

Thesis protocol for the award of
Diplomate of National Board in Nephrology

From

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**Outcome of MICA antibody on immediate
graft function in live related renal
transplantation**

Guide
Dr. Ashok Sarin
MD, FRCP
Senior Consultant
Department of Nephrology
Apollo hospital
New Delhi

Submitted by

Dr. Riteshkumar Banode
D.N.B. Post Graduate Trainee

INTRODUCTION

Renal transplantation is the treatment of choice for patients with end-stage renal disease, facilitating a return to near normal health and extending life expectancy. Improvements in immunosuppressive therapy, aimed at limiting the effects of T-cell-mediated immune responses to the graft, have increased overall graft survival [1] and reduced acute rejection (AR). However, rejection caused by antibody-mediated graft damage arising from B-cell responses to mismatched human leukocyte antigen (HLA) antigens remains a problem. The production of post-transplantation, *de novo* donor-specific antibodies (DSA) to HLA antigens is associated with acute and chronic allograft rejection [2,3]. Despite renal transplant rejection being strongly associated with HLA antibodies, some 11–20% of patients without HLA antibodies develop chronic allograft dysfunction [3,4]. Furthermore, hyperacute rejection can occur in the absence of HLA antibodies, thus implicating other non-HLA alloantigens [5,6] including the major histocompatibility complex (MHC) class I-related chain A (MICA) and MHC class I-related chain B (MICB) antigens also encoded by genes within the human MHC [7].

Major-histocompatibility-complex (MHC) class I-related chain A (MICA) antigens are surface glycoproteins with functions related to innate immunity. 2-4 MICA antigens are expressed on endothelial cells, dendritic cells, fibroblasts, epithelial cells, and many tumors, but not on peripheral-blood lymphocytes. As with the HLA antigens, which MICA antigens resemble only remotely in terms of structure, exposure to allogeneic MICA during transplantation can elicit antibody formation. 5 It is well known that antibodies against HLA antigens, especially those recognizing donor antigens, can severely damage kidney allografts. 6 Transplantation performed in the presence of a positive donor crossmatch may result in hyperacute vascular rejection. 7,8 Antibodies against HLA antigens have also been associated with both acute vascular rejection 9,10 and chronic rejection. 11 Since MICA antigens are not expressed on lymphocytes, 12 the cells commonly used for cross-matching, antibodies directed against MICA are not detected with the methods generally used. However, polymorphic MICA antigens are expressed on endothelial cells 12 and have been found to be cytotoxic in the presence of serum complement, 13 so it is likely that such antibodies are harmful to vascularized organ allografts. Preliminary studies with small numbers of patients indicated that MICA antibodies detected after transplantation might be associated with impaired survival of kidney allografts, 13-17 and an analysis of eluates from kidneys undergoing immunologic rejection has suggested that MICA antibodies may be involved in the pathogenesis of kidney-allograft rejection. 18. The mechanisms by which individuals develop antibodies to MICA are largely unknown. It has been suggested that pregnancy can induce MICA antibodies [20]. By contrast, the role of blood transfusions in the induction of MICA antibodies is unclear [23,29]. However, the effect of MICA mismatching in organ transplantation has not been fully addressed, largely because of a paucity of information on donor and recipient MICA types. In this study we will study the serum samples for antibodies against MICA antigens in live related renal transplant patient and outcome of MICA antibody on immediate graft function will be studied.

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 β_2M 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).

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).

