THE ROLE OF PRE-TRANSPLANT ANTIBODIES IN PREDICTING CHRONIC RENAL ALLOGRAFT INJURY

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Medicine, University of Sydney

August 2009

PREFACE

Statement of originality

Except where duly acknowledged, the studies contained in this thesis were planned and executed by the author.

Dr Brian Nankivell, Associate Professor Philip O'Connell, Professor Jeremy Chapman, Dr Richard Allen and the author collected renal Biopsy materials. Tissue for electron microscopy were embedded and archived by staff of the Electron Microscopy Unit Westmead Hospital. The database from which some of the histological and clinical data were extracted is maintained by Dr Brian Nankivell, Department of Renal Medicine, Westmead Hospital. Light microscopy analysis for this data base was performed by Dr Caroline Fung. Narelle Watson and Frederika Abou-Daher of The Tissue Typing Laboratory, Australian RedCross and Blood Service, Sydney, performed the Luminex and CDC assays. Jane Milliken of Institute of Clinical Pathology and Medical Research (ICPMR), Westmead hospital, performed C4d staining. Dr Brian Nankivell performed light microscopy data analysis for chapter 6. Statistical advice was obtained from Dr Karen Byth. All analyses were performed by the author except general linear mixed models.

With these exceptions the originality of this work lies with the author

ACKNOWLEDGEMENTS

I would like to thank my supervisors Professor Jeremy Chapman, Professor Philip O'Connell and Dr Brian Nankivell for their inspiration advice, patience and support during my candidature and throughout my training years. I particularly want to thank them for the opportunities they have given me most of which I had never dreamt of or imagined.

I acknowledge Mr Ross Boadle and the staff of the Electron Microscopy unit for their assistance and encouragement during the preparation and analysis of specimens for the EM study. I thank Mr Matthew Vitalone and Ms Elvira Jiminez for being excellent sounding boards for ideas and their encouragement during my candidature.

To Drs. Robert and Elizabeth Kaziro, thank you for introducing the idea of coming to Australia many years ago, for your encouragement, advice, and support through the ups and downs of this journey. This would not have happened without you. To my partner in life Sandra, thank you for your patience and for keeping things together during this time.

I dedicate this thesis to my dad, the late Frederick Wavamunno Mpagi and my mother Esther Mpagi who sacrificed a lot for my education and to Joshua and Elizabeth the bright new stars for the future.

ABSTRACT

Continued rise in the incidence of late graft loss despite better immunosuppression and improvement in acute rejection rates has led to renewed interest in the effect of HLA antibodies on long term graft outcomes. The significance of pre–transplant HLA antibodies detected by the widely used CDC assay on early graft outcomes is well studied. However, a lot remains unknown about effects of pre-transplant antibodies detected by sensitive solid phase assays on early and late graft outcomes. This is due to increased use of these assays resulting in better detection of HLA and non HLA antibodies. Such antibodies are thought to be of low affinity and avidity. In addition, low sensitivity of conventional techniques for diagnosis of antibody mediated graft injury results in late diagnosis when interventions are unlikely to reverse graft failure.

This thesis examines 3 aspects of antibody mediated graft injury,

1) effects of pre-transplant antibodies detected by Luminex (a solid phase assay) on histology and graft function outcomes,

2) use of electron microscopy in early diagnosis of chronic antibody mediated injury, and

3) the potential role of mycophenolate mofetil in amelioration of chronic graft injury.

In chapters 3 and 4, pre-transplant sera from kidney-pancreas recipients was analysed for HLA and MICA antibodies using the Luminex assay. In addition, antibody affinity and avidity were determined using molecules of equivalent fluorchromes (MESF) as a

measure of antibody strength. Presence of donor specific antibodies (DSA) was assessed for a predictive role of allograft histology and graft function outcomes.

DSA were associated with graft dysfunction and inferior death-censored graft survival. Graft dysfunction in recipients with DSA was associated with early peri-tubular capillary C4d deposition and transplant glomerulopathy. High MESF values, (of more than 100,000 units), predicted graft dysfunction, increased C4d deposition, and inferior graft survival. MICA antibodies did not have any effect on graft function or histology.

In chapter 5, early histopathological abnormalities associated with chronic antibody mediated injury were evaluated by comparing protocol biopsy ultrastructure and C4d deposition in transplant recipients with transplant glomerulopathy (TXG) and a matched cohort without glomerulopathy. TXG was predated by early glomerular endothelial cell activation which was characterised by vacuolation, hypertrophy, serration, and expansion of sub-endothelial space (lamina rara interna). Endothelial cells were transformed into an activated phenotype, with morphological transition from normal fenestrated endothelium to continuous endothelium. Glomerular and peri-tubular capillary basement membrane duplication accompanied endothelial cell activation. Endothelial abnormalities were evident before appearance of light microscopy abnormalities or detection of graft dysfunction.

In chapter 6, the effect of MMF on chronic transplant histology was evaluated by comparison of sequential prospective protocol kidney biopsies from two historical cohorts treated with cyclosporine and either Mycophenolate mofetil (MMF) or Azathioprine (AZA). Banff chronic interstitial fibrosis scores, global glomerulosclerosis and peri-glomerular fibrosis were reduced in the MMF treated group. Transplant glomerular morphology and markers of CNI toxicity were ameliorated in the MMF treated group.

In conclusion, pre–transplant DSA, detected by sensitive Luminex assays result in inferior histological and functional outcomes and chronic graft loss. Strength of antibody binding, defined by MESF values, modifies these outcomes. In patients with DSA who subsequently develop transplant glomerulopathy, ultra-structural abnormalities are associated with C4d deposition, involve the entire transplant capillary bed and can be detected before graft dysfunction occurs. MMF is an option for modification of immunosuppression in these patients. Given the poor outcomes, all patients should be screened for antibody using Luminex or other solid phase assays. In all those with DSA, antibody strength measured by MESF values and electron microscopic evaluation of transplant biopsies should be undertaken to identify patients at increased risk for adverse outcomes. This thesis describes the biological events in patients with donor specific antibody which is a starting point for clinical trials designed to alter patient outcomes.

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LIST OF ABBREVIATIONS

ANZDATA	Australia and New Zealand Dialysis and Transplant registry
ARCBS	Australian Redcross and Blood Service
ARF	Acute renal failure
ASHI	American Society of Immunogenetics and Histocompatibility
ATG	Antithymocyte globulin (Fresenius, Germany)
Avg	Average
AZA	Azathioprine
C4d	Complement split product
CAMR	Chronic antibody mediated rejection
CAN	Chronic allograft nephropathy
CDC	Cytotoxic dependent cross-match
cg	Chronic glomerular score
ci	Chronic interstitial score
CL	Capillary lumen
CNI	Calcineurin inhibitor
CSA	Cyclosporine
ct	Chronic tubular score
CVA	Cerebral vascular accident
DSA	Donor specific antibody
DTPA	Diethylenetriaminepentaacetic acid
ELISA	Enzyme Linked Immunosorbent assay
FI	Fluorescent intensity
GBM	Glomerular basement membrane

GFR	Glomerular filtration rate
HLA	Human Leucocyte antigen
i	Interstitial inflammation
IMPDH	Inosine 5-monophosphate dehydrogenase
IQR	Inter quartile range
KAT	Kidney anastomotic time
LD	Lamina densa
LRI	Lamina rara interna
LSM	Luminex labscreen
MESF	Molecules of equivalent soluble flourochrome
MICA	Major Histocompatibility complex class 1 related chain A antigens
MICB	Major Histocompatibility complex class 1 related chain B antigens
mm	Mesangial matrix
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
NS	Not significant
OGTT	Oral glucose tolerance test
OKT3	Orthoclone OKT3 (murine monoclonal antirejection agent)
PBMC	Peripheral Blood monocytes
PTC	Peri-tubular capillary
PTC-BMML	Peri-tubular capillary basement membrane multi-lamination
SAG	Single antigen

SCR-A	Subclinical rejection acute
SCR-B	Borderline subclinical rejection
SD	Standard deviation
SEM	Standard error of the mean
SPK	Simultaneous pancreas and kidney
t	Tubulitis
TAC	Tacrolimus
TEM	Transmission electron microscopy
TSANZ	Transplantation Society of Australia and New Zealand
TXG	Transplant glomerulopathy
US	Urinary space

PUBLISHED WORK AND PRESENTATIONS ARISING FROM THIS THESIS

Manuscripts

Wavamunno, M. D., O'Connell, P. J., Vitalone, M., Fung, C. L., Allen, R. D., Chapman, J. R., and Nankivell, B. J. (2007), 'Transplant glomerulopathy: ultra-structural abnormalities occur early in longitudinal analysis of protocol biopsies', *American Journal of Transplantation,* 7 (12), 2757-68.

Nankivell, B. J., **Wavamunno, M. D**., Borrows, R. J., Vitalone, M., Fung, C. L. S., Allen, R. D. M., Chapman, J. R., and O'Connell, P. J. (2007), 'Mycophenolate mofetil is associated with altered expression of chronic renal transplant histology', *American Journal of Transplantation,* 7 (2), 366-76.

Book Chapter

Wavamunno M. D., O'Connell P. J., Chapter 103, Chronic Allograft Dysfunction, Comprehensive Clinical Nephrology, 4th Edition, Editors Johnson R.J., Feehally J (in press).(adapted from literature review).

Abstracts and presentations

Pre-transplant DSA detected with Luminex are associated with inferior graft outcomes, impact is modified by antibody strength. **Moses D Wavamunno,** Phillip J O'Connell1 ,Caroline L-S Fung, Richard D.M. Allen, Narelle Watson, Frederika Abou-Daher, Jeremy

R. Chapman, Brian J. Nankivell, O'Connell P.J, Caroline L-S Fung, Allen R.D.M., Narelle Watson, N, Abou-Daher F, Chapman J.R Brian J. Nankivell B.J.*The American Transplant Congress, Boston, 2009.*

Pre-transplant DSA detected with Luminex are associated with inferior graft outcomes, impact is modified by antibody strength. **Moses D Wavamunno,** Phillip J O'Connell1 ,Caroline L-S Fung, Richard D.M. Allen, Narelle Watson , Frederika Abou-Daher, Jeremy R. Chapman, Brian J. Nankivell. *Transplantation Society of Australia and New Zealand, 27th Annual Scientific Meeting, Canberra 2009.*

The Impact of Pre-Transplant Anti-HLA And MICA Antibodies On Long-Term Graft Outcomes. **Moses D Wavamunno**, Phillip J O'Connell ,Caroline L-S Fung, Richard D.M. Allen, Narelle Watson, Frederika Aboud-Daher, Jeremy R. Chapman, Brian J. Nankivell *The XXII International Congress of The Transplantation Society, Sydney 2008*

Glomerular and Peri-tubular Capillary Ultrastructure in Transplant Glomerulopathy. **Moses D. Wavamunno**, Philip J. O'Connell, Matthew Vitalone, Caroline L-S Fung, Richard D.M. Allen, Jeremy R. Chapman, Brian J. Nankivell. *The Transplantation society of Australia and New Zealand, 25th Annual Scientific Meeting, Canberra 2007.*

Glomerular and Peri-tubular Capillary Ultrastructure in Transplant Glomerulopathy. *The Transplantation Society, new Key Opinion Leader meeting, Sydney, Australia 2007.*

Mycophenolate Mofetil Reduces The Histological Expression Of Chronic Allograft Nephropathy. **Moses D Wavamunno**, Matthew Vitalone, Richard J Borrows, Caroline L-S Fung, Richard D.M. Allen, Phillip J O'Connell, Jeremy R. Chapman, Brian J. Nankivell. *The First International Joint Transplant Conference, Boston, 2006.*

Awards and Prizes

The TSANZ Young Investigator Travel Grant for the American Transplant Congress 2009

The TSANZ Young Investigator Award 2009

The TSANZ Young Investigator Award 2008

TSANZ International Travel Grant to attend and present at WTC 2006 Boston, USA.

CHAPTER 1

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 Introduction

This chapter reviews current knowledge on the definitions, classification, and pathogenesis of chronic renal allograft dysfunction. Various aspects of antibody mediated graft injury and its contribution to chronic graft dysfunction are reviewed. These include mechanisms of antibody production, methods of antibody detection, and clinico-pathological expressions of antibody mediated graft injury. Recent developments in these areas are reviewed with emphasis on how they have contributed to the growing body of evidence on the role of antibodies in chronic graft injury.

1.2 Chronic Allograft Dysfunction

1.2.1 Registry data

Introduction of immunosuppressive agents such as Calcineurin Inhibitors (CNI) and Mycophenolate Mofetil (MMF) resulted in reduced acute rejection rates and better short term graft survival. However, there has not been significant improvement in long term survival (Meier-Kriesche et al. 2004). Over the last 2 decades, 1 year renal allograft survival in Australia has increased from 50% to 94% in deceased donor kidney transplants and up to 97% in living related transplants (Campbell 2008). Late graft loss continues to be the "Achilles heel" of kidney transplantation, with 5 and 10 year survival rates as low as 91% and 58% for deceased donor transplants respectively and 90% and 77% respectively, for living donor

transplants (Anonymous 2004; Campbell 2008). Data from the Australia and New Zealand registry (ANZDATA) shows that in the years 1991 to 2007, 1 year primary deceased donor graft survival improved from 85% to 91% while only modest improvements in 5 year graft survival, from 72% to 82% were observed (Campbell 2008).

1.2.2 Definitions and nomenclature

The majority of late graft losses are preceded by a clinical syndrome characterised by slow progressive decline in graft function, proteinuria, and hypertension. Tubular atrophy and interstitial fibrosis are predominant histopathological hallmarks of this syndrome. Increasing prevalence of end stage renal disease (ESRD), shortage of organ donors and the high numbers of patients returning to dialysis after transplant with biopsy evidence of chronic injury (Paul 1999) have led to renewed interest in this clinical syndrome. The overall aim is to identify transplant recipients at risk for chronic graft dysfunction before irreversible pathological abnormalities are established.

The constellation of clinical and pathological abnormalities described above was initially referred to as chronic rejection and later as chronic allograft nephropathy (CAN). For many years, it was believed that late allograft dysfunction was primarily the result of immune mediated injury. This belief led to confusion in the transplant community as to the exact nature of chronic allograft injury. The perception that late graft loss was caused by chronic immune mediated allograft injury

emerged in the 1950s when several authors described arterial and glomerular abnormalities in transplant recipients who had late acute rejection episodes and circulating alloantibody (Hume et al. 1955; Jeannet et al. 1970; Porter et al. 1963). Subsequent studies reported severe, recurrent, and late cellular rejection, in the context of inadequate immunosuppression, as the major risk factors for late graft loss. Occurrence of these patterns of cellular rejection was named "chronic rejection" (Almond et al. 1993b; Almond et al. 1993a; Monaco et al. 1999). These studies promoted the view that all late graft loss/dysfunction was caused by "chronic rejection" which was considered a separate diagnosis from acute and late rejection. As interstitial fibrosis and tubular atrophy were common findings in transplant recipients with graft dysfunction, the presence of these histological abnormalities, even in the absence of arteriopathy, glomerular abnormalities (presumed to be immune mediated), or acute interstitial inflammation was taken as evidence for a chronic immune mediated allo-response. Thus, the term "chronic rejection" came to represent the clinico-pathological syndrome of progressive dysfunction, interstitial fibrosis, and tubular atrophy observed in failing allografts (Halloran 2002). In the 1990s, it was clear that these pathological features and clinical patterns previously attributed to chronic rejection were a consequence of multiple different aetiologies of which the allo-immune response was but one of the causes. Chronic allograft nephropathy, a term first used in 1991, then became the all encompassing description for various aetiologies believed to cause

fibrosis and tubular atrophy. These included, chronic rejection, calcineurin inhibitor toxicity, hypertensive vascular diseases and chronic infections (Solez 1994). Recently, there has been a change in terminology toward defining specific aetiological diagnoses within the entity of CAN as will be discussed below.

1.2.3 Classification

By the early 1990's, there was a need to develop a classification system to standardize reporting of transplant pathology and to facilitate better understanding of the pathophysiology of injury in late graft dysfunction. The Banff schema developed by a group of renal pathologists, nephrologists and transplant surgeons in Banff, Canada, incorporated other classification systems (Isoniemi et al. 1994), and with subsequent refinements a scoring system for renal transplant pathology emerged. The schema categorised acute lesions such as interstitial inflammation, tubulitis, and chronic lesions such as interstitial fibrosis, glomerulopathy, mesangial matrix, vascular fibrous intimal thickening, and arteriolar hyaline thickening along a semi-quantitative severity score developed on a scale of 0, 1, 2, or 3. Patterns of scored lesions, supported by specific pathological features, were then classified into a clinico-pathological diagnosis (Racusen et al. 1999; Solez et al. 1993; Solez 1994).

A review of the Banff criteria noted that use of the non-specific term "CAN" undermined recognition of morphological features, which could

enable diagnosis of specific causes of chronic graft dysfunction (Solez et al. 2007). The schema has been consequently modified to include various aetiological causes of chronic graft injury such as chronic antibody mediated rejection and chronic T-cell mediated rejection. The former is characterised by glomerular and peri-tubular capillary basement membrane duplication in the presence of complement component deposition (C4d) and anti-donor HLA antibody. In contrast, chronic active T-cell mediated rejection is characterised by arterial intimal fibrosis in the presence of a mononuclear cell infiltrate. A third category of interstitial fibrosis and tubular atrophy not otherwise specified (IF/TA_{nos}) is also recognized in addition to non-immune causes of injury such as calcineurin inhibitor toxicity. Although this classification provides a standardized framework for histological analysis and reporting of renal allograft pathology, the interaction of all factors that contribute to graft injury is still not fully understood.

1.2.4 Pathophysiology of Chronic Renal Allograft Injury.

1.2.4.1 Limitations

The pathophysiology of chronic renal allograft injury/dysfunction is not well understood due to several reasons. One of the limitations to understanding mechanisms of chronic injury is that pathology of a failing allograft represents a final common pathway of damage and injury resulting from multiple overlaid causes. In this context, identification of a specific cause is difficult. Moreover, evolution of transplant practices with time indicate that histologic changes are

likely to be influenced by factors which vary with transplantation era such, as donor/recipient mix, ethnicity, immunological risk, proportion of marginal donors and immunosuppressive protocol. As an example, the classical histology of "chronic rejection" from the prednisolone and azathioprine era is different to that of the CNI era where there is less acute and subclinical rejection, but more nephrotoxicity and polyoma viral infection. These factors result in lack of clarity in mechanisms of injury.

1.2.4.2 Role of Protocol Biopsies

Current understanding of the pathogenesis of chronic allograft injury is attributed to the increasing use of surveillance or protocol biopsies in several transplant centres (Dimeny et al. 1994, 1995; Nankivell et al. 2003; Paul et al. 2000; van Es et al. 2000). Protocol biopsies enable assessment of individual risk factors for allograft injury through detection of early pathological abnormalities and are considered the preferred tool to screen for subclinical injury (Rush 2006; Schaub et al. 2007). In contrast, "biopsies for cause" often reflect injury from the causes of acute dysfunction together with multiple aetiologies. Data from protocol biopsies enables pathological features to be used as surrogate markers for chronic injury (Mengel et al. 2007; Thaunat et al. 2007). This concept is well supported by several studies which demonstrate that chronic pathology detected by protocol biopsies at 3 months after transplant, predicts poor long term graft survival (Nankivell et al. 2003) (Nickerson et al. 1998; Seron et al. 2002a;

Seron et al. 2002b; Seron 2003). In addition, Nankivell et al demonstrated that sub-clinical rejection, (defined as occurrence of histologic signs of acute rejection in patients with stable graft function), preceded long term deterioration of graft function (Nankivell et al. 2001). Randomized control studies incorporating protocol biopsies point to the usefulness of this procedure in determining efficacy of immunosuppression therapy (Rush et al. 2007; Rush et al. 2009). The studies described above provide insights into the individual causes of graft injury and how these interact within the graft to result in chronic graft injury.

1.2.4.3 Causes of Chronic Renal Injury

Individual factors associated with chronic graft injury are broadly categorized into allo-immune dependent and independent factors. Allo-immune-independent factors include peri-transplant events such as prolonged cold and warm ischemic times, donor related characteristics like donor age and donor–recipient size matching (Monaco et al. 1999; Shoskes et al. 1998; Terasaki et al. 1997), factors related to recipient morbidity such as post transplant hypertension, hyper-lipidemia (Fernandez-Miranda et al. 1997; Paul et al. 1995; Ponticelli et al. 1993) and toxicity from immunosuppression (Nankivell et al. 2004a; Solez et al. 1998). Allo-immune dependent factors associated with chronic injury include multiple and severe late acute rejection episodes and acute vascular rejection (Bellamy et al. 2000; Kasiske et al. 1991; Matas et al. 1994; van Saase et al. 1995). There is compelling evidence

that sub-clinical rejection is an important factor in early and chronic graft injury (Legendre et al. 1998; Nankivell et al. 2004c; Rush et al. 1995; Veronese et al. 2004). Pre-sensitisation to donor HLA, the subject of this thesis, is also an important allo-immune factor for graft injury discussed in more detail in section 1.2.5 (Davenport et al. 1994; Mauiyyedi et al. 2001; Regele et al. 2001).

1.2.4.4 Histological Progression of Chronic Graft Injury

The natural history and progression of chronic renal allograft injury was well described in a protocol biopsy series examining the causes and correlates of chronic allograft dysfunction. Nankivell et al evaluated over 900 protocol biopsies from recipients of simultaneous kidney-pancreas transplants. Results from this study provided valuable insight into the relationships between interstitial fibrosis, arteriolar hyalinosis, and glomerular sclerosis (Nankivell et al. 2003). Data from this and other studies demonstrates that allograft injury occurs in 2 distinct phases: an early phase characterised by tubular-interstitial damage (Kuypers et al. 1999; Nankivell et al. 2004b), and a late phase which involves the microvascular compartments (i.e. glomerular, peri-tubular and interstitial capillary networks). Injury during the late phase leads to progressive interstitial fibrosis, tubular atrophy and glomerulosclerosis (Cosio et al. 1999; Kasiske et al. 1991; Nankivell et al. 2003; Sijpkens et al. 2003; Solez et al. 1998; Veronese et al. 2004). Factors associated with early tubular-interstitial injury include pre-

existing conditions in the donor kidney, peri-operative events such as prolonged ischemic time, and post-implantation events such as early rejection, delayed graft function and immunosuppressive toxicity. In contrast, graft injury in the late phase is attributed to persistent, progressive subclinical inflammation (Nankivell et al. 2006b) and the effects of CNI toxicity expressed as nodular arteriolar hyalinosis and striped tubulointerstitial fibrosis (Benigni et al. 1999; Davies et al. 2000; Mihatsch et al. 1988c, 1988a, 1988b; Mihatsch et al. 1988d). While the series published by Nankivell et al clearly demonstrated the role of cellular rejection and immunosuppression toxicity in the pathogenesis of chronic graft injury, the contribution of HLA antibodies to these pathological processes was not clearly elucidated. The main conclusion from studies examining chronic graft injury is that late graft dysfunction results from cumulative tissue injury due to several pathogenic insults. Graft injury occurs in specific histological compartments, is modified by the kidney's healing response to the injury, and immunosuppression regimen. However, mechanisms of graft injury still lack clarity. The following section reviews postulated mechanisms of injury.

1.2.4.5 Mechanisms of Graft Injury and Dysfunction

The nephron is the functional unit of the kidney. Injury to any component of the nephron, results in functional failure of the whole unit. In renal allografts, dysfunction occurs due to direct nephron injury, chronic ischemia, and impairment of the healing process

following graft injury. The starting condition of the transplanted kidney and subsequent immune and non-immune stressors discussed in section 1.2.4.3 contribute to loss of structural integrity of the graft and subsequent failure (Nankivell et al. 2006a).

Individual nephrons fail due to either glomerular injury as a result of glomerulosclerosis and transplant glomerulopathy, or loss of tubular components. Glomerular injury leads to capsular adhesions, mis-direction of ultra-filtrate and reduction in overall filtration efficiency (Kriz et al. 2001). On the other hand, tubular injury caused by localized apoptosis, tubular atrophy, or luminal obstruction from cellular debris leads to loss of basement membrane integrity (Bonsib et al. 2000). Inflammatory necrosis and impaired healing eventually develop into local obliterative fibrosis and structural compromise.

Chronic ischemia plays a major role in the initiation and perpetuation of glomerular and tubular-interstitial injury described above. Vascular obliteration manifests as glomerulosclerosis, CNI-induced arteriolar hyalinosis (Nankivell et al. 2004a) fibro intimal hyperplasia of small arteries and cyclosporine-induced vasoconstriction. Similar process occur in the peri-tubular capillary network (PTC) causes interstitial ischemic injury and peri-tubular capillary loss (Ishii et al. 2005).

