396BS	6.5.4	M	26	DD	Yes	10/11/2006	29	23
194AH	6.5.5	M	52	DD	No	06/12/2004	36	-
881AM	6.5.6	M	37	DD	Yes	12/07/2007	41	-
690MO	6.5.7	M	38	DD	Yes	15/05/2007	43	28
728JP	6.5.8	F	37	DD	No	22/03/2008	36	-
057SR	6.5.9	M	33	DD	Yes	09/08/2009	-	-
599EM	6.5.10	F	63	DD	No	30/04/2008	30	-
197TW	6.5.11	M	22	LD	No	20/02/2008	42	47
977HB	6.5.12	F	55	DD	Yes	02/05/2009	-	-
495RT	6.5.13	F	40	DD	Yes	20/09/1994	-	-
561JB	6.5.14	F	25	DD	No	24/07/1995	-	-
953OW	6.5.15	F	41	DD	No	06/06/1997	-	-
598AM	6.5.16	M	53	-	No	01/01/1988	-	-

[1]eGFR – estimated glomerular filtration rate ml/min/1.73m^2

[2]Morphometric analysis - index of % chronic damage in renal cortex (Howie *et al*., 2004)

Table 6.5.2: Summary of MICA and HLA antibody profiles for longitudinal study patients

ID	Fig. No.	No. sera tested	MICA Ab first found	MICA-DSA	MICA-NDSA	HLA-DSA	HLA-NDSA	Acute Rejection
986EL	6.5.1	14	9-12m	008, 009	007, 019	DQ9	A11, 66; DQ6, 7, 8	ACR
300RA	6.5.2	11	1-1.5m	002	001,007,012,017,018	-	A30,31; B35,51,52,53,75,78	ACR
521HJ	6.5.3	6	8-10m	001	007, 012, 018	-	-	ACR
396BS	6.5.4	7	-	008	001,004,007,009,012,018,019	-	A2,34,A68,69; Cw6; DQ4,5,6,8,9	No
194AH	6.5.5	3	-	004	-	-	-	No
881AM	6.5.6	5	21d	016	004, 006, 009, 019, 008	-	A23,25; B13,38,39,44,51,53,58, 67	No
690MO	6.5.7	9	6m	009	004	A2; DQ2	A34,66,68,69; B57; DQ4,5,7,8,9	unknown
728JP	6.5.8	6	12m	008	007, 019	-	A68; B13,44,45,46,57	No
057SR	6.5.9	4	-	-	001, 002, 007, 012, 017, 018	-	-	No
599EM	6.5.10	7	-	004	001, 009, 008, 019	-	-	No
197TW	6.5.11	10	3-9m	009	004, 008, 019	-	-	No
977HB	6.5.12	7	-	008, 018	002, 004, 006, 007, 009, 017, 019	A24	A29	No
495RT	6.5.12	5		unknown	008, 019	B7	A23,24,66,69; B13,27,42,46,48,57,58,61,73	No
561JB	6.5.13	4		unknown	002, 004, 007, 008, 009, 018,019	A30; B44	A1,23,24,31,74,80;B76,44,82,45,50,38,49	No
953OW	6.5.14	6		unknown	001, 007, 012, 018	B27, 35	A66, multi-B, multi-DR	No
598AM	6.5.15	4		unknown	002, 017	-	B46, 62 (wk)	No

6.6 Results and Discussion

For my investigation into the relationship of MICA antibodies and renal transplant clinical course, I performed a longitudinal analysis of sixteen renal graft recipients with a total of 108 serial serum samples collected over a period of around two years. Among these patients, twelve had MICA typing information for patient and donor and six of these were negative for HLA antibodies as assessed by SAg Luminex testing. The finding that so many recipients with MICA antibodies did not have HLA antibodies is unusual as most previous studies report nearly all those with MICA antibodies as having HLA antibodies as well. Nevertheless, the HLA antibody status was confirmed as accurately as possible and fortuitously affords the opportunity to assess the role of MICA antibodies alone with regard to graft function measured by eGFR.

6.6.1 Association of MICA antibody production and change in eGFR

The results of the longitudinal analysis, presented in Figures 6.5.1-6.5.16, include eGFR measurements taken yearly for up to three years post-transplant and is a more accurate measurement of graft function than serum creatinine values (Marcen *et al.*, 2010). A common observation was that increasing MICA antibody fluorescence intensity often accompanied decreasing eGFR in the first two years of transplantation. Eight of a possible nine recipients showed this association with seven of them involving MICA-DSA and one with MICA-NDSA. Additionally, patients 521HJ (Figure 6.5.3), 194AH (Figure 6.5.5), 599EM (Figure 6.5.10) and 197TW (Figure 6.5.11) had no positive results with SAg HLA Luminex analysis and the other four recipients did not have HLA-DSA detected with the exception of 986EL where donor-specific DQ9 was detected.

Patient 986EL received a re-transplant from a living related donor in January 2006 and is an interesting case for several reasons. Despite a 010 A-B-DR mismatch, two MICA alleles were mismatched and the patient was negative for MICA antibodies in five serum samples tested in the first nine months of transplantation. This was followed by a slight rise in MICA-DSA and NDSA fluorescence intensity and coincided with a diagnosis of ACR at twelve months post-transplant. Additionally, this patient had HLA-DQ9 DSA with MFI rising to positive levels shortly after a rise in MICA antibody MFI. After an initial peak there was a sharp rise in MFI for MICA*008 and *019 antibodies. MICA*008 antibody was donor-specific and MICA*019 shares an epitope expressed at residues 213 (I) and 251

(R) and is known to react with MICA*008 antigens (Zou *et al*., 2009). MICA*004 and MICA*009 were also detected and MICA*009 is donor-specific but had lower intensity values which rose more gradually. MICA*004 is an autoantibody and may be reactive because of the shared polymorphism with MICA*009 at residue 122 (V). The increase in MICA antibody MFI occurred after ACR and no MICA antibodies were detected three months previous, therefore it is difficult to assess whether their presence was the cause or the consequence. The eGFR measurement taken at twelve months post-transplant also coincides with the ACR episode and could explain why it was so low at this time (19 ml/min/1.73m^2). At 24 months post-transplant there was a further decline of 3 ml/min/1.73m^2 and after 36 months, there was more loss of function which technically resulted in graft failure - with eGFR <15 ml/min/1.73m^2 return to dialysis was imminent. Furthermore biopsy evidence showed chronic damage in the renal cortex with an index of 59% using methodology as described (Howie *et al*., 2004), a CD index >40% is considered highly indicative of CAD. It is possible that the ACR was caused by the single HLA-B or DQ mismatch but also possible that allorecognition of mismatched MICA antigens, resulting in MICA antibody production was responsible. It should also be considered that the ACR episode itself, especially if severe, could be sufficient to irreversibly damage the graft and lead to loss of adequate function although ACR is not thought to be progressive (P.F. Halloran, 2010).

Another patient also had reduced eGFR associated with production of *de novo* MICA antibodies but did not experience AR or develop detectable HLA antibodies. Patient 197TW (Figure 6.5.11) was transplanted in February 2008 with a kidney donated by his mother (aged 49 years). The patient MICA type was MICA*002, - and the donor was MICA*002, 009:01 and there was one HLA-A and one HLA-B mismatch (HLA-DQ was matched). Pre-transplant sera for this patient was negative for MICA antibodies and further monthly serum samples taken post-transplant were also negative. Between three and nine months post-transplant, patient 197TW developed MICA*004, 008, 009, 019 antibodies that increased in strength over the next twelve months. When MICA antibody levels peaked at 24 months there was a decline in eGFR of 7 ml/min/1.73m^2 to 42 ml/min/1.73m^2 and equivalent to stage 3 CKD. A protocol biopsy taken at twelve months post-transplant showed an index of 47% chronic damage in the renal cortex. These results strongly indicate MICA antibodies may be implicated with CAD in the absence HLA antibodies.

Patient 194AH received a kidney from a deceased donor in 2004. There was an HLA class I haplotype mismatch including one MICA allele mismatch. Patient MICA type was

MICA*008, - and the donor was MICA*008, 004 and there was no diagnosis of AR. The patient did not produce HLA antibodies but MICA*004 DSA was produced by three months post-transplant remaining steady for around two years before disappearing completely. By this time there was a sharp decline in eGFR from 50 to 38 ml/min/1.73m^2 and a biopsy at eleven months showing 32% CD index. MICA antibodies were still undetected at three years post-transplanted and longer analysis would be required to know whether this is permanent.

Patient 521HJ had reduced eGFR, MICA-DSA but no HLA antibodies and was diagnosed with ACR just before a steep rise in fluorescence intensity for MICA antibodies. A reduction in eGFR of 7 ml/min/1.73m^2 to 25 ml/min/1.73m^2 occurred after 12 months and remained the same the following year.

Patient 057SR (Figure 6.5.9) did not have detectable HLA antibodies and lost his graft after almost twenty years. High levels of MICA antibodies were detected prior to failure. Interestingly, the fluorescence intensity of MICA antibodies decreased sharply shortly after graft failure and may have been absorbed by the graft. Following re-transplantation, MICA antibodies had almost returned to their original levels. It is possible that MICA antibodies were a major factor leading to chronic dysfunction and graft loss in this patient.

Although published studies of MICA antibody longitudinal analysis using Luminex assays do not provide MICA typing details of the donor, they do show similar results to my study. A longitudinal study of mainly renal graft recipients with HLA antibodies by Van Den Berg-Loonen and co-workers (van den Berg-Loonen *et al.*, 2006) showed results of one recipient with MICA antibodies but no HLA antibodies. After detection of low levels of MICA antibodies at five months post-transplant, a very large increase in fluorescence intensity occurred by two years and was associated with an increase in serum creatinine and graft failure after three years post-transplant. In another study (Kinukawa *et al*., 2006) longitudinal analysis up to ten years post-transplant of eight patients with MICA antibodies was provided. Seven of these renal graft recipients had lost their grafts to rejection and one died with a functioning graft. In four recipients, no HLA antibodies were detected. One patient produced MICA*012 antibody twelve months post-transplant with increasing MFI that correlated with an increase in serum creatinine and the graft failed by sixteen months. Another patient developed antibodies to MICA*004, 008 and 009 by 42 months post-transplant and followed a rise in serum creatinine. MICA antibody strength continued to increase along with serum creatinine and the graft failed at six months. Another patient

with no HLA antibodies suddenly developed antibodies to MICA*001, and 018 in the eighth year of transplant when serum creatinine levels began to rise and the graft was lost two years later. Finally, a patient who had MICA*001, 012, 018 antibodies throughout the course of their transplant had graft failure after ten years following a steady increase in serum creatinine in the five years previous.

Taken together the results of this and published studies show that even when patients have no detectable HLA antibodies, the presence of MICA antibodies is associated with reduced eGFR (or increased serum creatinine) with evidence of CAD from biopsies or graft failure. In the next chapter I will perform univariate and multivariate analysis of GS and mean eGFR in patients with and without MICA antibodies and MICA-DSA to investigate whether these observations are statistically significant.

6.6.2 MICA antibody specificity

In Chapter 4 various MICA epitopes were identified in MICA positive recipients that have been shown in published studies (Zou *et al*., 2009) to react with MICA antibodies and which share particular amino acid polymorphisms. The MICA amino acid alignment from Chapter 4 is reproduced here to aid interpretation (Table 6.6.1).

Table 6.6.1: Protein alignment of polymorphic MICA residues shown experimentally to be involved in allorecognition by MICA antibodies.. Modified from Zou *et al.,* 2009 (Permission granted by Springer).

Domain:	α-1			α-2								α-3				
	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2
Residue:	1	2	3	9	2	2	2	5	7	7	8	0	1	1	1	5
	4	4	6	1	2	5	9	6	3	5	1	6	0	3	5	1
Antigen												MICA-G1				
MICA*001	W	T	C	Q	L	K	M	H	K	G	T	G	W	T	S	Q
MICA*012	-	-	-	-	-	E	-	L	-	-	-	-	-	-	-	-
MICA*018	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*007	-	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*002	G	A	-	-	-	E	-	-	-	-	-	-	-	-	-	-
MICA*017	G	A	-	R	-	E	-	-	-	-	-	-	-	-	-	-
												MICA-G2				
MICA*004	-	A	Y	-	V	E	V	-	E	S	R	S	R	-	T	-
MICA*009	-	A	Y	-	V	E	V	-	E	S	-	S	R	-	T	-
MICA*008	-	A	Y	-	-	E	V	-	E	-	-	S	R	I	T	R
MICA*019	-	A	Y	-	-	E	V	-	E	S	-	S	R	I	T	R

The two largest groups of MICA antibodies were called MICA-G1 and MICA-G2 and divide the MICA antibodies into two broad groups, in a similar way to the Bw4 and Bw6 epitopes of class I HLA molecules, and is thought to represent two ancestral lineages of MICA alleles (Choy and Phipps, 2010). There are also amino acid differences with individual MICA antigens, as well as groups of two or three antigens.

In some patients only one MICA specificity was identified for example 194AH (Figure 6.5.5) where only one antibody, MICA*004 was detected and antibodies may be specific for R at residue 181. Another patient, 690MO (Figure 6.5.7) had MICA*004 and MICA*009 antibodies detected and both antigens uniquely express V at residue 122. Other patients seemed to have more than one specificity which differed by their fluorescence intensity and a good example is 396BS (Figure 6.5.4) who has MICA*001, 012, 018 (T at residue 24) which have declining fluorescence intensity while the other group-specific epitope, MICA-G2, contains a MICA-DSA and fluorescence intensity increased after transplantation. This can also be clearly seen with patient 300RA (Figure 6.5.2) who has antibodies to MICA*001, 012, 018 (T at residue 24) that have half the fluorescence intensity of MICA*002, 017 antibodies that may be reactive with G at residues 14.

The reactivity with MICA*007, observed in 50% of these cases is unexpected and perplexing. This antigen does not have any unique amino acids or polymorphisms that may make it cross-reactive in any way other than would be expected. MICA*007 is detected in some sera where only MICA-G2 antibodies are otherwise present as seen with 986EL (Figure 6.5.1) or when only MICA-G1 antibodies are detected, for example 521HJ (Figure 6.5.3) or 057SR (Figure 6.5.9). Possibly, MICA*007 has a unique conformational epitope that is recognised by antibodies. Unfortunately there were no examples of patients with grafts expressing a mismatched MICA*007 so its 'true' reactivity cannot be assessed. It may be that the antibody binding associated with the MICA*007 bead is the result of misfolded MICA molecules on the bead exposing epitopes that are reactive with non-MICA antibodies as I discussed in Chapter 4 but this occurs with all three Luminex bead assays and, as can be seen by this analysis, it is consistently detected.

These data extend and enhance the observations noted in Chapter 4. Not only are antibodies specific for epitopes based on unique or shared polymorphism in the extracellular domains but are consistently detected in serial serum samples with no disappearance of individual MICA antibodies or the sudden appearance of other MICA

antibodies. It is also evident that the epitope specificity can result in differing fluorescence intensities by MICA antibodies giving credence to the shared epitope reactivity hypothesis.

6.6.3 First appearance of MICA antibodies and evolutionary course

Seven patients in this series produced *de novo* MICA antibodies post-transplant. The earliest post-transplant time-point for MICA positivity in one patient was three weeks as seen with patient 881AM (Figure 6.5.6) and another early appearance of MICA antibodies occurred with patient 300RA (Figure 6.5.2). The longest time for MICA antibodies to appear was twelve months as observed with patients 986EL (Figure 6.5.1) and 728JP (Figure 6.5.8). Three patients became positive for MICA antibodies at around six months post-transplant as shown in Figures 6.5.3, 6.5.7 and 6.5.11. Therefore these results show that MICA antibodies can be produced as early as three weeks but up to twelve months post-transplant or perhaps even longer. This is very similar to the first appearance of HLA antibodies in renal transplantation (van den Berg-Loonen *et al*., 2006). However, in a longitudinal study up to ten years post-transplant, some patients did not develop MICA antibodies until after many years and in one case MICA antibodies did not appear for eight years (Kinukawa *et al*., 2006).

In three patients (Figures 6.5.1, 6.5.2 and 6.5.7) the initial detection of low-titre MICA antibodies disappeared in the next serum but returned in the subsequent sample with high fluorescence intensity, producing a characteristic small peak followed by a larger peak. This may be the result of antibody class switching and affinity maturation giving rise to much more efficient antibody binding.

Another three patients had MICA antibodies with increased fluorescence intensity post-transplant, which then declined steadily or sharply within two years to negative or undetectable levels. Patient 300RA (Figure 6.5.2) suddenly became negative for MICA antibodies eighteen month post-transplant and the subsequent sample was also negative. Patient 396BS (Figure 6.5.4) had very low levels of MICA antibodies by fifteen month post-transplant and patient 194AH lost reactivity with MICA*004 beads by 24 months post-transplant and was still negative after three years of engraftment. However, all three patients associated with a decrease in eGFR during the period when MICA antibodies were detected.

6.6.4 MICA antibody 'strength'

Mizutani demonstrated a highly significant correlation of HLA antibody strength (high MFI) with graft failure, in particular patients with incremental increases in antibody strength (Mizutani *et al.*, 2007). Terasaki and colleagues previously found that strong HLA antibodies were highly associated with graft failure in the first year of transplantation compared to recipients with moderate HLA antibodies (Terasaki *et al*., 2007) and in another study increasing strength of both HLA and MICA antibodies was associated with graft failure (Mizutani *et al*., 2005). The authors of these studies suggest that the strength of the antibodies measured by MFI is as important as donor-specificity in antibody-mediated graft loss. In Chapter 5 I found that strong MICA antibodies were highly significantly associated with ACR but it was difficult to ascertain whether the antibodies were a consequence or the cause. The results presented in this chapter show that increasing fluorescence intensity for *de novo* MICA antibodies usually coincides with a reduction in eGFR but most patients also had MICA-DSA so it is difficult to assess whether the strength of the antibody was an additional factor. This is also the case in the published study where graft failure was implicated with a rise in MICA antibody fluorescence intensity (Kinukawa *et al*., 2006). More studies involving larger numbers of patients are required to prove this hypothesis but it may be worthwhile monitoring patients for MICA in addition to HLA antibodies, at least in the first year of transplantation. If *de novo* MICA antibodies specific for donor antigens are found with increasing strength there may be time for therapeutic intervention to prevent aAMR, chronic loss of function or graft failure.

6.6.5 Long-term renal graft recipients and MICA antibodies

I also carried out longitudinal analysis of four patients who were transplanted between thirteen and 25 years ago and have MICA antibodies but otherwise enjoy good graft function. In all of these patients, MICA antibodies remained detectable throughout the period of investigation, although there were fluctuations, and in all cases the same MICA specificities were detected. It was not possible to test serum from the beginning of the transplants as they were such a long time ago but it is possible that these patients had MICA antibodies for many years and yet have had a functioning graft much in excess of the usual half-life for renal transplants. This is a paradox if MICA antibodies are deleterious to GS, and is also observed in patients with HLA antibodies and good long-term graft function. At the 15th IHIWC in Brazil, Paul Terasaki attempted to explain this phenomenon during a

presentation about HLA and MICA antibodies. He used the analogy that smoking has been well documented as causing lung cancer BUT not all smokers develop lung cancer. In other words, some people may be more susceptible to graft damage by antibodies than others just as some people are more resistant to developing lung cancer. Another theory is that antibody 'accommodation' could be the reason. In this situation the organ becomes resistant to antibody-mediated injury evidenced by C4d deposition on renal biopsies in the absence of other signs of rejection. The detection of complement fixation shows that antibodies still recognise and bind to their antigens and the lack of lysis may implicate a regulatory mechanism (Yu *et al.*, 1996). Whatever the reason, it is clear that some patients are not affected by the presence of MICA antibodies and do not lose their grafts as a consequence.

In the next chapter I will carry out univariate and multivariate analysis to investigate whether there is a statistically significant relationship between the production of MICA antibodies and reduced GS or function. I also analyse graft recipients for soluble MICA and MICB production, which may be implicated with improved GS.