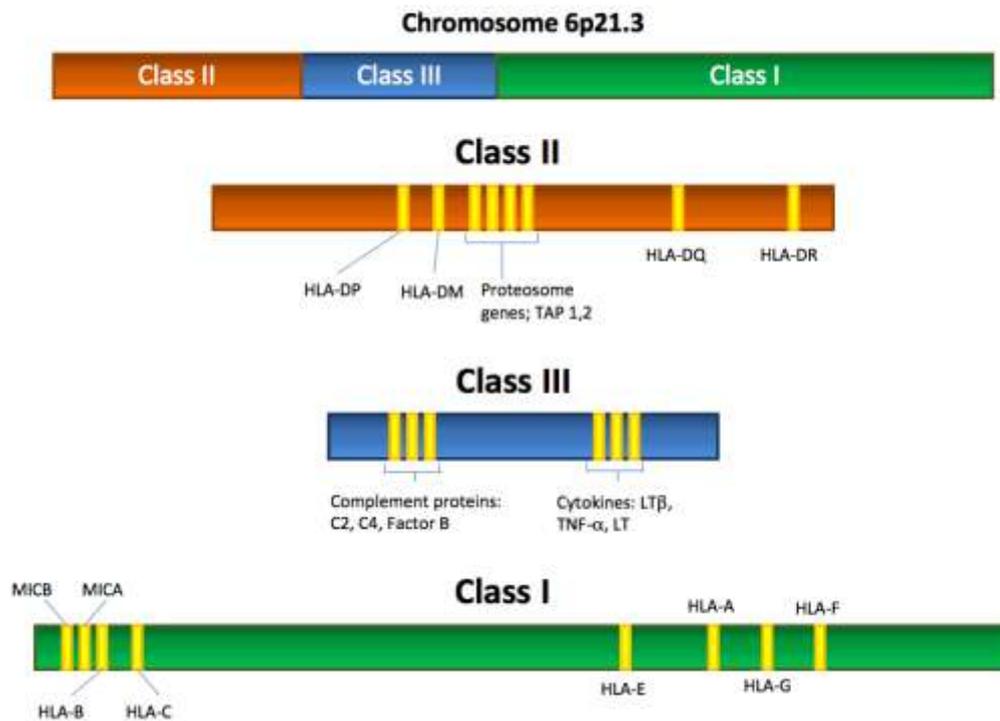


Figure 1.3.1: Genomic organisation of the MHC region. The human MHC region encompasses 3.6 Mb on chromosome 6, contains over 200 gene loci including all HLA genes and many immune regulatory genes. MICA and MICB are situated centromeric to HLA-B in the class I region. (Klein and Sato, 2000).

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 α 1- α 3 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

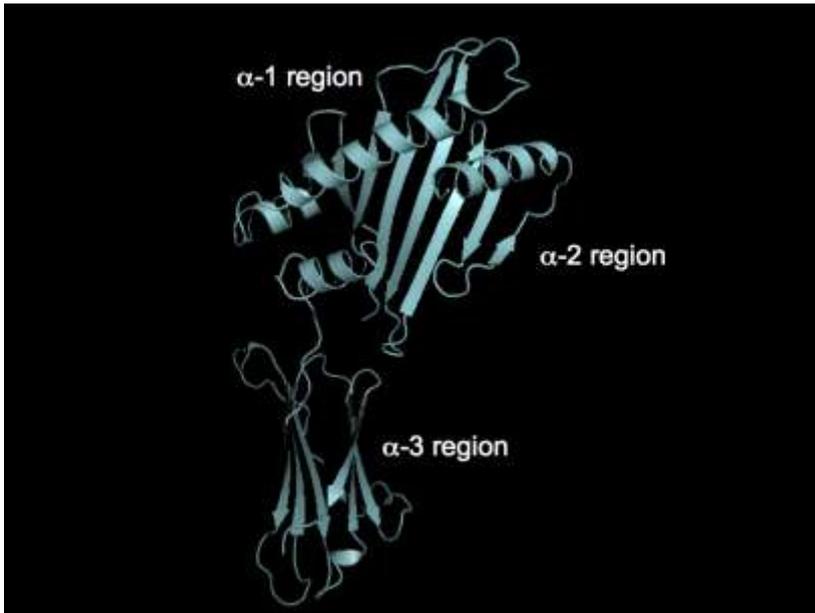


Figure 1.3.2: Ribbon model derived from X-ray crystallographic analysis of the extracellular domains of the MICA molecule. The three alpha regions are indicated with α -3 nearest the cell membrane

In further contrast to HLA class I molecules, the platform formed by the α 1 and α 2 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 α 2 helix becomes ordered by a further two alpha-helical turns and the α 1 and α 2 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

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 α 2M 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 Chapter 1 60

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

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 α 1- α 3 domains of MICA and MICB are polymorphic with the greatest variability in the α 2 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 α 3 domain. Position 684 is synonymous and substitutions at

nucleotide positions 685, 697 and 713 lead to amino acid substitutions at residues 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 Chapter 1 62

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

MICA antibodies and solid organ transplantation

Transplantation is the treatment of choice for patients with end-stage renal disease (ESRD), 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 Graft survival (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), Chapter 1 70 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 Chapter 1 71 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 Chapter 1 72

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 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 Chapter 1 73

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). Chapter 1 74 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 23 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. 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. 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 Chapter 1 78 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 Chapter

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.