Another important aspect in the mechanisms of chronic graft injury is the process of tissue repair. Recurrent episodes of acute injury are followed by partial or incomplete resolution of inflammation, resulting

in a vicious cycle of non-specific injury, which causes more inflammation. The ongoing process leads to enhanced expression of allo-reactive molecules and further injury (Halloran et al. 1999). Presence of inflammatory cells within areas of fibrosis ("activated fibrosis") and their associations with progressive damage, functional impairment and reduced graft survival is evidence for this mechanism of chronic injury (Nankivell et al. 2004c; Shishido et al. 2003). The vicious cycle discussed above is further perpetuated by impaired capacity to withstand stress, as a result of factors such as increased donor age which contribute to limited ability to repair structural damage, causing further amplification of external insults by pre–existing abnormalities (Land 2004). Other factors known to contribute to progression of chronic renal disease in the non-transplant population such as hypertension, smoking, and hyperlipidemia, when present in the recipient, create an environment that favours continued attrition of the allograft.

In summary, the transplanted kidney gradually malfunctions from the effects of catastrophic failure of many individual nephrons combined with incremental loss of architectural integrity and global vascular insufficiency. The aetiological factors are superimposed onto the effects of intrinsic donor variables and peri-operative events (Figure 1.1 overleaf). These are categorised into immune and non immune factors. The role of HLA antibodies in pathophysiology of this condition is discussed in section 1.2.5.

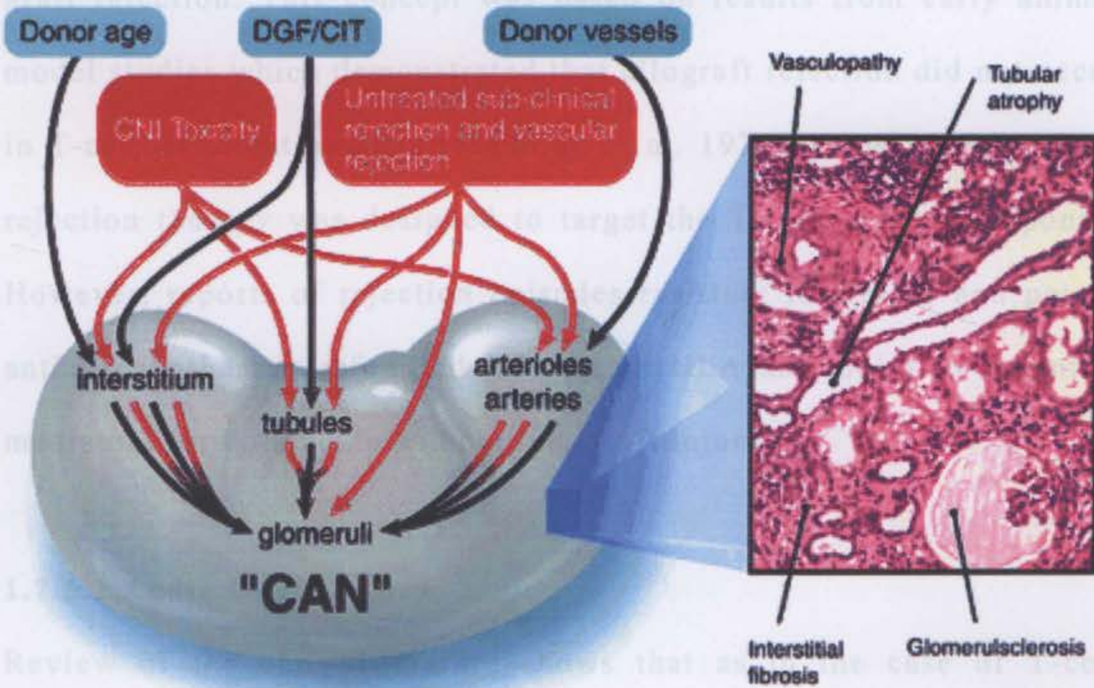


Figure 1.1: A summary of interactive factors, which result in chronic allograft nephropathy. The transplanted kidney fails due to multiple factors affecting individual compartments of the allograft (Chapman et al. 2005).

1.2.5 The Role of HLA Antibodies in Graft Injury

As outlined in section 1.2.4.3, causes of allograft injury are categorised into immune and non-immune factors. Immune factors include cellular and antibody mediated responses to the graft. This section reviews the literature on the role donor antibodies play in acute and chronic graft injury.

For decades, the contribution of HLA antibody to graft injury was unrecognised because T-cells were regarded as the main mediators of graft rejection. This concept was based on results from early animal model studies which demonstrated that allograft rejection did not occur in T-cell deficient animals (Manning et al. 1973). Consequently, anti-rejection therapy was designed to target the T-cell immune response. However, reports of rejection episodes resistant to steroid and potent anti T-cell therapy refocused interest on HLA antibodies as potential mediators for both acute and chronic graft injury.

1.2.5.1 Acute Graft Injury

Review of the early literature shows that as in the case of T-cell rejection, evidence that antibodies are involved in early allograft rejection has been available for several decades. Experiments performed in animal models demonstrated that mouse skin allografts were not only capable of inducing antibodies but in addition, passive transfer of antibodies within the first week after transplant resulted in graft rejection (Gerlag et al. 1975). Patel and Terasaki had earlier reported that presence of HLA antibody in a recipient at time of transplant was associated with hyperacute rejection. This study established the concept that antibodies are involved in the early rejection of grafts (Patel et al. 1969). Since then, several studies have demonstrated a high incidence of primary non-function and decreased short term graft survival in graft recipients pre-sensitised with HLA antibodies (Bryan et al. 2000; Iwaki et al. 1987b; Iwaki et al. 1987a;

Karpinski et al. 2001; Mahoney et al. 2002; O'Rourke et al. 2000; Terasaki et al. 1971).

1.2.5.2 Chronic Graft Injury

Evidence in support of the hypothesis that HLA antibody contributes to chronic graft injury has also been available for decades. Jeannet and colleagues found that circulating donor specific antibodies correlated with presence of arterial stenosis in renal biopsies (Jeannet et al. 1970). This lesion is currently considered as one of the hallmarks of chronic antibody mediated injury. In subsequent studies, data from animal models demonstrated that B cell deficient mice were incapable of developing similar lesions (Russell et al. 1997). This study provided further evidence for the contribution of antibody in the pathogenesis of this lesion.

More recent studies have confirmed that circulating HLA antibodies are present in a substantial proportion of renal allografts with chronic vascular damage and late graft loss (Davenport et al. 1994; Piazza et al. 2001b; Piazza et al. 2001a). A large prospective multi-centre trial, reported that post-transplant HLA antibodies were detected in 20% of 2,278 renal allograft recipients. Graft failure at 1 year occurred more frequently in patients who developed de novo allo-antibodies than in those who did not (Terasaki et al. 2004). In another study, de novo production of donor HLA–specific antibodies was shown in 51% of 112 renal transplant recipients with graft failure compared with 2% of 123

stable controls, presence of antibody predicted subsequent graft loss (Worthington et al. 2003). A temporal relationship between appearance of HLA antibodies and subsequent graft rejection was demonstrated by El- Awar and colleagues who reported that almost 90% of 826 patients waiting for a regraft, had circulating HLA antibody (El-Awar et al. 2002). Another study which performed serial antibody testing found that all 14 patients who rejected their grafts developed HLA antibodies years prior to rejection (Lee et al. 2002).

These studies were limited by evaluation of only post transplant antibodies for a role in chronic graft injury. This is because earlier work on pre-transplant antibodies (discussed in section 1.2.5.1) led to two assumptions on the role of antibodies in graft injury. The first assumption was that, antibody present at time of transplant led to immediate antibody mediated rejection and the second was, that antibody present "early" i.e. in peak serum but not current represented a pre-existing sensitisation which implied only T-cell memory and subsequent intense allo-immune cellular rejection or accelerated rejection usually at day 3 to 5 after transplantation. Improved methods of antibody detection have resulted in increased sensitivity in detection of pre-transplant HLA antibodies and renewed research interest in the effects of pre-transplant HLA antibody. This has shifted the focus from early to long term outcomes as these antibodies are presumed to be of lower strength by virtue of the tests required to detect them. The following section reviews antibody detection techniques and how they

have revolutionised current understanding of the effects of antibodies on graft outcomes.

1.2.5.3 Antibody Detection Techniques

Sensitive techniques for antibody detection are a major advance in transplantation. In addition to improved monitoring of post-transplant antibody, they have renewed debate on the interpretation and clinical relevance of antibody present at the time of transplantation. Antibody detection methods have evolved from membrane based serological assays such as complement dependent cytotoxic assays (CDC) and flow cytometry which require lymphocyte preparation, to assays which incorporate purified HLA antigens attached to plates such as enzyme linked immunosorbent assays (ELISA) or micro-bead particles (Luminex). These assays are more sensitive compared to the CDC assays and can be used to identify novel specific antigenic targets (Gibney et al. 2006). However, increasing use has generated questions about their clinical relevance and interpretation especially when used in pre-transplant screening (Gebel et al. 2003).

1.2.5.3.1 Serological based assays

The complement dependent cytotoxic cross-match (CDC-XM)(Terasaki et al. 1964) based on work by Patel and Terasaki is still the most widely used test to detect antibody to HLA antigens for more than 30 years. In this assay, patient serum is incubated with donor lymphocytes, if antibodies specific for HLA antigens are present, cell

death will occur on addition of rabbit serum as a source of complement (Figure 1.2). Cell death, can be microscopically visualised after staining with cell dyes to asses the degree of cell death. Variations to this technique such as addition of anti-human globulin (Fuller et al. 1978) and extended incubation (Ting et al. 1973) have been used to improve sensitivity. The degree of pre-sensitisation is determined by testing patient serum against a random panel of lymphocytes from HLA typed individuals. The frequency of positive reactions described as the panel of reactive antibodies (PRA) gives the percentage of local donors with whom the patient is likely to have a positive cross-match. Variations in PRA may occur due to changes in the panel composition. HLA specificity can be determined with this assay if selected panels do not contain common combinations of HLA antigens. The drawbacks of the test include subjectivity, difficult interpretation in the presence of auto-reactive antibody and lower sensitivity and specificity in comparison to new cross-match techniques (Duquesnoy et al. 2003; Worthington et al. 1998).

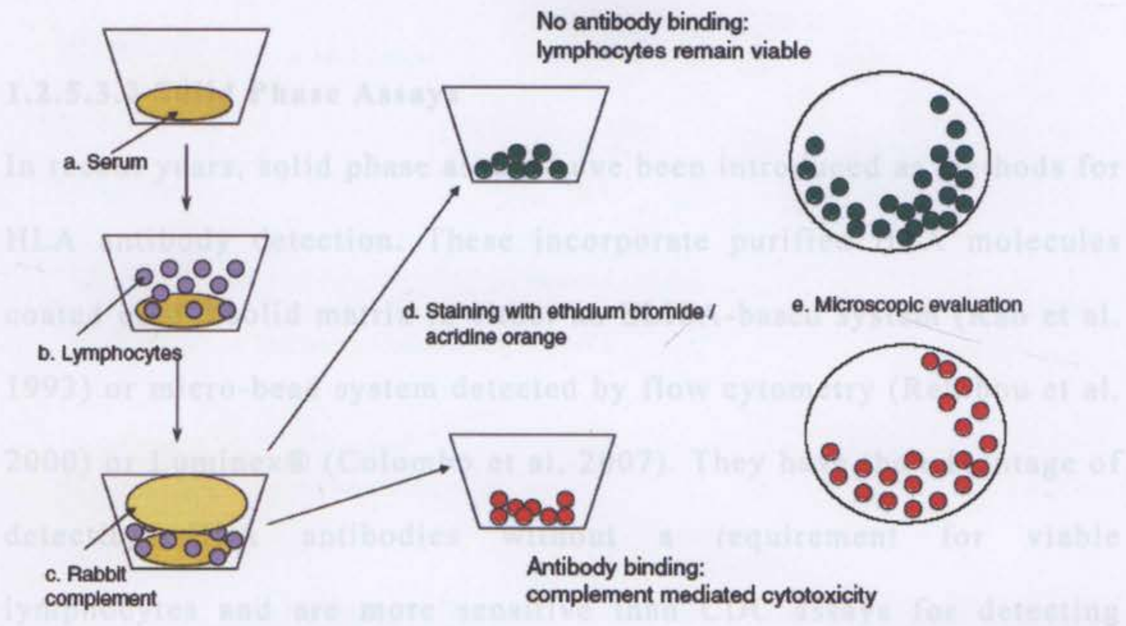


Figure 1.2: Schematic representation of the complement dependent cytotoxicity test (Fuggle et al. 2008).

Due to drawbacks of the CDC assay, many laboratories have adopted assays that offered greater specificity and sensitivity in antibody detection. Introduction of the flow cytometry technique increased ability to detect donor-specific HLA antibodies associated with rejection in the presence of a negative donor CDC-XM (Bryan et al. 1998; Karpinski et al. 2001). In the flow cytometry cross-match (FCXM) donor reactive antibodies independent of complement fixation are detected by incubation of patient sera with pooled cells. With this assay, antibody reactivity to T and B cells can be independently evaluated while avoiding detection of auto-reactive antibodies, which is a significant advantage over the CDC-XM. However, as in the CDC-

XM, determination of HLA specificities can be cumbersome and time consuming.

1.2.5.3.2 Solid Phase Assays

In recent years, solid phase assays have been introduced as methods for HLA antibody detection. These incorporate purified HLA molecules coated onto a solid matrix in either an ELISA-based system (Kao et al. 1993) or micro-bead system detected by flow cytometry (Rebibou et al. 2000) or Luminex® (Colombo et al. 2007). They have the advantage of detecting HLA antibodies without a requirement for viable lymphocytes and are more sensitive than CDC assays for detecting HLA antibodies (Leffell et al. 2005; Saidman 2007; Zeevi et al. 2006).

The Enzyme Linked Immunosorbent Assay

Enzyme linked Immunosorbent assay (ELISA) introduced more than a decade ago, utilises HLA molecules from pooled donors bound to the surface of plastic wells on an ELISA plate (Kao et al. 1993; Zachary et al. 2001). Antibody against added patient sera are detected by addition of a secondary antibody (an enzyme–conjugated anti human immunoglobulin) that causes a colour change on addition of the enzyme substrate (Figure 1.3). Antibody-antigen interactions are detected by measuring changes in optical density. When compared to CDC assays, ELISA have the advantages of being more sensitive although non-specific binding of other immunoglobulins may occur in patients with autoimmune disorders.

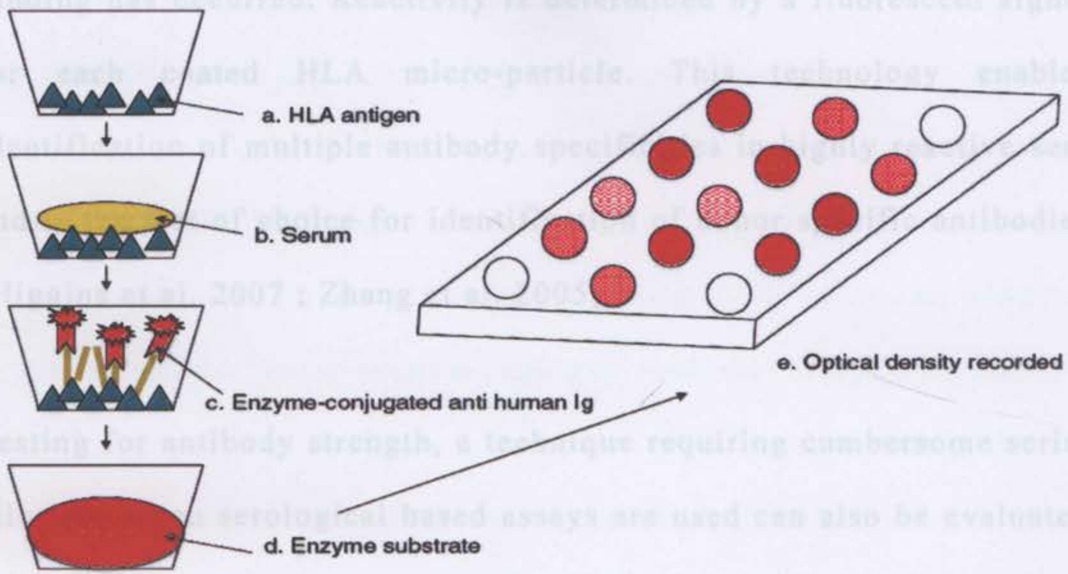


Figure 1.3: Schematic presentation of the Enzyme linked immunosorbent assay (Fuggle et al. 2008).

Luminex

Luminex®, the platform used for antibody detection in this thesis incorporates micro-bead particles coated with purified HLA antigens derived from lymphoblastoid cell lines (Figure 1.4). Fluorochromes embedded within each bead give it a unique signal to enable identification of different beads in sets. Beads are bound with a large number of class I and class II molecules which provides a negative or positive result, this test is referred to as the Labscreen 1 and 2. Alternatively, a single HLA molecule may be attached to one bead, the assay is then referred to as a single antigen assay (SAG). Antibody binding to the micro-particles is detected using R-Phycoerythrin conjugated anti human Ig. A laser beam within a flow analyser excites

each bead to determine its identity and whether antibody–antigen binding has occurred. Reactivity is determined by a fluorescent signal for each coated HLA micro-particle. This technology enables identification of multiple antibody specificities in highly reactive sera and is the test of choice for identification of donor specific antibodies (Higgins et al. 2007 ; Zhang et al. 2005).

Testing for antibody strength, a technique requiring cumbersome serial dilutions when serological based assays are used can also be evaluated using beads with known quantities of fluorochrome and antigen density. Mizutani et al demonstrated that antibody titre correlates directly with fluorescence intensity values and molecules of equivalent fluorochromes (Mizutani et al. 2007). This variation to the Luminex® assay has a potential to provide useful information in post-transplant monitoring and pre–transplant assessment of risk in patients with DSA.

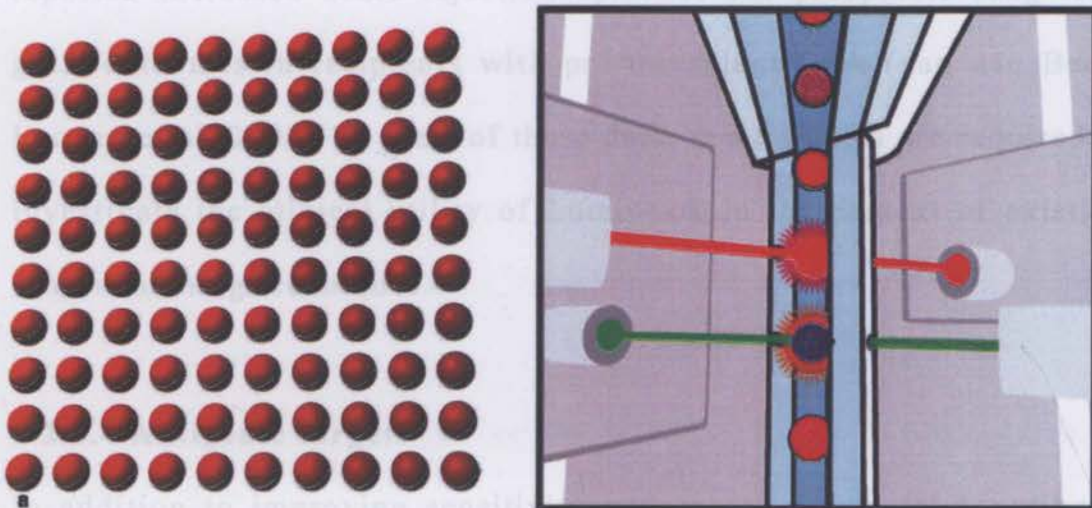


Figure 1.4: Schematic representation of the Luminex® assay (Fuggle et al. 2008).

There is little data about the significance of antibodies detected by solid phase testing in the post-transplant setting. Increased sensitivity of these assays has generated concerns regarding their clinical relevance. This is because antibody is presumed to be of low titre, non-complement binding and of low binding capacity (affinity and avidity) in comparison to antibody detected with the CDC assay. In addition, specificities to cross-reactive antigen can be obtained, but the significance of such antibody is unknown. The fundamental question is whether a positive Luminex® test, when obtained in the pre-transplant setting, should be a contraindication to transplant or a risk stratification test. Two studies have addressed this question with variable results. Outcomes in 121 patients were evaluated according to DSA status at day of transplant, presence of DSA had little impact on early graft outcomes although long-term graft outcomes were inferior (Gupta et al. 2008). Another study in highly sensitised recipients reported increased acute rejection episodes but acceptable long term graft outcomes in recipients with pre-transplant DSA (van den Berg-Loonen et al. 2008). In view of these data, more studies are required to investigate the clinical utility of Luminex® in the context of existing cross-matching techniques.

1.2.5.4 Antigenic targets

In addition to improving sensitivity and specificity in HLA antibody detection, solid-phase assays have enabled detection of novel antigenic targets. The well known targets for antibody production in

transplantation are class I and class II MHC molecules (Erlich et al. 2001) and the ABO blood group antigens. Class I molecules are expressed on surfaces of all nucleated cells, whereas expression of class II molecules is limited to B cells, dendritic cells and endothelial cells. HLA specific allo-antibodies are produced following exposure to HLA molecules during pregnancy, after blood transfusion or transplantation. These antibodies are predominantly of the IgG class although several subclasses are recognised with varying ability to trigger complement activation (Bindon et al. 1988). Blood group antigens are carbohydrate epitopes present at the surface of erythrocytes and endothelial cells, antibodies are usually of the IgM class, and arise naturally in normal individuals who are not of the A and /or B blood group in response to environment antigens. Transplantation across the ABO barrier, once considered a contraindication, is now an evolving field.

Non-HLA antigenic molecules are potential targets of the allo-response (Chao 2004; Derhaag et al. 2000; Wu et al. 2002). Terasaki reported that non-HLA antibodies were detectable in up to 38% of recipients whose grafts failed (Terasaki 2003b). In this category, non-classical HLA molecules, referred to as MHC class I-related chain A (MICA) and B (MICB) have emerged as potential targets for the allo-immune response. MICA, one of the targets widely investigated to date, is a polymorphic molecule expressed on endothelial, dendritic, fibroblasts and epithelial cells but not on peripheral blood lymphocytes.

Antibodies to MICA antigens are not detected by conventional cross-matching techniques because these require lymphocytes, which do not express MICA antigens. Using Luminex® based platforms; purified MICA antigens can be attached onto micro-beads and tested against patient sera. Preliminary studies report association between preformed MICA antibodies and early immunological complications in the absence of donor specific HLA antibodies (Mizutani et al. 2005; Sumitran-Holgersson et al. 2002). Presence of pre-transplant MICA antibodies was associated with increased rates of acute rejection and reduced 1 year graft survival in a study of 1910 kidney transplant recipients (Zou et al. 2007). Donor specificity was not established in any of the studies and data on mechanisms by which antibodies develop before transplant is lacking. Antibody against targets such as angiotensin 2 type 1 receptor have also been associated with graft dysfunction in renal allografts (Dragun et al. 2005). In cardiac transplant recipients, antibody to vimentin and myosin are associated with obliterative arteriopathy (Jurcevic et al. 2001), although their exact role in renal graft injury remains unknown. In view of these studies and the increasing ability to detect such antibodies in patient sera, more studies are required to investigate mechanisms of action and any impact on graft outcomes.

1.2.5.5 Mechanisms of Antibody Production

Antibodies are produced by B lymphocytes and plasma cells. T-cells have a central role in the immune response by initiating the recruitment

of alloantibody producing B cells. Presentation of antigen to recipient T cells, an essential part of the immune response, results in recruitment of antibody producing cells. B cell activation is thought to occur through either the indirect pathway in which donor antigen is processed and presented to activated T cells by recipient antigen presenting cells or through a direct pathway in which antigen is processed by donor antigen presenting cells prior to presentation to recipient T cells (Baratin et al. 2004; Chen et al. 1996; Gallon et al. 1995). Recent studies demonstrating maintenance of indirect and loss of direct responses in recipients with chronic allograft injury suggest that this is one of the mechanisms through which stimulation of antibody production occurs and subsequent graft injury is mediated (Baker et al. 2001; Poggio et al. 2004).

Activated T-cells provide for B-cell memory, antibody class switching and affinity maturation through secretion of cytokines and co-stimulatory factors. B-cell activation results in production of plasma cells, which continue to provide antibody indefinitely without requiring T-cell help. Presence of plasma cell rich infiltrates in rejecting allografts and detection of C4d suggests that the graft may be a site for T-cell and B-cell interaction and subsequent long-term generation of plasma cells thus perpetuating antibody production.

The concept of T and B cell memory is important particularly in multi-parous females who are candidates for transplantation. These patients

may be negative for HLA antibody when screened by CDC assays but are likely to have covert sensitisation when screened by sensitive assays such as Luminex® or ELISA discussed in section 1.2.5.3. This observation suggests that although the HLA antibody is no longer detectable by CDC, memory cells yielding low level antibody may still present. Luminex® provides the technology to detect these low level antibodies. The critical question is whether these antibodies are of any clinical significance.