CHAPTER 7

Soluble MICA/B in renal transplantation and association of MICA antibodies with graft survival and function

7.1 Introduction and aims

Much evidence has been generated over the past ten years associating expression of MICA and MICB on tumours with regulation of anti-tumour immune responses (Groh *et al*., 1999). These studies have led to the concept that MIC molecules can be shed from tumours in the soluble form leading to decreased surface expression of MIC molecules on the tumour, reducing immunogenicity and circumventing NK-mediated lysis. In addition, the soluble forms of MICA and MICB (and other NKG2D ligands) can engage with NKG2D activatory receptors on NK-cells and T-cells resulting in internalisation and degradation of the NKG2D complex, thereby decreasing NKG2D expression on NK-cells and T-cells and impairing their anti-tumour function (Groh *et al*., 2002). This mechanism has important implications for immunotherapy in cancer patients and attempts at prevention of MIC molecule shedding by identifying the biochemical pathways leading to MIC cleavage are evident from the literature (Salih *et al.*, 2006; Zwirner *et al.*, 2007). These studies have led to the hypothesis that the soluble form of MICA may have a protective effect against rejection in solid organ transplantation (Suarez-Alvarez *et al.*, 2006a; Suarez-Alvarez *et al.*, 2006b). However, the study by Suarez-Alvarez and colleagues involved less than 50 heart transplant recipients and there are currently no published studies investigating the role of soluble MICA in renal transplantation.

Despite improvements in immunosuppression reducing the incidence and severity of AR (Meier-Kriesche *et al*., 2004), little has changed in 30 years with respect to late graft failure due to chronic decline in graft function. Chronic damage in renal transplantation has a multifactorial aetiology involving both immunological and non-immunological mechanisms. Non-immune factors such as donor or patient gender or age, the source of the donor (living or deceased), race and primary kidney disease can all affect graft function and foreshorten GS. Immunological mechanisms associated with chronic damage include ACR and aAMR. The traditional way to assess these risk factors is by time-dependent statistical analysis of GS by the Kaplan-Meier method for univariate analysis and Cox regression multivariate analysis can then reveal independent risk factors. These studies are important

in understanding which factors impact on GS and whether different techniques or therapies can improve OS.

Measurement of renal function is a non-invasive method of monitoring changes that may relate to infection or rejection or other complications that may require therapeutic intervention or further investigation by biopsy. Tests for renal function assess how efficiently the kidneys filter blood and as this cannot be measured directly, clearance of a filtration marker is used. Measurement of serum creatinine was until recently the most common method of measuring renal function. Creatinine is a breakdown product of creatine phosphate in muscles and is produced at a constant rate by the body, dependent on body mass. Creatinine is filtered out of the blood by the kidneys and rising levels in the blood can be measured and used to calculate the creatinine clearance, reflecting the glomerular filtration rate (GFR) and hence renal function. Blood creatinine clearance is now recognised as an unreliable measure of GFR as serum creatinine is affected by age, weight, muscle mass, race and various medications. To compensate for variations of creatinine production between individuals, several equations have been developed to take into account anthropometric factors including age, gender ethnicity and body mass. Two formulae are widely used in clinical practice for adults: the Cockcroft-Gault (C-G) equation (Cockcroft and Gault, 1976) and the more recently developed MDRD equation (A. S. Levey *et al.*, 1999). The C-G equation was based on 249 patients and estimates creatinine clearance but is not normalised for body mass, uses an out-dated assay for creatinine measurement and the results should be considered with caution. The original MDRD equation was based on 1628 patients with CKD and estimates GFR adjusted for body surface area using age, gender, ethnicity, serum creatinine, urea, nitrogen and albumin (A. S. Levey *et al*., 1999). In 2000, a simplified method was introduced that performed as well as the original MDRD equation but required only serum creatinine, age, gender and ethnicity (African-Caribbean descent) (A. S. Levey *et al.*, 2000) and is currently the most widely used equation, particularly in the UK.

Estimated GFR (eGFR) is expressed as ml/min/1.73m^2 and an eGFR >90 ml/min/1.73m^2 is considered normal. CKD can be categorised by eGFR values and also correlates with stages of rejection in renal transplantation. Stage 1 CKD is where eGFR is greater than 90 ml/min/1.73m^2 but urine analysis, structural abnormalities or a genetic trait indicate kidney disease. Stage 2 CKD shows mildly reduced kidney function with eGFR between 60-89 ml/min/1.73m^2 with other indicators of kidney disease (as in stage 1). Stage 3 CKD reflects moderately reduced function of 30-59 ml/min/1.73m^2. Stage 4 CKD is where eGFR is

severely reduced and between 15-29 ml/min/1.73m^2 and stage 5 is very severe or end-stage renal failure (<15 ml/min/1.73m^2). Most kidney transplant recipients have eGFR in stage 3 CKD and progression to stage 5 indicates graft failure, and the patient is prepared to return to dialysis (Burden and Tomson, 2005). The measurement of eGFR in renal transplant recipients provides a factor that can be used for clinical management and also allows analysis of transplantation variables to be correlated with renal function in transplantation outcome studies as an alternative to GS.

In this chapter I evaluate soluble MICA and MICB production in renal transplant recipients and correlate the presence or absence with transplantation variables to assess whether the soluble form of MIC products associate with variables such as HLA or MICA mismatching, production of antibodies or, importantly, with less AR. This has not been reported in renal transplantation but a small study of heart transplant recipients did associate less AR with the presence of sMICA (Suarez-Alvarez *et al*., 2007). A GS study of our renal transplant cohort was also carried out, primarily to assess whether MICA antibodies are associated with reduced GS and to also to investigate which factors were responsible for graft loss in this cohort. Finally, I carried out a thorough univariate and multivariate statistical evaluation of graft function assessed by eGFR using the MDRD method to investigate whether MICA antibody production in renal graft recipients is independently associated with reduced graft function and therefore implicated with CAD. Most results presented in this chapter have been published in a peer-reviewed journal (Cox *et al.,* 2011).

7.2 Soluble MICA and MICB in renal transplantation

To investigate whether soluble MICA or soluble MICB (sMICA or sMICB) present in the serum of renal transplant patients has a positive impact in terms of less AR as has been reported in heart transplant recipients, sera from renal graft recipients was tested approximately one year post-transplant.

Using a sandwich ELISA method to detect sMICA and sMICB, as detailed in Chapter 2, 201 graft recipients transplanted since 2003 were tested for sMICA and 200 patients were tested for sMICB. This test is very sensitive, capable of detecting as little as 62.5 pg/ml soluble MICA protein (156.25 pg/ml soluble MICB). I analysed association with general clinical characteristics, HLA and MICA mismatching, MICA/HLA antibodies and ACR or aAMR.

7.2.1 Association of sMICA and sMICB with general clinical characteristics

The overall frequency of sMICA (n=201) was 12.5% and sMICB (n=200) was 41%; higher levels of sMICB were detected in renal graft recipients compared to sMICA as shown in Figure 7.2.1. There was no statistical significance with any of the variables in association with sMICA as listed in Table 7.2.1, although there was a statistical trend with younger recipient age (P=0.061) and transplant number (P=0.095).

Table 7.2.1: Clinical characteristics of 201 renal graft recipients and association with sMICA detected in serum one year post-transplant

Variable	sMICA pos (n=25)	sMICA neg (n=176)	χ^2	P-value
Gender			0.5	0.470
Male	12 (48%)	98 (56%)		
Female	13 (52%)	78 (44%)		
Recipient Age[1]	38.5 ± 13	44 ± 14		0.061
Donor Age[1]	46 ± 10	46 ± 15		0.889
Donor status			1.7	0.189
Living	10 (40%)	48 (27%)		
Deceased	15 (60%)	128 (73%)		
Transplant No.			2.8	0.095
Primary	18 (72%)	150 (85%)		
Re-transplant	7 (28%)	26 (15%)		

[1]Age ± SD, groups were compared using Student's t-test (independent variables).

In association with sMICB, (Table 7.2.2) recipient age was significantly different between the two groups (P=0.015) and a trend was observed for donor status (P=0.056) and transplant number (P=0.088). Levels of sMICB were generally higher with a mean of 300 pg/ml compared with sMICA which showed a mean concentration of 100 pg/ml (Figure 7.2.1).

Table 7.2.2: Clinical characteristics of 200 renal graft recipients and association with sMICB detected in serum one year post-transplant

Variable	sMICB pos (n=82)	sMICB neg (n=118)	χ^2	P-value
Gender			0.1	0.751
Male	44 (53%)	66 (56%)		
Female	38 (47%)	52 (44%)		
Recipient Age[1]	41 ± 14	46 ± 14		0.015
Donor Age[1]	44 ± 15	48 ± 15		0.106
Donor status			3.6	0.056
Living	17 (21%)	39 (33%)		
Deceased	65 (79%)	79 (67%)		
Transplant No.			2.9	0.088
Primary	65 (79%)	104 (88%)		
Re-transplant	17 (21%)	14 (22%)		

[1]Age ± SD, groups were compared using Student's t-test (independent variables).

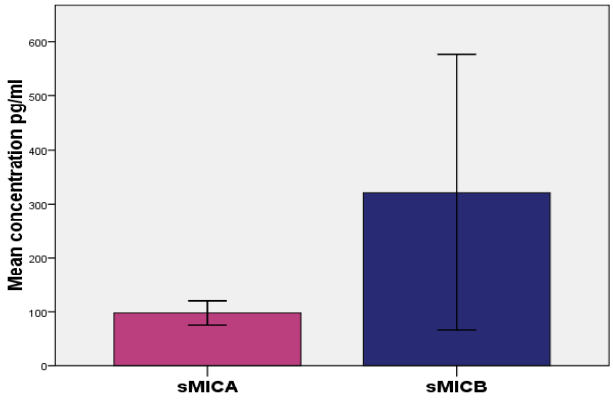

Figure 7.2.1: Comparison of mean concentration of sMICA and sMICB (pg/ml) detected in renal transplant recipient serum one year post-transplant.

7.2.2 Association of sMICA and sMICB with HLA antigen and MICA allele and antigen mismatches

As shown in Table 7.2.3, there was no significant correlation between the presence of sMICA and HLA class I mismatches and analyses of individual loci also revealed no association. HLA-DR mismatching did not associate with detectable sMICA but analysis of HLA-DQ mismatching revealed highly significant association (P=0.003). MICA allele or residue mismatching was not associated with the presence of sMICA.

Table 7.2.3: HLA antigen and MICA allele/antigen mismatching in 201 renal graft recipients and association sMICA detected in serum one year post-transplant

Variable	sMICA pos (n=25)	sMICA neg (n=176)	χ^2	P-value
HLA-A, -B, -C mm			5.5	0.479
0	1 (4%)	16 (9%)		
1-3	16 (64%)	86 (49%)		
4-6	8 (32%)	74 (42%)		
HLA-DR mm			0.1	0.937
0	11 (44%)	74 (42%)		
1	12 (48%)	84 (48%)		
2	2 (8%)	18 (10%)		
HLA-DQ mm				
0	11 (44%)	105 (60%)	11.4	0.003
1	11 (44%)	69 (39%)		
2	3 (12%)	2 (1%)		
MICA allele mm			0.2	0.924
0	8 (32%)	58 (33%)		
1	13 (52%)	85 (48%)		
2	4 (16%)	33 (19%)		
MICA amino acid mm				0.849
Matched	11 (44%)	81 (46%)		
Mismatched	14 (56%)	95 (54%)		

mm = mismatch

Table 7.2.4 reveals significant association of sMICB and HLA class I mismatching (P=0.010). A detailed analysis of individual HLA loci showed mismatching for HLA-B was responsible (P=0.045). There was no significant association of sMICB with mismatching HLA class II loci or MICA allele or residue mismatching.

Table 7.2.4: HLA antigen and MICA allele/antigen mismatching in 200 renal graft recipients and association with sMICB detected in serum one year post-transplant

Variable	sMICB pos (n=82)	sMICB neg (n=118)	χ^2	P-value
HLA-A, -B, -C mm			16.8	0.010
0	10 (12%)	7 (6%)		
1-3	40 (49%)	60 (51%)		
4-6	32 (4%)	51 (43%)		
HLA-B mm				
0	17 (21%)	13 (11%)	6.9	0.045
1	47 (57%)	87 (74%)		
2	18 (22%)	18 (15%)		
HLA-DR mm			0.7	0.701
0	36 (44%)	62 (53%)		
1	39 (48%)	57 (48%)		
2	3 (4%)	14 (12%)		
HLA-DQ mm			4.4	0.113
0	53 (65%)	62 (53%)		
1	26 (32%)	54 (46%)		
2	3 (4%)	2 (2%)		
MICA allele mm			1.6	0.452
0	30 (37%)	37 (31%)		
1	35 (43%)	61 (52%)		
2	17 (21%)	20 (17%)		
MICA amino acid mm				0.936
Matched	38 (46%)	54 (46%)		
Mismatched	44 (54%)	64 (54%)		

7.2.3 Association of sMICA and sMICB with MICA or HLA antibodies and AR

There was no significant association of sMICA/B with HLA antibodies, MICA antibodies, ACR or aAMR (Tables 7.2.5 and 7.2.6).

Table 7.2.5: HLA antibodies, MICA antibodies and rejection in 201 renal graft recipients and association with sMICA detected in serum one year post-transplant

Variable	sMICA pos (n=25)	sMICA neg (n=176)	χ^2	P-value
HLA class I antibodies			0.4	0.509
Positive	5 (20%)	46 (26%)		
Negative	20 (80%)	130 (74%)		
HLA class II antibodies				0.842
Positive	4 (16%)	31 (18%)		
Negative	21 (84%)	145 (82%)		
MICA antibodies				0.913
Positive	2 (8%)	13 (7%)		
Negative	23 (92%)	163 (93%)		
ACR				0.807
Positive	5/24 (21%)	33 (19%)		
Negative	19/24 (79%)	143 (81%)		
aAMR				0.875
Positive	2/24 (8%)	13/175 (7%)		
Negative	22/24 (92%)	162/175 (93%)		

Table 7.2.6: HLA antibodies, MICA antibodies and rejection in 200 renal graft recipients and association with sMICB detected in serum one year post-transplant

Variable	sMICB pos (n=82)	sMICB neg (n=118)	χ^2	P-value
HLA class I antibodies			1.1	0.302
Positive	17 (21%)	32 (27%)		
Negative	65 (79%)	86 (73%)		
HLA class II antibodies			0.6	0.430
Positive	16 (20%)	18 (15%)		
Negative	66 (80%)	100 (85%)		
MICA antibodies			0.4	0.513
Positive	6 (7%)	6 (5%)		
Negative	76 (93%)	112 (95%)		
ACR			0.1	0.727
Positive	16/81 (20%)	21 (18%)		
Negative	65/81 (80%)	97 (82%)		
aAMR			1.3	0.259
Positive	4/80 (5%)	11 (9%)		
Negative	76/80 (95%)	107 (91%)		

The concentration (pg/ml) of sMICA and sMICB was compared in recipients with and without ACR or aAMR. Analysis by Student's t-test (not shown) revealed no statistical difference in mean concentration of sMICA or sMICB between recipients with biopsy confirmed rejection and those with no rejection as can be clearly seen in Figure 7.2.2.

The mean concentration of sMICA was 296 pg/ml for patients without rejection and 236 pg/ml for those with ACR. The concentration mean of sMICB was 1431 pg/ml for recipients without rejection and 1687 pg/ml for patients with ACR. For aAMR, the mean concentration of sMICA was 301 pg/ml for patients without rejection and 95 pg/ml for those with aAMR. The mean concentration of sMICB was 1473 pg/ml for recipients without rejection and 2060 pg/ml for patients with aAMR.

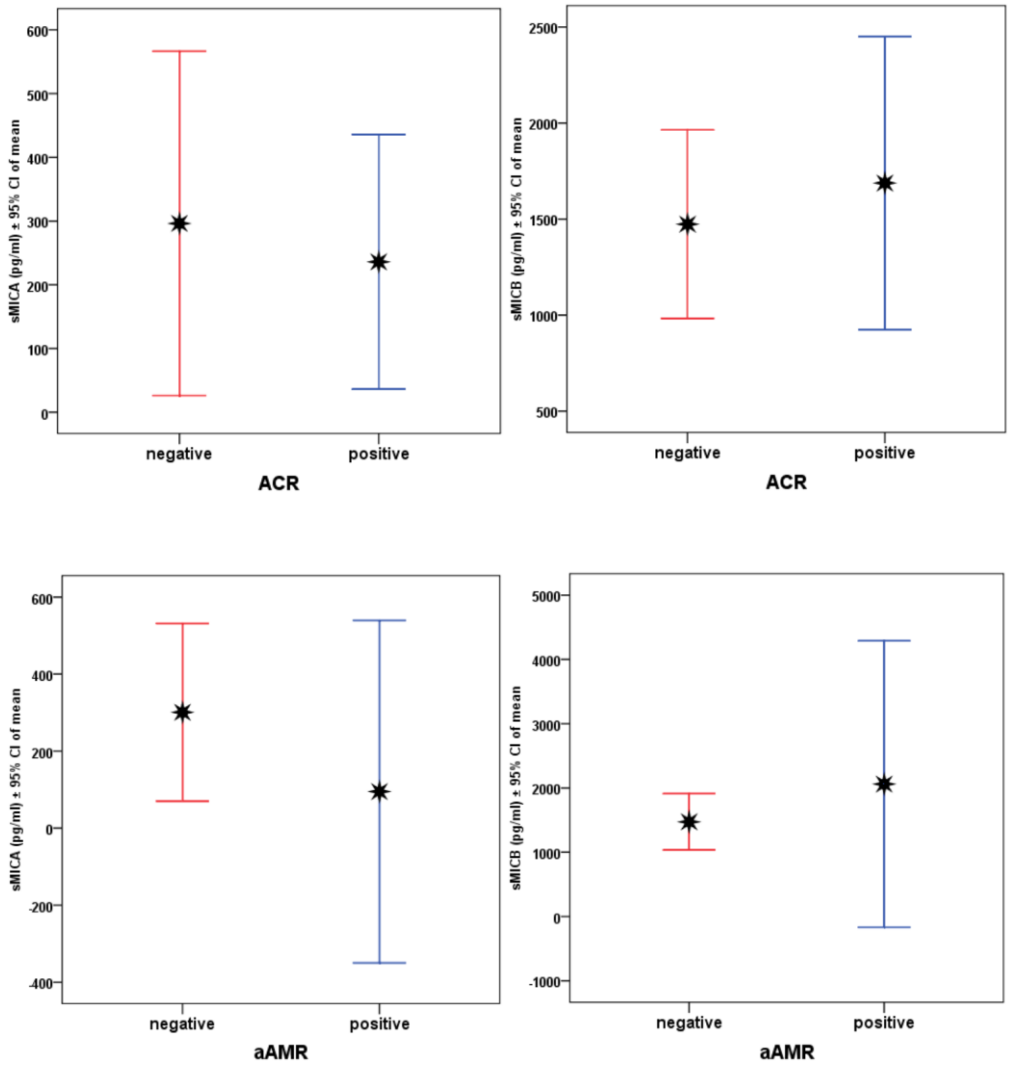

Figure 7.2.2: Effect of sMICA/B concentration on occurrence of AR. Comparison of mean concentration of soluble MICA (n=24) and soluble MICB (n=81) (pg/ml) detected one year after transplantation for those with or without ACR and aAMR.

7.2.4 Association of sMICA and sMICB with reduced graft function

To assess graft function in patients with and without soluble MIC protein detected in serum, comparisons of eGFR measurement taken at one, two and three years post-transplant were made, but revealed no significant difference. However, if patients were categorised according to eGFR < 30 ml/min/1.73m^2 (stage 4 CKD) or ≥ 30 ml/min/1.73m^2, a statistical trend (P=0.078) was observed for patients without sMICA having poorer function, five years post-transplant. Ninety percent of 19 patients with sMICA had eGFR ≥ 30 ml/min/1.73m^2 compared to 45% of 156 patients with no detectable sMICA. No statistical significance was associated with sMICB (Figure 7.2.3).