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.

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 Chapter 1 80in 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: Cell line or Nationality
Relative sizes of exon 5 in different MICA alleles
MICA microsatellite

A4	BTBa	179
A5	MLF	182
A5.1	CGM1a	183
A6	MOUa	185
A7	Spanishb	188
A8	Italianc	191
A9	YARa	194
A10	25/1506d	197

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

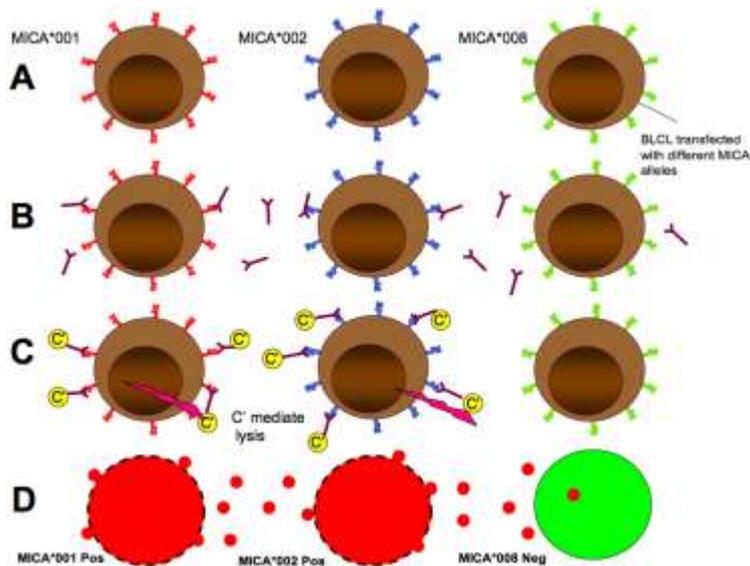


Figure 1.4.6: MICA antibody detection using CDC. In this example, three MICA alleles have been transfected into BLCLs – MICA*001, 002 and 008. A. Cells are placed into individual wells of a „Terasaki“ micro-well plate and B. patient serum is added. C. Following incubation, complement is added to the 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 Chapter 1 88 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

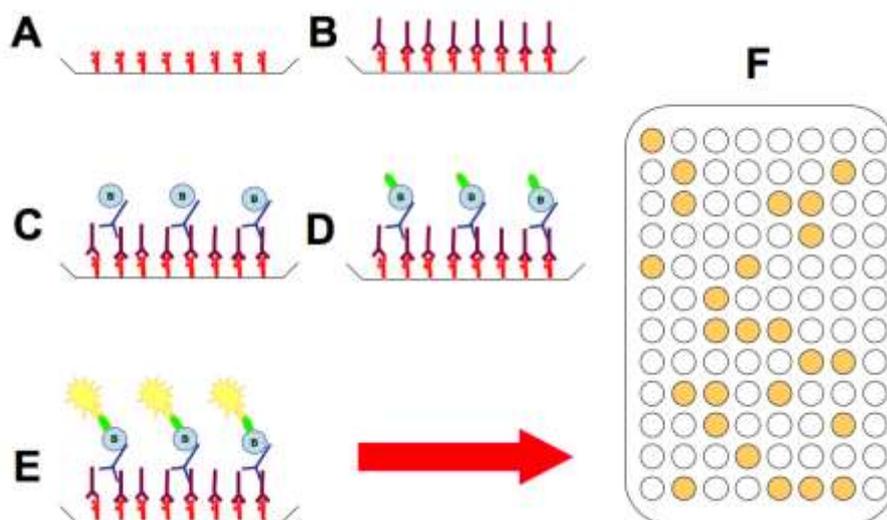


Figure 1.4.7: MICA

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.