In summary, Luminex® has increased the ability to detect HLA antibodies. In addition, this assay has facilitated identification of novel targets of the allo-immune response. The effect of antibody detected using these techniques on graft outcomes needs further clarification. The basic question is to what extent these antibodies are detrimental to the graft and if they are, should patients screened with these assays prior to transplantation be excluded from transplantation if the assay is positive. Answering this question necessitates a review of mechanisms and histopathological expression of antibody mediated graft injury.

1.2.5.6 Mechanisms of Antibody Mediated Graft Injury

1.2.5.6.1 The Role of Complement

Studies from xeno-transplantation indicate that complement fixation by antibody is essential for pathogenesis of acute rejection (Auchincloss et al. 1998). More recent studies show that locally produced

complement is capable of triggering complement receptors present on antigen presenting and infiltrating T cells. This results in T-cell activation, expansion, differentiation and class switching of allo-antibody to high affinity IgG. The effect of locally synthesised components appears to be more important than that of circulating complement components (Sacks et al. 2003a; Sacks et al. 2003b; Sacks et al. 2003c; Vieyra et al.). The discovery of staining techniques for complement fragment C4d, one of the end-products of complement activation, provides a visible link between presence of circulating antibody and allograft tissue injury. This discovery has enabled researchers to characterise histological patterns of antibody mediated graft injury, which will be reviewed in section 1.2.5.7

Complement activation occurs via a series of activation and regulation steps involving complement proteins, initiated through either alternate or classical pathways. Activation ultimately results in formation of a membrane attack complex (MAC), capable of cell lysis (Figure 1.5). Alternate pathways have not been shown to participate in acute or chronic rejection. Classical pathway activation occurs when IgG antibodies bind antigenic targets on graft endothelium and interact with c1q, a component of C1, leading to activation of c1r, c1s, and subsequent activation of C2 and C4. C4 is cleaved by C1s into C4a and C4b, C4b is later inactivated to C4d, which is covalently bound to graft endothelium by thio-ester and amide bonds.

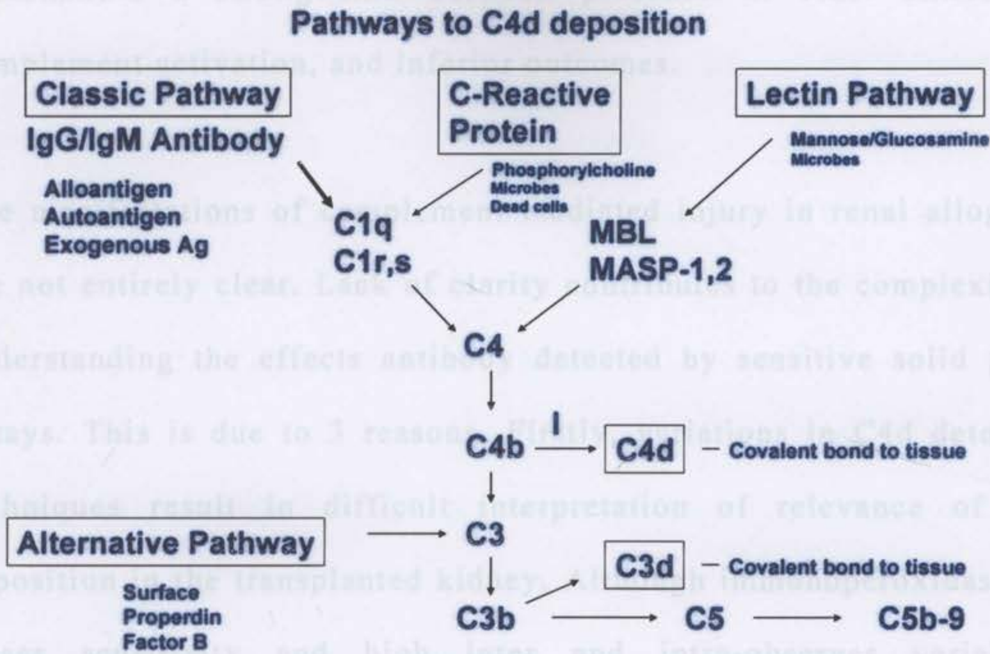


Figure 1.5: Pathways to C4d deposition

Immune-fluorescence techniques using monoclonal C4d antibody developed by Feutch et al (Feucht 2003) and an immuno-peroxidase variant using polyclonal antibody, developed by Regele at al (Regele et al. 2001), have allowed detection of C4d on graft endothelium. Using either one of these techniques, C4d can be demonstrated on peri-tubular capillary endothelium and on Type 4 collagen in capillary basement membranes (Collins et al. 1999) in addition, immuno-electron microscopy studies localise C4d deposition on capillary endothelial surfaces and in endothelial cell intracytoplasmic vesicles (Regele et al. 2002). Presence of C4d in peri-tubular capillaries is associated with inferior graft survival (Feucht et al. 2005) and circulating donor specific HLA (Collins et al. 1999) antibodies. These studies

demonstrate a strong link between presence of HLA antibodies, complement activation, and inferior outcomes.

The manifestations of complement mediated injury in renal allografts are not entirely clear. Lack of clarity contributes to the complexity of understanding the effects antibody detected by sensitive solid phase assays. This is due to 3 reasons. Firstly, variations in C4d detection techniques result in difficult interpretation of relevance of C4d deposition in the transplanted kidney. Although immunoperoxidase has lesser sensitivity and high inter and intra-observer variability compared to immunofluorescence stains (Seemayer et al. 2007), it enables evaluation of glomerular C4d deposits. These deposits have previously been considered irrelevant when detected with immunofluorescence.

Secondly, the definition, interpretation, and relevance of a positive stain are evolving. The Banff 2007 schema (Solez et al. 2008) classifies staining as diffuse ($C4d_3$) when more than 50% staining is found in cortical and/or medullary peri-tubular capillaries(PTC), focal ($C4d_2$) when staining is 10-50%,minimal ($C4d_1$) when staining is 1 to 10%, and negative when there is no staining in peri-tubular capillaries. In earlier classifications, a threshold of 50% PTC staining was considered clinically relevant. This was based on associations of more than 50% staining in acute rejection and early graft loss (Poduval et al. 2005). The most recent classification of C4d staining is challenged by

studies which show indistinguishable outcomes between diffuse and focal staining (Magil et al. 2006). These studies also demonstrate associations between focal C4d and peri-tubular capillary inflammation. In fact some postulate that focal C4d may have intermediate prognosis between diffuse and negative C4d (Lorenz et al. 2004; Magil et al. 2006; Poduval et al. 2005).

Thirdly, the specific role of C4d in tissue injury is unknown. It has been postulated that C4d is not pathogenic thus pointing to the possibility of other factors mediating tissue injury. This hypothesis is supported by studies from protocol biopsies in ABO incompatible renal allografts which demonstrate C4d deposition without histological evidence of antibody mediated rejection (Haas et al. 2006). Knowledge of the complement pathway suggests that tissue injury occurs after formation of a membrane attack complex (MAC) capable of cell lysis. Downstream events, involve activated C4b combining with C2a to form a C4b-C2a complex, which activates C3 by cleavage to c3a and c3b. The C3b–C2a complexes together with C4b constitute C5 convertase, initiating formation of the membrane attack complex (C5-C9). Cell lysis, endothelial and B cell activation, production of pro-inflammatory molecules such as cytokines, chemokines, adhesion molecules, and growth factors occur mediated by the membrane attack complex leading to further tissue injury. Therefore, the committed step to complement mediated tissue injury is at the C3 stage, C4d deposition is a by-product of MAC formation following C3 activation. It is logical to

hypothesize that both C3d and C4d should be detectable in grafts with antibody mediated rejection. However, studies investigating utility of C3d staining have shown inconsistent results to the extent that up to 20% of biopsies with C3d do not have C4d. Recognized histological markers of acute antibody mediated graft injury are not consistently observed in C3d positive biopsies and no association with DSA has been proven (Haas et al. 2006; Herman et al. 2005). In addition, MAC deposits have not been detected in peri-tubular capillaries (Nishi et al. 2004).

Other mechanisms independent of complement activation have been proposed in the pathogenesis of antibody mediated graft injury. Animal studies demonstrate that cultured mouse endothelial cells can be activated by non-complement fixing antibodies (Smith et al. 2000). In addition, in vitro studies show that MHC class 1 molecules promote proliferation of human microvascular endothelial cells in the absence of complement or inflammatory cells (Rahimi et al. 2004). These mechanisms require further evaluation particularly in the context of sensitive assays, capable of detecting low level antibody.

In summary, in addition to the lack of clarity on the clinical relevance of antibodies detected using sensitive techniques, some aspects of complement mediated injury as a mechanism for antibody mediated graft injury are still unclear in part due to deficiencies in detection of complement activation at various levels of the complement pathway.

The above studies suggest that the concept of complement activation is not an "all or none" phenomenon. Different levels of complement activation could mediate varying expressions of tissue injury. These variations could result from presence of low level antibody only detectable by sensitive assays. The next section reviews histopathological expressions of antibody mediated graft injury.

1.2.5.7 Clinico-Pathological Entities of Antibody Mediated Graft Injury

Antibody mediated graft injury is categorised into 3 main clinico-pathological groups based on clinical characteristics, C4d staining patterns and Banff histology. These categories are accommodation, acute antibody mediated rejection, and chronic antibody mediated rejection.

1.2.5.7.1 Accommodation

Accommodation defines a state of graft resistance to the effects of complement fixation and antibody (Koch et al. 2004). This phenomena was first observed in ABO incompatible transplant programmes when grafts continued to function despite high titres of circulating antibody (Park et al. 2003; Shishido et al. 2001; Terasaki et al. 2008). Similar observations have been reported in HLA mismatched ABO compatible grafts although some authors are of the view that it is only a matter of time until these grafts fail and that any appearance of graft resistance

to injury is likely to be transient or insufficient to prevent chronic injury and subsequent graft failure (Terasaki et al. 2008).

Expression of protective proteins by graft endothelium and regulation of complement activation are some of the postulated endothelial events believed to result in accommodation. Cyto-protective proteins such as (B cell lymphoma 2) and Bcl-x, (both anti-apoptotic proteins) initially described in accommodated rodent xenografts (Tabata et al. 2003), were later found to be highly expressed on endothelium from stable human renal allografts in presence of circulating antibody (Salama et al. 2001). Low HLA antibody concentrations increase expression of these proteins (Narayanan et al. 2004) supporting the hypothesis that accommodation could occur as a result of low antibody titre.

Up regulation of complement regulatory factors, such as Delay Accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and protectin (CD 59) in cultured endothelial cells exposed to C reactive protein (Grubbs et al. 2003) was associated with inhibition of complement mediated cytotoxicity due to endothelial antibody. Protocol biopsy studies show that although C4d deposition is common in ABO incompatible grafts a small proportion of biopsies have histological evidence of acute humoral (Fidler et al. 2004; Haas et al. 2006) and graft dysfunction (Mengel et al. 2005). These findings and failure to demonstrate MAC in biopsies with C4d and/or C3d (Haas et al. 2006) suggests that in some patients, complete activation of

complement pathways does not occur or regulatory factors effect endothelial resistance. These findings are of special significance for antibody detected by Luminex® assay. Because antibody detected using these assays is thought to be of low affinity and avidity compared to that detected by CDC assays the phenomena of accommodation could explain why some grafts continue to function in the presence of circulating antibody.

1.2.5.7.2 Acute Antibody Mediated Rejection (AAMR)

AAMR is defined by clinical evidence of acute graft dysfunction, histological evidence of acute tissue injury, immunopathological evidence of complement activation and serological evidence of donor specific antibodies at time of biopsy (Racusen et al. 2003; Racusen 2003; Takemoto et al. 2004). The morphological features of acute injury consist of neutrophil and/or mononuclear cell margination within PTC or glomerular capillaries, micro- thrombi, fibrinoid necrosis or acute tubular injury. Immuno-pathological injury is defined by complement component 4d deposition in peritubular capillaries as discussed previously. HLA Antibodies are present in up to 95% of patients with C4d deposition and acute graft dysfunction (Bohmig et al. 2002; Haas et al. 2006; Mauiyyedi et al. 2002b) confirming the role of antibodies in acute graft injury. AAMR may develop within the first few weeks after transplant or after several years, together with acute cellular rejection, acute tubular injury, or thrombotic microangiopathy. Pre-sensitization due to blood transfusion, pregnancy, or previous

transplants is one of the major risk factors for acute antibody mediated graft injury. Pre-transplant HLA antibody detected by CDC is associated with acute graft loss due to antibody mediated graft injury. This effect is probably due to high affinity and avidity antibody. In contrast, the effect of antibody detected using Luminex® may have different expression due to low affinity and avidity.

1.2.5.7.3 Chronic Antibody Mediated Rejection

1.2.5.7.3.1 Transplant Glomerulopathy

Transplant glomerulopathy and arteriopathy are the histopathological abnormalities attributed to chronic antibody mediated injury in renal allografts. The term Transplant Glomerulopathy was coined by Zollinger in 1970 in reference to glomerular abnormalities specific to renal allografts observed to be distinct from recurrent or de novo glomerulonephritis. The latter is characterised by immune complex deposition and presence of inflammatory cells although its descriptive features had been reported in the early 1960s (Porter et al. 1963). Interest in this lesion has increased in the last decade for two reasons. Firstly, it is one of the specific diagnoses arising out of the histological group originally described as chronic allograft nephropathy, and secondly, it is a recognised cause of renal allograft loss and late dysfunction (Suri et al. 2000). TXG is clinically accompanied by progressive decline in graft function, substantial or nephrotic-range proteinuria, hypertension, and shortened graft survival. In patients with glomerulopathy, loss of graft function occurs before

widespread glomerulosclerosis and its appearance is followed by an accelerated rate of graft loss (Suri et al. 2000).

1.2.5.7.3.2 Pathology

Transplant glomerulopathy (TXG) is characterised by duplication of glomerular capillary basement membrane, mesangial matrix expansion, and absence of immune deposits. The Banff 2003 criteria requires presence of duplicated GBM in more than 10% of glomerular capillary loops in the most affected non-sclerotic glomeruli to make the diagnosis of TXG (Racusen et al. 2003).

Clinico-pathological studies suggest that TXG is the final manifestation of capillary injury occurring in conjunction with interstitial inflammation, peri-tubular capillary and glomerular inflammation (Gloor et al. 2007 ; Maryniak et al. 1985; Sis et al. 2007) although it may also occur independently from interstitial fibrosis, tubular atrophy and or transplant arteriopathy (Gloor et al. 2007 ; Sis et al. 2007). On ultra-structural examination, this lesion appears as expansion of the sub-endothelial space with deposition of flocculent or fibrillary material, expansion of mesangial matrix and interposition of mesangial cell cytoplasm within the lamina densa (Zollinger et al. 1973). Endothelial cell swelling and acute glomerulitis precede GBM duplication suggesting that endothelial injury is the initial insult that results in GBM remodelling.

The evolution of ultra-structural changes is not well elucidated. Early studies reported acute endothelial cell swelling, mesangiolysis, and glomerular hyper-cellularity which is later followed by a chronic lesion characterised by reduplication of glomerular basement membrane (Habib et al. 1987; Habib et al. 1993a; Habib et al. 1993b). Maryniak et al described "evolving, intermediate and chronic" phases in the development of transplant glomerulopathy based on electron microscopy studies (Maryniak et al. 1985). These studies were limited by, heterogeneity of study groups making it impossible to exclude glomerular abnormalities due to recurrent disease, and lack of sufficient consecutive EM biopsy material to demonstrate ultra-structure progression through each of the different stages. It is also envisaged that the disease entity as described then is different to what we now see due to differences in immunosuppressant regimens and other time dependent factors.

More recent studies have described the association between light microscopy features of TXG and duplication of the peri-tubular capillary basement membrane suggesting that the process that results in TXG involves the entire glomerular and peri-tubular capillary beds of the transplanted kidney (Ivanyi et al. 2000; Ivanyi 2003; Regele et al. 2002). Peri-tubular capillary basement membrane multi-lamination (PTCBMML) in association with TXG was first described in the 1990s (Monga et al. 1990; Monga et al. 1992). It is classified as mild, moderate and severe depending on the number of basement membrane

layers. PTCBMML of more than 6 layers is associated with TXG (Drachenberg et al. 1997; Ivanyi 2003).

1.2.5.7.3.3 The triad of Transplant Glomerulopathy, HLA Antibodies, and Complement Activation

Glomerular basement duplication and PTCBMML are believed to be a consequence of allo-immune injury in the kidney capillary network. This hypothesis relies on establishing the complex relationship between glomerular and peri-tubular capillary abnormalities, evidence of complement activation in PTC, in the presence of circulating donor specific antibody.

Until recently, it was unclear whether TXG was the result of humoral or cellular immune responses. Initial studies in animal models showed that antibodies against donor glomerular and tubular basement membranes antigens were produced in Lewis rats with glomerulopathy (de Heer, 1994). However, others reports described glomerular infiltration of activated T cells in patients with glomerulopathy and higher incidence of glomerulopathy following human sub-clinical rejection and experimental chronic rejection (Akalin et al. 2003). Recent human studies, report that TXG is more common in sensitized patients with HLA antibodies, those with prior acute antibody mediated rejection and presence of donor specific class II antibodies. The incidence is higher if both class I and Class II antibodies are present (Colvin et al. 2005; Gloor et al. 2007).

Association with capillary endothelial C4d deposits provided the link between mechanisms of tissue injury, histopathology, and circulating antibody further strengthening the hypothesis. C4d deposition in peri-tubular capillaries was found in patients with glomerulopathy but not in patients with IgA nephropathy (Vongwiwatana et al. 2004). Mayueddi et al demonstrated C4d deposition in over 50% with Transplant glomerulopathy and /or arteriopathy. Over 80% of these cases with C4d had circulating anti-donor HLA antibody, and there was strong association between C4d deposits and presence of peri-tubular capillary multi-lamination of more than 5 layers (Mauiyyedi et al. 2001). Regele et al found C4d deposits in 34% of 213 allograft recipients and also demonstrated a strong association with TXG and PTCBMML (Regele et al. 2002).

A few studies do not support such a strong association between transplant glomerulopathy, graft complement activation, and circulating HLA antibody. Cases of glomerulopathy without detectable HLA antibody or C4d at the time of diagnosis have been reported (Al Aly et al. 2005; Namba et al. 2006; Nickeleit et al. 2002; Nickeleit et al. 2003, Sijpkens et al. 2003). C4d deposition did not predict development of glomerulopathy raising concerns about specificity in chronic antibody mediated rejection (Akalin et al. 2003). It is unclear whether these findings can be explained by variations in antibody strength, effects of complement regulatory factors, the natural history

of TXG or technical factors relating to methods of antibody detection, complement detection, and diagnosis of TXG.

In summary, antibody mediated graft injury may have variable morphological features within the renal allograft. It seems plausible that low level antibody detected with sensitive assays may result in accommodation due to incomplete activation of complement rather than acute antibody mediated rejection. However, the effect of such antibodies on the occurrence of chronic antibody mediated injury is unclear. Limitations in the techniques used for the diagnosis of transplant glomerulopathy are likely to contribute to this uncertainty.

1.2.5.8 Treatment of Antibody-mediated rejection

Antibody mediated rejection can be resistant to therapy and carries a poor prognosis. Treatment protocols incorporate multiple approaches which include suppression of the T cell dependent antibody response using anti-thymocyte globulin, calcineurin inhibitors and mycophenolic acid, removal of donor reactive antibody using plasmapheresis or immuno-adsorption, blockade of residual antibody with intravenous immunoglobulins and depletion of naïve and memory B cells with rituximab. In comparison to acute antibody mediated rejection (AAMR), treatment for chronic antibody mediated rejection (CAMR) is not well described. In AAMR large titres of allo-antibody result in massive complement activation and lytic injury to the graft endothelium requiring aggressive removal of the alloantibody. In contrast, it is postulated that CAMR occurs because of low level

antibody with complement activation causing sub-lytic injury in a slow progressive manner. Consequently, the adequate therapeutic approach to CAMR remains to be determined.

Combinations of TAC and MMF have been proposed as rescue therapy based on the beneficial effects of MMF demonstrated in transplant recipients with chronic rejection (Allison et al. 2000; Dudley et al. 2005; Halloran et al. 1997). TAC and MMF are thought to have an effect on B cell function (Fulton et al. 1996; Halloran 2004). In a small case series, where TAC/MMF replaced CSA/AZA in patients with biopsy proven CAMR, there was a reduction of DSA titres (Theruvath et al. 2001), although these results were not reproduced in other studies (Schwarz et al. 2006). It is therefore not clear whether these agents are the treatment of choice for acute AMR and CAMR. The roles of plasma exchange, intravenous immunoglobulin (IVIG) and rituximab are still undergoing evaluation.

1.3 Rationale for Thesis

The rationale for this thesis is that despite the extensive body of evidence supporting the hypothesis that HLA antibodies have a significant role in the pathogenesis of chronic renal allograft injury, there are issues that remain unresolved. These issues are outlined below.

Firstly, the clinical relevance of a cross-match test performed with solid phase assays in the pre-transplant setting is still uncertain. It is unclear whether a positive test should constitute a contraindication to

transplantation or simply reflect a degree of risk. The lack of clarity arises from observations, which suggest that sensitive assays reveal covert sensitisation, which is below thresholds of detection by CDC and other less sensitive assays.

Secondly, although histological patterns of graft injury have been described, it is difficult to predict which patients are likely to exhibit these abnormalities. As an example, some grafts continue to function even in the presence of circulating of HLA antibody (Terasaki et al. 2007). There are differing explanations for this observation. One opinion suggests that in time, these grafts eventually fail whereas another suggests that there are factors that determine whether graft injury in the presence of circulating antibody. Cumulative endothelial damage is likely to be a gradual process and the extent of injury sustained may be modified by regulatory mechanisms leading to accommodation as discussed in section 1.2.5.7.1. In fact Colvin et al proposed that chronic antibody mediated rejection occurs through a series of stages initiated by alloantibody production, followed by interaction of antibody with endothelium resulting in C4d deposition. Complement activation then leads to graft injury and irreversible by histo-pathologic abnormalities (Colvin et al. 2005). Factors likely to contribute to a delay in appearance of detrimental effects of antibody include, antibody characteristics such as class, isotope, binding capacity, time of appearance (pre-transplant *versus* de novo) and the ability to fix complement (Bohmig et al. 2008). Recent advances in

antibody detection techniques provide an opportunity to investigate these factors further.

Thirdly, the effect of non-HLA antibodies on graft outcomes and histology is still under evaluation. Of special interest are MICA and MICB antibodies discussed in section 1.2.5.4. Detection of these antibodies is now possible with new techniques. Their role in antibody mediated injury is of particular interest because antibodies are expressed on vascular endothelium, which is the primary interface of antigen-antibody interactions.

Fourthly, histological components of chronic antibody mediated rejection (transplant glomerulopathy, peri-tubular capillary multi-lamination, and C4d deposition) are not consistently observed in patients with HLA antibody. Although protocol biopsies have enabled early evaluation of histological graft damage in specific groups of patients before end-stage graft damage occurs, transplant glomerulopathy remains a late diagnosis and its evolution is not well elucidated. Sensitive diagnostic techniques applied at earlier time points are likely to identify pathology predictive of later graft damage. This would improve diagnosis, provide better correlation with aetiological factors, and potentially allow early intervention before irreversible graft damage occurs.

Finally, effective interventions in patients with chronic antibody mediated rejection remain elusive. Current modalities aim to reduce

levels of antibody prior to transplant through desensitisation protocols, treatment of acute antibody mediated rejection episodes, and identification of patients at risk through post transplant DSA monitoring. However, as discussed in section 1.2.5.8, there are no specific options available for the treatment of chronic antibody mediated rejection.

1.4 Hypotheses

Several hypotheses are generated from this review.

1) Solid phase assays identify patients with donor specific antibodies who appear to be unsensitised by CDC testing. When present, antibodies predict graft dysfunction, histopathological abnormalities and ultimately graft loss. However, their effect is determined by several factors such as antibody strength and immunosuppression regimen. In addition to HLA antibodies, presence of MICA antibodies in pre-transplant sera results in histological abnormalities and subsequent graft dysfunction.

2) In recipients with HLA pre-sensitisation, specific ultra-structural abnormalities which predict histopathological features of chronic antibody mediated graft injury (described in section 1.2.5.7.3), can be detected before graft dysfunction and light microscopy features are evident. Recognition of these ultra-structural abnormalities results in better correlation between complement activation and HLA antibody.

3) Modification of maintenance immunosuppression ameliorates the effects of chronic graft injury. In the absence of specific therapies for chronic antibody mediated injury, modification of immunosuppression can be used to reverse the effects of long term antibody mediated injury.