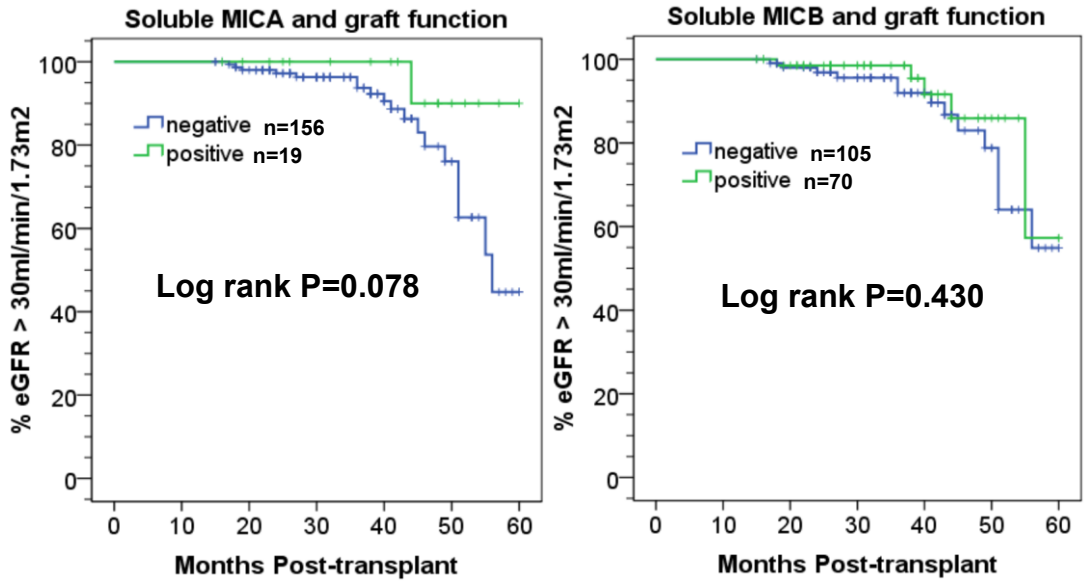

Figure 7.2.3: **Analysis of graft function in patients with eGFR < 30 ml/min/1.73m^2 (stage IV CKD) or ≥ 30 ml/min/1.73m^2 and sMICA or sMICB.** At five years post-transplant, 90% of patients with sMICA had eGFR or ≥ 30 ml/min/1.73m^2 compared to 45% of patients with no detectable sMICA. No difference was observed with sMICB.

7.2.5 Five-year renal graft survival and the impact of sMICA and sMICB

Association of sMICA or sMICB with five-year GS is shown in Figure 7.2.4. Although not significant, a decline in OS was observed and occurred around three years post-transplant. Patients with sMICA and/or sMICB had 98% OS after five years compared to the negative group who had OS of 83%. There was less of a difference for graft recipients with sMICA. Five-year OS was 90% for those with sMICA compared to 86% of recipients with no

sMICA. Patients with sMICB had five-year OS of 98% compared to 85% of patients with no detectable sMICB.

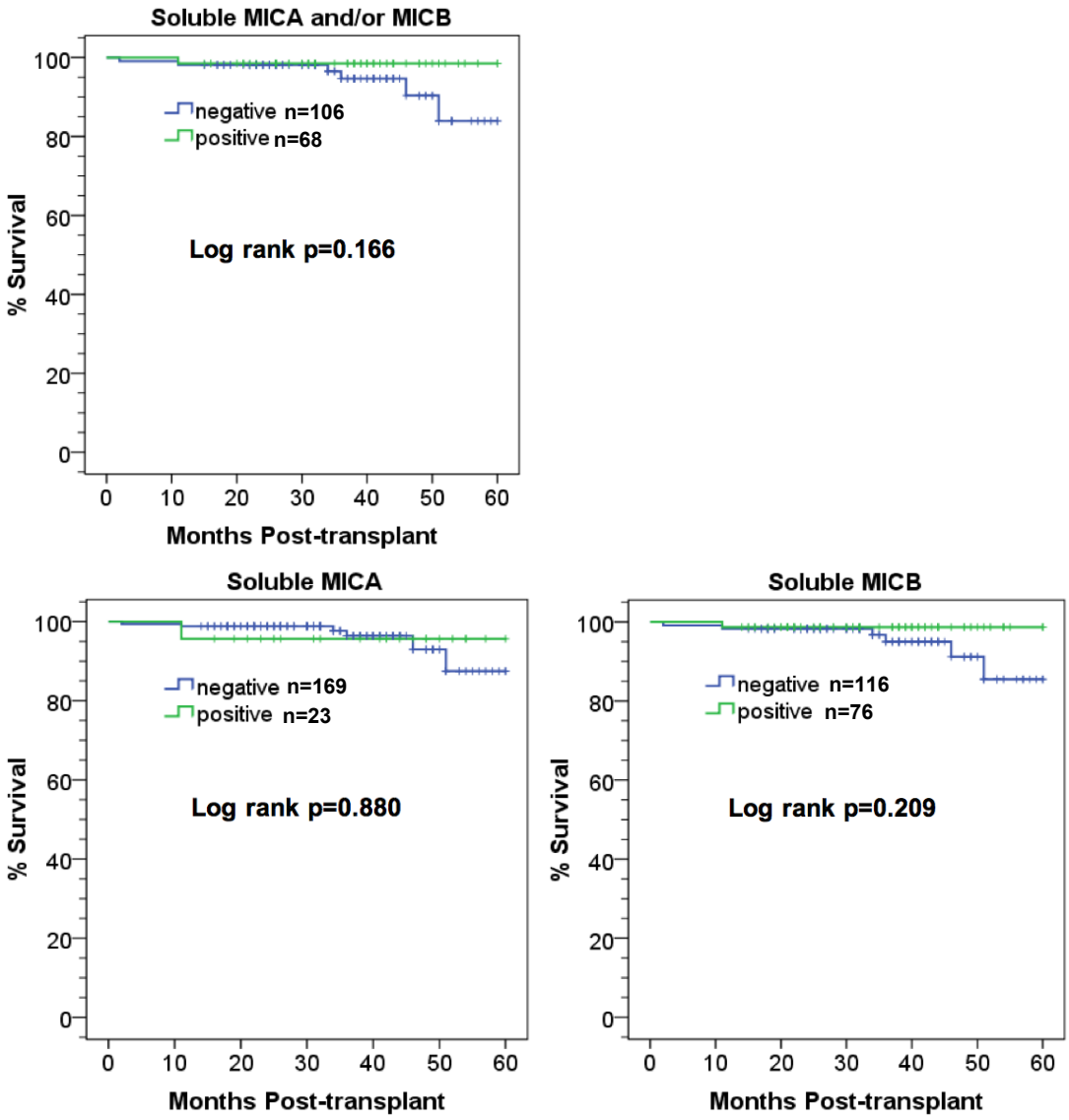

Figure 7.2.4: Association of sMICA and sMICB with five-year renal graft survival.

7.3 Five-year renal graft survival analysis and association with transplantation variables

7.3.1 Inclusion criteria for survival analysis

For this analysis it was necessary to gather clinical information that was not available on local databases or paper records. This required help from the clinical renal consultant at the Royal Free Hampstead Trust, Dr Mark Harber. I chose to focus this study only on patients where MICA typing information was available for the donors and patients. Secondly, all patients in the analysis were transplanted no earlier than 31st October 2003 and no later than 31st October 2008. Using these criteria, a total of 227 patients were included in the request for information regarding loss of graft and the date when it occurred as study end-points. In addition, patients with functioning grafts with eGFR less than 15 ml/min/1.73m^2 were also included as a graft loss at the time when the eGFR was first recorded as <15 ml/min/1.73m^2. Patients were omitted if lost to follow-up (moved to a different region) or the graft was lost due to non-compliance with immunosuppressive regime or technical problems. A valid graft loss was counted if the patient returned to haemodialysis, was re-transplanted or eGFR was less than 15 ml/min/1.73m^2. A total of 216 patients were included in analysis for five-year GS. Ten patients had graft failure - five patients returned to haemodialysis, two patients were re-transplanted and three patients had eGFR <15 ml/min/1.73m^2. There were six patients with graft failure that were not included in the analysis - two were non-compliant, one had an infected graft removed, one had a cancerous graft removed and two lost grafts for technical reasons i.e reperfusion problems or primary non-function. A further five patients died within the study period with a functioning graft.

7.3.2 Transplant variables included for survival analysis

Transplant variables considered in this analysis were patient and donor gender, patient and donor age, source of donor (living or deceased), number of transplants (primary or re-graft), HLA class I and class II antibodies and MICA antibodies. MHC mismatching was considered for antigenic mismatches of HLA-A, -B, -C, -DR, -DQ loci and allelic mismatches of the MICA locus. Rejection was considered for ACR and aAMR.

7.3.3 Univariate statistical analysis

Univariate analysis was performed for OS estimates using the Kaplan-Meier method and level of significance was assessed using the log rank statistic. P value of ≤ 0.05 was considered significant.

7.3.4 Overall five-year survival and the impact of source of donor, gender and age

Figure 7.3.1 shows five-year survival associated with donor source (living or deceased), patient or donor gender and patient or donor age. No significant difference was found with any of these variables. However, male patients with grafts from male donors had survival of 90% and survival was 61% with grafts from female donors, revealing a statistical trend (log rank P=0.060). The patient age group 50-59 years had 100% survival and lowest survival of 74% was observed with patients over 60 years. The donor age group with highest graft survival in recipients was 18-34 years.

7.3.5 Overall five-year survival and the impact of number of transplants, MICA antibodies or HLA antibodies

Significantly decreased GS (Figure 7.3.2) was observed with repeat transplantation (log rank p=0.002) with 80% GS in re-transplanted patients compared to 88% for patients with no previous grafts. No significant correlation was found for GS in patients with MICA antibodies. HLA class I antibodies associated with significantly reduced GS of 82% versus 90% for patients with no class I antibodies (log rank p=0.001). Similarly, but more significantly, after five years post-transplant, recipients with HLA class II antibodies had reduced GS of 75% compared to 90% for those with no class II antibodies (log rank p<0.001). The effect of HLA antibodies on GS was much stronger when recipients had both HLA class I and class II antibodies as observed in 54% compared to 91% (log rank p<0.001). HLA donor-specific antibodies also correlated with reduced survival (log rank p=0.020).

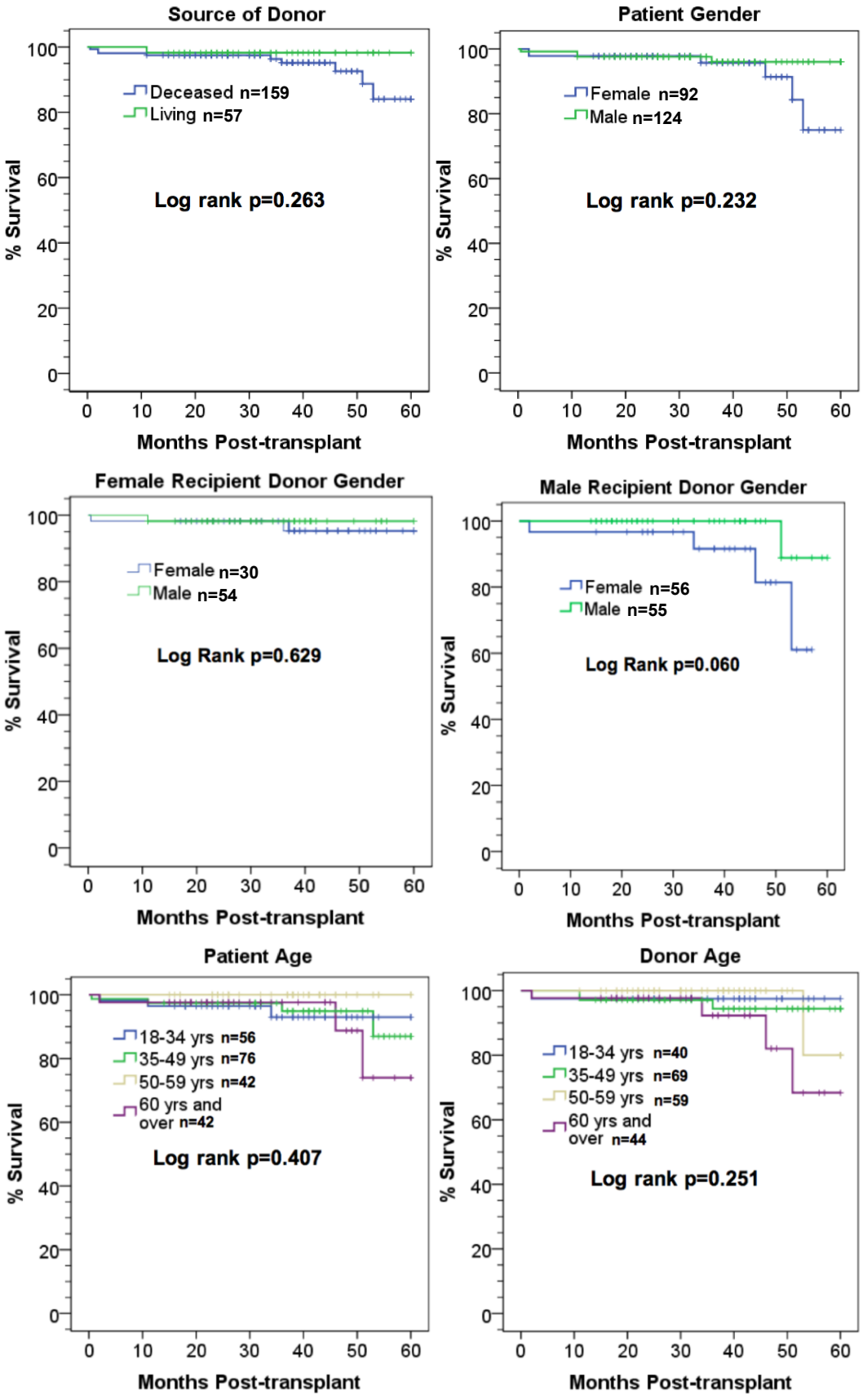

Figure 7.3.1: Association of source of donor (deceased or living), patient or donor gender and patient or donor age with five-year overall renal GS.

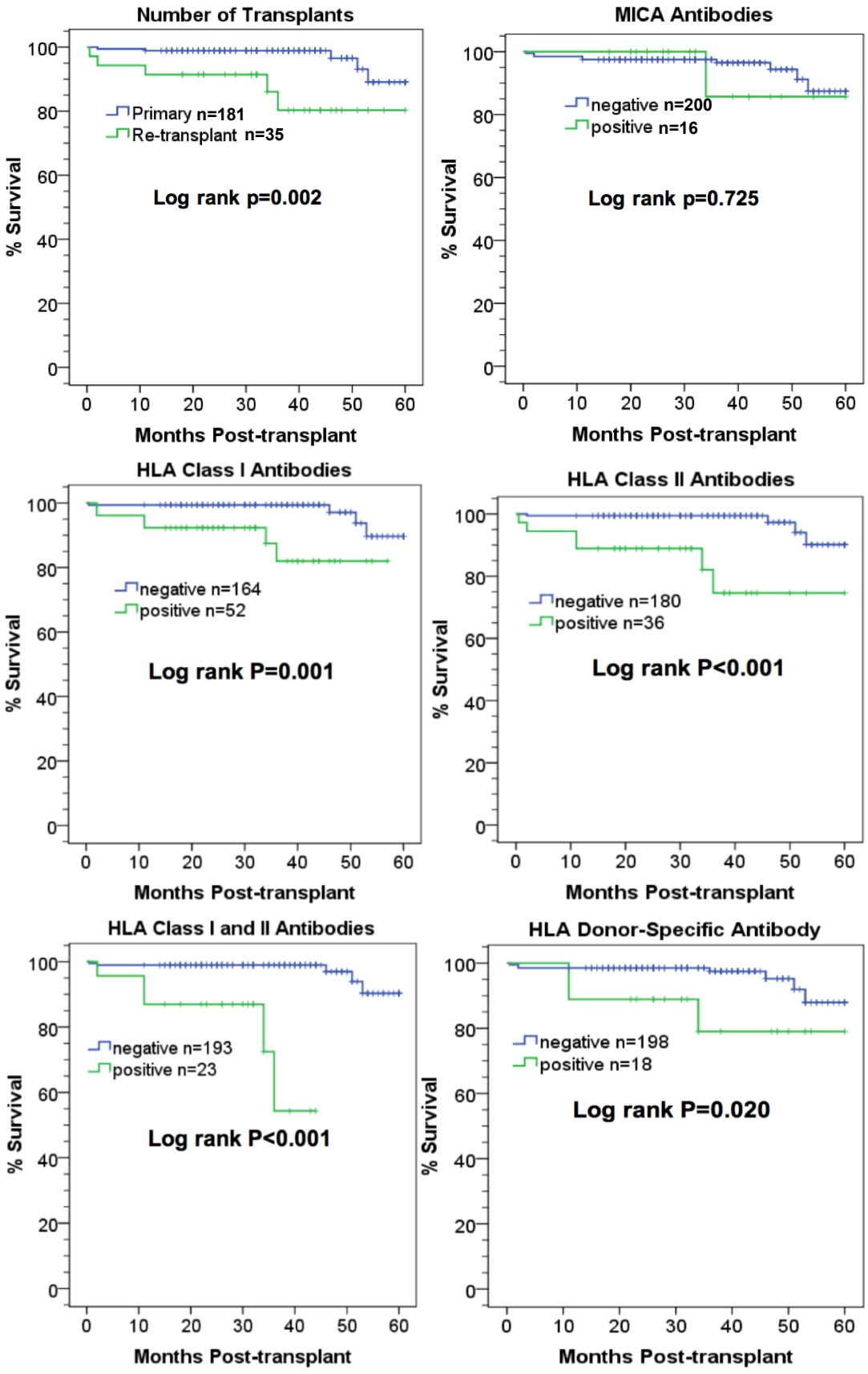

Figure 7.3.2: Association of the number of transplants, MICA antibodies, HLA class I antibodies, HLA class II antibodies, HLA class I and class II antibodies or HLA-DSA with five-year overall renal GS.

7.3.6 Overall five-year survival and the impact of ACR and aAMR

Figure 7.3.3 shows the effect of AR on five-year GS. ACR was significantly associated with reduced survival (log rank p=0.001) affecting 68% compared to 91% of patients with no ACR. There was also a significant correlation with aAMR and decreased OS (log rank p=0.048). Patients having aAMR had 87% survival compared to those with no aAMR having OS of 88%, although after three years this group had OS of 98%.

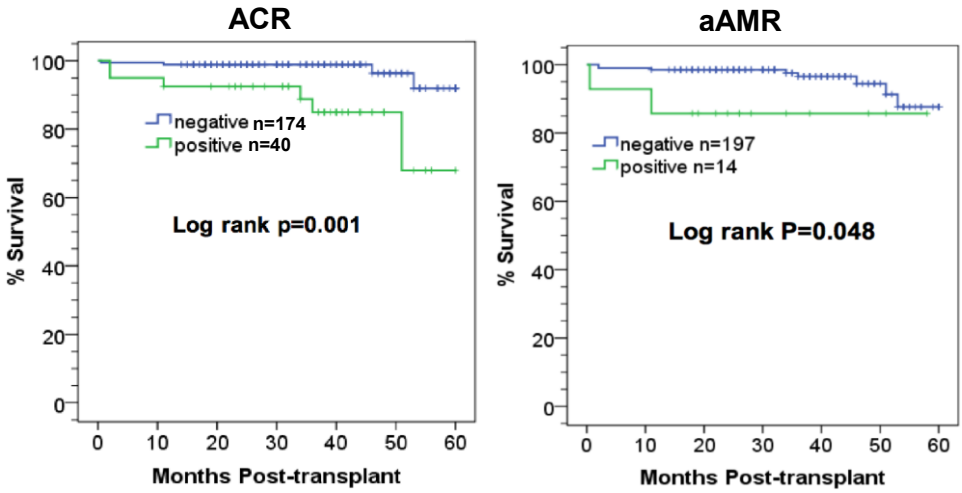

Figure 7.3.3: Association of ACR or aAMR with five-year overall renal GS.