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 Chapter 1 91

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

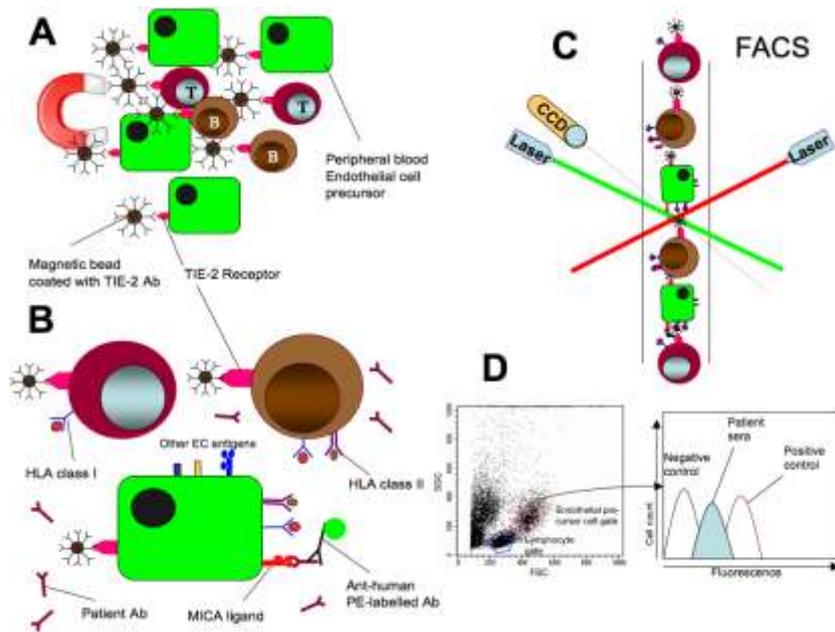


Figure 1.4.9: XM-ONE[®] crossmatch assay to detect non-HLA antibodies directed against EC antigens. A. Magnetic isolation of EC and lymphocytes from peripheral blood, targeting the TIE-2 receptor. B. Cells are incubated with patient serum followed by antihuman IgG labelled with PE. C. Flow cytometry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

Methods

- **Aims and Objectives**

- This will be a prospective study of 50 MICA Antibody positive kidney transplants performed at our institute between 2016 - 2018
- The outcome of the transplant will be assessed with respect to the following parameters:
 - graft function
 - incidence of rejection
 - incidence of major infections
 - graft failure and cause of graft failure

 - mortality and cause of death

To investigate associations between MICA antibodies, renal function and clinical course, eGFR was calculated from serum creatinine values (expressed as ml/min/1.73 m²) according to the four-point MDRD .

Type of study : hospital based cross sectional observational study

Duration of study : 2 years

Sample size : 50

**Inclusion criteria: 1) Live related renal transplant patient with positive MICA antibody
2) Patient willing to participate in study.**

**Exclusion criteria: 1) Cross match positive
2) DSA positive
3) ABO incompatible transplant
4) Second transplant**

Specimen Collection:

1) Pre transplantation serum samples from kidney recipients who are undergoing transplantation with organs from live donors will be collected.

2) Laboratory Tests: IgG anti-HLA class I reactivity in the serum samples will be tested with the use of Quick screen enzyme-linked immunosorbent assay (ELISA) kits and for IgG anti-HLA class II reactivity with the use of B-Screen ELISA kits.

3) HLA typing and panel-reactive HLA antibodies will be determined .

4) Tests for IgG antibodies against MICA antigens will be performed with the use of soluble MICA antigens produced in insect cells coupled to polystyrene microbeads.

5) Depending on level of MICA antibody level patient will be divided in three groups (group A –MICA Antibody level between 500 to 1000 MFI, group B MICA antibody level 1000 to 2000 and group c level more than 2000.

Patient graft function during post-operative period will be monitored and outcome at the time of discharge with respect to MICA antibody level and type of desensitization therapy will be studied.

1.1 Study Performa

I. Pre-transplant Evaluation & Demography of Patient

- Recipient Name, Age and Sex
- Recipient Ethnicity
- Donor Age and Sex
- Donor Type- LD
 - First Degree Relatives
 - Spousal Donors
 - Others
- Native Kidney Disease- defined c clinically or biopsy proven
- Co-morbidities
- Pre-transplant Dialysis
 - Type of Dialysis
 - Duration of Dialysis
 - Blood transfusion
- Pre-emptive Transplant

- PRA
- CDC Crossmatch
- FC Crossmatch
- Luminex
- MICA Antibody

TEST	SPECIFICITY	MFI VALUE
MICA AB		

▪

II. Desensitization Protocol

- IV Ig
- PLEX
- Rituximab

III. Immunosuppression

- Induction
- Maintenance

IV. Prophylaxis received post transplant

V. Outcome

Post operative days	1	2	3	4	5	6	7
Hb							
TLC							
PLATELETS							
Blood urea							
Serum creatinine							
Sr sodium							
Sr potassium							
Tac levels							
Urine output							
Graft doppler							