To examine these hypotheses, 4 studies were undertaken. Chapters 3 describes a longitudinal study undertaken to evaluate the effects of pre-transplant HLA antibodies detected using the Luminex® assay. The aim of the study was to evaluate incidence and patterns of antibody mediated graft injury in transplant recipients with donor specific HLA antibodies. Factors that influence outcomes were investigated. Functional and histological data from sequential biopsies was analysed against pre-transplant antibody status. The relationships between antibody strength, graft dysfunction, complement deposition, and histological abnormalities were examined. Chapter 4 describes a longitudinal study evaluating the effects of non-HLA antibodies on graft histology and functional outcomes. MICA antibodies were assessed using the Luminex® assay. MICA antibody status was evaluated against sequential protocol biopsy histology and isotopic GFR. In chapter 5, evolution of histological features of chronic antibody mediated rejection was evaluated using electron microscopy. The aim of the study was to examine ultra-structural features predictive of transplant glomerulopathy. Sequential protocol biopsies from patients with established transplant glomerulopathy were analysed and

correlated with presence of HLA antibody, complement deposition, and graft function. In chapter 6, the effects of immunosuppression on chronic glomerular histology were evaluated using light and electron microscopy techniques. Sequential protocol biopsies in patients treated with azathioprine were compared against patients treated with mycophenolate mofetil.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

This chapter describes the study group and methods used to test the hypotheses described in section 1.4. The 4 analytical tools are light microscopy, electron microscopy, C4d immunoperoxidase staining and evaluation for donor HLA antibody.

2.1 Study Group

The study group consisted of 241 consecutive patients with type 1 diabetes mellitus who received a simultaneous kidney and pancreas transplant at Westmead from 1987-2007 (Nankivell et al. 2003). This group had a protocol post-transplant follow-up involving a transplant biopsy and graft functional studies including isotopic GFR and OGTT. Results from the biopsies were entered into a data base referred to as the Westmead longitudinal histological database in this thesis. Demographic and other clinical data were collected from patient medical records, the Westmead post-transplant flow chart, and the RISC data base. Patients were treated with immunosuppression therapy which incorporated either Cyclosporine or Tacrolimus with Azathioprine or Mycophenolate mofetil and prednisolone depending on protocols in use at time of transplant. From this core study group, specific criteria were used to recruit patients into each of the studies.

2.2 Renal Biopsy

Each of the patients had three or more sequential kidney transplant biopsy specimens performed and processed for light and electron

microscopy studies with separate cores preserved in paraffin sections for later use. Biopsies were performed at 0, 1, and 3 months and then 1,3,5,7 and 10 years after transplantation. Needle core biopsies were obtained from the cortex within the upper-pole of the allograft, using an automated sterilised biopsy gun (Promag, Northbrook, Il) and an 18 gauge single use disposable biopsy needle (World Wide Medical Technologies, Oxford, CT). Implantation biopsies were obtained from the donor allograft after dissection of surface adipose tissue in the operating theatre during perfusion. Post transplant biopsy procedures were performed as out-patient services.

2.3 Light Microscopy analysis

Renal biopsy light microscopy analysis was performed by 2 blinded observers according to the Banff working classification (Racusen et al. 1999). Transplant glomerulopathy was defined using the Banff schema and scored by the extent of persistent peripheral capillary loop involvement in the most affected non sclerotic glomeruli. A score of cg0 is no glomerulopathy, cg1 is 10-25% of the most affected peripheral capillary loops, cg2 is 26-50% and cg3 is greater than 50% of affected loops (Racusen et al. 1999; Racusen et al. 2003; Solez 1994).Thrombotic microangiopathic processes, which may produce similar glomerular histology, were excluded by appropriate clinical and blood testing. When diagnosed by 1 of the 2 blinded observers, glomerulopathy was confirmed by electron microscopy. Although contemporaneous immunofluorescence was not routinely undertaken in

the protocol biopsy studies, retrospective EM review excluded *de novo* glomerulonephritis (all recipients' experienced renal failure from diabetic nephropathy and so recurrent glomerulonephritis was excluded as a confounding factor). Assessment of global glomerulosclerosis and quantification of other Banff qualifiers, including mesangial matrix (mm), arteriolar hyalinosis (ah), chronic interstitial fibrosis (ci) and tubular atrophy (ct) scores were also undertaken. Acute rejection was defined clinically by a 25% increase in serum creatinine supported by biopsy. Subclinical rejection was defined as proven acute rejection without immediate functional deterioration and categorised as acute subclinical rejection (SCR-A) when acute rejection was present (Banff grade IA and above) and borderline subclinical rejection (SCR-B) when borderline changes were present (Solez et al. 1993). Cyclosporine nephrotoxicity was defined by the presence of striped cortical fibrosis or de novo or increasing arteriolar hyalinosis (not attributable to renal ischemia or donor hyalinosis from implantation biopsies), which was often supported by the presence of tubular micro-calcification (not related to preceding biopsy evidence of acute tubular necrosis). Biopsies were evaluated for peri-tubular capillary inflammation using the PTC score based on the highest number of inflammatory cells observed in 10 hpf fields (Gibson et al. 2008).

2.4 C4d Staining

C4d staining was performed retrospectively on paraffin sections from available sequential samples obtained at implantation, 0, 1, 3, 6 months

and annually up to 5 years post transplant. This analysis was performed independently from the Banff light microscopy analysis. After initial de-waxing with histolene and re-hydration in graded alcohols, antigen retrieval (Universal Decloaker, Biocare Medical, Concord, CA, USA) was followed by primary polyclonal anti-human C4d antibody (Biomedica, Vienna) and immunoperoxidase then counter-stained with hematoxylin (Regele et al. 2001). Severe antibody-mediated rejection and tissue without primary antibody were used as positive and negative control, respectively. Positive PTC staining was defined as bright linear circumferential staining along PTC in renal cortex and/or medulla. The proportion (%) of peri-tubular capillaries staining for C4d was derived by the number of positive PTC relative to the total number of PTC evaluated from 8-10 contiguous high power fields. Positive C4d staining was defined by diffuse (>50%), circumferential PTC staining (Poduval et al. 2005; Racusen et al. 2003; Solez et al. 2008). Positive glomerular staining was defined as positive bright linear staining of glomerular capillaries. A biopsy was classified as positive when more than 50% of glomerular capillaries had C4d staining in each of 4 glomeruli per biopsy. (Figures 2.1, 2.2, 2.3 and 2.4)

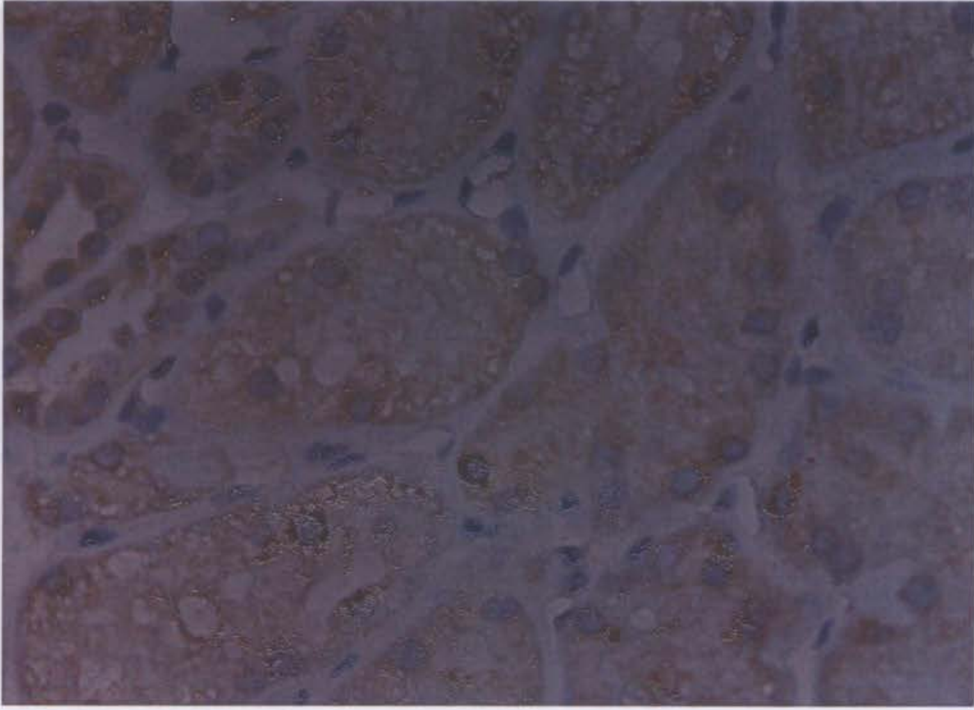


Figure 2.1: Renal biopsy, showing negative C4d staining in peri-tubular capillaries

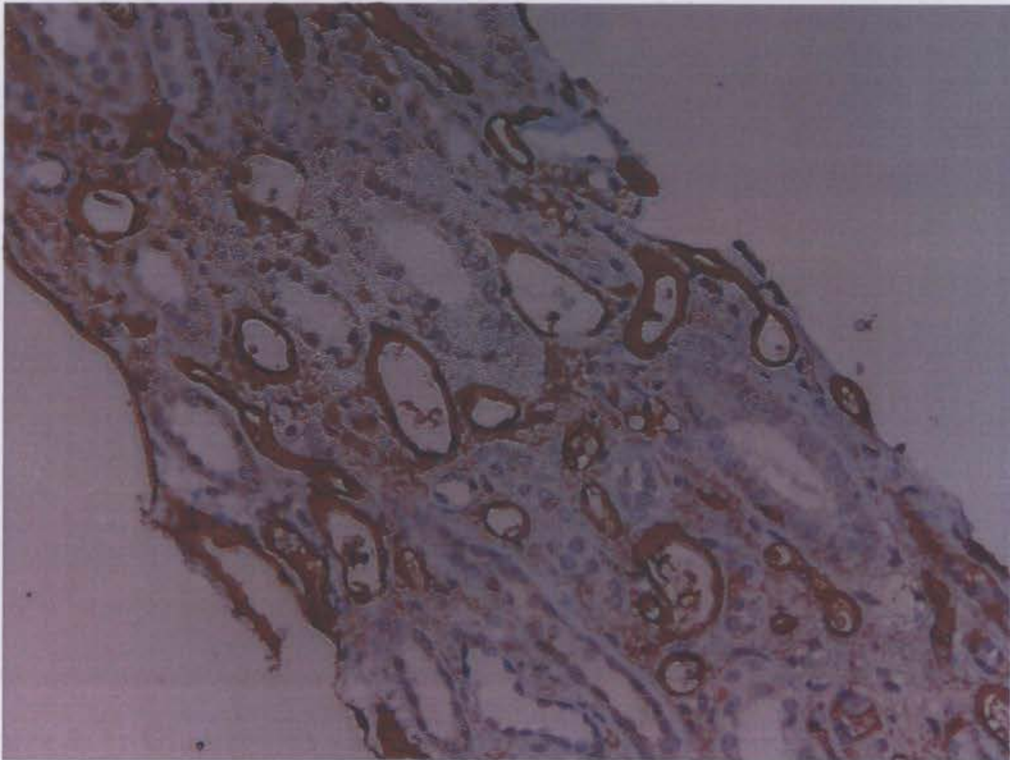


Figure 2.2: Renal biopsy showing circumferential C4d staining in peri-tubular capillaries.

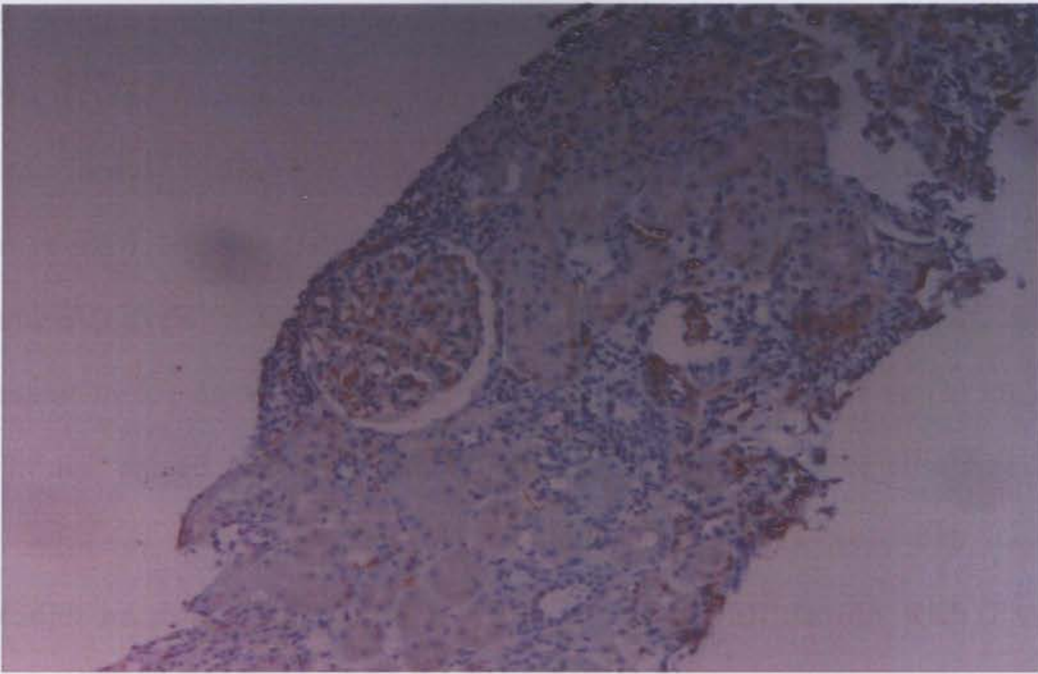


Figure 2.3: Renal cortical tissue showing negative glomerular and peri-tubular capillary C4d staining.

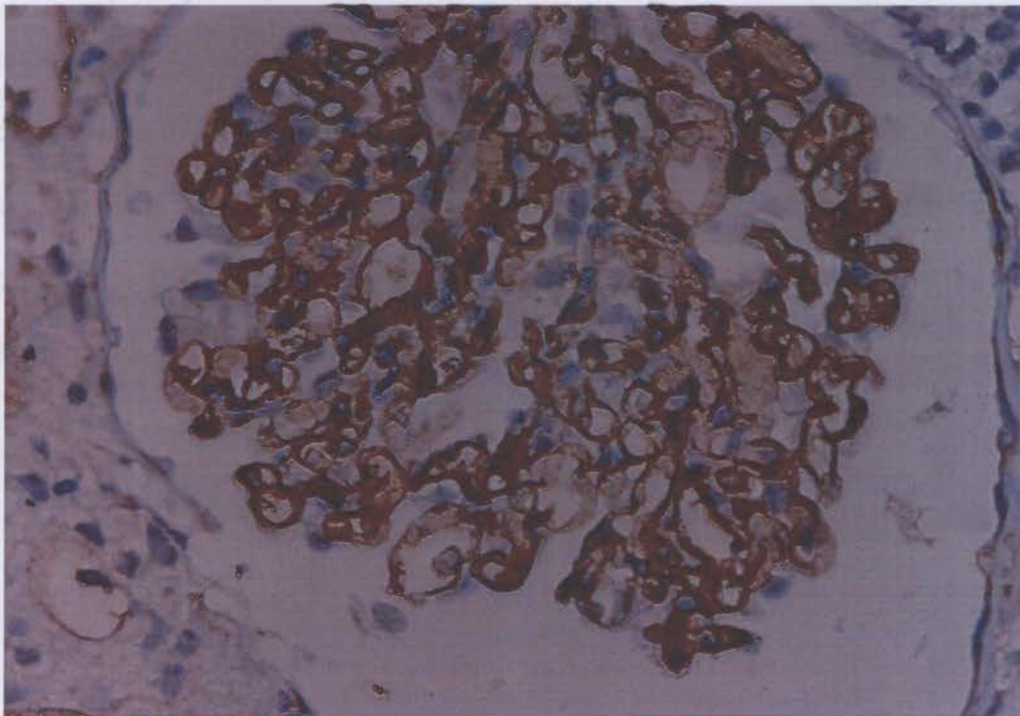


Figure 2.4: Glomerulus showing positive C4d staining.

2.5 Transmission Electron Microscopy

2.5.1 Tissue fixation and embedding

After renal biopsy, tissue for electron microscopy studies was transported in modified Karnovsky fixative (2% formalin, 2.5% glutaraldehyde in 0.1M Mops buffer, pH 7.4). Blocks of $1mm^3$, were washed in 0.1 M MOPS buffer, post-fixed in 2 % osmium tetroxide for 2 hours, rinsed briefly in distilled water and then further post fixed in 2% aqueous uranyl acetate for one hour. The blocks were dehydrated through an ethanol series (50-100%), prior to infiltration with TAAB TLV ™ (TAAB Laboratories Equipment Ltd, Aldermaston) epoxy resin. Blocks were embedded in Easy–Molds ™ (Leica Microsystems, Vienna) and polymerised at 70^0 C for ten hours (Figure 2.5). From each biopsy, up to 7 blocks were available for section preparation. A block was chosen at random for preparation of ultra thin sections. This process was done in two stages, with the initial stage involving cutting and staining of a semi-thin section (500nm thickness) to identify glomeruli, followed by cutting and staining of ultra-thin sections (89nm thickness) for transmission electron microscopy.

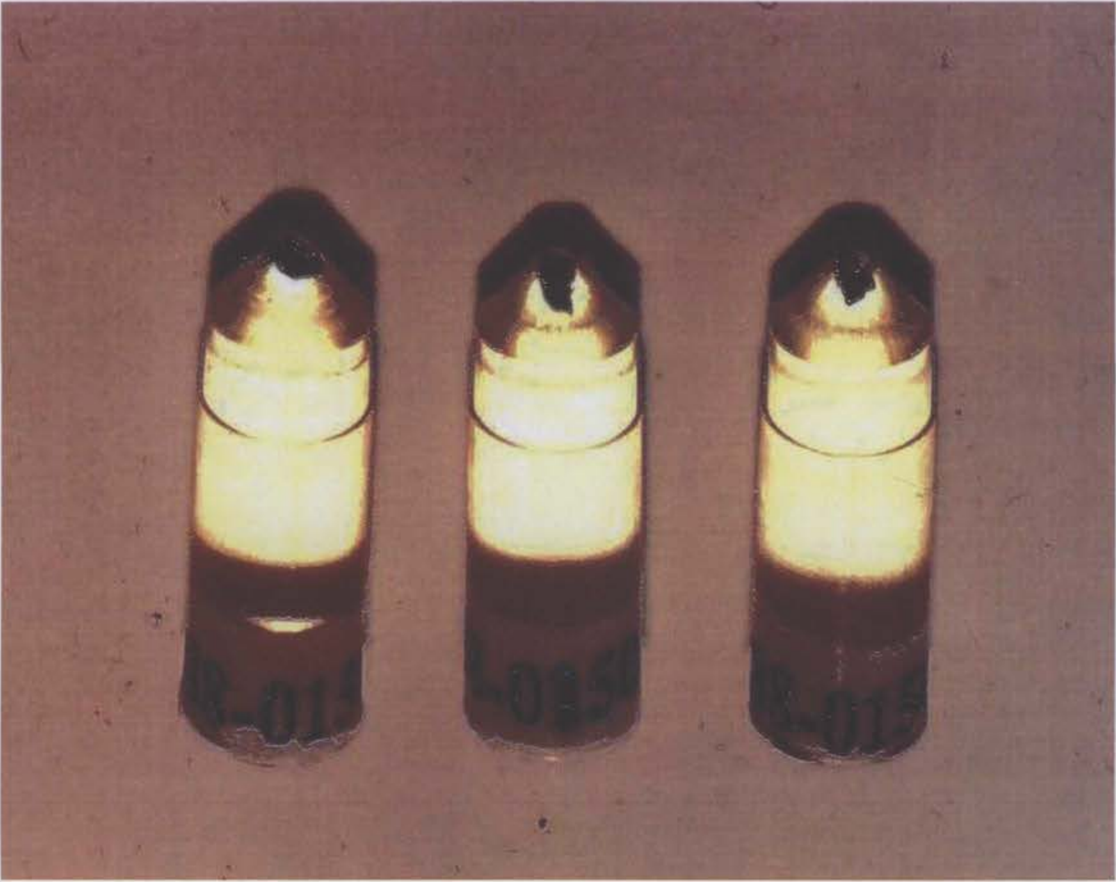


Figure 2.5: Epoxy resin blocks, embedded with renal cortical tissue

2.5.2 Block trimming

The resin block was mounted into a block holder and specimen head on an Ultracut UCT^{TM} ultramicrotome (Leica Microsystems, Vienna) presented in (Figure 2.6). The block head was "faced up" using a "Pittsburgh" glass knife (prepared from glass strips broken on a LEICA KMRO Knife maker) to smoothen it, then removed and clamped into a trimming stand under the microscope. Using a sharp knife, resin was trimmed to produce a trapezoidal block face.

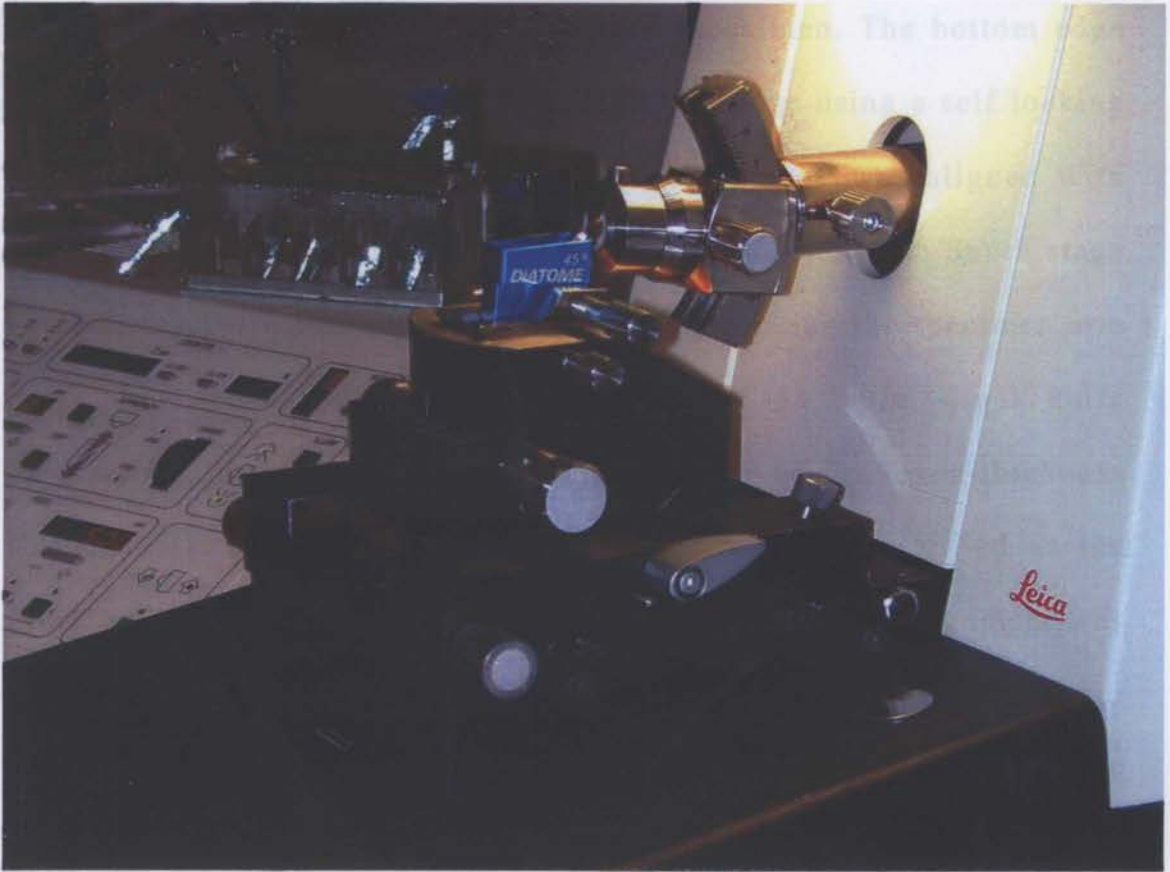


Figure 2.6: Leica ultra-microtome with block in specimen holder

2.5.3 Semi-Thin Sections

After trimming, the block holder was remounted into the specimen head, the trimming stand was replaced with a knife stage, and a glass knife with a water bath was mounted onto the knife stage at a clearance angle of 6°C. The water bath was filled with filtered distilled water until a negative meniscus was formed at the knife edge. The glass knife and water bath were manually advanced toward the specimen, the stage locked at a minimal distance between specimen and knife, and the knife aligned laterally using the east-west control to have the first 1/3

portion of the knife in contact with block specimen. The bottom edge of the block was aligned parallel to the knife edge using a self locking drive on the specimen head, whilst the block face was aligned with direction of cutting using the arc adjustment and the North/South stage control, the knife was advanced while gently rocking the specimen arm using the hand wheel to move the block past the knife. Block knife alignment was confirmed by appearance of a bright, even-thickness sharp line (a reflection of the knife edge), which diminished as the block and knife where advanced towards each other until alignment was achieved. Trimming was continued gently until the first complete sections appeared onto the water surface. After initial sectioning, the semi-thin feed was set to 500 nm to cut more sections. Sections were then collected using a teflon probe, dropped onto a slide with filtered water and then placed on a hotplate at 80^0C degrees and allowed to dry.