7.3.7 Multivariate analysis using Cox regression

Multivariate statistical analysis was performed by Cox regression. Variables considered in the multivariate model were source of donor, patient gender, donor gender, age of patient and donor, number of transplants, MICA antibodies, HLA class I antibodies, HLA class II antibodies, HLA antigen mismatch (HLA-A, -B, -DR), MICA allele mismatch and ACR and aAMR. Variables were selected for inclusion in the Cox regression model if the univariate analysis using the Kaplan-Meier method achieved a log rank statistic of $P \leq 0.100$. The results are presented in Table 7.3.1.

Table 7.3.1: Kaplan-Meier and Cox regression analysis of overall survival data

Variable	Univariate P Value	Multivariate hazard ratio (95% CI)	Multivariate P Value
HLA class I antibodies	0.001	2.6 (0.4 –17.5	0.330
HLA class II antibodies	<0.001	17.2 (2.2 – 132)	0.006
HLA-DSA	0.020	0.8 (0.2 – 3.8)	0.766
ACR	0.001	8.5 (1.7 – 42.0)	0.009
aAMR	0.048	9.7 (1.3 – 71.7)	0.025
Number of transplants	0.002	0.8 (0.2 – 3.8)	0.978

The variables achieving statistical significance with Kaplan-Meier univariate analysis were HLA class I and II antibodies, HLA-DSA, ACR and aAMR and number of transplants (primary or re-graft). HLA class I antibodies, HLA-DSA and number of transplants were not independent significant factors affecting OS by Cox regression multivariate analysis. Variables significantly and independently affecting OS were the presence of HLA class II antibodies (hazard ratio 17.2; P=0.006), ACR (hazard ratio 8.5; P=0.009) and aAMR (hazard ratio 9.7; P=0.025).

7.4 Renal graft function analysis

7.4.1 Assessment of chronic renal damage and renal function

Assessment of chronic renal damage in confirmed MICA antibody positive recipients in the same cohort was made on renal biopsies using a morphometric index of chronic damage (CD), which expresses the cross-sectional area of chronically damaged cortex as a percentage of the cross-sectional area of the renal cortex. An index >40% was strongly predictive of renal failure (Howie *et al*., 2004).

To assess renal function and associations of antibodies and other transplant variables with clinical course, eGFR was calculated from serum creatinine values and expressed as ml/min/1.73m^2 according to the 4-point MDRD as described (Burden and Tomson, 2005; A.S. Levey *et al*., 2006). Measurements of eGFR were taken one, two and three years post-transplant for patients available for follow-up and alive (205/227 MICA-typed pairs).

Analysis of eGFR using the MDRD method has been described as the 'gold standard' for assessment of renal function and is a relatively recent development. Previously, and still widely used, function was assessed using serum creatinine (SCr) values. I also analysed the data using SCr values to compare the two types of measurement, although this data was only available for one and two years post-transplant.

Statistical analysis was performed using Student's t-test for independent variables (2-tailed) to compare eGFR means for dichotomous variables (most variables were normally distributed). Where Levene's test for equality of variances achieved a probability of $\leq$0.100, the statistic for unequal variances was used. Non-parametric univariate testing (Mann-Whitney U) was used for serum creatinine (SCr) analysis, as most variables did not show a normal frequency distribution. The Z value statistic is included in this analysis, where values of significant association lie outside the 95% confidence interval of -1.96 to +1.96 standard deviations.

General transplantation variables included were patient and donor gender and age, source of donor, number of transplants (primary or re-transplant), ACR and aAMR. Categories of MICA and/or HLA antibodies used for analysis were HLA antibodies overall, MICA antibodies overall, HLA antibodies only, MICA antibodies only, HLA and MICA antibodies together, HLA-DSA, MICA-DSA, class I-DSA, class II-DSA and strong MICA antibodies. All factors achieving a probability of P $\leq$0.100 were included in a multivariate analysis model (antibody categories chosen were MICA-DSA, HLA class I-DSA and class II-DSA). Analysis was performed for each eGFR or SCr measurement time-point using linear regression. Significant independent variables revealed by linear regression had a probability of P $\leq$ 0.05.

7.4.2 Association of general transplantation variables with reduced graft function

Analysis of post-transplant eGFR revealed significant association with donor gender, patient and donor age, donor source, re-transplantation and ACR/aAMR as shown in Table 7.4.1. Significantly decreased eGFR occurred with grafts from female donors after one year (P=0.006) and two years (P=0.007) post-transplant with a mean difference of 7.9 and 8.9 ml/min/1.73m^2, respectively. Patient age $\geq$50 years significantly associated with decreased eGFR after one-year post-transplant (P=0.020) but then stabilised. The age of the donor associated with highly significantly decreased graft function (P<0.001) at all three time-points and donors $\geq$50 years old resulted in an average decrease of 14 ml/min/1.73m^2

compared with donors <50 years old. Patients with grafts from deceased donors had significantly decreased eGFR after one year post-transplant (P=0.021) but not after the second year, with a mean difference of 7.0 ml/min/1.73m^2.

Table 7.4.1: Comparison of mean eGFR in renal transplant recipients with risk factors for CAD at one, two and three years post-transplant

Variable	Yr	eGFR[1] mean ± SD (n)		P Value[2]	Mean Diff.
		Male	**Female**		
Patient	1	51.2 ± 17.2 (117)	54.4 ± 21.9 (88)	NS	3.1
	2	51.4 ± 17.9 (107)	54.3 ± 24.3 (73)	NS	2.9
	3	49.3 ± 19.9 (66)	50.5 ± 20.9 (45)	NS	1.2
Donor	1	55.8 ± 20.4 (104)	47.9 ± 18.0 (82)	0.006	7.9
	2	57.0 ± 22.1 (88)	48.1 ± 19.0 (75)	0.007	8.9
	3	52.8 ± 20.8 (48)	47.3 ± 19.6 (54)	NS	5.6
		<50 years	**≥50 years**		
Patient	1	54.9 ± 20.9 (128)	48.8 ± 16.0 (77)	0.020	6.1
	2	55.3 ± 22.1 (115)	49.5 ± 17.7 (65)	NS	4.8
	3	51.9 ± 21.1 (73)	45.8 ± 18.1 (38)	NS	6.1
Donor	1	58.0 ± 20.7 (106)	47.1 ± 15.4 (95)	<0.001	10.8
	2	60.5 ± 21.0 (95)	43.6 ± 16.4 (84)	<0.001	16.1
	3	57.2 ± 20.4 (59)	41.9 ± 16.3 (51)	<0.001	15.4
		Deceased	**Living**		
Donor Source	1	50.6 ± 20.0 (148)	57.6 ± 17.0 (57)	0.021	7.0
	2	51.9 ± 21.2 (131)	54.3 ± 19.4 (49)	NS	2.4
	3	48.9 ± 20.6 (83)	52.5 ± 19.1 (28)	NS	3.6
		Primary	**Re-transplant**		
No. Txps	1	53.2 ± 18.1 (173)	49.1 ± 25.6 (32)	NS	4.1
	2	53.1 ± 19.8 (152)	49.7 ± 25.0 (28)	NS	3.4
	3	51.9 ± 19.6 (92)	39.4 ± 20.6 (17)	0.013	12.5
		Negative	**Positive**		
ACR	1	54.7 ± 18.8 (168)	43.0 ± 19.6 (36)	0.001	11.7
	2	55.2 ± 20.4 (145)	41.5 ± 18.5 (34)	<0.001	13.7
	3	54.3 ± 19.8 (84)	35.6 ± 15.0 (26)	<0.001	18.7
aAMR	1	53.5 ± 18.7 (189)	36.2 ± 20.5 (13)	0.001	17.3
	2	52.7 ± 20.6 (169)	44.6 ±19.0 (8)	NS	8.1
	3	50.2 ± 20.4 (105)	37.7 ± 16.3 (4)	NS	12.5

[1] estimated glomerular filtration rate (ml/min/1.73m^2). [2]Student's t-test for independent variables

Re-transplanted patients had significantly reduced eGFR after three years post-transplant with eGFR of 39.4 ml/min/1.73m^2 compared with 51.9 ml/min/1.73m^2 (P=0.013) and a

mean difference of 12.5 ml/min/1.73m^2. ACR was highly significantly associated with reduced eGFR after one year (P=0.001), two years (P<0.001) and three years (P<0.001) with increasing mean differences of 11.7, 13.8, 18.7 ml/min/1.73m^2, respectively (illustrated in Figure 7.4.1). Incidence of aAMR associated with decreased eGFR after one year (P=0.001) with a mean difference of 17.3 ml/min/1.73m^2.

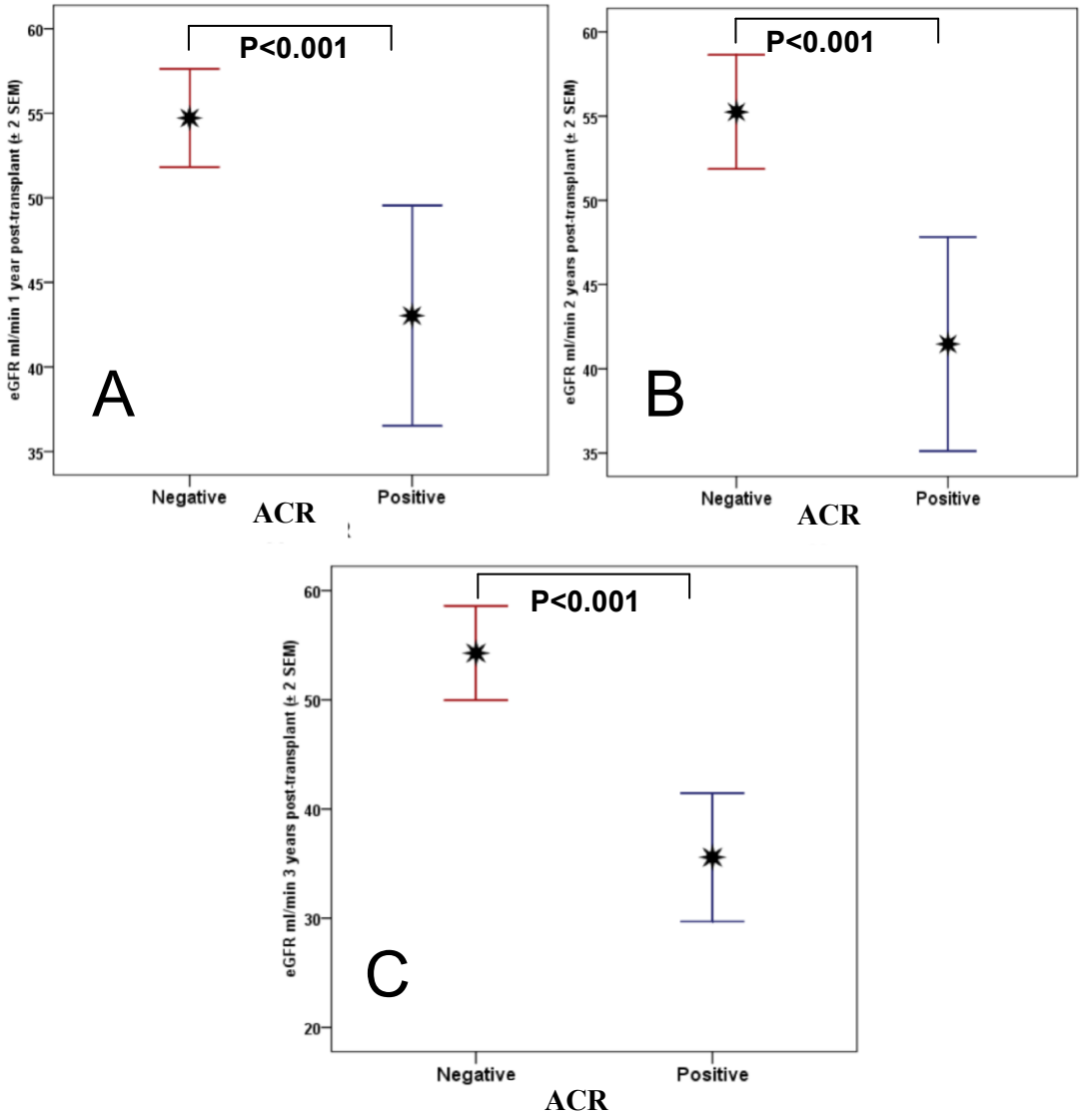

Figure 7.4.1: **Error bar charts showing eGFR (ml/min/1.73m^2) of patients with and without ACR episodes**. eGFR measurements taken 1 (A), 2 (B) and 3 (C) years post-transplant.

Analysis of SCr levels at years one and two post-transplant (Table 7.4.2) also showed significant correlation with several of the variables examined.

Table 7.4.2: Comparison of mean rank serum creatinine values with risk factors for CAD

Variable	Yr PT	Creatinine[1] mean rank[2] (n)		P Value	Z Value
		Male	**Female**		
Patient	1	107.1 (103)	73.9 (81)	<0.001	-4.2
	2	97.0 (107)	69.3 (65)	<0.001	-3.5
Donor	1	76.4 (96)	93.1 (70)	0.025	-2.2
	2	70.2 (82)	86.8 (73)	0.022	-2.3
		<50 years	**>50 years**		
Patient	1	93.5 (111)	91.0 (73)	NS	-0.3
	2	89.1 (109)	82.1 (63)	NS	-0.9
Donor	1	78.8 (94)	104.2 (87)	0.001	-3.3
	2	70.5 (89)	102.8 (82)	<0.001	-4.3
		Deceased	**Living**		
Donor Source	1	97.4 (134)	79.5 (50)	0.043	-2.0
	2	88.6 (125)	80.8 (47)	NS	-0.9
		Primary	**Re-transplant**		
No. Txps	1	90.7 (156)	102.5 (28)	NS	-1.1
	2	84.8 (145)	95.5 (27)	NS	-0.3
		Negative	**Positive**		
ACR	1	85.7 (149)	119.5 (34)	0.001	-3.4
	2	80.8 (138)	107.9 (33)	0.005	-2.8
aAMR	1	89.9 (170)	113.8 (11)	NS	-1.5
	2	84.7 (161)	99.1 (8)	NS	-0.9

[1] serum creatinine (μmol/l). [2]Mann-Whitney U test. Reproduced with permission from Elsevier (Cox *et al.,* 2001)

Patient gender associated with significant differences in SCr at one (Z=-4.2; P<0.001) and two (Z=-3.5; P<0.001) years post-transplant, where males had higher values. By contrast, donor female gender correlated with significantly higher creatinine values at one (Z=-2.2; P=0.025) and two (Z=-2.3; P=0.022) years post-transplant. Significantly higher SCr values were found with donors aged >50 years after year one (Z=-3.3; P=0.001) and year two (Z=-4.3; P<0.001), deceased donors after year one (Z=-2.0; P=0.043) and ACR after year one (Z=-3.4; P=0.001) and year two (Z=-2.8; P=0.005).

7.4.3 Association of HLA and/or MICA antibodies with reduced graft function

Table 7.4.3 shows univariate mean eGFR comparisons with different categories of HLA and/or MICA antibodies and reveals patients with MICA antibodies overall had significantly decreased eGFR of 14.3 ml/min/1.73m^2 (P=0.002) after two years post-transplant. HLA-DSA associated with a significantly decreased eGFR of 10.7 ml/min/1.73m^2 in the second year post-transplant (P=0.027) and 26.8 ml/min/1.73m^2 after the third year. There was a trend associating MICA-DSA with reduced eGFR after one year post-transplant but was highly significant in the second year (P=0.001) with a mean difference of 17.1 ml/min/1.73m^2 and significant after the third year with a mean difference of 17.5 ml/min/1.73m^2 (P=0.038). Patients with HLA-DSA only (no MICA antibodies) had significantly reduced eGFR after three years post-transplant with a mean difference of 27 ml/min/1.73m^2 (P=0.001). MICA-DSA only (no HLA antibodies) associated with a decreased eGFR of 18 ml/min/1.73m^2 after the second year of transplantation (P=0.001). HLA class I-DSA were significantly associated with a decreased eGFR at one (P=0.028), two (P=0.045) and three years (P=0.007) post-transplant with decreased eGFR and a mean difference ranging from 13-25 ml/min/1.73m^2. There was a significant correlation with HLA class II-DSA production and decreased eGFR after the first year of transplantation, of 10.6 ml/min/1.73m^2 and also after the third year (P=0.002) with a mean difference of 25.6 ml/min/1.73m^2. Patients with HLA class I+II-DSA, significantly associated with decreased eGFR at one year post-transplant (P=0.019) with a mean difference of 17.3 ml/min/1.73m^2 and a trend after three years (P=0.066). Strong MICA antibodies were associated with lower eGFR values at two (P=0.039) and three years (P=0.036) post-transplant with a difference of 19.2 and 21.5 ml/min/1.73m^2, respectively. Figure 7.4.2 shows error bar charts with levels of eGFR associated with the groups positive and negative for MICA antibodies.

Table 7.4.3: Comparison of mean eGFR in renal transplant recipients with different categories of HLA/MICA antibodies at one, two and three years post-transplant

Antibodies	Yrs PT	eGFR[1] mean ± SD 1 year N=205; 2 year N=180; 3 year N=112					
		Negative	n	Positive	n	P Value[2]	Mean Diff.
HLA	1	53.7 ± 18.5	146	43.3 ± 21.0	59	NS	4.4
	2	53.0 ± 19.4	129	50.3 ± 23.2	51	NS	2.7
	3	51.7 ± 19.6	83	44.1 ± 21.1	29	0.083	7.6
MICA	1	53.1 ± 19.7	189	44.4 ± 12.4	16	0.084	8.7
	2	53.4 ± 20.6	165	39.1 ± 14.4	15	0.002	14.3
	3	50.6 ± 20.2	104	38.1 ± 16.6	8	0.091	12.5
HLA & MICA	1	52.8 ± 19.5	197	43.6 ± 14.4	8	NS	9.2
	2	52.7 ± 20.5	172	42.0 ± 18.6	8	NS	10.7
	3	50.2 ± 20.1	107	40.4 ± 20.6	5	NS	9.8
HLA-DSA	1	53.3 ± 18.8	186	43.8 ± 23.0	19	NS	9.5
	2	53.3 ± 20.1	164	41.4 ± 22.2	16	0.027	11.9
	3	51.6 ± 19.2	104	24.8 ± 15.4	8	<0.001	26.8
MICA-DSA	1	53.1 ± 19.6	192	43.2 ± 12.1	13	0.073	9.9
	2	53.4 ± 20.5	168	35.7 ± 11.2	12	<0.001	17.7
	3	50.7 ± 20.1	106	33.2 ± 13.2	6	0.038	17.5
HLA-DSA Only	1	53.0 ± 18.8	189	45.6 ± 24.1	16	NS	7.4
	2	52.9 ± 20.2	167	43.4 ± 23.0	13	0.100	9.5
	3	51.2 ± 19.5	106	24.2 ± 14.9	6	0.001	27.0
MICA-DSA Only	1	52.7 ± 19.5	198	45.5 ± 11.8	7	NS	7.2
	2	52.8 ± 20.5	174	34.8 ± 7.6	6	0.001	18.0
	3	50.1 ± 20.2	109	34.3 ± 8.6	3	NS	15.8
Class I-DSA	1	53.2 ± 19.0	193	40.6 ± 21.5	12	0.028	12.6
	2	53.0 ± 20.4	170	39.6 ± 18.9	10	0.045	13.4
	3	50.8 ± 19.8	107	26.2 ± 13.5	5	0.007	24.6
Class II-DSA	1	53.2 ± 18.7	191	42.6 ± 24.8	14	0.047	10.6
	2	52.8 ± 20.0	169	42.4 ± 20.6	11	NS	10.4
	3	51.1 ± 19.5	106	25.5 ± 16.4	6	0.002	25.6
Class I + II-DSA	1	53.0 ± 19.0	198	35.7 ± 23.6	7	0.019	17.3
	2	52.6 ± 20.3	175	40.0 ± 24.5	5	NS	12.6
	3	50.3 ± 20.0	109	28.7 ± 13.6	3	0.066	21.6
Strong MICA	1	52.8 ± 19.3	200	37.6 ± 14.0	5	0.082	15.2
	2	52.8 ± 20.4	175	33.6 ± 15.8	5	0.039	19.2
	3	50.5 ± 20.0	108	29.0 ± 14.8	4	0.036	21.5

[1]estimated glomerular filtration rate (ml/min/1.73m^2). [2]Student's t-test for independent variables. Reproduced with permission from Elsevier (Cox *et al.,* 2001)

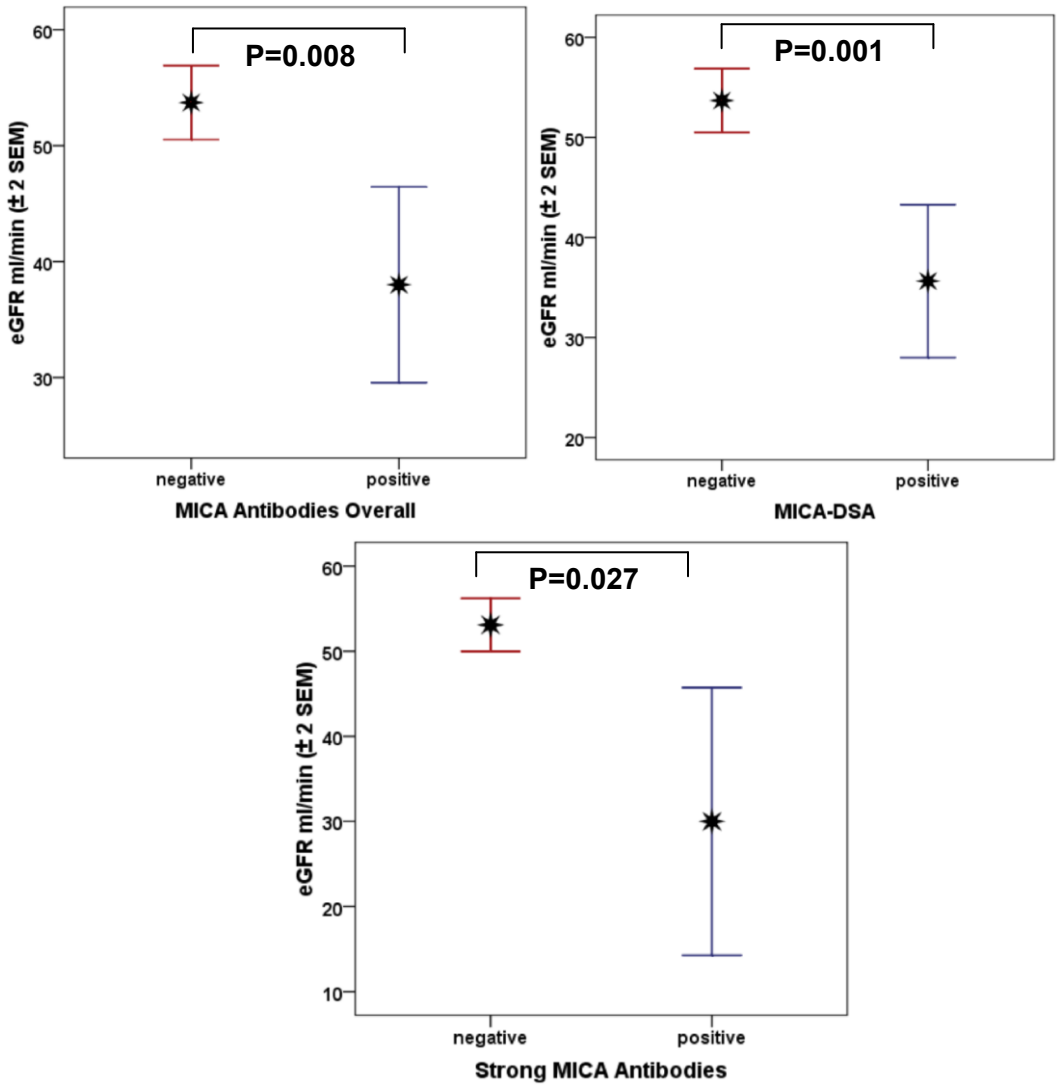

Figure 7.4.2: **Error bar charts showing mean eGFR (ml/min/1.73m^2) of patients with and without MICA antibodies overall, MICA-DSA and strong MICA antibodies.** eGFR measurements were taken at 2 years post-transplant.

Analysis using SCr values at one and two years post-transplant (Table 7.4.4) also revealed significant association of increased values in graft recipients with MICA and HLA antibodies. Significant differences were observed in patients with MICA antibodies after one year (Z=-2.0; P=0.046) and two years (Z=-2.9; P=0.004). Co-production of HLA and MICA antibodies associated with increased SCr after one year (Z=-2.4; P=0.017) with an equivalent trend after two years (Z=-1.9; P=0.054). There was a trend of association with HLA-DSA after one year of transplantation that became significant after the second year (Z=-2.5; P=0.014). Similarly, MICA-DSA associated with a trend for increased SCr after one year but correlated significantly after year two (Z=-3.2; P=0.001). Significant differences also associated MICA-DSA alone following the second year of transplantation

(Z=-2.0; P=0.044). Significant increases in SCr were observed with HLA class I-DSA after the first year (Z=-2.2; P=0.025) and second year (Z=-2.7; P=0.007) of engraftment, whereas HLA class II-DSA significantly associated with increased values only after two years (Z=-2.1; P=0.039). Production of both class I and II HLA-DSA correlated with increased SCr at one year (Z=-2.4; P=0.019) and two years (Z=-2.5; P=0.013) post-transplant. Strong MICA antibodies (producing high-MFI) also showed significant association with increased SCr after one year (Z=-2.4; P=0.015) and two years post-transplant (Z=-2.0; P=0.042).

Table 7.4.4: Mean rank serum creatinine comparisons with different HLA and/or MICA antibody categories at one and two years post-transplant

Antibodies	Yr PT	Creatinine[1] mean rank[2] (n) 1 year N=184; 2 year N=172			
		Negative	Positive	P Value	Z Value
HLA	1	89.5 (131)	100.1 (53)	NS	-1.2
	2	83.9 (125)	93.4 (47)	NS	-1.1
MICA	1	90.3 (171)	120.9 (13)	0.046	-2.0
	2	83.5 (160)	126.8 (12)	0.004	-2.9
HLA and MICA	1	90.8 (178)	143.7 (6)	0.017	-2.4
	2	85.1 (166)	125.0 (6)	0.054	-1.9
HLA-DSA	1	90.5 (169)	115.1 (15)	0.086	-1.7
	2	83.6 (157)	116.6 (15)	0.014	-2.5
MICA-DSA	1	90.7 (174)	123.3 (10)	0.060	-1.8
	2	83.6 (163)	138.6 (9)	0.001	-3.2
HLA-DSA Only	1	91.4 (171)	106.4 (13)	NS	-1.0
	2	84.5 (159)	110.7 (13)	0.068	-1.8
MICA-DSA Only	1	92.4 (178)	96.8 (6)	NS	-0.2
	2	85.2 (167)	130.7 (5)	0.044	-2.0
HLA class I-DSA	1	90.5 (175)	131.4 (9)	0.025	-2.2
	2	84.1 (163)	130.4 (9)	0.007	-2.7
HLA class II-DSA	1	91.0 (173)	116.4 (11)	NS	-1.5
	2	84.5 (161)	116.4 (11)	0.039	-2.1
Class I+II-DSA	1	91.0 (179)	147.4 (5)	0.019	-2.4
	2	84.9 (167)	140.9 (5)	0.013	-2.5
Strong MICA	1	91.1 (180)	156.4 (4)	0.015	-2.4
	2	85.3 (168)	136.6 (4)	0.042	-2.0

[1]serum creatinine (μmol/l). [2]Mann-Whitney U test.

7.4.4 Graft function multivariate analysis – linear regression model

Multivariate analysis of eGFR measurements taken at one (n=205), two (n=180) and three years (n=112) post-transplant and SCr data for one (n=184) and two years (n=172) post-transplant was performed using linear regression. Factors that were significant or a trend

(P≤0.100) by univariate analysis, were entered into the model at each time-point and results are shown in Tables 7.4.5 and 7.4.6.

Table 7.4.5: Linear regression analysis of factors associated with eGFR decrease in renal graft recipients

Year	Variable	Univariate P-Value	B[1]	CI (95%)	Multivariate P-Value
1	ACR	0.001	12.3	5.5 – 19.1	<0.001
	aAMR	0.001	17.2	3.6 – 30.7	0.022
	Donor ≥ 50yrs	<0.001	11.1	5.9 – 16.4	<0.001
	Patient ≥ 50yrs	0.020	3.7	-	0.172
	Donor source	0.021	6.9	1.0 – 12.7	0.022
	Donor female	0.006	7.1	1.9 – 12.2	0.007
	HLA class I-DSA	0.028	3.6	-	0.575
	HLA class II-DSA	0.047	8.8	-	0.166
	MICA-DSA	0.073	6.5	-	0.251
2	ACR	<0.001	12.8	5.4 – 20.1	0.001
	Donor ≥ 50yrs	<0.001	17.3	11.7 – 22.8	<0.001
	Donor female	0.007	6.3	0.8 – 11.9	0.026
	HLA class I-DSA	0.045	11.2	0.4 – 22.3	0.050
	MICA-DSA	<0.001	16.1	3.9 – 28.2	0.010
3	ACR	<0.001	15.9	8.2 – 23.6	<0.001
	Donor ≥ 50yrs	<0.001	14.5	8.1 – 20.9	<0.001
	Re-transplantation	0.013	1.0	-	0.985
	HLA class I-DSA	0.007	18.8	0.3 – 37.4	0.046
	HLA class II-DSA	0.002	1.7	-	0.860
	MICA-DSA	0.059	10.8	-	0.172

[1]B – coefficient of variation, refers to difference in eGFR between positive and negative cases. Covariates were included in the model where P≤ 0.100 by univariate analysis. Reproduced with permission from Elsevier (Cox *et al.,* 2001).

Linear regression analysis of factors associated with decreased graft function and measurements of eGFR taken at one-year post-transplant revealed association with several independent variables. At one year post-transplant, ACR and aAMR were independently correlated with decreased eGFR (P<0.001 and P=0.022 respectively), both with high coefficient of variation (B). Although ACR was more significant, aAMR associated with a

larger decrease in eGFR of 17.2 ml/min/1.73m^2 compared to 12.3 ml/min/1.73m^2. Donors aged $\geq$ 50 years old highly correlated with decreased eGFR of 11.1 ml/min/1.73m^2 (P<0.001) and the donor source (deceased) associated with a decrease of 6.9 ml/min/1.73m^2 (P=0.022). Female donor was also an independent risk factor with a reduced eGFR of 7.1 ml/min/1.73m^2 (P=0.007). MICA-DSA or HLA-DSA antibodies were not independent variables at one year post-transplant. At two years post-transplant, significant independent variables were ACR (P=0.001), donors aged $\geq$ 50 years old (P<0.001), female donor gender (P=0.026), HLA class I-DSA (P=0.050) and MICA-DSA (P=0.010). The independent variable with the lowest reduction in eGFR was female donor (6.9 ml/min/1.73m^2) whereas the largest difference was observed with donors aged $\geq$ 50 years old and resulted in a decrease of 17.3 ml/min/1.73m^2. MICA-DSA associated with a decrease of 16.1 ml/min/1.73m^2, compared with 11.2 ml/min/1.73m^2 with HLA class I-DSA. Among the variables entered into the regression model for the three-year post-transplant time-point, ACR (P<0.001), donors aged $\geq$ 50 years old (P<0.001) and HLA class I-DSA (P=0.046) were independent variables with eGFR differences of 15.9, 14.5 and 18.8 ml/min/1.73m^2, respectively

Linear regression analysis was also performed for variables showing significant association or a trend with increased SCr. After one year of transplantation, variables significantly and independently associated with a rise in SCr were ACR (P<0.001) and deceased donor (P=0.018). Following the second year of transplantation, significant, independent variables were ACR (P=0.024), donors $\geq$ 50 years (P<0.001), male patients (P=0.014), HLA class I-DSA (P=0.002) and MICA-DSA (P=0.001). Results are shown in Table 7.4.6.

Table 7.4.6: Linear regression analysis of factors associated with mean rank serum creatinine increase in renal graft recipients

Year	Variable	Univariate P-Value	B[1]	CI (95%)	Multivariate P-Value
1	ACR	0.001	64.1	31.8 – 96.4	<0.001
	Donor ≥ 50yrs	0.001	18.6	-	0.149
	Deceased donor	0.043	35.1	6.0 – 64.2	0.018
	Donor female	0.025	21.7	-	0.096
	Patient male	<0.001	2.8	-	0.825
	HLA class I-DSA	0.025	30.9	-	0.289
	MICA-DSA	0.060	21.5	-	0.461
2	ACR	0.005	24.0	3.3 – 44.7	0.024
	Donor ≥ 50yrs	<0.001	32.3	16.4 – 48.2	<0.001
	Donor female	0.022	14.7	-	0.071
	Patient male	<0.001	20.6	4.2 – 36.9	0.014
	HLA class I-DSA	0.007	62.7	22.7 – 103	0.002
	MICA-DSA	0.001	67.5	28.9 – 106	0.001

[1]B – coefficient of variation, refers to difference in SCr between positive and negative cases. Covariates were included in the model where P≤ 0.100 by univariate analysis.

7.4.5 Assessment of chronic renal damage by morphometric analysis

Of seventeen MICA antibody positive graft recipients, ten had biopsies taken for arbitrary reasons at an average of nine months post-transplant. Among the ten biopsies, three (30%) had an index >40% chronic damage in the renal cortex and these patients did not have HLA class I or class II antibodies detected by HLA SAg Luminex assay.

7.5 Discussion

7.5.1 Soluble MICA and soluble MICB in renal transplantation

It has become increasingly evident in recent years that the soluble forms of MICA and MICB molecules have the potential to engage the NK-cell activatory receptor NKG2D and down-regulate expression (Groh *et al*., 2002). This has the effect of reducing the potential of NK-cells to lyse target cells via NKG2D and is proposed as a tumour immune evasion mechanism. Soluble MICA and MICB can be detected at high levels in patients with various carcinomas (Marten *et al*., 2006) and has been associated with poorer prognosis. Soluble MICA levels have also been found to be higher in haematopoietic stem cell transplantation (HSCT) patients than in healthy controls, moreover higher levels of sMICA were significantly associated with chronic GvHD (cGVHD) (Boukouaci *et al.*, 2009). It is likely that sMICA found in patients with cGVHD is a consequence of inflammatory events taking place post-transplant rather than an involvement of sMICA with the pathogenesis of cGVHD. These authors also found that having MICA antibodies associated with a lower incidence of sMICA detection and may indicate that MICA antibodies can neutralise sMICA.

The hypothesis was made that sMICA detected in the sera of heart transplant recipients may have an immunosuppressive effect and decrease incidence of AR (Suarez-Alvarez *et al*., 2006a; Suarez-Alvarez *et al*., 2006b). Since there are no publications investigating the relationship of sMICA and rejection in renal transplant recipients and the study by Suarez-Alvarez was not very large (n=34 and only ten patients had rejection), I decided to analyse the smaller recipient cohort for the presence of sMICA (n=200, 53 with rejection) and sMICB (n=201, 52 with rejection). The results for this part of the study show that 12.4% of 200 graft recipients had detectable sMICA in their serum at one year post-transplant and 41% of 201 recipients had detectable sMICB. Generally the mean concentration of sMICB was higher than for sMICA. In this study there was no significant association of soluble MIC with any transplantation variable except for sMICB with younger recipient age (P=0.015) and number of HLA-A, -B, -C mismatches (P=0.010). The sMICB negative group curiously had more HLA mismatches than the sMICB positive group. There was also no significant association with sMICA or sMICB with the presence of HLA or MICA antibodies or with rejection in this study. The contrast in findings between the Suarez-Alvarez study and this one may relate to the type of transplantation, theirs was heart

transplants and sMICA expression may be more frequent than in renal transplants as their frequencies were much higher. More likely it is the size of the study as only 34 patients in total were analysed, compared to 200 patients in this study.

Analysis of eGFR in patients with or without sMICA or sMICB showed patients with sMICA had better graft function compared to those without. Although only a statistical trend (P=0.078), 45% less patients had eGFR $\geq$ 30 ml/min/1.73m^2 if they were negative for sMICA. Interestingly, although not significant, Kaplan-Meier analysis (discussed in the next section) revealed a tendency for recipients with sMICA or sMICB to have better overall GS (98%) at five years compared to sMICA or sMICB negative recipients (83%). Analysis of sMICA and sMICB individually showed less of a difference with sMICA (90% versus 86%) than with sMICB (98% versus 85%). Therefore it is possible that soluble NKG2D ligands detected in serum can protect against graft dysfunction and loss.

7.5.2 Five-year kidney graft survival analysis

Although it was of interest to analyse the kidney recipients for five-year overall GS, this is a relatively small cohort (216 patients). The ten patients eligible for the study (a small number for this kind of analysis) with graft failure represent a loss rate of 5% over five years and, therefore, a five-year graft OS of 95% for this cohort. Including all patients who lost grafts or died, overall GS for five years was 90%. There are many factors that associate with decreased renal GS, as discussed in Chapter 1, and I have included as many risk factors as possible where information was available and the numbers were not too small to render statistical analysis meaningless.

Live donors are associated with improved patient survival as well as GS. The results from this study revealed grafts from living donors had a survival of 98% after 5 years compared to 82% from deceased donors, however this was not statistically significant. Five-year patient survival was better than average in this cohort with 97% of patients with grafts from living donors and 97% of patients with deceased donor grafts still alive after five years. Thus, in terms of patient survival there was no difference between live and deceased donor transplants found in this study.

There are many studies investigating the effect of donor gender on GS. The largest European study was by the CTS and included 124,911 kidney transplants (Zeier *et al.*, 2002). Inferior graft outcome was apparent when male recipients received renal grafts from

female donors. Female patients also had poorer GS when the donor was female although the effect was more pronounced in male recipients and took several years to become apparent in both groups. My study also revealed a gender bias with GS based on gender of the donor. There was virtually no difference in survival when the recipient was female. The effect of donor gender was much stronger when the patient was male and the organ was from a female donor where 60% were still functioning after five years compared to 89% when the donor was male (Log rank p=0.06). Much of this difference only occurred after three years post-transplant and may explain why statistical significance was not achieved. A suggested reason is nephron under dosing with grafts from female donors (Brenner *et al.*, 1992). In allogeneic or syngeneic transplants, reduction of nephron mass had adverse effects on graft function and morphology (Azuma *et al.*, 1997). Therefore female donated kidneys have shorter survival because they are smaller, although this remains controversial, not least because it has been confirmed that males receiving livers from female donors also show poorer GS (Brooks *et al.*, 1997). Studies have also found that a significantly higher number of male recipients of female donor kidneys required treatment for rejection within the first year of transplantation (Vereerstraeten *et al.*, 1999; Zeier *et al*., 2002).