2.5.3.1 Staining Semi Thin Sections

Semi thin sections were allowed to dry for 15 minutes and then stained with 1% toluidine blue (Scharlau Chemie, Barcelona) in 1% borax while still on the hot plate for 2 minutes. Stain was washed off with running water and a dry cover-slip applied with mounting medium. Section quality and staining where checked with a light microscope and glomeruli for analysis were selected avoiding sclerosed and incomplete glomeruli. If glomeruli were inappropriate for ultra-thin sectioning or

the block had no glomeruli then another block was selected and the process above repeated to identify suitable glomeruli for analysis.

2.5.4 Ultra-thin Sections (86nm)

The block was trimmed to a smaller trapezium size, as described above then remounted into the specimen arm of microtome and aligned to a diamond knife (Diatome, Switzerland). Knife to specimen alignment was performed as described above. The final approach was made in increments of less than 200nm at a cutting speed of 1mm/second. Section thickness was judged by interference colour of the section on water. Usable sections are of silver to pale gold colour corresponding to thickness 60-90 nm. The sections were flattened using chloroform vapour and carried onto standard 400 mesh copper hexagonal grids (Gilder) by submerging the grid clamped onto forceps and withdrawing it through the ribbon of sections. Sections were held stationary if necessary with an eye lash or Teflon probe. Three grids were prepared for each biopsy specimen (Figure 2.7).

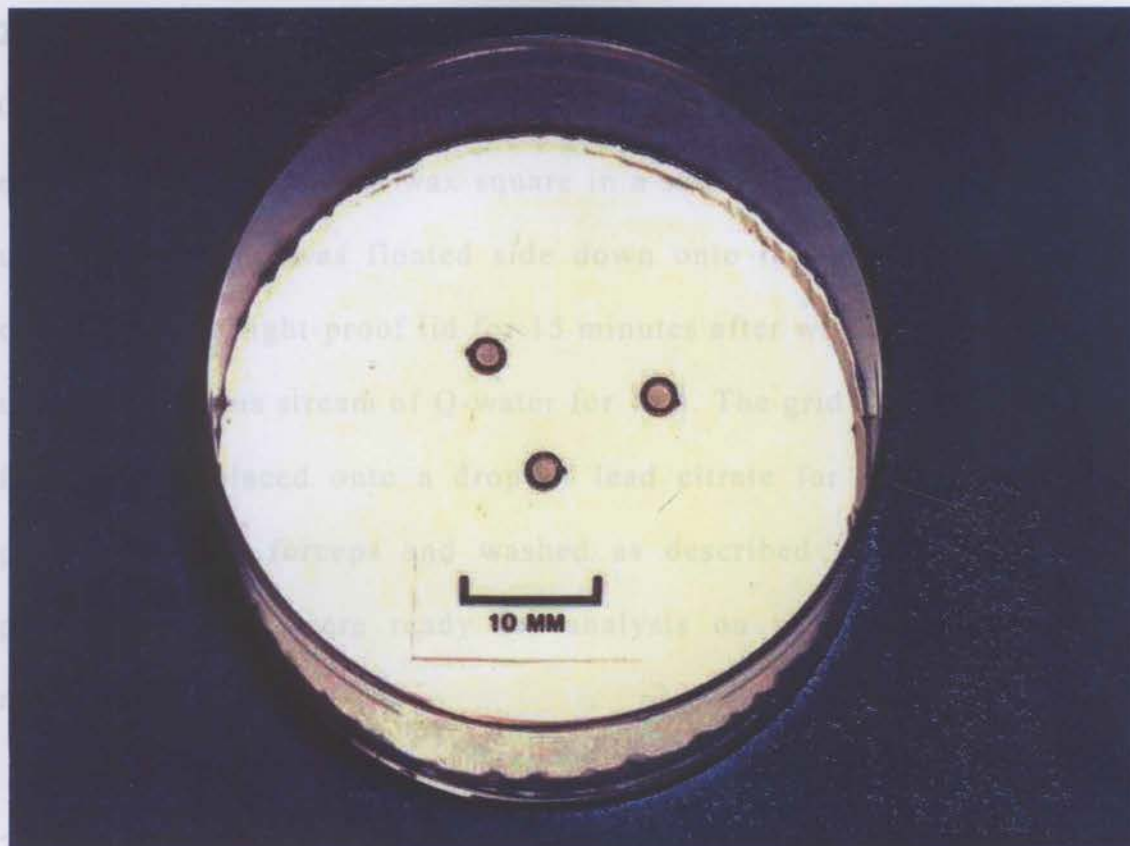


Figure 2.7: Cover dish showing copper grids used for transfer of ultra-thin sections for electron microscopy.

2.5.4.1 Staining Ultra-Thin Sections

Reagents

Two percent Uranyl acetate was prepared by adding 0.5 ml of 4% uranyl acetate to 0.5 mL of absolute ethanol and centrifuging at 6000 rpm for 5 minutes in a 1.5 mL Epperndorf tube before use. Reynold's lead citrate was prepared by adding lead nitrate AR 1.33 g to trisodium citrate 1.76 g and 30mL of distilled water. The lead citrate preparation was stored at 4° C.

2.5.4.2 Staining methods:

One drop of uranyl citrate per grid (2% Uranyl acetate and 50% ethanol) was placed on a wax square in a staining dish. A grid with an ultra-thin section was floated side down onto the drop and the dish covered with a light-proof lid for 15 minutes after which it was washed under continuous stream of Q-water for 15 s. The grid was blotted with filter paper, placed onto a drop of lead citrate for 4 minutes, then picked up with forceps and washed as described above. After this process, sections were ready for analysis on transmission electron microscopy.

2.5.5 Data Acquisition and Analysis

Sections were examined on a Philips CM120 BioTWIN (FEI Electron Optics, Eindhoven) transmission electron microscope at 80kV (Figure 2.8). Images were collected with a mounted Morada digital camera operating on the iTEM platform (Olympus-SIS, Munster Germany). Sections were identified by laboratory serial number. Images were acquired and stored as tiff files for post acquisition analysis.

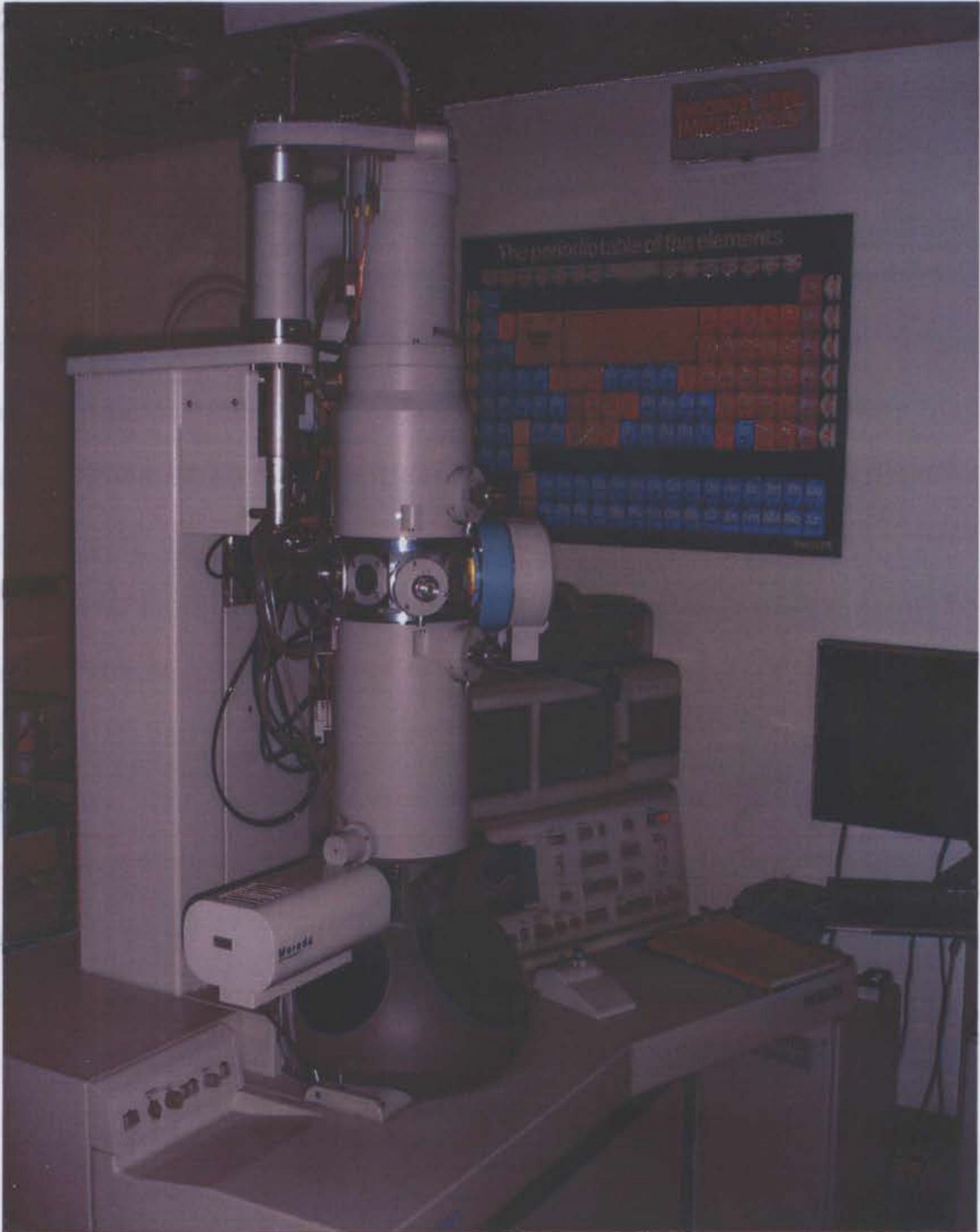


Figure 2.8: Philips CM120 BioTWIN Electron microscope

2.5.5.1 Glomerular Capillary Loop Ultrastructure

One to two glomeruli were examined per biopsy time-point. Intra-glomerular sampling comprised selection of 8 to 10 glomerular capillary loops, using a random start point by assignment of the top left-hand corner of the hexagonal grid to a number 1, and then proceeding anticlockwise to avoid image overlap at lower magnification. Images were obtained from the peripheral capillary loop at 3, 9, and 12 o'clock positions at 17,500x and 24,500x magnification (depending on the capillary loop orientation). These images were used to assess, morphological changes of the endothelial membrane, lamina rara interna (LRI), lamina densa (LD), lamina rara externa and epithelial cell foot processes. The morphological features of the capillary loop endothelial membrane were evaluated for the following characteristics; loss of fenestrae, endothelial thickening, vacuolation, and extensions of endothelial membrane into the lamina interna giving rise to a serrated appearance. LRI was assessed for structural expansion, presence of osmiophilic inclusions, and formation of new layers of LD.

2.5.5.2 Glomerular Basement Membrane diameter

Images at 3,400 magnifications were used for estimation of the glomerular basement membrane (GBM) width. To eliminate operator bias, capillary loops were photographed at 3, 9 and 12 o'clock positions. The orthogonal intercept method was used to measure GBM (Caramori et al. 2003; Dische 1992; Jensen et al. 1979). A 2000 nm x

2000nm grid was randomly superimposed on the photomicrograph using imaging software (Soft Imaging Systems, analySIS, GmBh, Germany). An intercept point was defined by the intersection between a gridline and the capillary loop endothelium. The basement membrane thickness was assessed as the diameter from the intercept point, to the epithelial cell membrane, in a line perpendicular to the plane of the GBM. The mean GBM width was expressed as the harmonic mean of orthogonal intercepts and expressed in nm (Figure 2.9).

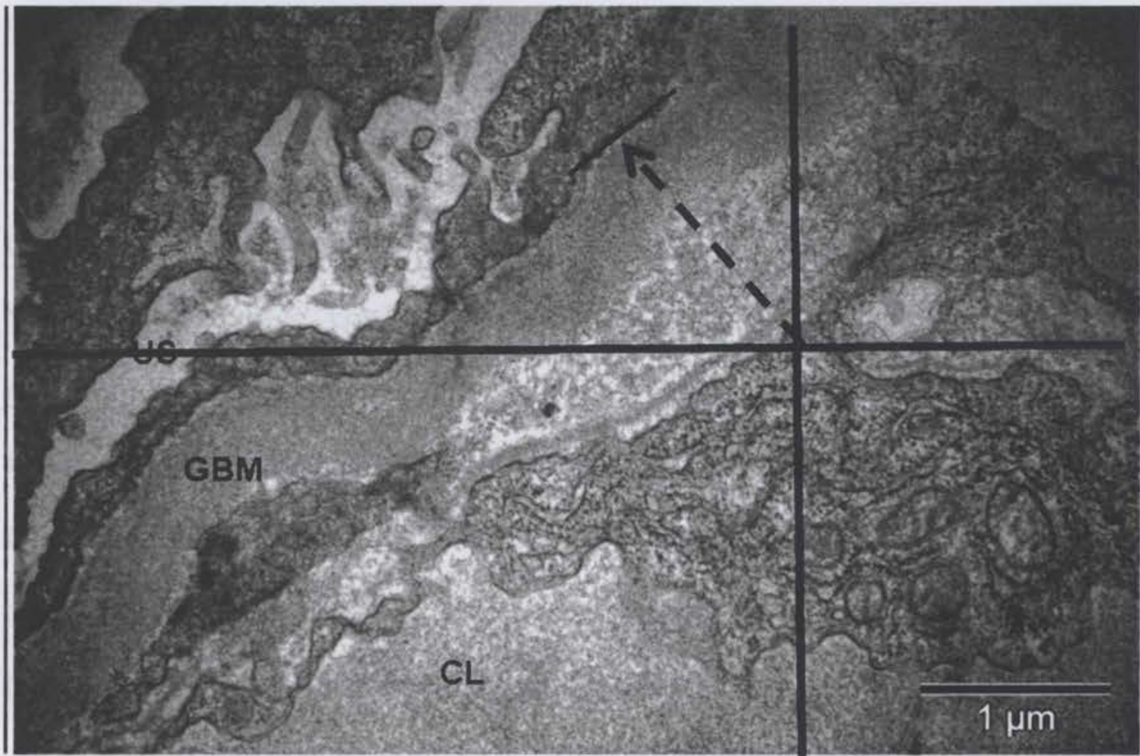


Figure 2.9: EM photomicrograph demonstrating measurement of GBM width. Intercept point = intersection between grid lines and capillary endothelium (thick). GBM width is distance from intercept point to epithelial membrane (dashed arrow) expressed as harmonic mean.

2.5.5.3. Mesangial morphometry

Mesangial matrix and mesangial cell area-fractions were estimated from photomicrographs of whole glomeruli taken at 4200x magnification to identify mesangial areas, and at 9700x to identify mesangial matrix and cellular fractions. Mesangium was defined by the following criteria: loss of parallelism between the endothelial and epithelial surface; presence of basement membrane like material; identification of a mesangial cell or mesangial cytoplasm; and presence of mesangial GBM. Mesangial cell bodies were identified by nuclear notching and dense cytoplasmic contents. Endothelial cells were differentiated by the presence of a capillary lumen and fenestrated endothelial membrane. Point counting by a 126-point randomly overlaid grid from morphometric analysis software (Soft Imaging Systems, analySIS, GmBh, Germany) was applied. Area fractions of mesangial matrix and mesangial cells were computed with points falling on the entire sampled glomerular profile as a reference. The fractional area of total mesangium (Mes/glom); mesangial matrix, (MM/glom); and mesangial cells (MC/glom) were thus defined (Fioretto et al. 1994; Osterby 1986) (Figure 2.10).

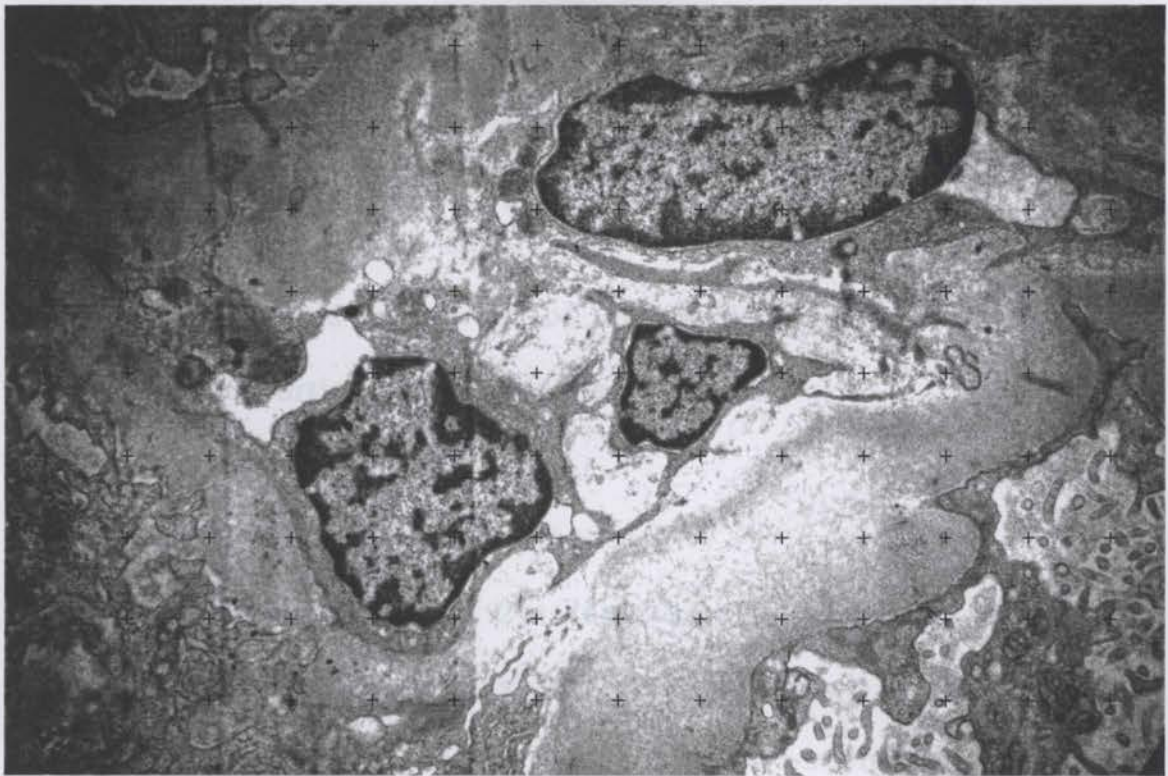


Figure 2.10: Electron micrograph showing a point grid overlaid on EM photomicrograph for assessment of mesangial area fractions.

2.5.5.4 Peri-tubular Capillary Ultrastructure

PTC ultrastructure was evaluated from 4 to 5 peri-tubular capillaries, selected from the same biopsy as the light microscopy time point. Where possible PTC located close to previously sampled glomeruli were used for this analysis. Image acquisition was performed as described in section 2.5.5. Peri-tubular capillary basement membrane layers were counted to asses for multi-lamination (PTC-BMML) and endothelial cell abnormalities such as thickening, fenestration, and serration using images taken at 7,400x magnifications. For these evaluations, the capillary circumference was divided into 4 quadrants

by the longest longitudinal axis and the longest axis perpendicular to this (Ivanyi et al. 2000). Basement membrane layers for each quadrant were counted as the highest number of layers observed and averaged for each capillary profile. Endothelial abnormalities where noted as presented if observed in any one of the capillary portions and results presented as proportions of the criteria of interest.

2.6 HLA and Donor-Specific Antibodies

2.6.1 Complement Dependent Cytotoxic Assay

A standard CDC cross-match test was performed on all donor-recipient pairs prior to transplant. Donor lymphocyte and recipient sera were incubated at room temperature for 30 minutes; freshly thawed cold rabbit complement was added and incubated at room temperature for 60 minutes, after which fluorescent stain was added. Trays were covered with foil for at least 15 minutes. Test reactions were scored using the recommended ASHI scoring system by estimating percentage of cell death beyond that of background or negative control. Results were validated using positive and negative control sera. Extended incubation time was performed to increased sensitivity.

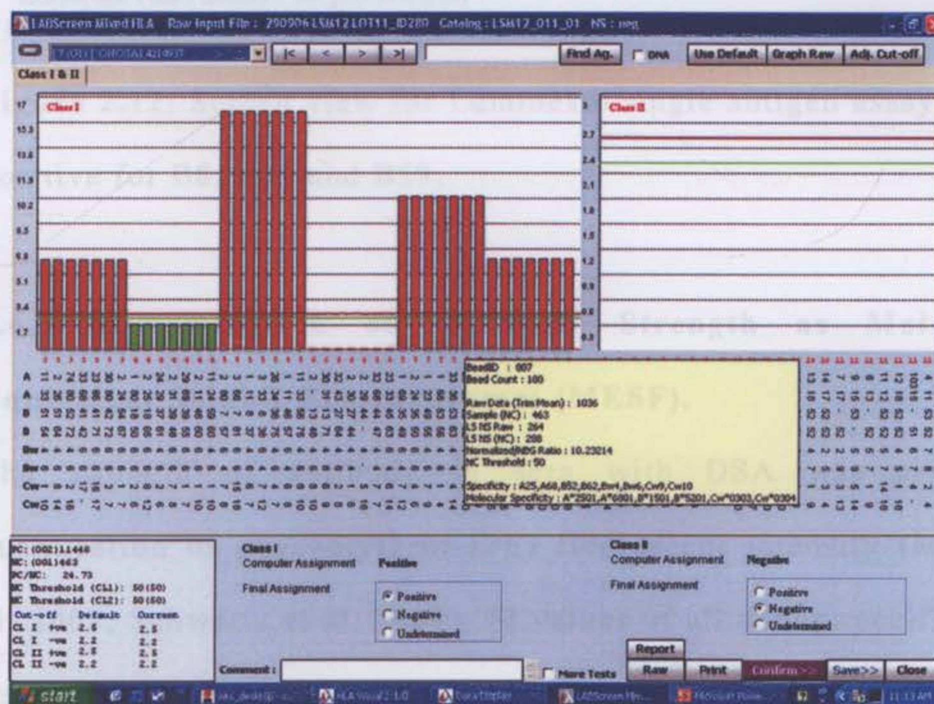
2.6.2 Luminex assay

The Luminex® assay (El-Awar et al. 2005) was performed at the Tissue Typing laboratory, Australia Redcross and Blood service (ARCBS). Sera from all SPK recipients was collected and stored at the ARCBS cold room prior to transplantation. Sera were screened for HLA

antibody using HLA class I and II mixed beads (LABScreen®, One Lambda Inc. Canoga Park CA), as described below: 5µl of LABScreen® beads were incubated with 20 µl of patient serum and incubated in the dark for 30 minutes at 20-25°C with gentle shaking. A negative control serum was tested to establish background values. After incubation150 µl of diluted wash buffer was added to the well plate, and then centrifuged at 1300 g for 5 minutes followed by addition of 200µl of 1x wash buffer and centrifuged at 1300 g for 5 minutes. After second wash, 100 µl of 1x R-Phycoerthrin(PE)-conjugated goat antihuman IgG was added to each well. The well plate was then incubated in the dark for 30 minutes at room temperature then centrifuged at 1300 g for 5 minutes. Conjugated anti human IgG was removed by vacuum aspiration, the plate washed with wash buffer then 80 µl of 1X PBS was added prior to data acquisition.

The LABScan™ 100 flow analyser was used to detect fluorescent emission of PE from each bead after calibration according to the Luminex® user's manual. Serum reactivity was assessed by the fluorescence intensity signal (FI) for each HLA bead after correction for non-specific binding to the negative control bead. The fluorescent signal value used was the trimmed mean according to the user's manual. The positive/negative cut-off values were determined according to manufacturer instructions. Sera positive for HLA antibody on Labscreen I and II were then tested by single antigen beads (One Lambda Inc,) and bead specificity was assigned according to

manufacturer's specification (The Luminex 100TM Users manual Luminex® Corporation PN-89-00002-00-00-5). Donor specific antibody was defined as a positive Luminex® SAG test for mismatched donor antigen. Serological techniques were used for donor and recipients HLA typing. Documented mismatches at HLA A, B and DR loci were used for determination of donor specificity. Antibodies to HLA C, DRB3, 4, 5 and DQB1, were not used to assign specificity. MICA antibody testing was performed using Luminex® beads according to manufacturer's specification. Figures 2.11 and 2.12 show screen views of results from the Luminex® assay.



Luminex HLA class I & II screen – class I positive

Figure 2.11: Screen view of Luminex® assay output for Labscreen Mixed HLA class I and II. Sera was positive for class I antibody but negative for class II antibody.