Another non-immunological factor that may influence GS is age of the donor or recipient. It has been recognised for some time that there may be a negative impact on long-term kidney GS with increasing donor age (CTS, 1991) and was verified in a very large cohort of kidney transplants (Opelz, 2000). Donor age is now recognised as a major risk factor for long-term GS. In general older renal graft recipients and donors over 60 years of age do less well. Both age-related immune and non-immune factors can influence GS in elderly patients. The results from my study are not significant but show decreased GS when the recipient is over 60 years and a stepwise reduction in survival with increasing donor age associating with decreased survival. Donors aged between 50 and 59 years associated with five-year renal GS of 80% and donors over 60 years, 68%. This compares to donors aged 18-34 years associating with GS of 96% and donors aged 35-49 years associating with GS of 94%. Again, these differences were only noticeable after three years post-transplant.

The association of re-transplantation with significantly decreased GS of 80% compared to 90% for primary transplants (Log rank p=0.002) may correlate with pre-sensitisation to HLA and/or MICA antigens. Chi-squared analysis showed MICA antibodies associated with re-transplantation ($P<0.001$) as discussed above and in a separate analysis, HLA class I and II antibodies were also highly significantly associated with re-transplantation ($P<0.001$). It is therefore likely that the decreased GS associated with re-transplanted

patients is a consequence of HLA and/or MICA antibody production. However, MICA antibodies did not significantly associate with decreased GS in this study. This is possibly due to the relatively small number of MICA positive individuals and only one patient reaching the study end-point (graft failure), which has reduced statistical power. Previous studies have demonstrated association of MICA antibodies with inferior GS (Mizutani *et al*., 2005; Panigrahi *et al*., 2007b; Panigrahi *et al.*, 2007c; Zou *et al*., 2007). However, in a recent study of 425 patients (Lemy *et al*., 2010) using the Gen-Probe Luminex assay, the authors also found no significant difference in GS after ten years between patients with and without MICA antibodies. Moreover, patients without MICA antibodies actually had slightly worse GS. Although the study by Zou and co-workers (Zou *et al*., 2007) revealed convincing evidence for association of pre-formed MICA antibodies with decreased GS, it remains unclear whether MICA antibodies produced post-transplant associate with decreased GS and highlights a need for large, collaborative, multicentre studies.

In the early days of kidney transplantation, matching donor and recipient for class I HLA-A and HLA-B and class II HLA-DR antigens had a major impact on both patient and GS (Persijn *et al.*, 1978). A study by the CTS shows a direct correlation with the number of HLA mismatches and GS. A stepwise reduction in GS clearly shows completely matched grafts (HLA-A, B, DR) have superior survival and a six-antigen mismatch has the poorest GS. Results from my study did not show a significant correlation between the degree of HLA matching and five-year GS. This could be because it is a relatively small single-centre study and the six categories of 0-6 mismatches render each group even smaller for example, only three patients had six mismatches. Analysis of each locus (including HLA-C and –DQ) also revealed no significant association with mismatching and GS. I also carried out analysis for MICA allele mismatching in relation to five-year GS. This too did not reveal significance, although a survival rate of 76% was observed with two MICA allele mismatches compared to 90% with one mismatch or 88% with no mismatches. This is, to my knowledge, the first time analysis with MICA matching and GS has been carried out and only future multicentre and collaborative studies will be able to address this issue with any reliability.

The final variable considered for analysis of GS was ACR or aAMR. Early AR (within 6 months) has been shown by many studies to be an important prognostic factor for reduced GS. A study of 219 primary transplants from living related donors found early AR was significantly correlated with the subsequent detection of biopsy proven CAD (Basadonna *et al.*, 1993). Interestingly, Cox regression analysis of my data revealed significant risk

factors for graft loss to be HLA class II antibodies, ACR and aAMR leading to eventual chronic loss of function and loss of the graft. This would make sense as cellular rejection can be successfully treated with immunosuppressive drugs but causes permanent damage to the graft and little can be done routinely to prevent antibody-mediated graft injury from accumulating. I was unable to obtain data of diffuse C4d staining in peritubular capillaries for all patients diagnosed with aAMR but many were not C4d positive, possibly because the method for detection was immunoperoxidase staining rather than the more sensitive immunofluorescence technique. There was, however, a highly significant correlation between HLA class I and II-DSA and aAMR in univariate analysis and 50% of recipients with both types of HLA DSA had C4d deposition detected.

7.5.3 Renal graft function analysis

Known risk factors affecting long-term renal allograft survival include source of the donor (deceased or living), gender and age of the donor or patient, AR and sensitisation to HLA antigens. Other major risk factors known to have a negative impact on GS and function are calcineurin inhibitor use (CNI) and delayed graft function (DGF). DGF is very frequent with deceased donors, occurring with 50% of non-heart beating donors and 30% of heart beating donors, compared with only 5% of living donors (personal communication, Mark Harber). Unfortunately, details of DGF and CNI use were not available for this analysis and their contribution to reduced graft function and CAD cannot be assessed. However, as most patients with DGF received grafts from deceased donors, the impact of DGF would be included by analysis of donor source.

The mean eGFR for all patients at one and two years post-transplant was 52.6 ml/min/1.73m^2 and after three years was 49.8 ml/min/1.73m^2. This represents a gradual decline over the years with most patients having the equivalent of CKD stage 3, similar to other studies (Karthikeyan *et al.*, 2004; Marcen *et al.*, 2005).

Donor gender was discussed in the previous section in the context of GS and the poorer survival of female donor organs also correlates with decreased graft function. Female donor organs significantly associated with reduced eGFR after one and two years post transplant. Based on averages of eGFR over the three years of follow-up, there was a significant difference of 7.5 ml/min/1.73m^2 in favour of male kidneys although this was only significant in the first two years post-transplant, perhaps indicating that although responsible for lower eGFR, this situation is not progressive. Analysis of serum creatinine

values indicated female kidneys associated with higher values but was not as highly significant as analysis of eGFR. Interestingly, there was no significant difference of eGFR between male or female recipients but comparison of mean rank serum creatinine levels revealed highly significantly increased levels in male recipients. This probably relates to the fact that men have a higher muscle mass than women and produce more creatinine, highlighting the usefulness of the eGFR MDRD method of analysis. Furthermore, the decreased significance of female organs by SCr analysis, compared to eGFR analysis is likely to be related to higher SCr values in male recipients, reducing the differential.

Older patients associated with a decline in graft function but only significantly in the first year post-transplant and this was not verified by serum creatinine analysis. By contrast, older donors significantly associated with worse outcomes by both eGFR and SCr analysis, in agreement with previous studies (Almond *et al.*, 1993; Jain *et al.*, 2010; Oien *et al.*, 2007; Pessione *et al.*, 2003; Zeier *et al*., 2002). Both eGFR and SCr analysis identified significant outcome differences with deceased donors after one year of transplantation. Conversely, re-transplantation only associated with significantly reduced eGFR after three years and could indicate a correlation with the increased presence of HLA antibodies in these patients. ACR was highly correlated with reduced function at each time-point with increased eGFR mean difference and SCr. Although ACR is injurious to the graft, causing nephron cell death if untreated, this also is thought to be non-progressive once the AR episode is alleviated (Halloran, 2010). The further deterioration that is observed in these patients may relate to the production of antibodies after the rejection episode. Acute AMR was only associated with significantly reduced eGFR after one year post-transplant by univariate analysis. There was no significant association of aAMR with increased SCr, although levels were higher. The significant reduction of eGFR could be related to both pre-formed antibodies as well as sensitisation to donor antigens occurring in the early post-transplant period when the organ is more inflamed due to cold or warm ischaemia-reperfusion injury.

As my study did not show an association of MICA antibodies with significantly decreased GS as observed by some other studies, I focussed on investigating correlation of MICA and HLA antibodies with impaired graft function using SCr and eGFR measurements taken post-transplant. Analysis of all patients eligible for this part of the study revealed no significant correlation with HLA antibodies overall, although there was evidence of a trend after three years for reduced eGFR. Interestingly, significant association of decreased graft function, with large eGFR and SCr differences, was observed in recipients with MICA antibodies. The strongest associations were after two years post-transplant, particularly

patients with MICA-DSA antibodies, resulting in a mean eGFR difference of 17.7 ml/min/1.73m^2 (P<0.001). Recipients with strong MICA antibodies also associated with decreased function although only four patients were included in the positive group, accounting for the lower P values. It is interesting that among six recipients with MICA-DSA but no HLA antibodies, there was highly significant association (P=0.001) with a decreased eGFR of 18 ml/min/1.73m^2 following the second year of transplant by both eGFR and SCr analysis. This contrasts with HLA-DSA where significance with reduced eGFR was not reached until after the third year of engraftment (P<0.001), also involving six patients. The reduction in eGFR was, however, higher than MICA-DSA resulting in a reduced eGFR of 27 ml/min/1.73m^2.

HLA class I-DSA associated with a significant and steady decrease in graft function with a mean eGFR difference ranging from 12.6 to 24.6 ml/min/1.73m^2 between years one and three post-transplant, most significantly after three years. HLA class II-DSA associated with significantly decreased graft function after one-year post-transplant and may relate to aAMR, as there was no progressive damage evident at two years post-transplant. However, by three years post-transplant, HLA class II-DSA was highly significantly associated with a decrease of 25.6 ml/min/1.73m^2 and could indicate that these antibodies require several years to cause chronic damage and CAD as reported in studies discussed above. There was a significant correlation with reduced eGFR and increased SCr if patients had both HLA class I and II-DSA, particularly after the first year of transplantation and possibly indicates damage caused by pre-formed and *de novo* HLA antibodies, leading to aAMR. However, both types of HLA-DSA associated with a trend for reduced eGFR after three years of engraftment, whereas individually, class I or II-DSA where highly significant at three years post-transplant.

Linear regression analysis of transplantation variables revealing significant or trend association with reduced eGFR or increased SCr by univariate analysis was performed. The results from the eGFR statistical model indicated independent transplant variables at one-year post-transplant were ACR, aAMR, donors $\geq$ 50 years old, deceased donors and female donor gender but not MICA or HLA antibodies. The SCr model also identified ACR and deceased donors as independent variables and revealed no independent relationship with male recipients. However, donor age or gender were not independently significant factors as revealed by eGFR measurement, which may relate to limitations of SCr analysis. The SCr linear regression model failed to identify MICA or HLA antibodies as independent variables associated with graft dysfunction after one year of transplantation, confirming

results of eGFR analysis. This finding seems to indicate that in the first year of transplantation, it is factors relating to the donor (age, gender, deceased or living) in addition to AR that are the main factors affecting graft function.

Risk factors for reduced eGFR after two years post-transplant found to be significant independent variables were ACR, donors $\geq$ 50 years old, female donors, HLA class I-DSA and MICA-DSA. All variables entered into this model remained as significant independent factors associated with graft dysfunction. The SCr linear regression model revealed similar results, however male recipients also independently associated with reduced graft function but this may be due to males producing more SCr. Importantly, both methods of analysis showed independent association of MICA-DSA with graft dysfunction and this is a novel finding.

After three years post-transplant, significant independent variables associated with decreased eGFR were ACR, donors $\geq$ 50 years old and HLA class I-DSA. It is likely that a lack of association with MICA-DSA is due to only six individuals with MICA-DSA remaining in the study after three years. Although not significant, there was still a reduction of 10.8 ml/min/1.73m^2 in recipients with MICA-DSA compared to an eGFR reduction of 18.8 ml/min/1.73m^2 for recipients with HLA class I-DSA.

Overall, the effect of MICA antibodies had a persistent association with decreased eGFR and accounted for a large difference in function between those with or without MICA antibodies. Significant independent association with graft dysfunction correlated with MICA-DSA, becoming apparent at two years post-transplant. HLA class I-DSA also have a role in CAD and appears to occur around the same time as MICA-DSA association with reduced function. The results show HLA class II antibodies take longer to correlate with reduced function as HLA class II-DSA had more of an effect at the three-year time-point in contrast to MICA-DSA or HLA class I-DSA. These data show that it takes time for antibodies to have an independent effect and different types of antibody associate with a decline in graft function at different time points, possibly reflecting independent mechanisms of graft injury.

The finding that recipients with MICA-DSA are likely to have decreased eGFR, the apparent differential time kinetics of MICA or HLA antibody-mediated graft damage and their significant independent association with reduced function, indicates that two or more separate immunological mechanisms may be at play. Although there was less of a

reduction in eGFR with MICA-DSA compared to HLA-DSA, the reduction caused by MICA-DSA was almost as severe as that caused by donor organs ≥ 50 years of age, as revealed by linear regression analysis. Additionally, assessment of chronic renal damage by morphometric analysis showed that three patients of the ten with biopsies taken at nine months post-transplant had an index of >40% chronic damage in the renal cortex, strongly indicative of CAD. Importantly, these three patients only had MICA antibodies as HLA antibodies were undetected and verified using an HLA SAg Luminex assay.

7.5.4 Possible mechanisms of allograft injury by MICA antibodies

A correlation between MICA antibodies and ACR suggests cellular mechanisms culminate in MICA sensitisation. Further multivariate analysis revealed only patients with strong MICA antibodies developed AR. It is likely that T-cell indirect allorecognition of mismatched MICA epitopes can give rise not only to T-cell help for the production of IgG antibodies against MICA, but also cell-mediated immunity resulting in cellular rejection. By comparison, a study investigating MICA matching and GvHD in hematopoietic stem cell transplantation found a significantly higher rate of grade II-IV acute GvHD in MICA mismatched patients, indicating a T-cell response to mismatched MICA antigens (Parmar *et al*., 2009). This mechanism is illustrated in Figure 7.5.1 (A) showing uptake of cell debris or sMICA molecules by recipient DCs or B-cells and subsequent presentation to naïve alloreactive CD4+ T-cells to produce T-effector cells and antibody-producing B-cell clones. Analysis of serial serum samples from patients with strong MICA antibodies who experienced ACR revealed that antibodies were not present before the ACR episode and therefore not the cause. The higher level of MICA antibodies in patients who had ACR may relate to an increased expression of MICA during an inflammatory alloresponse. Nevertheless, *de novo* or pre-existing MICA antibodies may be implicated in the longer process of CAD.

Evidence from recent studies investigating the role of NK-cells and its activatory receptor, NKG2D, in solid organ transplantation suggest these innate immune cells participate in the allo-immune response directly and indirectly by cross-talk with cells of the adaptive immune response. The dogma that has been upheld is that NK-cells do not participate in rejection as earlier studies showed rats depleted of NK-cells still rejected skin, heart or liver allografts (Heidecke *et al.*, 1985). Additionally, T-cell depletion resulted in allografts being accepted, indicating that only T-cells are important. However since then phenotypic characteristics of NK-cells relating to inhibitory and activatory receptors have been

revealed and among them, NKG2D has emerged as an important activatory receptor (Bauer *et al*., 1999).

As already discussed, MICA is abundantly expressed in renal and pancreatic grafts following transplantation (Hankey *et al*., 2002) and a more recent study has shown that in mice, NKG2D ligands are rapidly up-regulated in response to injury associated with cardiac transplantation and associate with an innate immune response. This was followed by a second wave of NKG2D ligand up-regulation mediated by the adaptive immune response (Kim *et al.*, 2007). Blockade of NKG2D in CD28 negative mice or in the context of B7 blockade resulted in extending the allograft survival. NK-cells can become activated in response to ligands expressed on allografts and secrete cytolytic enzymes such as granzyme A and B and perforin as well as inflammatory cytokines IFN-γ and TNF-α that enhance antigen-specific T-effector cell activity and DC function (Obara *et al.*, 2005). Thus, NKG2D ligation with MICA molecules expressed on allografts may provide a link between the innate and adaptive immune responses leading to acute and chronic graft rejection (Kitchens *et al.*, 2006).

The definitive role of NK-cells in allograft rejection awaits clarification but NK-cells have been implicated with both transplant rejection and tolerance depending on their activation status and cross-talk between NK and other cells (Suarez-Alvarez *et al.*, 2009b). It appears from the literature that in solid organ transplantation, MHC class-I mismatching between donor and recipients is necessary but not sufficient to mediate rejection of allografts via KIR receptors by NK-cells (Uehara *et al.*, 2005), possibly because of the requirement for co-expression of other receptors as well as KIR, unlike NKG2D where no co-stimulation is required for activation. In a mouse skin transplant model, NK-cells stimulated with IL-15 differentiated and acquired additional effector functions that mediated graft rejection independent of an adaptive immune response (Kroemer *et al.*, 2008). However, in the resting state or in the absence of inflammation, NK-cells can favour tolerance by killing donor DCs or suppressing T-cell activation. In addition, pro-inflammatory cytokines such as IL-12, IL-15 and IL-18 can up-regulate expression of NKG2D and its ligands, thus enhancing NKG2D effector functions. NKG2D ligands may also be upregulated on DCs in response to TNF-α or IFN-γ and the reciprocal production of IL-15 by DCs could further amplify NKG2D-mediated immunity. Macrophages are prominent in rejecting tissues and are highly responsive to TNF-α and IFN-γ, resulting in activation and up-regulation of NKG2D expression, which may enhance effector functions and lysis of graft cells via engagement with MICA (Figure 7.5.1 (C)) and provide a further source of IL-15 (Liu and

Li, 2010). Cytokines produced by activated NK-cells can also stimulate MHC class I and II expression on target cells, rendering them susceptible to attack by antigen-specific effector T-cells as shown in Figure 7.5.1. Conversely, NK-cells may lyse activated CD4+ and CD8+ T-cells via engagement of NKG2D with MICA, in a perforin-dependent manner as described by Molinero and co-workers (Molinero *et al.*, 2002a), thereby participating in transplant tolerance. NK-cells exposed to IL-15 may also be able to influence T-regulatory (Treg) cell involvement by engagement of NKG2D with ULBP1 (only NKG2D ligand to be found on expanded Tregs) and direct lysis by the NK-cells, thereby reducing their numbers (Roy *et al.*, 2008) as shown in Figure 7.5.1 (D). Another mechanism that could lead to graft tolerance and already discussed is down-regulation of NKG2D by engagement with sMICA that can be detected in the serum of some patients (Figure 7.5.1 (E)). My own experiments did not show reduced AR in patients with sMICA in contrast to a published study (Suarez-Alvarez *et al*., 2006b) although I have confirmed that this molecule induces down-regulation of NKG2D and when detected in renal graft recipients, associates with a trend showing higher eGFR.

The results of these more recent studies indicate a role for NK-cells in acute and chronic graft rejection by amplifying adaptive immune responses via cross-talk between cells of innate and acquired immunity. NK-cells have different functions depending on certain conditions and can either participate in and enhance rejection processes or support tolerance. Moreover, studies have revealed that CsA does not inhibit alloreactive NK-cell cytotoxicity or proliferation (Petersson *et al.*, 1997). In addition, tacrolimus, MMF and short-term azathioprine are ineffective in suppressing NK-cells (Alamartine *et al.*, 1994; Pedersen and Beyer, 1986; Shapira *et al.*, 2005). However, a recent publication has shown that proliferation of IL-2 activated healthy donor NK-cells is inhibited in the presence of these drugs and MMF can reduce surface expression of NK-cell activatory receptors, including NKG2D (Ohata *et al.*, 2011). More studies are clearly required to elucidate the effect of immunosuppression on NK cell activity. Attempts have been made to limit NK-cell activation in transplantation by blocking activatory receptors. Blockade of NKG2D in CD28 negative mice or in the context of B7 blockade resulted in extending allograft survival (Kim *et al*., 2007) and may also be applicable to humans. Importantly, these authors found blockade of NKG2D did not deplete CD8+ T-cells or NK-cells or prevent their infiltration into cardiac grafts, suggesting NKG2D blockade results in effector function inhibition. Thus, MMF use or NKG2D blockade in renal transplantation in humans may have the potential to tip the balance in favour of tolerance and prevent or limit rejection.

Integrative genomics analysis of serological responses against MICA antigens after renal transplantation identified the glomerulus as a specific target (L. Li *et al*., 2009). Immunohistochemistry (IHC) was performed which localised expression of MICA to podocytes within the glomeruli (L. Li *et al*., 2010). In addition, the presence of infiltrating mononuclear cells, B-cells, CD8+ T-cells and NK-cells in renal transplant biopsies confirming AR was indicated by IHC staining with antibodies against cell-specific markers. Post-transplant, functionally significant damage in the renal cortex include AR, infection and allorecognition of compartment-specific antigens. It is feasible that MICA antibodies reacting with over-expressed MICA antigens in these compartments activate complement-dependent cytotoxicity and may also facilitate direct lysis by degranulation of NK-cells engaging NKG2D with MICA ligands or activated via FcγRIIIA (CD16) through antibody interaction (Figure 7.5.1 (G)). Alternatively, binding of antibodies with MICA molecules may initiate complement-independent mechanisms of graft damage by inducing a prothrombotic phenotype resulting in vascular thrombosis and loss of graft function (Sumitran-Holgersson *et al*., 2002). These authors observed that antithrombotic molecules such as perlecan and thrombomodulin were decreased and prothrombotic molecules increased when endothelial cells were incubated with sera from patients who were positive for MICA antibodies. Furthermore, complement-independent mechanisms of graft injury may explain association of MICA antibodies with rejection in the absence of C4d deposition.

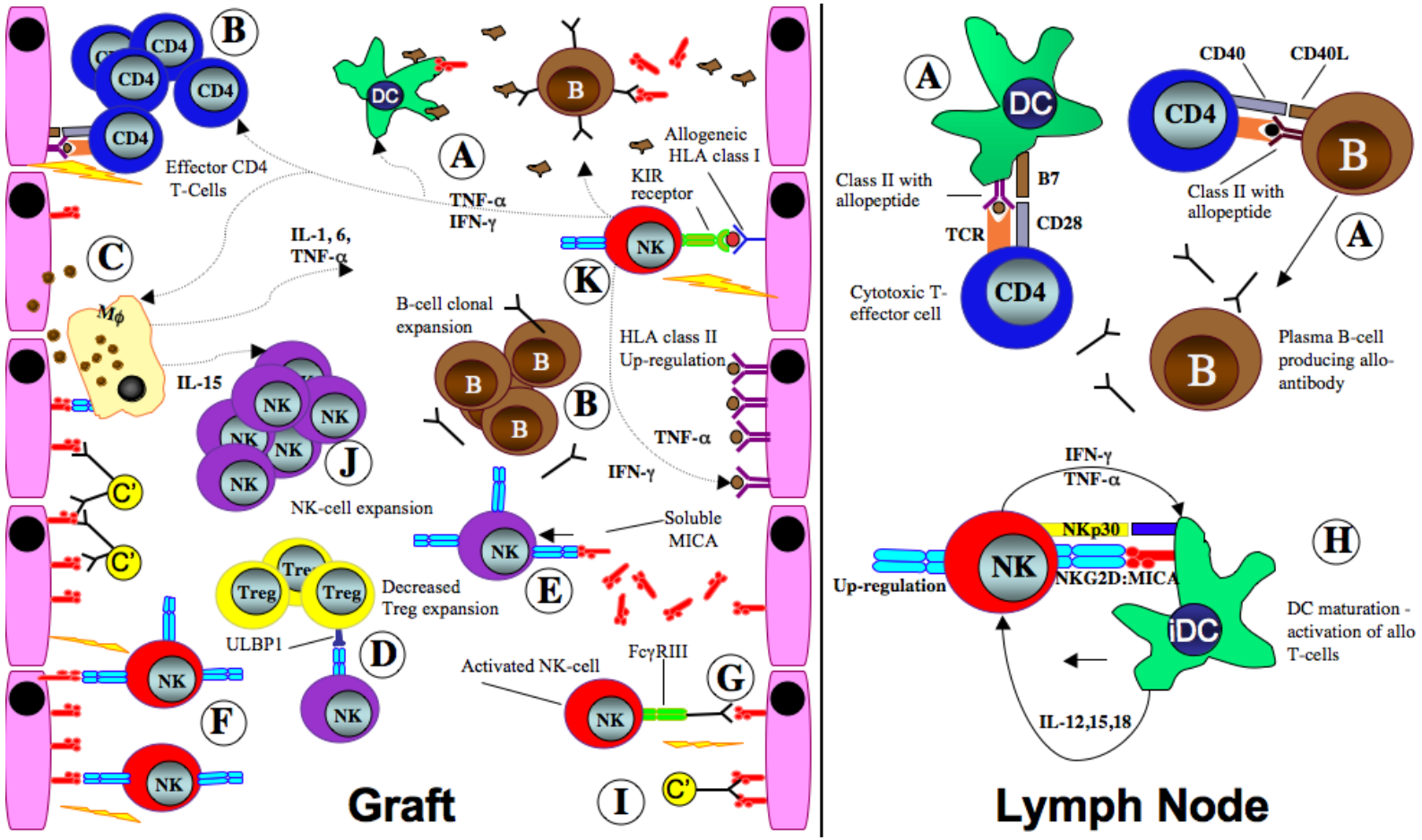

Figure 7.5.1: Indirect allorecognition, MICA, NKG2D and the immune response in transplantation. (A) Allogeneic MICA peptides from apoptotic cell material or shed sMICA is presented to CD4+ T-cells by recipient DCs or B-cells resulting in effector T-cells and MICA antibody producing B-cells. (B) Expansion of alloreactive T-cells and B-cell clones. (C) Cytokines produced by activated NK-cells upregulate NKG2D on macrophages enhancing the lytic function. (D) NK-cells limit Treg expansion by engagement of NKG2D with ULBP1 and NK-cell-mediated lysis. (E) Soluble MICA shed from the graft causes down-regulation of NKG2D receptors. (F) Over-expression of MICA in grafted tissue enables NK-mediated direct lysis. (G) Deposition of antibodies enables ADCC via the NK FcγRIII receptor. (H) NK-cells can engage NKG2D with MICA expressed on immature DCs leading to DC maturation. (I) Antibody-mediated lysis by complement deposition (J) NK-cell expansion and additional effector functions via IL-15. (K) NK-cell activation by KIR recognition of mismatched MHC class I. IFN-γ and TNF-α amplify the immune response by enhancing T-effector function and up-regulating MHC class I and II expression.

7.5.5 Limitations of this study

This study has certain limitations, firstly analysis within different groups, such as deceased or living donors and primary or re-transplanted patients, was not possible as this considerably reduced the size of the cohorts and would not be statistically robust. Also, I have not considered the effect of immunosuppression protocol differences due to the data not being available to me, and it is known that the use of CNI associates with nephrotoxicity and chronic renal damage (Nankivell *et al.*, 2004). However a study of 161 kidney recipients found no association with drug therapy and MICA antibody status (Suarez-Alvarez *et al*., 2009a) and a slight, but significant, increase in mycophenolic acid use in MICA antibody positive recipients, but not CNI was found by Zou (Zou *et al*., 2007). The possibility remains, however, that CNI use may impact on long-term eGFR and function. We have also not considered recipient primary disease, however in a similar study this too was not significant in terms of MICA antibodies (Suarez-Alvarez *et al*., 2009a) but may also have an effect on long-term function. DGF is a known risk factor for acute and chronic graft rejection (Troppmann *et al.*, 1995) but again, this information was not available. The definition of MICA antibody positivity was dependent on concordant results from two out of three tests and was only performed for sera that tested positive by MICA Luminex screening (One Lambda, CA, USA), therefore I may have underestimated the prevalence of MICA antibody positivity due to variations within the methodologies used. In addition, the mean time of testing for MICA and HLA antibodies was only seven months post-transplant and many of the patients transplanted in 2008 (when most of the testing was done) only had sera available between one and three months post-transplant. Therefore some patients may not have been transplanted for a sufficient period to allow time for sensitisation to donor antigens. Finally, although the immobilised MICA proteins used for detection of MICA antibodies cover the most common MICA alleles defined, it is possible that I may have missed detection of antibodies reactive with rare MICA proteins, again underestimating the prevalence of MICA antibody positivity.

The results from this study highlight a need for further work to clarify the role of MICA antibodies and MICA mismatching in renal AR and CAD and future, larger, multi-centre studies addressing the issues discussed in this and previous chapters may confirm my findings with greater certainty

CHAPTER 8

Conclusions

It is now more than half a century since Murray and colleagues performed the first successful kidney transplant and over this time refinements of HLA typing, HLA antibody detection and crossmatching, immunosuppression and organ preservation have improved both patient and GS. In the UK, around 2519 renal transplants are performed each year, increasing by 8% per year. UK one-year GS rates of 95% for living donor and 93% for deceased kidney donors are now achieved and continue to improve gradually year on year. However, 2-4% of renal transplants fail each year, half of these are a consequence of death of the patient with a functioning graft and the other half due to progressive graft dysfunction. For these reasons the longer term outcomes in renal transplantation are not as impressive, showing ten-year GS rates of 78% for living and 67% for deceased donors and in contrast to one-year GS, these figures have remained unchanged (statistics from UK Transplant Activity Report, 2009/10, reproduced with kind permission).

As described in Chapter 1, many factors both immunological and non-immunological can contribute to progressive graft dysfunction, termed chronic allograft dysfunction (CAD), in organ transplantation. Evidence has been increasing of involvement of HLA antibodies with CAD progression (Terasaki and Ozawa, 2004) and results of the chronic rejection component of the 13th IHIWC project revealed 40% of chronic rejection graft loss could be attributable to HLA antibodies (Ozawa *et al*., 2007). As there remain a proportion of chronic graft failures that have not resulted from death with a functioning graft or HLA antibodies, attention has also focussed on non-HLA antigens as targets of immune destruction by antibodies. Moreover, HAR has been documented in patients without HLA antibodies, implicating other alloantigens (Sumitran-Karuppan *et al*., 1997). Several non-polymorphic antigens expressed on endothelial cells have been identified in organ transplantation and are possible candidates, including angiotensin type I receptor, vimentin and glutathione-S-transferase T1. More importantly, the antigenic products of the MICA and MICB genes are expressed on endothelial tissues and become upregulated due to stress and inflammation. Furthermore, these genes are highly polymorphic, in close proximity to HLA-B, expressed in a co-dominant manner and their products are ligands for the NK-cell activatory receptor, NKG2D. For these reasons investigations of MICA and MICB alleles

and antigens in the field of transplantation have been carried out since their discovery almost twenty years ago.

It has now been established that the presence of MICA antibodies detected in organ transplant recipients either pre- or post-transplantation can result in AR, CAD or reduced GS. However, no study has provided detailed analysis of MICA matching between recipients and their donors, therefore the relevance of MICA polymorphism in transplantation and the development of MICA antibodies has not been fully investigated. MICA typing of donors is also essential to determine whether MICA-DSA is detrimental to GS, as is the case with HLA-DSA, or whether MICA-DSA associate with graft dysfunction.

The aim of my PhD project was to attempt to resolve some of these issues by carrying out a study investigating MICA allele mismatching, development of MICA antibodies post-transplant and correlations with AR, GS and CAD in a large, single centre renal graft recipient cohort. To evaluate the relevance of MICA diversity in the transplant setting, I developed a SBT approach for MICA typing to enable high-resolution characterisation of MICA alleles in 227 kidney recipients and their donors.

MICA typing of over 600 individuals for this analysis revealed two novel MICA alleles, further increasing the diversity of the MICA gene. This suggests that the MICA allelic repertoire could be much larger than the 76 variants characterised so far (IMGT/HLA database release 3.4.0, April 2011), for two main reasons. Firstly, MICA typing by SBT is not widely carried out and is the only method capable of identifying novel alleles whenever they are encountered (with the exception of RSCA). Most studies involving MICA typing now use the commercially available Luminex-based SSOP method (One Lambda) or concentrate only on the number of GCT repeats in the transmembrane region. Therefore nucleotide mutations outside probe target regions or in the extracellular domains will not be detected with these methods. Secondly, in contrast to HLA typing, characterisation of MICA alleles in recipients and donors is not a requirement for transplantation and subsequently carried out in only a few centres, again impeding the elucidation of the extent of MICA polymorphism. For these reasons, and bearing in mind the exponential rise of new HLA alleles that resulted from the introduction of molecular HLA typing methods, the number of different MICA alleles in the human population could be far greater than is currently appreciated. Nevertheless, my analysis confirmed previous observations that the majority of expressed MICA molecules were limited to a few allelic variants common in various populations (Gao *et al*., 2006). The MICA typing carried out for this project was

performed with as many patient and donor pairs as possible, utilising a DNA resource that was established at Anthony Nolan. I was able to analyse MICA allelic profiles for 227 pairs (from a total of 301) transplanted between 2003 and 2008 and examine statistical relationships with MICA mismatching and the development of MICA antibodies and the impact of MICA antibodies and MICA-DSA on AR, graft dysfunction and five-year GS.

The analysis detailed in Chapter 5 showed highly significant association of MICA allele and MICA amino acid residue mismatching with the development of MICA antibodies post-transplant. Further analysis showed MICA amino acid mismatching was independently associated with the presence of MICA antibodies. Moreover, analysis of mismatched MICA amino acid residues revealed strong, significant association with mismatches involving residues that differ between two MICA ancestral lineages, or unique polymorphisms associated with particular MICA alleles. There was also a significant correlation with the presence of MICA antibodies detected in patients who had previous transplants as previously reported (Lemy *et al*., 2010), suggesting prior MICA mismatching resulted in MICA sensitisation, as is the case with HLA antibodies. It is therefore highly likely that mismatching MICA alleles between recipient and donor in renal transplantation is a factor resulting in the development of antibodies directed against MICA antigens.

There is evidence that HLA antibodies not only correlate strongly with aAMR, but also associate with ACR (Halloran *et al*., 1992; Q. Zhang *et al*., 2005b). The analysis of MICA antibodies discussed in Chapter 5 revealed a similar trend with ACR. However, the risk of developing ACR increased if graft recipients developed both MICA and HLA antibodies and was highly significant if patients had MICA antibodies showing high MFI values. By contrast, the incidence of aAMR did not associate with MICA antibodies while HLA class I, class II and DSA were highly significant. These results may indicate that it takes longer to develop MICA antibodies and may also occur due to up-regulation of MICA antigens during ACR and aAMR responses to mismatched HLA antigens. Therefore this process may not have fully developed at the time of testing for HLA and MICA antibodies, often within three months post-transplant, and could also explain the low incidence of MICA antibodies compared to other studies. If testing was performed, for example, one year after transplantation, there may have been an association of both ACR and aAMR with MICA antibodies. Nevertheless it is possible from these results that indirect allorecognition of mismatched MICA antigens can lead to cellular immune responses and production of high-titre MICA antibodies. Furthermore, the longitudinal analysis of MICA antibody

production discussed in Chapter 6 shows three patients who clearly developed high-MFI MICA antibodies directly after an ACR episode.

The next part of this study investigated the relevance of MICA antibodies and impact on longer-term graft outcomes, assessed by five-year GS and eGFR measurement. There was no significant association of MICA antibodies with reduced GS although only sixteen of the 216 patients analysed had MICA antibodies and only one of those had graft failure. Analysis for factors independently associated with decreased GS in this cohort was performed using Cox regression and revealed significant correlation with HLA class II antibodies, ACR and aAMR.

By contrast, analysis of post-transplant graft function assessed by eGFR measurement revealed strong significant association of deceased eGFR and increased SCr in the presence of MICA antibodies, especially MICA-DSA. HLA class I and class II antibodies also associated with reduced function but significantly decreased eGFR also occurred in patients with only MICA antibodies, suggesting an independent role. It became apparent from this analysis that reduction in eGFR associating with HLA or MICA antibodies occurred at different times. The strongest association of MICA antibodies with graft dysfunction occurred after two years whereas HLA antibodies showed stronger association after year three post-transplant. Additionally, decreased eGFR associated with HLA class I antibodies before HLA class II antibodies. In terms of the amount of graft dysfunction caused by the different antibodies, HLA-DSA antibodies associated with larger decreases in eGFR than MICA-DSA but if the MICA antibodies had high MFI values the damage caused was about the same as with HLA antibodies and also occurred sooner. These observations indicate differences in expression of antibody targets correlating the ubiquitous expression of HLA class I molecules and up-regulation of MICA molecules in the early post-transplant period with earlier graft dysfunction. Linear regression analysis of both eGFR and SCr parameters revealed that by the end of the second year post-transplant, both HLA class I-DSA and MICA-DSA were independently associated with graft dysfunction. Furthermore, MICA-DSA was more highly significantly associated and resulted in more dysfunction. This is the first study to show an independent and significant correlation of MICA-DSA with graft dysfunction and larger, multicentre studies are required to confirm these findings and establish whether the presence of MICA-DSA associate with progressive graft damage over a longer time-period.

For this study I used a very stringent method to characterise MICA antibodies in renal graft recipients. This arose from initial testing using the One Lambda Luminex MICA antibody detection assays, which revealed unexpectedly high frequencies of particular MICA antibodies. These patterns of MICA antibody detection, also noted by other researchers, were not reproduced when using a similar assay developed in Peter Stastny's laboratory using recombinant MICA molecules produced in insect cells. Furthermore, the results obtained using the One Lambda assays were not confirmed using a third Luminex assay for MICA antibody detection, produced by Gen-Probe. The Gen-Probe MICA antibody Luminex assay results were generally in agreement with those generated using Professor Stastny's Luminex beads, although some minor variations existed. It appears that the method of rMICA production may lead to these variations as discussed in Chapter 3. Large amounts of linear or incorrectly folded MICA protein on Luminex beads may be responsible for false positive reactions. For these reasons I would recommend using the Luminex assay manufactured by Gen-Probe, which also has a larger array of MICA antigens.

The differences in results obtained using different Luminex assays highlights the need for international exchange and characterisation of MICA antibody-containing sera. Fortunately efforts are underway as part of the 16th IHIWC to fulfil this need. A program of MICA antibody testing using well characterised sera as part of an international, multicentre collaborative project has been organised by Peter Stastny. The results should identify problem areas in MICA antibody testing and could lead to the development of more reliable assays.

Based on the findings of this study, the following recommendations may lead to improved outcomes in renal transplantation and further understanding of MICA antibody involvement in graft rejection and dysfunction:

MICA antibody detection

- Patients with ESRD should be screened for MICA antibodies prior to transplantation.
- If MICA antibodies are detected they should be characterised by SAg Luminex methods.
- H&I laboratories should participate in international serum exchange schemes for MICA antibody testing.

- Patients with MICA antibodies, and more importantly those showing high MFI values, should be crossmatched with potential donors using an endothelial cell crossmatch assay such as XM-ONE® (Absorber, Sweden) to minimise the risk of hyperacute or AR.
- All patients should be monitored for MICA antibodies post-transplant. For patients with high-risk MICA mismatches (see below) this should be carried out at three months post-transplant. All other renal graft recipients should be tested for MICA antibodies 6-12 months post-transplant.
- Recipients diagnosed with AR should be tested for MICA antibodies using a SAg Luminex technique to identify MICA-DSA.
- Recipients with MICA-DSA should be closely monitored for changes in graft function assessed by eGFR.
- Patients with high-MFI MICA antibodies showing signs of declining graft function may benefit from plasma exchange and drug therapy aimed at limiting antibody production.

MICA genotyping and matching

- Renal graft recipients and their donors should ideally be typed for MICA alleles prior to transplantation to minimise mismatching, however this may not be possible. As a compromise, closer matching for HLA-B antigen and cross-reactive groups may result in improved MICA matching through haplotypic association and linkage disequilibrium.
- Post-transplant, renal graft recipients and their donors should be typed for MICA alleles, preferably to allele level resolution using MICA-SBT and MICA exon 5 microsatellite analysis.
- H&I laboratories should participate in international DNA exchange schemes for MICA allele typing.
- Mismatching of MICA alleles belonging to MICA-G1 or MICA-G2 ancestral lineages or mismatches involving unique amino acid polymorphisms may indicate increased risk for developing MICA antibodies. Patients with high risk MICA allele mismatches should be closely monitored for the development of MICA antibodies (see above).