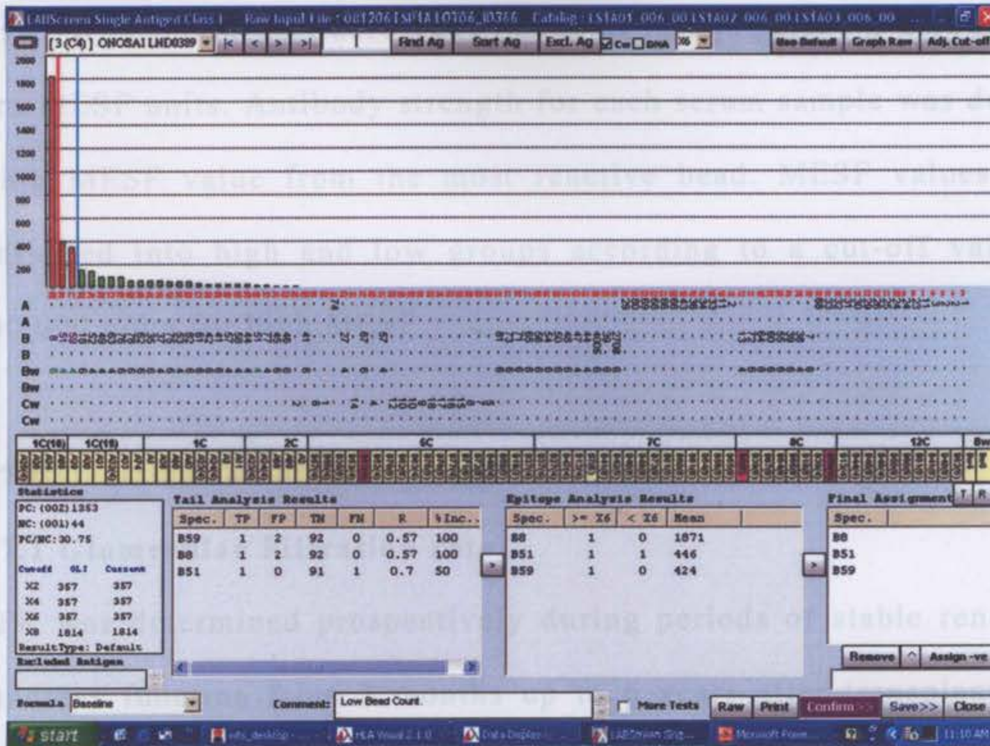


Figure 2.12: Screen view for Luminex® single antigen assay. Sera was positive for B8, B51 and B59.

2.6.3 Determination of Antibody Strength as Molecules of Equivalent Soluble Fluorochromes (MESF).

The strength of antibody in sera with DSA was assessed by quantisation of phycoerythrin (PE) fluorescent intensity (Mizutani et al. 2007; Schwartz et al. 2004). FI values of all donor specific reactive beads were converted into molecules of equivalent soluble fluorochromes (MESF) values using pre-calibrated Quantiplex™ beads (One Lambda, Canoga Park) with known graduated levels of fluorochromes similar to PE ranging from 0 to 50,000 units. A standard linear curve was generated using the LABMAS system. From this

curve, trimmed mean fluorescent values of test samples were converted into MESF units. Antibody strength for each serum sample was derived using MESF value from the most reactive bead. MESF values were classified into high and low groups according to a cut-off value of 100,000 units for high MESF.

2.7 Determination of Graft Function

2.7.1 Glomerular Filtration rate

GFR was determined prospectively during periods of stable renal and pancreas function from 3 months up to 6 years after transplantation. GFR was calculated after a single injection of Tc^{99m} DTPA from the disappearance of radioactivity of two timed plasma samples. This method is precise, accurate and reproducible and independent of variations in the volume of distribution of isotope. Serum creatinine was measured by Autoanalyzer (Hitachi 747 automatic analyser, Tokyo, Japan; Boehringer Mannheim reagents, FRG).

2.7.2 Oral Glucose Tolerance Test

Oral glucose tolerance tests (OGTT) were performed at 1, 3 and 5 years after transplant. OGTT were performed after overnight fasting, following ingestion of 75 g dextrose in 300 mL water (Glucaide, Histolabs, Sydney Australia) and collection of blood samples on ice at baseline and at 30 minute intervals until 3 hours. Samples were rapidly separated, frozen, then analysed in batches for C–peptide and insulin. Radioimmunoassays were used to measure insulin and C-peptide.

CHAPTER 3

PRE-TRANSPLANT DONOR SPECIFIC ANTIBODIES

3. PRE-TRANSPLANT DONOR SPECIFIC ANTIBODIES

3.1 Introduction:

This chapter examined the effect of pre-transplant donor specific antibodies detected using the Luminex® assay on graft outcomes in simultaneous kidney-pancreas transplant recipients. Although several studies have shown significant associations between the development of post-transplant HLA antibodies and inferior graft outcomes (McKenna et al. 2000; Terasaki 2003a; Terasaki et al. 2005), few have documented the impact of pre-transplant antibodies detected by sensitive solid phase assays.

Patel and Terasaki demonstrated that presence of antibodies against antigens expressed on donor white cells at the time of transplant is a major risk factor for hyperacute rejection (Patel et al. 1969). Findings from their landmark study are the basis for the lympho-cytotoxic cross-match test (CDC-XM), which is still widely used to assess immunological risk prior to transplantation. Currently, several techniques are available for detection and definition of HLA antibodies. These include solid phase membrane independent assays such as the enzyme linked immunosorbent assay (ELISA) and Luminex®. Luminex® incorporates purified HLA antigens attached to micro-particles. These assays provide increased sensitivity and specificity in antibody detection and donor recipient cross-matching (El-Awar et al. 2005; Pei et al. 2003; Taylor et al. 2009) compared with the widely used CDC assay, which is considered subjective,

cumbersome and occasionally difficult to interpret in the highly sensitised patient (Fuggle et al. 2008; Opelz et al. 1972; Opelz 1992; Taylor et al. 1989; Taylor et al. 1991).

The clinical relevance of low-titre antibodies detected by these highly sensitive assays is controversial. (El-Awar et al. 2007; El-Awar et al. 2007; Gebel et al. 2003). Whereas a positive pre-transplant CDC-XM is an established risk factor for early hyperacute rejection from presumably high-titre complement-fixing DSA (Patel et al. 1969), the risk associated with low titre and possibly non-complement fixing antibodies detected using solid phase assays prior to transplantation is unknown. It is not clear whether these antibodies constitute a contraindication to transplantation, or merely reflect a degree of risk. Furthermore, their impact on long term outcomes is uncertain, recent studies using the Luminex® assay for pre–transplant antibody detection have yielded conflicting results. Gupta et. al found little impact on early graft outcomes although long-term graft survival was inferior (Gupta et al. 2008), whereas van den Berg-Loonen reported increased acute rejection episodes but no effect on long term graft outcome (van den Berg-Loonen et al. 2008).

3.2 Materials and Methods:

The screened population was selected from 241 type 1 diabetic recipients of a combined kidney-pancreas transplant performed between January 1987 and December 2007 at Westmead hospital described in

section 2.1. Inclusion criteria for the study were availability of stored pre-transplant sera for the Luminex® assay. Of the 241 transplants, 121 donor recipient pairs had pre-transplant sera available for evaluation. Stored pre-transplant sera for 120 donor recipient pairs were unavailable because prior to transplantation, recipients resided out of the Westmead catchment area. Regulatory protocols did not allow access to these sera. Immunosuppression consisted of either tacrolimus (TAC) or cyclosporine (CSA), combined with azathioprine (AZA) or mycophenolate mofetil (MMF) and prednisolone. Immunosuppression was determined according to transplant era without prior knowledge of pre-transplant antibody status. Anti-lymphocyte therapy was used for treatment of steroid resistant cellular rejection episodes and not for induction therapy.

Kidney biopsies undertaken at implantation, 1, 3, 6 and 12 months and then annually until 5 years, were analysed. The Banff light microscopy analysis, C4d immunoperoxidase staining and assessment for HLA and donor specific antibodies were performed as described in sections 2.3, 2.4, 2.6.1 and 2.6.2 respectively. Antibody strength was assessed using molecules of equivalent fluorochrome. The detailed technique is described in section 2.6.3. Renal allograft function was assessed using serum creatinine and yearly isotopic GFR measurements described in section 2.7.1.1. Pancreas function was evaluated by sequential oral glucose tolerance tests as described in section 2.7.2.1.

3.2.1 Statistical analysis

Data was analysed according to pre-transplant DSA status as described in section 3.3.1. The statistical software packages SPSS and S-PLUS Version 8 were used for data analysis. Generalised Linear mixed effects models were used for analysis of repeated measurements and evaluation of within and between group variations. Data are expressed as mean±SD for patient demographics; and as mean ±SEM for other data, as indicated. Additional analysis was prformed comparing the study group (n=121) to the group where pre-transplant sera was unavailable (n=120).

3.3 Results

3.3.1 HLA and Donor Specific antibodies

121 donor-recipient pairs had stored pre-transplant sera available for DSA evaluation. This became the principal study group for antibody and histological analysis. All recipients had a negative peak and current cytotoxic T-cell cross-match at time of transplantation. Of the 121 sera samples screened, 57 were sensitised to HLA antibody when tested with Luminex® single antigen beads. Of the sensitised patients, 33 were sensitised with specificities against mismatched donor HLA (Group I) while 24 were sensitised but HLA specificities were not donor specific (Group II). Of the sensitised patients with DSA, Class I antibodies were found in 18 recipients, class II in 9 and both Class I and Class II in 6 recipients. 64 recipients were not sensitised to any

HLA antibody (Group III). Overall, DSA were found in 33 of 121 (27%) donor-recipient pairs screened using Luminex®.

3.3.2 Demographic Characteristics of Study Group

Mean donor age in combined study group was 26.9±10.4 years with 36% of donors being females. Of 121 recipients 43 (36%) were females, mean recipient age was 38±9 years. Mean historical and current PRA were 14% (range 0 to 96%) and 2% (range 0 to 6 %) respectively. Mean HLA A, B, AB and DR mismatches were 1.4±0.6, 1.7±0.5, 3±1, and 1.5±0.6 respectively whereas mean total HLA mismatch was 4.5±1.

Group I patients had a higher mean historical PRA (24%), compared to 14% in group II and 9% in group III (P=0.004) although they would not be considered highly sensitised by conventional definitions. A higher proportion of patients in group I were females compared to groups II and III; 70% *versus* 14% *versus* 34% (P=0.01). Presence of pre-transplant DSA was associated with peak PRA more than 25% (χ^2=4, P=0.04) and female gender (χ^2=6, P=0.01).

Table 3.1: Demographic characteristic according to pre–transplant DSA status (Group I =33, Group II= 24, Group III = 64).

	Group I Sensitised DSA positive	Group II Sensitised DSA negative	Group III Unsensitised	P
(Patient/biopsies)	33 (192)	24 (73)	64 (306)	NS
Donor age (mean± SD	30±10	21±8	27±10	NS
Donor sex (n, %female)	14 (36)	6 (25)	23 (36)	NS
Donor CVA	13 (39)	5 (26)	21 (32)	NS
Donor Hypertension	2 (6)	2 (10)	1 (2)	NS
KAT (mean±SD)	35±7	34±8	33±8	NS
Ischemic time hr (mean±SD)	12±3	12±3	11±3	NS
Recipient age (mean±SD)	36±8	39±7	38±10	NS
Recipient sex (n % female)	14 (36.8)	10 (62.5)	44 (67)	0.01
HLA A mm	1.4±0.8	1.4±01.1	1.4±1.1	NS
HLA B mm	1.7±0.6	1.6±0.6	1.7±0.5	NS
HLA DR mm	1.6±0.6	1.47±0.7	1.42±0.7	NS
Total HLA mismatch	4.6±0.8	4.5±1.1	4.6±1.1	NS
Peak PRA (% mean)	24	14	9	0.004
PRA at transplant (% mean)	4	3	0.7	0.02
Duration of dialysis (years)	3.4±0.6	1.7±1.3	1.6±1.4	0.03

KAT = kidney anastomotic time, PRA = panel reactive antibodies, CVA = cerebral vascular accident

Immunosuppression consisted of a calcineurin inhibitor, anti proliferative agent as either MMF or AZA and prednisolone. Immunosuppression protocol was not determined according to pre-transplant DSA status, as this was not known at the time of transplant. Of 33 patients in group I, 12 (36%) were treated with TAC/MMF and 21 (64%) with CSA/AZA. In group II, 19 (79%) were treated with TAC/MMF and 5 (21%) received CSA/AZA. Of the group III patients 47 (73%) received TAC/MMF whereas 18 (27%) were treated with CSA/AZA (all P=0.001). A higher proportion of patients in group I 72% were treated with ATG or OKT3 compared to 25% in group II and (33%) in group III. Trough levels of cyclosporine and tacrolimus at 1, 3, and 5 years after transplant years were comparable in both groups (Table 3.2).

Table 3.2: Immunosuppression according to DSA status.

	Group I Sensitised DSA positive	Group II Sensitised DSA negative	Group III Unsensitised	P
TAC/MMF (n, %)	11 (34.4)	14 (58)	49 (73)	0.001
CSA/AZA (n, %)	21 (65)	4 (17)	18 (27)	0.001
ATG/OKT3	23 (72)	6 (25)	20 (33)	0.001
CSA trough 1 yr (ng/ml)	252±33	222±95	209±96	NS
CSA trough 5 yr (ng/ml)	199±28	185±59	170±23	NS
TAC trough 1 yr (ng/ml)	7.7±1.0	9±1	8.7±0.9	NS
TAC trough 5 yr (ng/ml)	5.5±2	8.6±0.9	10.5±0.7	NS

Legend: TAC=Tacrolimus, CSA=cyclosporine, MMF=mycophenolate mofetil, AZA = azathioprine

3.3.3 Graft Function

3.3.3.1 Serum Creatinine

The presence of pre-transplant DSA was associated with reduced renal function in the first 3 months after transplant and beyond 2 years after transplant (Figure 3.1). Mean serum creatinine at weeks 1, 2, 3 and 4 were 211±29 μmol/l, 202±26 μmol/l, 175±18 μmol/l, 166±17 μmol/l in group I, compared to 121±12 μmol/l, 121±7 μmol/l, 122±10 μmol/l, 120±9 μmol/l in group II and 154±12 μmol/l, 145±8 μmol/l, 133±8 μmol/l, 125±5 μmol/l in group III (all $P<0.05$ for differences between

group I and other groups). At 3 months after transplant serum creatinine was comparable between all 3 groups as 128±7 μmol/l in group I, 111±6umol/l in group II and 115±11 μmol/l in group III (P=NS). Beyond 3 years after transplant, progressive deterioration in graft function occurred in group I, serum creatinine increased by 16 μmol/l/yr, while remaining unchanged in groups II and III (P=0.03). At 5 years, mean serum creatinine was higher in group I as 201±24 μmol/l compared to 125±12 μmol/l in group II and 130±11 μmol/l in group III (P<0.05).

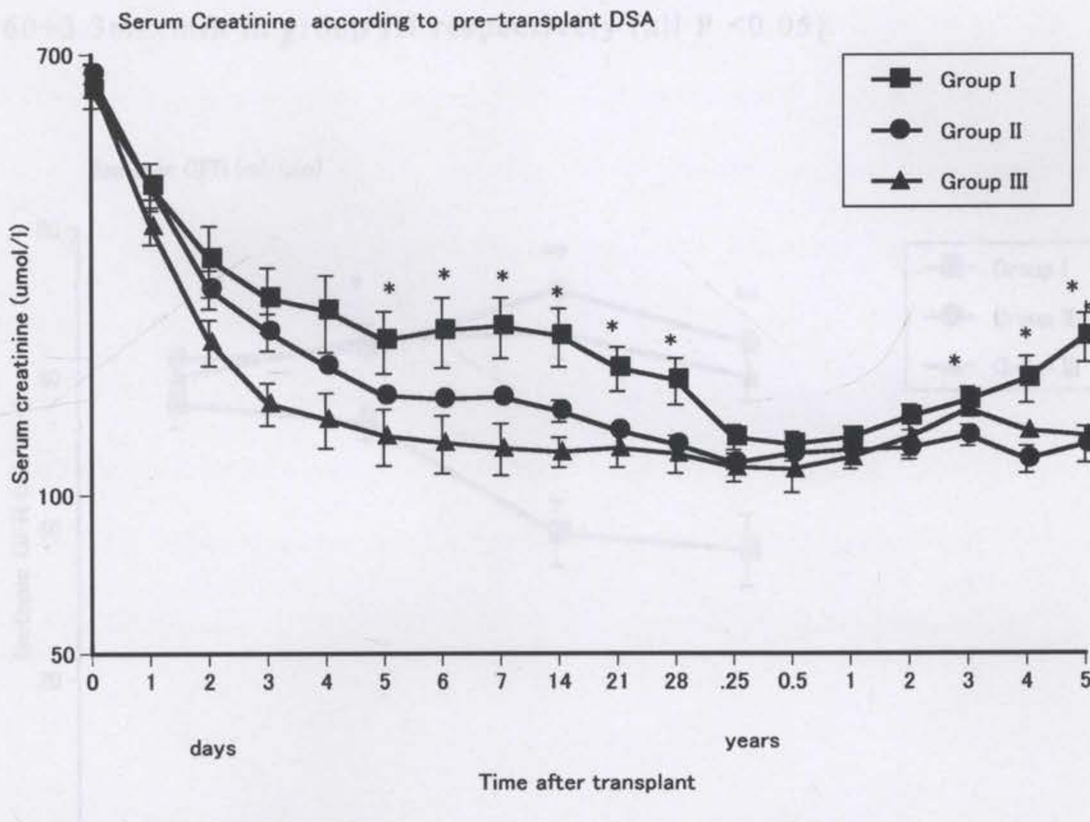


Figure 3.1: Serum creatinine in the first month after transplantation was higher in recipients with pre-transplant DSA (group I) compared to recipients without DSA groups II and III. Between 3 months and 2 years serum creatinine was comparable and after 2 years significant

differences were observed between groups I, group II and III. (Legend: * P<0.05).

3.3.3.2 Isotopic GFR

Late graft dysfunction was confirmed by Tc^{99m} DTPA isotopic GFR (Figure 3.2). GFR decreased at a rate of 9mL/min/year in group I compared with 1mL/min/yr in group II and III (P<0.0001). At 1, 3 and 5 years, GFR in group I was 54±3mL/min , 39±3mL/min and 37±3.3mL/min compared to 63±3.2 mL/min, 71±6mL/min and 65±2.9mL/min in group II and 65±2mL/min, 67±3mL/min and 60±3.3mL/min in group III respectively (all P <0.05).

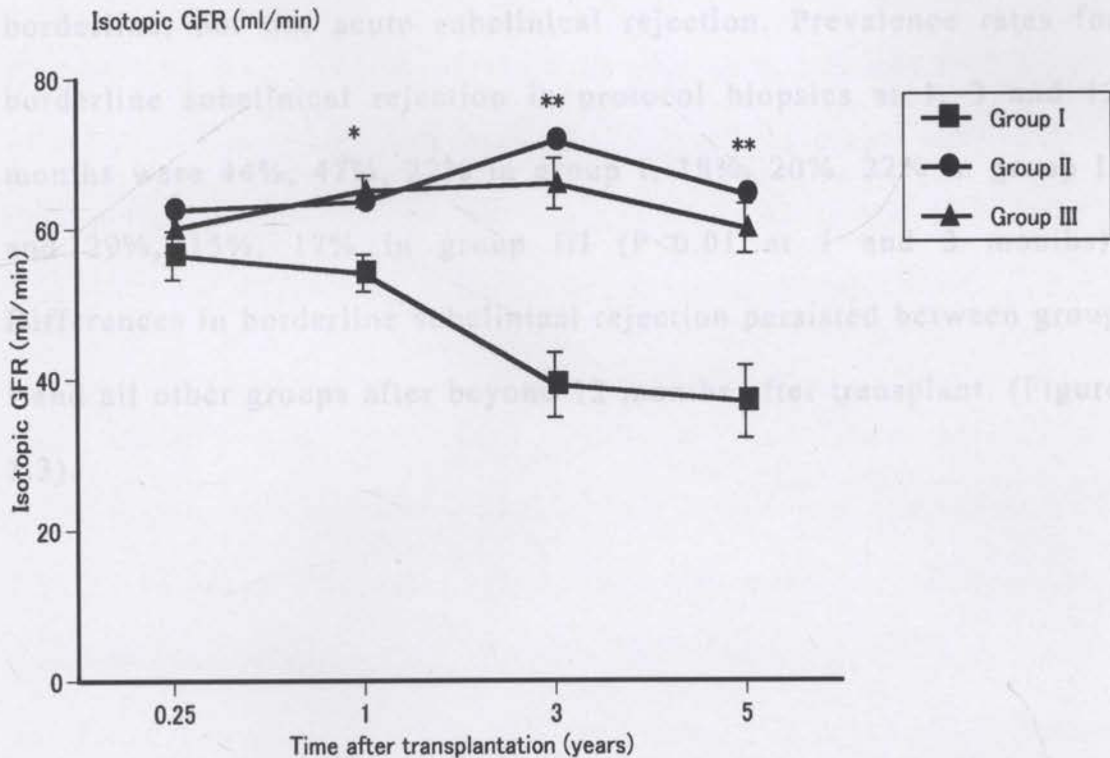


Figure 3.2: GFR at 1, 3 and 5 years is lower in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). (Legend: * P<0.05, **P<0.01).

3.3.4 Histological Outcomes

3.3.4.1 Acute Banff Histological Scores

From the Westmead longitudinal histological database, 714 biopsies were available for Banff analysis. Of these, 104 were indication biopsies performed within the first month after transplantation and these where analysed separately. Median biopsy follow-up in group I was 5 years (IQR 3-8 years) compared to 3 years (IQR 1-4) in group II and 5 years (IQR 1-5 years) in group III. Acute cellular and vascular rejection rates in indication biopsies were comparable between the groups as 21% and 23% in group I *versus* 21% and 12% in group III (all P=NS). Early graft dysfunction was associated with higher rates of borderline, but not acute subclinical rejection. Prevalence rates for borderline subclinical rejection in protocol biopsies at 1, 3 and 12 months were 44%, 47%, 22% in group I, 18%, 20%, 22% in group II and 29%, 15%, 17% in group III (P<0.01 at 1 and 3 months). Differences in borderline subclinical rejection persisted between group I and all other groups after beyond 12 months after transplant. (Figure 3.3).

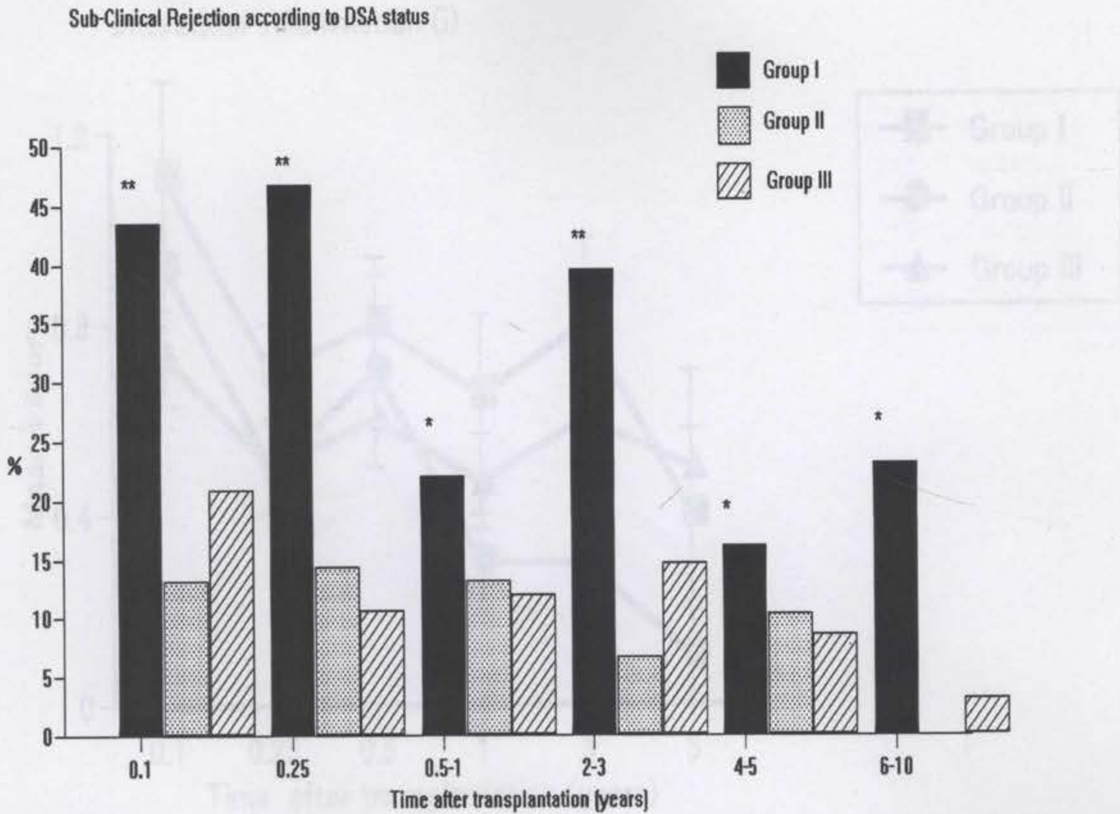


Figure 3.3: Sensitised recipients with DSA (group I) had higher rates of subclinical rejection compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). (Legend: * P<0.05, ** P<0.01 for differences in borderline sub-clinical rejection).