In conclusion, this study has shown that mismatching MICA alleles is a significant, independent factor leading to the development of MICA antibodies in some renal graft

recipients. The presence of MICA antibodies associated with ACR and MICA-DSA independently associated with decreased graft function. Graft recipients with MICA antibodies also showed a correlation with graft dysfunction even when HLA antibodies were absent. Despite a proportionately small number of patients developing MICA antibodies, these observations indicate that these antibodies, in addition to HLA antibodies, associate with poorer graft outcome warranting further investigation in larger collaborative studies. Furthermore, MICA typing of patients and donors together with MICA antibody screening, especially for re-transplanted patients, may identify those at risk of AR and chronic allograft dysfunction, allowing therapeutic intervention to influence the management of transplant survival.

Appendix A

Publications and Presentations

Journal Articles

1. "SEQUENCE OF A NOVEL HLA-A*0301 INTRONIC VARIANT (A*03010103)." N. Mayor, **S. Cox**, A. McWhinnie, J. Arguello, B. Shaw, A-M. Little, J. A. Madrigal, S. G. E. Marsh. Tissue Antigens. 2005, 65:1 107-109.

2. "HIGH-RESOLUTION MOLECULAR CHARACTERISATION OF THE HLA CLASS I AND II IN THE TARAHUMARA AMERINDIAN POPULATION." J. Garcia-Ortiz`, L. Sandoval-Ramirez, H. Rangel-Villalobos, H. Maldonado-Torres, **S. Cox**, C. Garcia-Sepulveda, L. Figuera, S.G.E. Marsh, A-M. Little, J. A. Madrigal, J. Moscoso, A. Arnaiz-Villena and J. Arguello. Tissue Antigens. 2006, 68:135-146.

3. "A NEW HLA-A*31 NULL ALLELE, A*3114N." D. Smith, W. Gardner, J. Baker, **S. Cox**, and L. Kresie. Tissue Antigens. 2006, 68:526-527

4. "AN UNLIKELY RARE RESULT OF HLA-A*0236, *3601 MASKING THE PRESENCE OF A NOVEL ALLELE A*0114." J. Crowley, C. Dunne, **S. Cox** and A-M. Little. Tissue Antigens. 2007, 69:200-1

5. "ADENOVIRUS E3/19K PROMOTES EVASION OF NK-CELL RECOGNITION BY INTRACELLULAR SEQUESTRATION OF THE NKG2D LIGANDS MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I CHAIN-RELATED PROTEINS A AND B." B. McSharry, H-G. Burgert, D. Owen, R. Stanton, V. Prod'homme, M. Sester, K. Koebernick, V. Groh, T. Spies, **S. Cox**, A-M. Little, E. Wang, P. Tomasec and W. Wilkinson. Journal of Virology. 2008, 82:9 4585-4594.

6. "IDENTIFICATION OF TWO NOVEL MICA ALLELES: MICA*054 AND MICA*056." **S. Cox**, H. Stephens, R. Fernando, J. Grant, A. J. Madrigal and A-M Little. Tissue Antigens. 2009, 73(1): 85-87.

7. "MICA ALLELE MISMATCHING, MICA ANTIBODIES AND REJECTION IN RENAL TRANSPLANTATION." **S. Cox**, H. Stephens, R. Fernando, A. Karasu, M. Harber, S. Powis, Y. Zou, P. Stastny, J. A. Madrigal, and A-M. Little. Human Immunology. 2011, 72(10): 827-834.

Presentations and Published Abstracts

1. "15 NEW CLASS I AND CLASS II ALLELES DISCOVERED IN HSCT PATIENTS AND DONORS TYPED BY SEQUENCE BASED TYPING." **S. T. Cox**, F. Partheniou, A. McWhinnie, R. Adams, J. Dey, M. Gibson, S. Wallis-Jones, J. A. Madrigal & A-M. Little. 17th Annual BSHI Conference, Sheffield, UK. 2006. Poster Presentation. International Journal of Immunogenetics. 2006, 33(4).

2. "MICA ANTIBODY SCREENING IN RENAL TRANSPLANT PATIENTS." **S. T. Cox**, R. Fernando, H. A. F. Stephens, J. A. Madrigal and A-M. Little. 18th Annual BSHI Conference, Birmingham, UK. 2007. Best Abstracts, Oral Presentation. International Journal of Immunogenetics. 2007, 34(4).

3. "MICA ANTIBODY SCREENING IN RENAL TRANSPLANT PATIENTS." **S. T. Cox**, R. Fernando, A. Karuso, J. Grant, A-M. Little, and H. A. F. Stephens. 21st Annual European Federation of Immunogenetics Conference, Barcelona, Spain. 2007. Poster Presentation. Tissue Antigens. 2007, 72(3).

4. "MICA ALLELE MISMATCHES AND PRODUCTION OF MICA ANTIBODIES IN RENAL TRANSPLANT PATIENTS." **S. T. Cox**, R. Fernando, H. A. F. Stephens, J. A. Madrigal and A-M. Little. 22nd Annual European Federation of Immunogenetics Conference, Toulouse, France. 2008. Poster Presentation. Tissue Antigens. 2008, 71(4).

5. "A SIGNIFICANT CORRELLATION BETWEEN MICA ANTIGEN MISMATCHES AND PRODUCTION OF MICA ANTIBODIES IN RENAL TRANSPLANT PATIENTS." **S. T. Cox**, H. A. F. Stephens, R. Fernando, M. Harber, S. Powis, J. A. Madrigal and A-M. Little. 15th International Histocompatibility and Immunogenetics Workshop and Conference, Rio de Janeiro, Brazil. 2008. Oral Presentation. Tissue Antigens. 2008, 72(3).

6. "A SIGNIFICANT CORRELLATION BETWEEN MICA ANTIGEN MISMATCHES AND PRODUCTION OF MICA ANTIBODIES IN RENAL TRANSPLANT PATIENTS." S. T. Cox, H. A. F. Stephens, **R. Fernando**, M. Harber, J. A. Madrigal and A-M. Little. 34th Annual Meeting of the American Society for Histocompatibility and Immunogenetics, Toronto, Canada. 2008. Oral Presentation (R. Fernando). Human Immunology. 2008, 69, Supplement 1.

7. "MICA ALLELE MISMATCHING, MICA ANTIBODIES AND REJECTION IN RENAL TRANSPLANT RECIPIENTS." **S. T. Cox**, H. A. F. Stephens, R. Fernando, M. Harber, A. Howie, S. Powis, Y. Zou, P. Stastny, J. A. Madrigal and A-M. Little. 24th Annual European Federation of Immunogenetics Conference, Florence, Italy. 2010. Oral Presentation. Tissue Antigens. 2010, 75(5).

8. "MICA ANTIBODY DETECTION IN RENAL TRANSPLANTATION USING FLUORESCENT BEAD TECHNOLOGY: FREQUENCIES, EPITOPE SPECIFICITY AND LONGITUDINAL ANALYSIS." **S. T. Cox**. Gen-Probe user meeting, Primosten, Croatia. May 29th – June 1st 2011. Oral Presentation.

Appendix B

Staff Consent Form

RESEARCH INSTITUTE

ANTHONY NOLAN

BE A MATCH, SAVE A LIFE

CONSENT & REQUEST FOR BLOOD

THE ANTHONY NOLAN TRUST RESEARCH INSTITUTE
The Royal Free Hospital, Fleet Road, London NW3 2QG
Tel: 020 7284 8315 Fax: 020 7284 8331, www.anthonynolan.org.uk

DEPARTMENT:		PROJECT MANAGER:
		SCIENTIST REQUESTING BLOOD:
SURNAME:		DATE OF BIRTH:
FIRST NAME:		Extn:
GENDER:	ETHNIC ORIGIN:	
COMMENTS AT TIME OF COLLECTION:		
DATE AND TIME OF BLOOD COLLECTION:		
CONSENT: **Please ensure the consent form (on the reverse of this form) is completed.**		
TESTS REQUESTED: Routine tests for (i) HLA typing: 2 x 4ml blood in EDTA (ii) CMV/ABO: 1x 4ml tube clotted. (iii) 20 - 50ml blood in anticoagulant. Please ensure your blood tubes are labelled with your name and DOB.		
(i) HLA typing ☐		**ii) CMV/ABO** ☐
(iii) Research project ☐		
Blood will be used at the laboratory above and will be processed between 9am-7pm Mon-Fri **DO NOT REFRIGERATE BLOOD.**		

For laboratory use only

Project Name ----------

Consent Form Number ANRI/000? Date of Sample taken ----------

Sample Number ---------- Signature of staff ----------

Phlebotomist Signature ---------- Form to Health & Safety Manager YES ☐ NO ☐

H/S Manager Signature ----------

Please ensure the accompanying request form (on the reverse of this form) is completed.

Name of member of staff	
I agree to my blood and or DNA being used in research tests related to: Histocompatibility factors CMV virology Blood group type Cellular assays for immune function All are important factors that are being investigated in relation to haematopoietic stem cell transplantation. I agree that a small quantity of my blood and or DNA will be retained by the laboratory for any future tests that relate to transplantation techniques.	yes ☐ no ☐ yes ☐ no ☐
The Institute may undertake studies of immunological markers that may be of importance in transplantation. I agree that some of my stored blood or DNA may be used anonymously in these studies for the benefit of patients.	yes ☐ no ☐
I give explicit consent to the Anthony Nolan Trust to store any sensitive personal data* relating to me, held by it. * *For the research tests that we are conducting we need to collect data, which the Data Protection Act defines as sensitive, such as ethnicity).* I agree that during the course of laboratory tests, my blood may be screened for markers for infectious diseases including Hepatitis B & C and HIV and I agree to this as an automatic procedure. I understand and accept that in the event of a needlestick injury my blood may become a source of contamination and I would therefore be routinely approached for a blood sample for further testing on blood borne virus. The results of these tests would then be disclosed to the source recipient and the information obtained would be stored confidentially.	yes ☐ no ☐ yes ☐ no ☐

Signature of consenting individual:

Date:

Appendix C

Renal graft recipient and donor MICA types

Patient	MICA_1	MICA_2	Donor	MICA_1	MICA_2
524JA	004		530NM	004	00901/049
533CB	008	012	601RB	00702	008
877LB	00901/049	010	634JC	008	010
961PB	008	018	124WK	001	00902
696IC	008		320RH	004	019
336CC	004	016	705BK	008	
976ES	016	018	992EB	002	007
488DG	008	00901/049	072KG	007	008
861YH	010	019	504GH	017	019
449JH	002	008	453JW	002	
502SJ	004	008	570CJ	004	00901/049
521HJ	004		397CW	001	00901/049
133JK	008	010	793TK	00701	010
368JL	002	010	755JU	008	010
074HM	008		247DM	008	
619KM	008	015	134BD	008	017
519NP	004	008	368TP	004	011
220PP	002	030	423KC	002	008
396BS	002		350SM	002	008
548NS	002	00901/049	675BR	002	010
149CW	002	008	853RH	004	007
733SA	00802	019	022AM	004	019
057GA	008		001SC	008	018
479BB	004		287DR	008	
977HB	002		212GS	008	01801
787TD	008	00901/049	291JM	00901/049	011
388GP	008	027	392RM	00201	008
350LO	004		223LN	008	00901/049
960MB	008	016	456LB	008	
727MM	008	035	052EK	002	008
182RO	002	004	686DT	008	011
181JR	004	019	382YR	002	019
381FC	008		579MR	008	00902
362MJ	002	004	915RJ	002	008
945VS	008	017	805NS	002	018
451PK	008		746CH	008	
367DZ	008	029	457DZ	008	029
173RO	004	019	171MC	008	019
562ST	002	008	779EF	008	010

970ED	004	012	471NC	008	01802
380CW	008		033AW	008	
603JB	008	035	261GC	002	010
219GM	011	018	834JL	004	008
379BB	002	00802	541SL	004	038
679RD	002	007	747KR	002	010
995GC	008	00901/049	229JC	002	008
159KC	002	019	085JJ	002	010
075PL	002	008	262JC	002	008
776RA	004	012	531MA	004	018
864FR	007	00901/049	748AB	008	00901/049
090KB	004	012	091MB	004	019
355EB	008	011	807FB	008	011
621CO	004	008	706JC	004	00802
669DC	008	00901/049	199TP	004	00901/049
050SC	002	00901/049	192JB	002	018
380RA	002	008	797JJ	002	008
157IT	008		522LK	008	011
870AL	008	010	634JC	008	010
372NF	002	016	562DC	002	018
134RD	002	008	159DB	00202	008
237CR	002	004	928TW	00902	012
295PM	008	019	868CE	002	018
059VC	004	010	171MC	008	019
435HH	004	008	263SR	004	018
987NI	016		151UF	008	011
852DI	00201	008	854DA	002	008
832HJ	008	00901/049	044PR	008	00901/049
051LM	006	008	623PS	008	
304JM	002	008	305BM	002	008
373AG	00901/049		123NM	004	00901/049
385GA	008		504GH	017	019
121BE	008		122DD	002	008
505PA	004	019	225IB	002	00901/049
118LB	002		345LA	004	008
185MB	00901/049	017	225IB	008	012
366PB	002	00702	992EB	002	007
528DB	002	017	255RW	008	017
139DB	002	018	140GB	002	010
535RB	002	010	756SM	007	019
329MB	007	011	522LK	008	011
461BB	008	018	223JF	004	00901/049
502CB	004	010	121AB	004	00901/049
293MC	008		697PC	008	
357DC	008	012	803DC	007	008
875CC	008		208SC	00702	008

756FC	008	027	375ML	008	
228RD	008	00901/049	216CL	008	00902
174BD	002	00901/049	154GC	008	016
621VD	008	00901/049	836DS	008	00902
218ND	008	00901/049	219LP	008	00902
054NE	004	008	392RM	002	008
219JE	008		848GM	002	041
020TF	001	008	522JP	004	007
210AF	008	00902	885HF	00901/049	
945EF	008	012	552AF	008	012
934PF	008	011	498FC	002	019
361JG	004	030	926MH	008	
347VG	002	008	192JE	008	
817IG	019		108SR	007	012
314DH	008	00901/049	739FO	004	008
374NH	004	008	002SC	004	008
994LH	008		865BF	008	
867RH	011	016	376SH	002	011
868DH	002	011	634CH	008	00901/049
462LJ	002	004	215AL	00902	012
917UK	002	004	587CE	002	008
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724FK	002	004	251GF	008	018
736MK	008		945NI	008	
880DK	004	008	545JK	008	007
264PL	004	008	002MN	007	008
171KL	004	008	311JB	00802	00901/049
035RL	008	012	372ML	008	
128BL	001	00901/049	968PL	001	00901/049
185AL	016	019	672LL	008	019
834HL	00802	019	562DC	002	018
693MM	002	007	162MB	008	
373SM	002		387JM	002	011
749SM	004	008	436JL	004	009
609PM	00901/049	027	240AS	004	012
971PM	004	008	003SC	008	
677VN	004	008	198CB	002	004
732SN	008	00901/049	889PM	008	
777AN	008		462DB	008	
822AO	002	008	823MT	008	015
892AO	002	004	XXXPJ	008	
284SP	018	019	462NH	008	018
738AP	017	01801	168PW	002	018
728JP	007	00901/049	394BQ	008	
727JQ	00901/049	029	136AF	008	
758AR	002	00901/049	004SC	002	010

498RM	001	008	449KY	001	007
736JR	008		564BT	008	
126MR	008	00802	851PL	008	018
910OS	008		916JS	011	018
516NS	008	00901/049	637FB	008	00901/049
286ES	001	004	205JR	008	
927PS	00901/049	019	144PH	008	
975NS	008		102KJ	008	
386JS	002	00901/049	579MR	008	00902
027TS	002	008	142TD	002	008
832DT	008	018	312PA	002	008
130AT	008	004	166ST	008	019
522DT	008		832JH	008	00901/049
731DU	008	018	251GF	008	018
039JV	002	019	160RT	002	00901/049
389AW	008		247MH	002	008
662AS	008	012	876JW	008	
447RA	002	00902	526JJ	004	
896HR	002	008	292BC	008	
962MA	00902		707HS	002	008
638YG	008	013	966DH	00902	018
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040PK	002	007	595JK	002	007
690SM	002	019	480DV	011	016
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881AM	002	018	075IK	011	016
039JP	008	00901/049	856AW	008	00901/049
057SR	008		207SM	008	00901/049
497AR	018		066MR	017	018
512RS	002	008	498FC	002	019
528JS	002	004	768DB	004	
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244JD	008	018	682SB	002	008
397SD	004	00901/049	768DB	004	
895SH	00901/049	027	812XE	008	029
029RL	008		262EM	008	
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733MO	007	011	906JT	002	008
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394EB	008	00902	501DW	008	054
833GD	00901/049	016	005SC	008	00901/049
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247AV	002	008	333AM	002	008
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053JJ	004	008	148DD	007	008
808HK	002	019	142SK	002	027
756MW	004		116HG	002	004
132AS	00901/049	019	002MG	008	017
354SA	007	008	102NA	004	008
269JN	008	011	718VR	011	
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140ME	004		717RS	008	00901/049
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796PW	004	008	211AN	008	016
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430SG	002	012	436JG	012	017
604DP	008		942DH	008	
920MH	002	027	262EM	008	
372JK	002	008	385PB	004	007
219BO	012	018	924LJ	001	004
187SD	002	008	928MG	002	008
281CT	002	008	504SC	002	008
501GO	002	004	894OA	008	011
792KC	002	008	620HS	008	
983TK	004	008	193EK	004	008
192MK	002	004	196AK	00901/049	018
803DG	008		995GC	008	
139KS	008	017	579NS	017	018
599EM	008		310PL	004	008
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Appendix D

EMBL flatfile Summaries

MICA*054 EMBL Flatfile

```
ID   AM899996; SV 1; linear; genomic DNA; STD; HUM; 1257 BP.
XX
AC   AM899996;
XX
DT   09-OCT-2007 (Rel. 93, Created)
DT   13-JAN-2009 (Rel. 99, Last updated, Version 3)
XX
DE   Homo sapiens partial MICA gene for MHC class I chain-related protein A,
DE   MICA*010v allele, exons 2-5
XX
KW   major histocompatibility complex; MHC class I chain-related protein A;
KW   MICA gene; MICA*010v allele.
XX
OS   Homo sapiens (human)
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia;
OC   Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae;
OC   Homo.
XX
RN   [1]
RP   1-1257
RA   Cox S.T.
RT
RL   Submitted (08-OCT-2007) to the EMBL/GenBank/DDBJ databases.
RL   Cox S.T., The Royal Free Hospital, Anthony Nolan Research Institute,Pond
RL   Street, Hampstead, London, NW3 2QG, UNITED KINGDOM.
XX
RA   Cox S.T., Stephens H.A.F., Fernando R., Grant J., Madrigal J.A., Little A.-.M.
RT   "Two novel MICA alleles, MICA*054 and MICA*056"
RL   Tissue Antigens 73(1):85-87(2009).
XX
DR   IMGT/HLA; HLA03157; MICA*054
XX
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XX
SQ   Sequence 1257 BP; 241 A; 245 C; 267 G; 204 T; 300 other
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     ctacggcgat atctagaatc cagcgtagtc ctgaggagaa cagnnnnnnn nnnnnnnnnn       660
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     agagggcaac atcaccgtga catgcagggc ttccagcttc tatccccgga atatcatact       840
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MICA*056 EMBL Flatfile

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DT   13-JAN-2009 (Rel. 99, Last updated, Version 3)
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DE   MICA*019variant allele, exons 2-5
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KW
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OC   Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae;
OC   Homo.
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RN   [1]
RP   1-1257
RA   Cox S.T.
RT
RL   Submitted (29-FEB-2008) to the EMBL/GenBank/DDBJ databases.
RL   Cox S.T., The Royal Free Hospital, Anthony Nolan Research Institute, Pond
RL   Street, Hampstead, London, NW3 2QG, UNITED KINGDOM.
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RN   [2]
RA   Cox S.T., Stephens H.A.F., Fernando R., Grant J., Madrigal J.A.,
RA   Little A.-.M.;
RT   "Two novel MICA alleles, MICA*054 and MICA*056"
RL   Tissue Antigens 73(1):85-87(2009).
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