Mean Banff i scores at 1 and 3 months in group I were 1.1±0.2 and 0.7±0.1 compared to 0.9±0.2 and 0.5±0.1 in group II and 0.7±0.1 and 0.5±0.1 in group III respectively (P=NS).The mean t scores score at 1 and 3 months in group I was 1.1±0.1 and 0.5±0.1 compared to 0.6±0.2 and 0.3±0.1 in group II and 0.7±0.1 and 0.4±0.1 in group III (P=0.18 at 1 month and P=NS at 3 months. Beyond 12 months, the mean i and t score were comparable between groups (Figures 3.4 and 3.5).

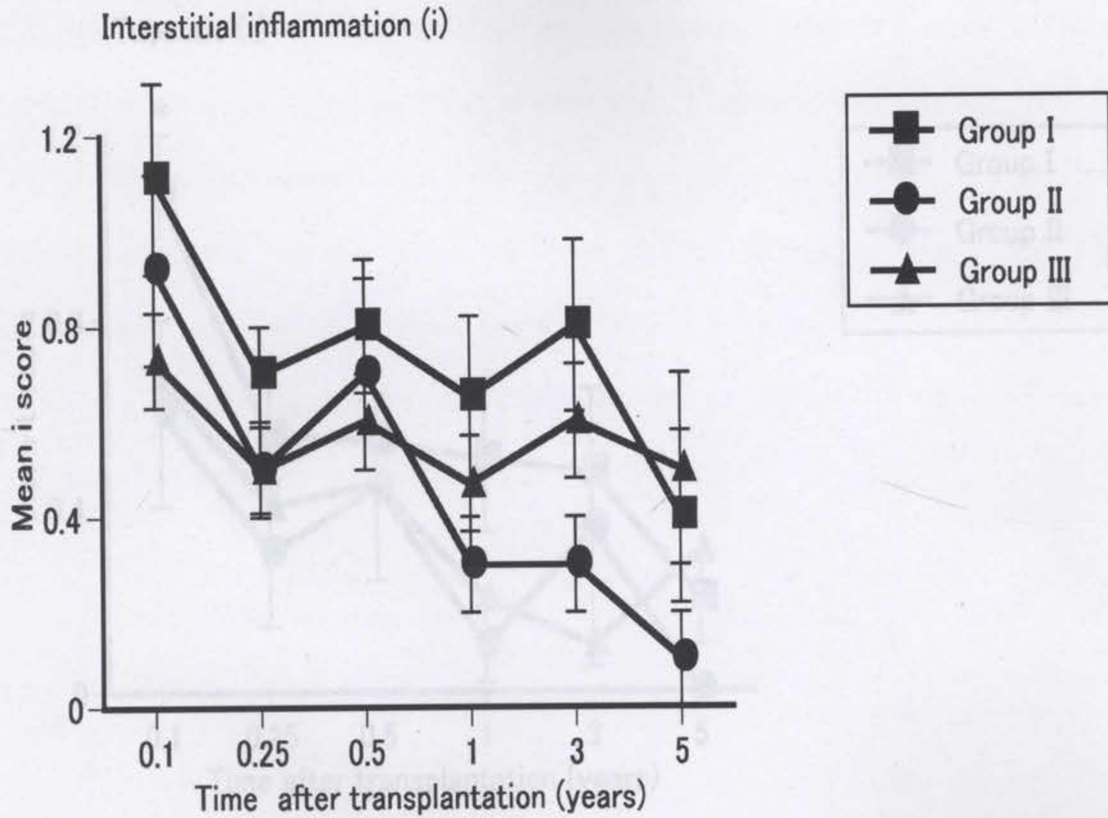


Figure 3.4: The Banff interstitial inflammation score (i), according to DSA status. Acute interstitial inflammation was comparable in all 3 groups. (Legend: * P<0.05).

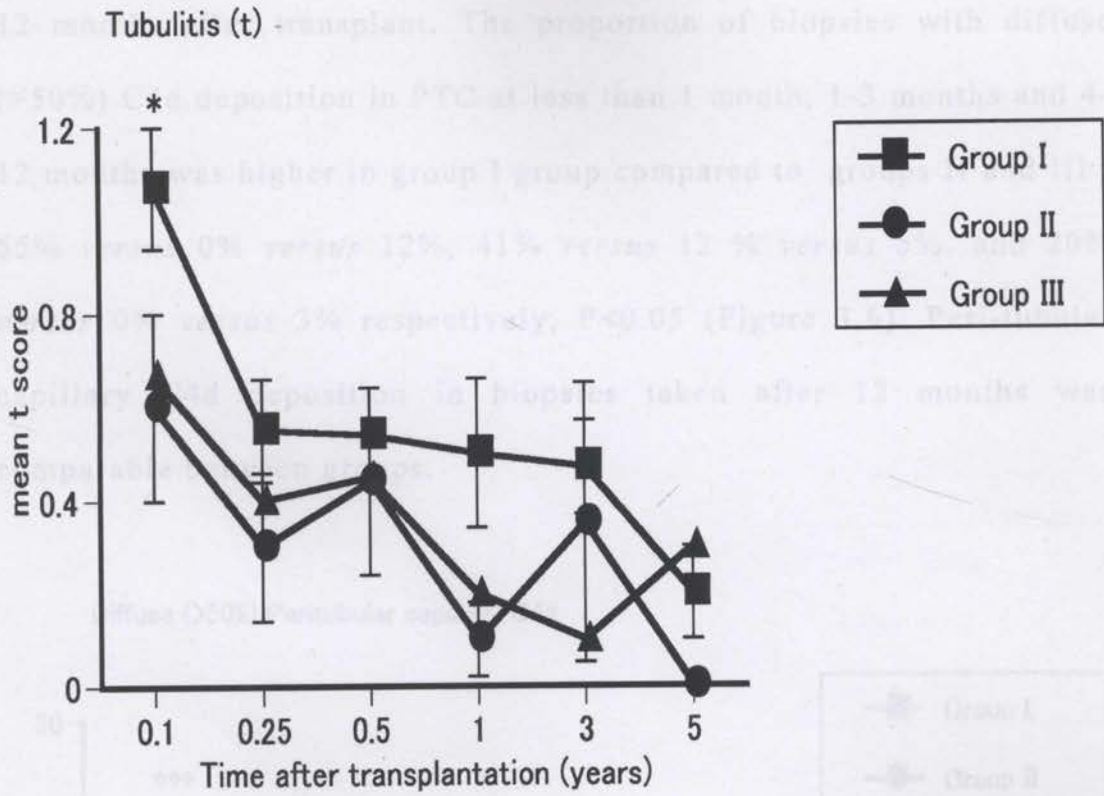


Figure 3.5: Banff tubulitis score according to DSA status. The t score was higher at 1 month after transplant in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). The mean t score was comparable in all 3 groups after 3 months. (Legend: * P<0.05).

Staining for C4d was performed on 415 paraffin sections. Biopsies performed before the first month of transplant were "for cause" (n=98) with protocol biopsies beginning at 1 month through to 5 years post transplant (n=317). Overall prevalence of C4d staining was 13% in PTC and 32% in glomeruli. The proportion of biopsies with diffuse (>50%) C4d deposition in group I increased rapidly within the first month, peaked at 3 months, and subsequently decreased between 4 and

12 months after transplant. The proportion of biopsies with diffuse (>50%) C4d deposition in PTC at less than 1 month, 1-3 months and 4-12 months was higher in group I group compared to groups II and III ; 55% *versus* 0% *versus* 12%, 41% *versus* 12 % *versus* 5%, and 20% *versus* 0% *versus* 3% respectively, P<0.05 (Figure 3.6). Peri-tubular capillary C4d deposition in biopsies taken after 12 months was comparable between groups.

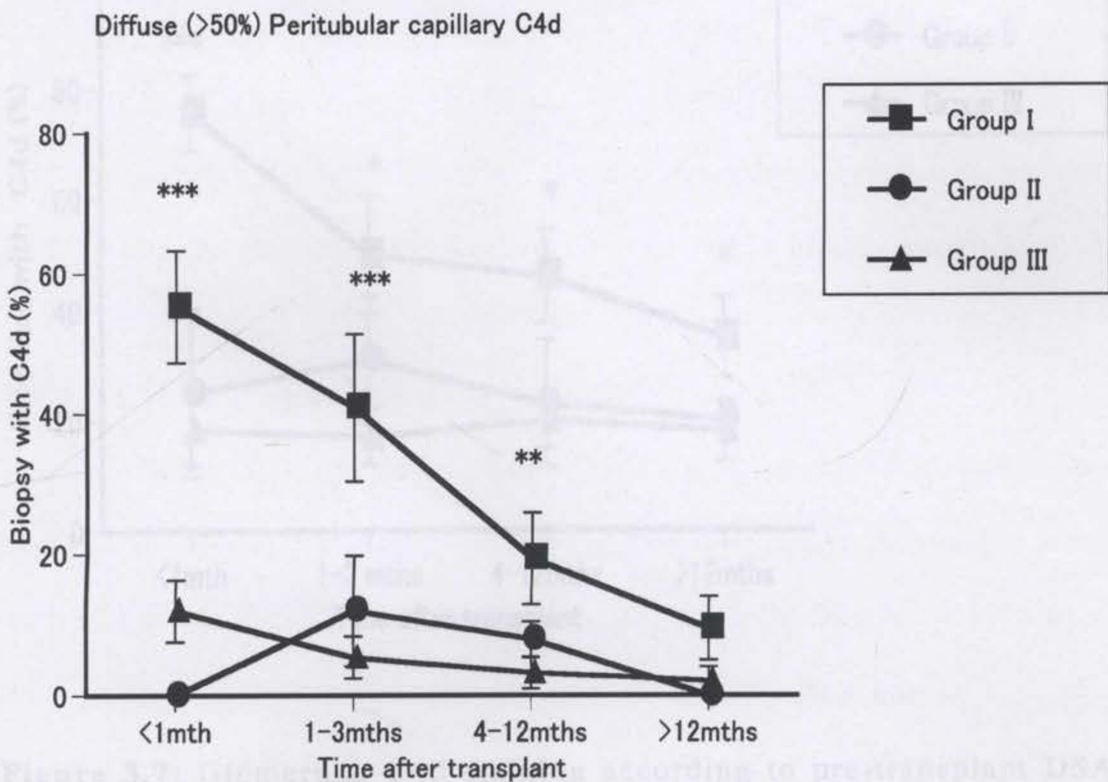


Figure 3.6: Peri-tubular C4d staining according to pre-transplant DSA status (n=415 biopsies). The proportion of biopsies with diffuse C4d PTC was higher in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA group II and unsensitised recipients (group III). (Legend: * P<0.05, ** P<0.01, *** P<0.001).

Glomerular C4d staining had a similar pattern, with higher proportions of staining present in indication biopsies from recipients in group I. However, in contrast to peri-tubular capillaries, C4d staining in glomerular capillaries persisted beyond 12 months in these patients throughout histological follow-up (Figure 3.7).

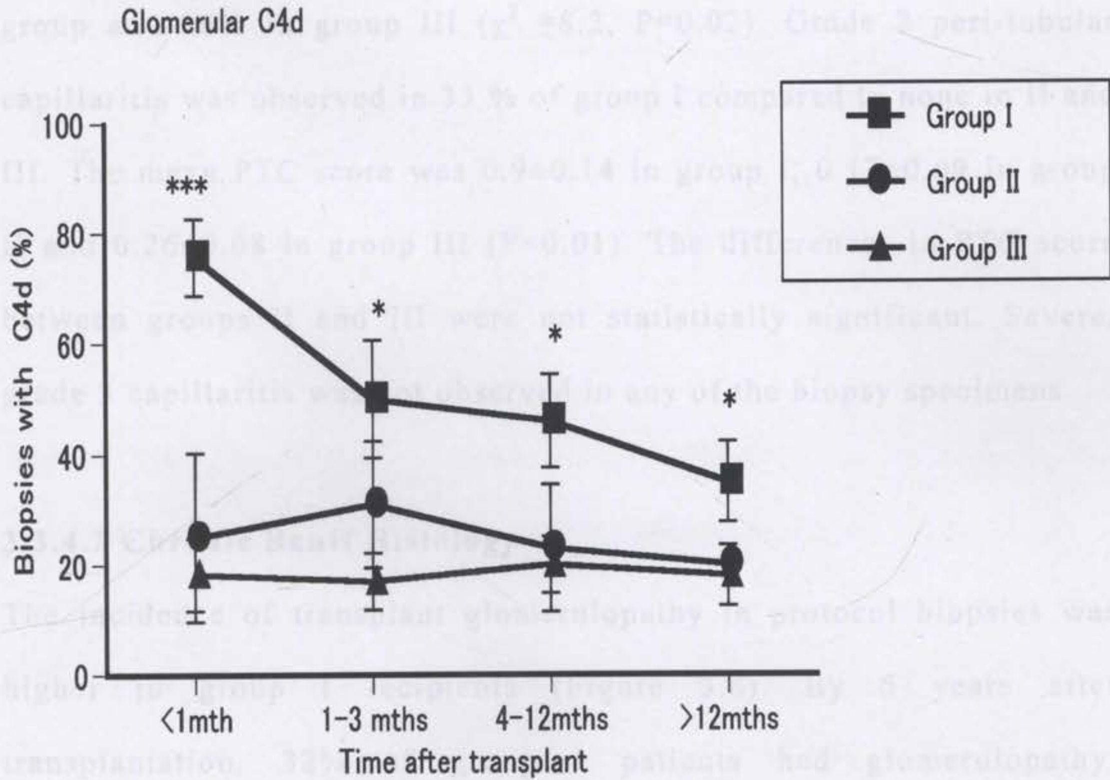


Figure 3.7: Glomerular C4d staining according to pre-transplant DSA status (n=415 biopsies). The proportion of biopsies with glomerular C4d staining is higher in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). Differences in proportion of biopsies with glomerular C4d staining persisted beyond 12 months after transplant. (Legend: * P<0.05, ** P<0.01, *** P<0.001).

There was a strong association between PTC C4d deposition and peri-tubular capillary inflammation. Among indication biopsies 76% of C4d positive biopsies demonstrated peri-tubular capillary inflammation compared to 13% of C4d negative biopsies (χ^2=26, P<0.001). A similar association was observed with respect to pre-transplant DSA. 56% of biopsies in group I exhibited capillaritis compared to 13% in group II group and 26% in group III (χ^2 =8.2, P=0.02). Grade 2 peri-tubular capillaritis was observed in 33 % of group I compared to none in II and III. The mean PTC score was 0.9±0.14 in group I, 0.12±0.09 in group II and 0.26±0.08 in group III (P<0.01). The differences in PTC score between groups II and III were not statistically significant. Severe, grade 3 capillaritis was not observed in any of the biopsy specimens.

3.3.4.2 Chronic Banff Histology

The incidence of transplant glomerulopathy in protocol biopsies was higher in group I recipients (Figure 3.8). By 5 years after transplantation, 32% of group I patients had glomerulopathy, increasing to 54% by 10 years, compared to 20% in group II at 5 and 10 years and 10% and 18% in group III respectively (P=0.01 by log rank test). Chronic glomerulopathy scores increased with time in group I, with significant differences occurring beyond 2 years after transplantation.

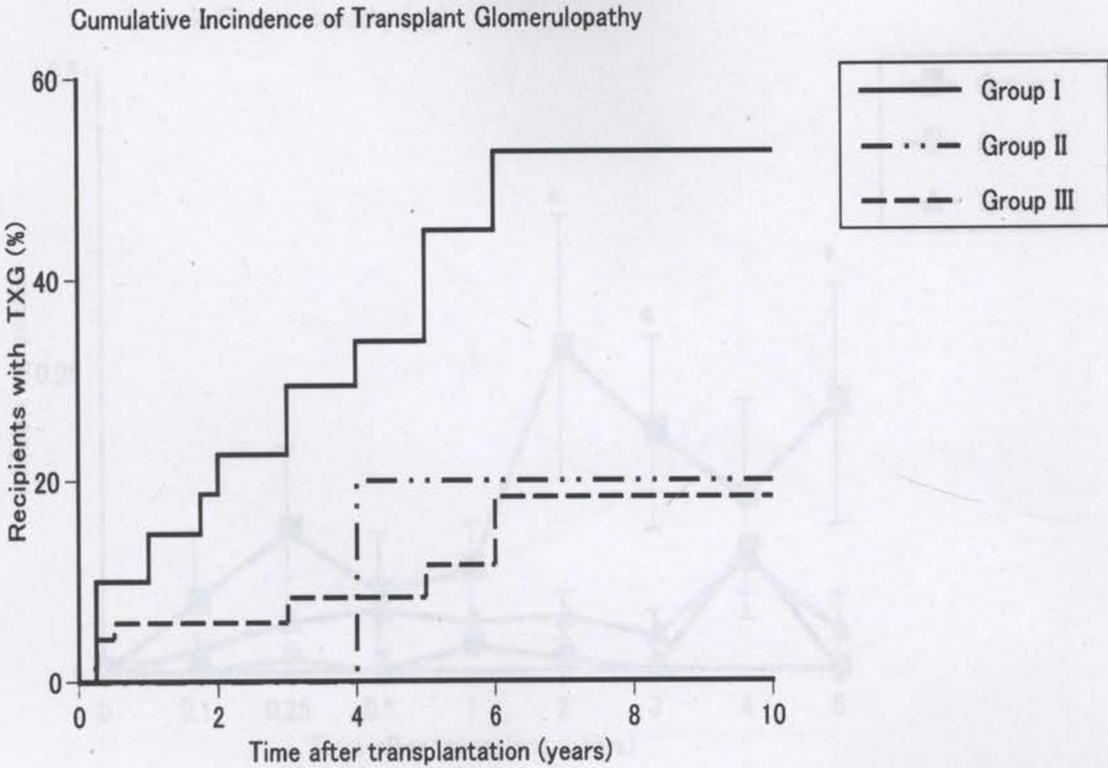


Figure 3.8: Association of pre-transplant DSA status and subsequent risk of transplant glomerulopathy: the cumulative incidence of transplant glomerulopathy was increased in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III) (P=0.004 group I *versus* group III by Log rank test).

Banff chronic histology scores for glomerular pathology are shown in figure 3.9. Mean Banff cg scores at 2, 3, and 5 years were 0.27±0.11, 0.20±0.08 and 0.22±0.1 in group I, compared to 0.04±0.02, 0.02±0.002, 0.03±0.03 in group II (P<0.001). Two patients in group II had a cg score of more than 1 at 4 years after transplant.

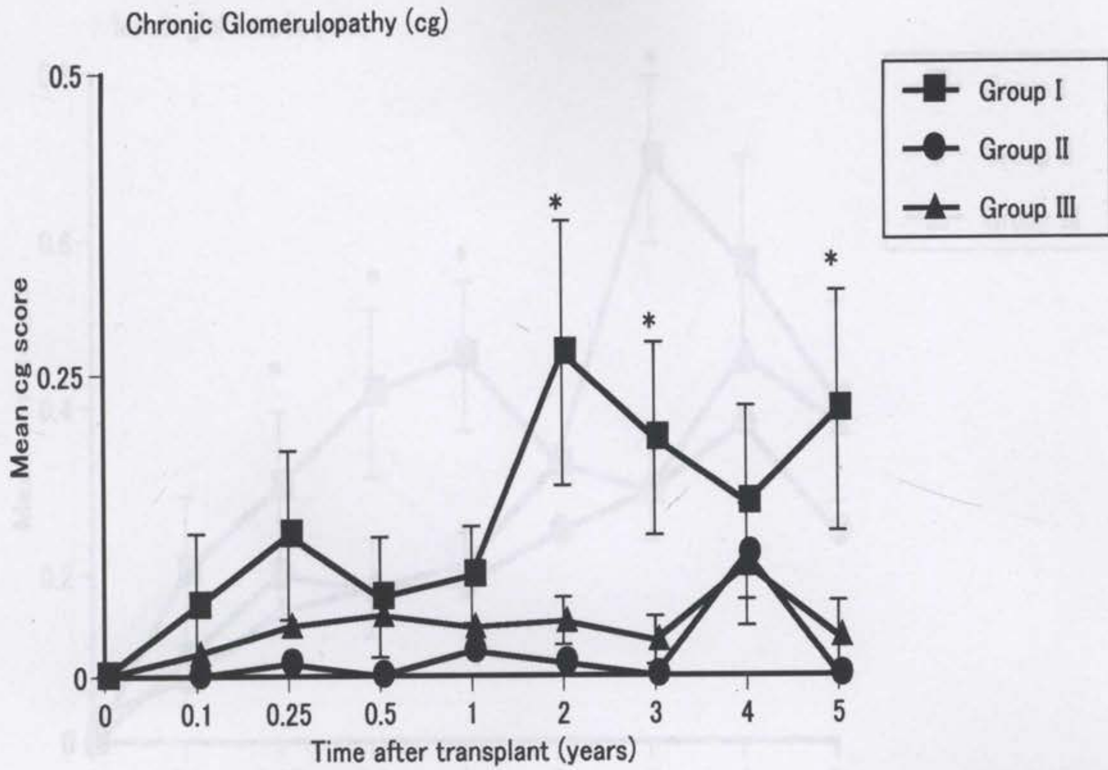


Figure 3.9: Mean Banff chronic glomerulopathy score (cg) according to DSA status. The cg score was higher at 2, 3, and 5 years after transplant in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). (Legend: * P<0.05, ** P<0.01, *** P<0.001).

In contrast to chronic glomerulopathy, differences in mesangial matrix occurred earlier after transplant. Mean mesangial matrix scores (mm) were higher in group I at 3 month, 6 months, 1 year and 3 years after transplant compared to groups II and III (Figure 3.10).

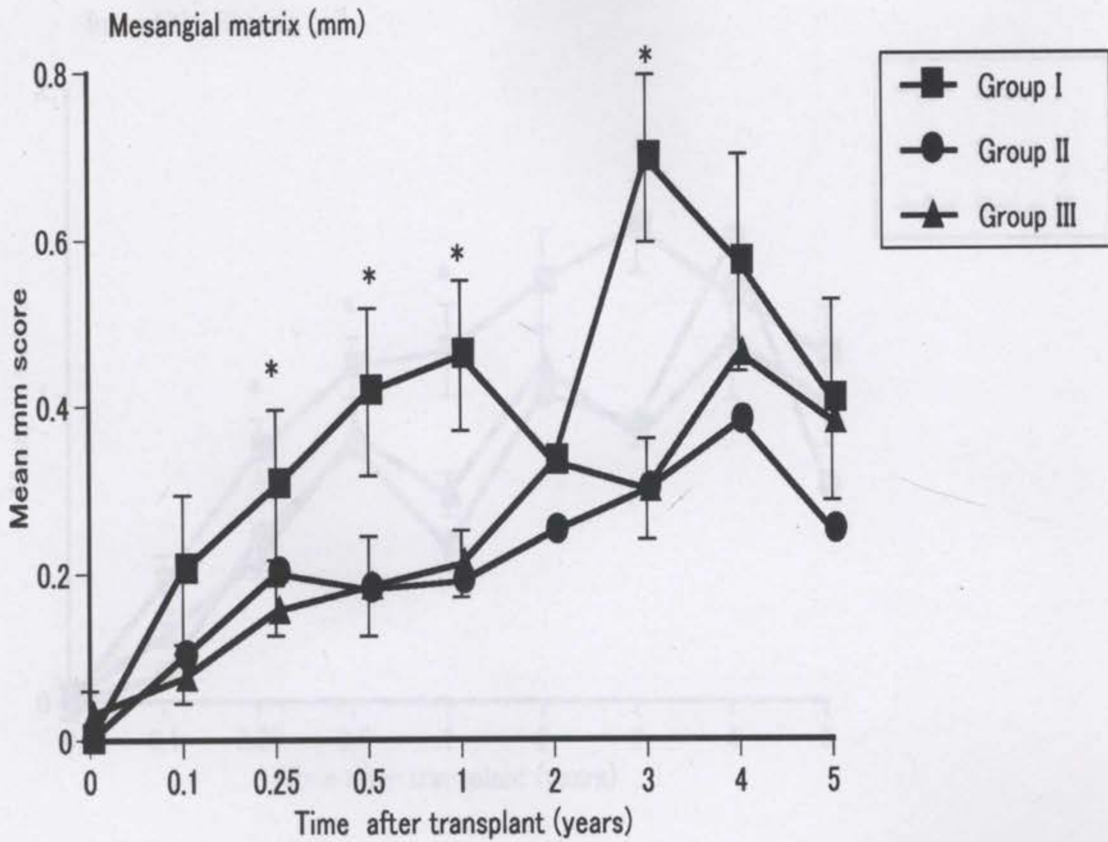


Figure 3.10: Banff mean mesangial matrix score (mm) score according to DSA status. The mm score was higher at 3 months 6 months,1 and 3 year after transplant in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). (Legend: * P<0.05).

The mean Banff chronic interstitial scores (ci) were higher in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) at 3, 6, 12 months and 3 years after transplant (Figure 3.11).

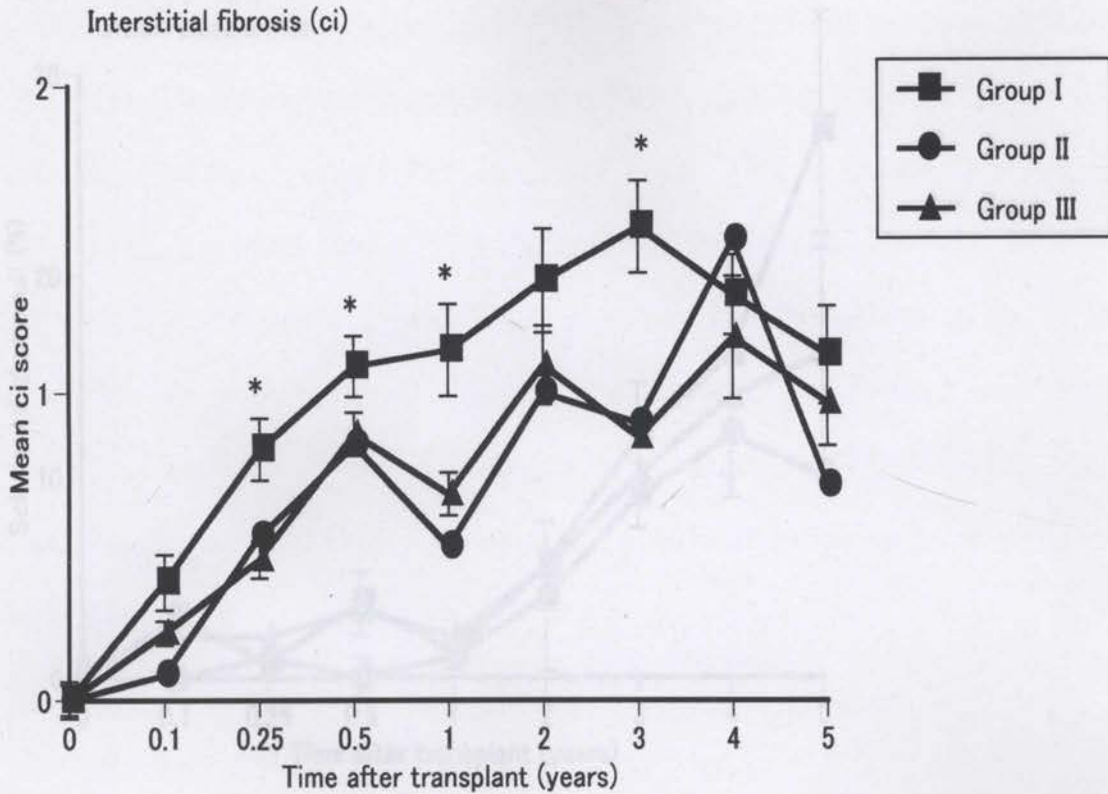


Figure 3.11: The mean Banff chronic interstitial score (ci) score according to DSA status. The ci score was higher in sensitised recipients with DSA (group I) compared to sensitised recipients without DSA (group II) and unsensitised recipients (group III). (Legend:* P<0.05)

The proportion of sclerosed glomeruli (Figure 3.12) was comparable between sensitised recipients with DSA (group I), sensitised recipients without DSA (group II) and unsensitised recipients (group III).

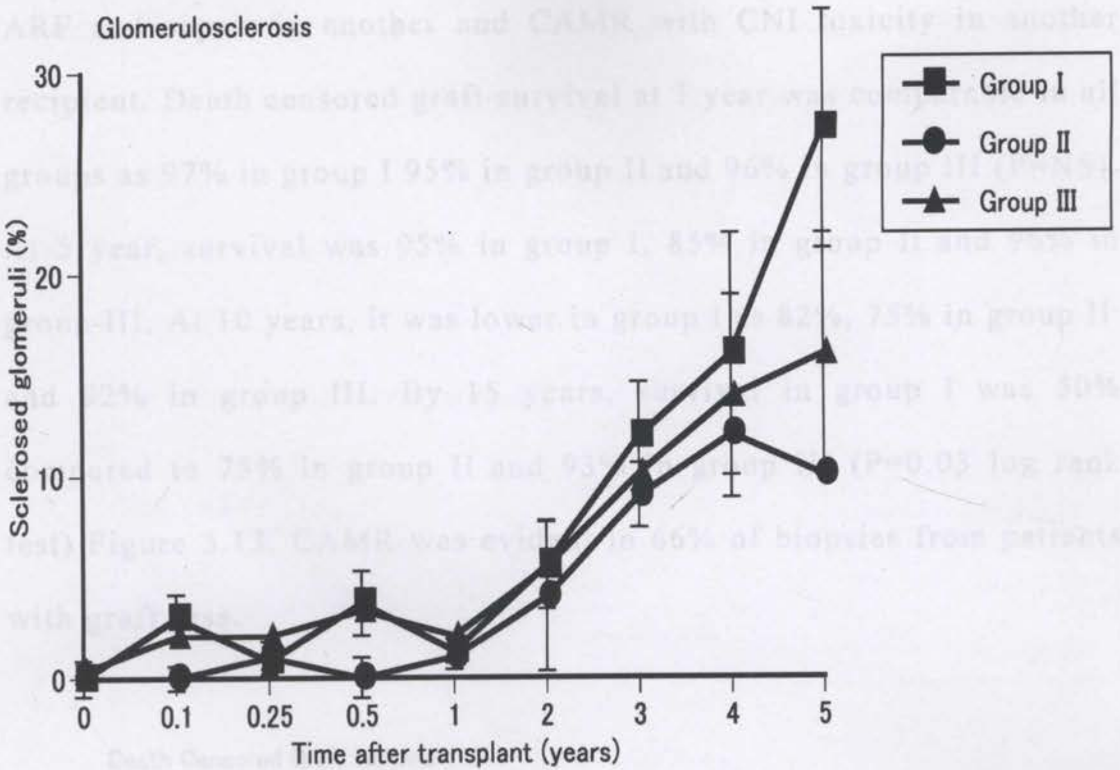


Figure 3.12: Proportion of sclerosed glomeruli according to DSA status. There was no difference in the proportion of sclerosed glomeruli in sensitised recipients with DSA (group I), sensitised recipients without DSA (group II), and unsensitised recipients (group III).

3.3.5 Graft Survival

Pre-transplant DSA was associated with increased graft loss. Graft loss occurred in 7 of 33 (21%) recipients in group I compared to 3 of 20 (15%) in group II and 2 of 66 (3%) in group III (P=0.02). The majority of graft losses occurred more than 5 years after transplant, early graft loss (under 4 months) occurred in 1 patient with DSA. In group II recipients, graft loss was attributed to BKV nephropathy in 1 recipient,

ARF and sepsis in another and CAMR with CNI toxicity in another recipient. Death censored graft survival at 1 year was comparable in all groups as 97% in group I 95% in group II and 96% in group III (P=NS). At 5 year, survival was 95% in group I, 85% in group II and 96% in group III. At 10 years, it was lower in group I as 82%, 75% in group II' and 92% in group III. By 15 years, survival in group I was 50% compared to 75% in group II and 93% in group III (P=0.03 log rank test) Figure 3.13. CAMR was evident in 66% of biopsies from patients with graft loss.

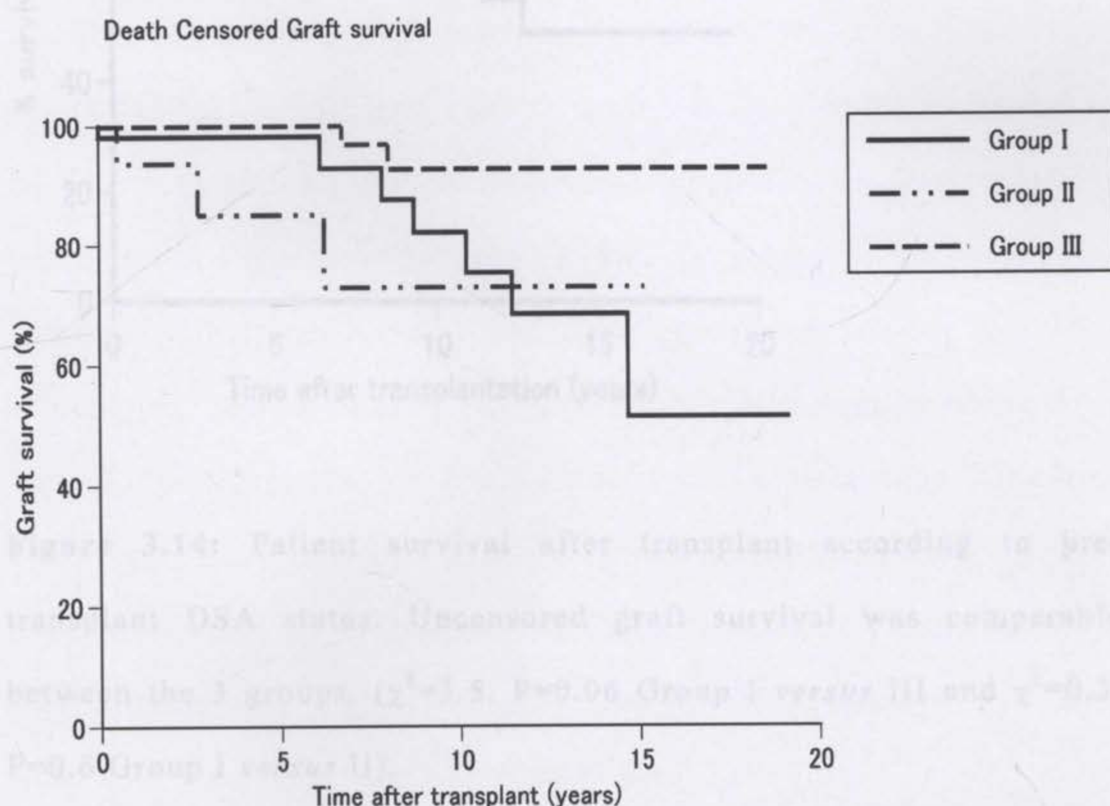


Figure 3.13: Graft survival at 10 and 15 years was lower in sensitised recipients with DSA (group I) compared to unsensitised recipients (group III) (P=0.02, χ^2=5.4 Log Rank test).

Uncensored graft survival was comparable between the 3 groups (Figure 3.14).Mean survival was 13 year SE 1.2 (CI 14-18 years) in group I 13 years SE 1.1 (CI 11-15 years) in group II and 16 years SE 0.9 (CI 14-18) in group III.

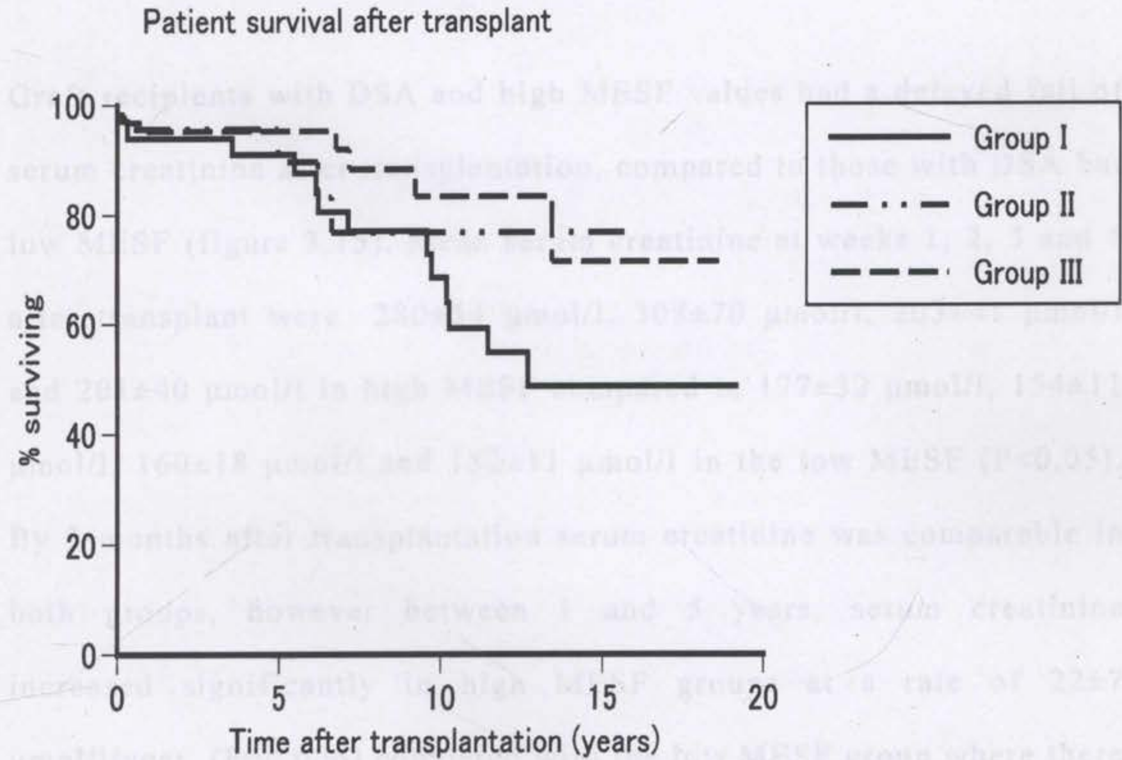


Figure 3.14: Patient survival after transplant according to pre-transplant DSA status. Uncensored graft survival was comparable between the 3 groups. (χ^2=3.5, P=0.06 Group I *versus* III and χ^2=0.3, P=0.6 Group I *versus* II).

3.3.6 Effect of Anti-Body Strength measured by MESF

Antibody strength in patients with DSA was assessed using MESF values, patients were classified into high MESF and low MESF groups

using a cut-off of 100,000 MESF units. Mean values for high MESF were 250,195±130,095 units and 33,597±26,483 for low MESF. High MESF values (>100,000 units) predicted early and late graft dysfunction, increased C4d deposition, worse graft survival and a trend towards higher incidence of transplant glomerulopathy.

Graft recipients with DSA and high MESF values had a delayed fall of serum creatinine after transplantation, compared to those with DSA but low MESF (figure 3.15). Mean serum creatinine at weeks 1, 2, 3 and 4 after transplant were 280±54 μmol/l, 308±70 μmol/l, 203±41 μmol/l and 201±40 μmol/l in high MESF compared to 177±32 μmol/l, 154±11 μmol/l, 160±18 μmol/l and 152±11 μmol/l in the low MESF ($P<0.05$). By 3 months after transplantation serum creatinine was comparable in both groups, however between 1 and 5 years, serum creatinine increased significantly in high MESF groups at a rate of 22±7 μmol/l/year, ($P=0.006$) compared with the low MESF group where there was no significant change. The time for serum creatinine to fall below 200 μmol/l was 9 days in high MESF group compared to 3 days in both medium and low MESF groups ($P=0.02$). The mean difference in serum creatinine between high MESF group and low MESF groups in the first week after transplantation was 115±43 μmol/l ($P<0.01$).

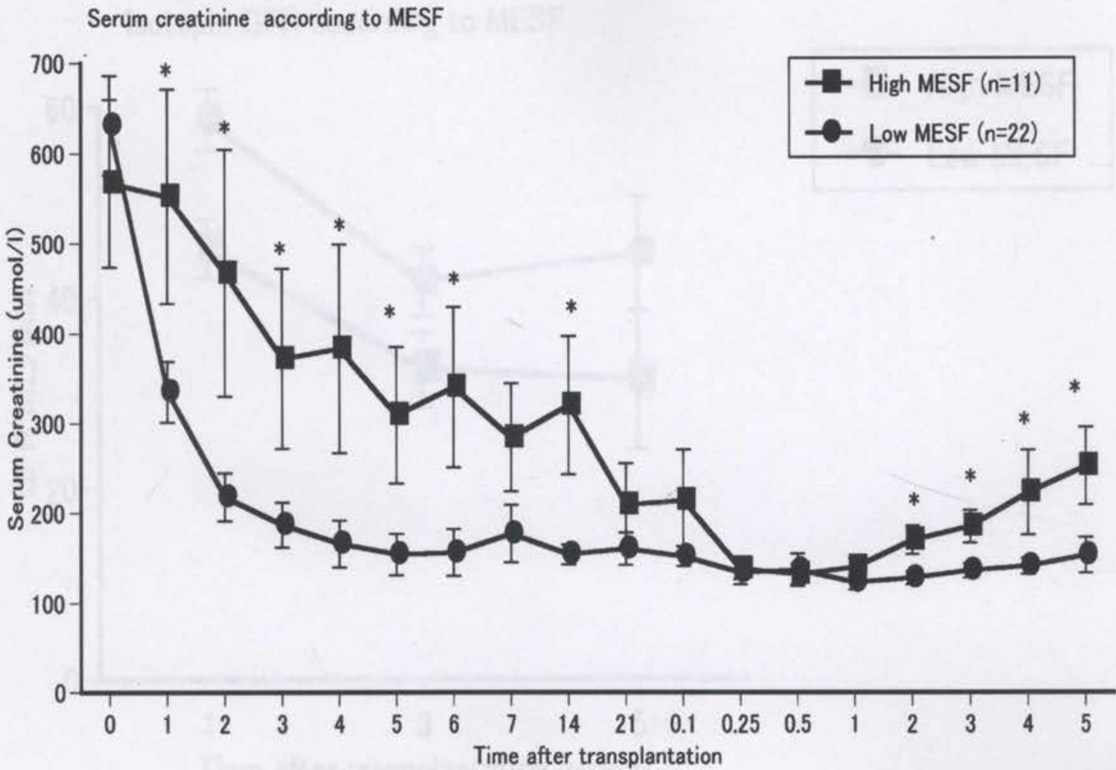


Figure 3.15: Graft Function in sensitised recipients with DSA according to antibody strength. Serum creatinine in graft recipients with High MESF was higher in the first month and after 1 year compared to recipients with low MESF. (Legend: * P<0.05)

There was trend towards lower GFR in patients with high MESF although this was not statistically significant (Figure 3.16).

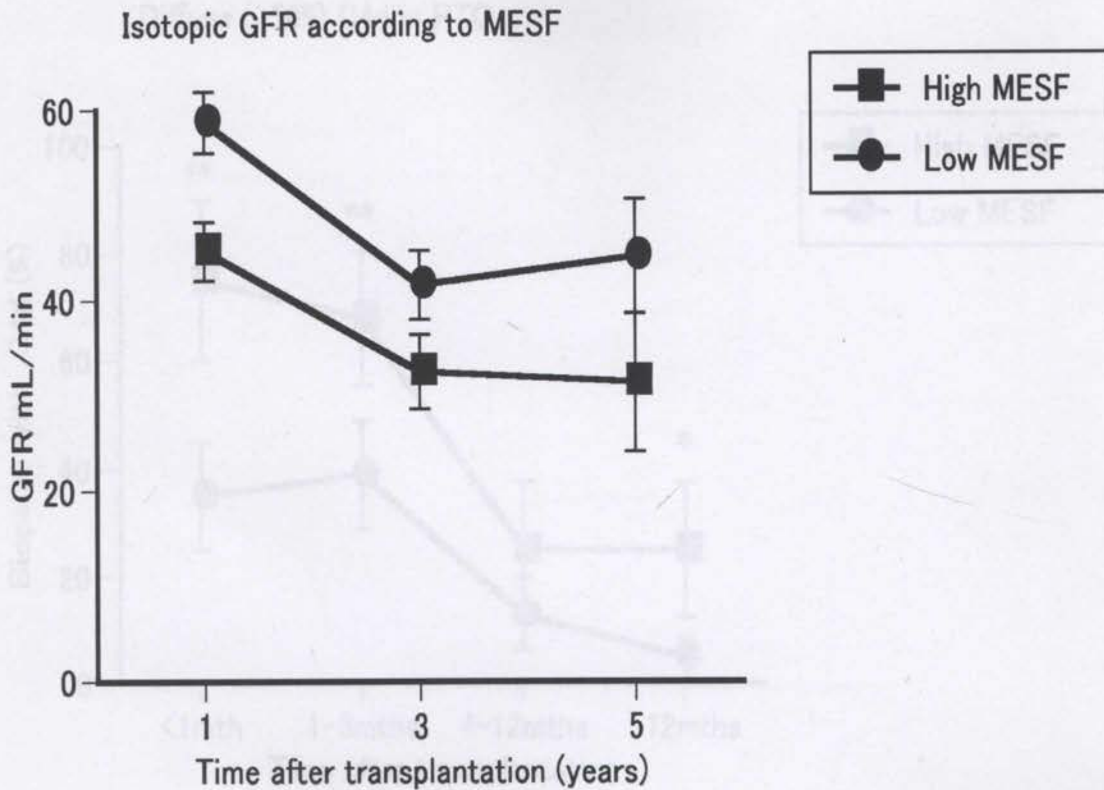


Figure 3.16: Graft Function in patients with DSA according to antibody strength. GFR at 1 year is comparable in both high and low MESF groups. (Legend: * P<0.05).

Among 138 biopsies available for C4d staining in group I, the proportion of biopsies with diffuse PTC-C4d was greater in the high MESF group, as 75% compared to 35% in the low MESF group in indication biopsies taken within the first month after transplant (P<0.001). C4d deposition in PTC decreased in both groups but remained higher in the high MESF throughout follow-up period (Figure 3.17).

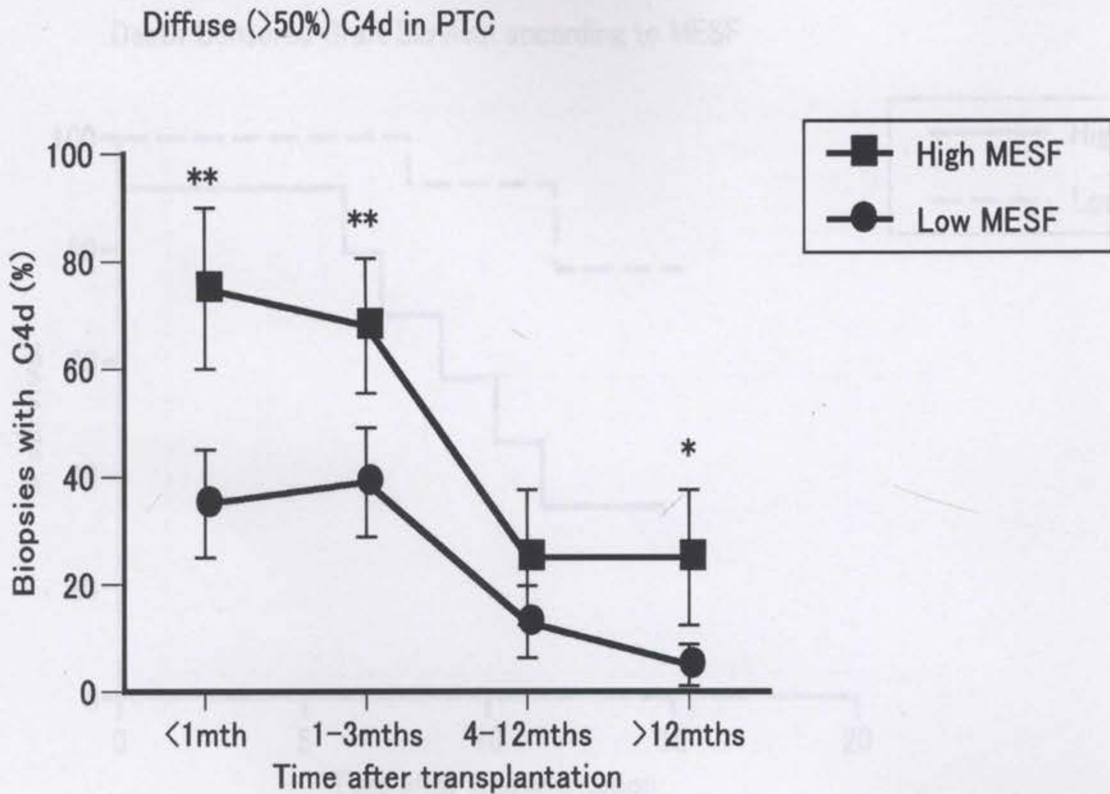


Figure 3.17: Diffuse C4d-PTC staining in sensitised recipients with DSA (group I) according to antibody strength measured as MESF units (n=205 biopsies). The proportion of biopsies with diffuse (>50%) C4d in PTC was higher in patients with high MESF compared to those with low MESF values. (Legend: * P<0.05, ** = P<0.001).

High MESF values were associated with increased graft loss. Death censored 1 year graft survival was 90% in the high MESF group compared to 100 % in the low MESF. Survival at 10 years was lower in the high MESF group as 42% compared to 80% in the low MESF group (P=0.02) (Figure 18).Mean survival was 10.5 years SE 1.5 (CI 7.5-14 years) in the high MESF compared to 17.4 years SE 1.8 (CI 15-19.6) in low MESF.

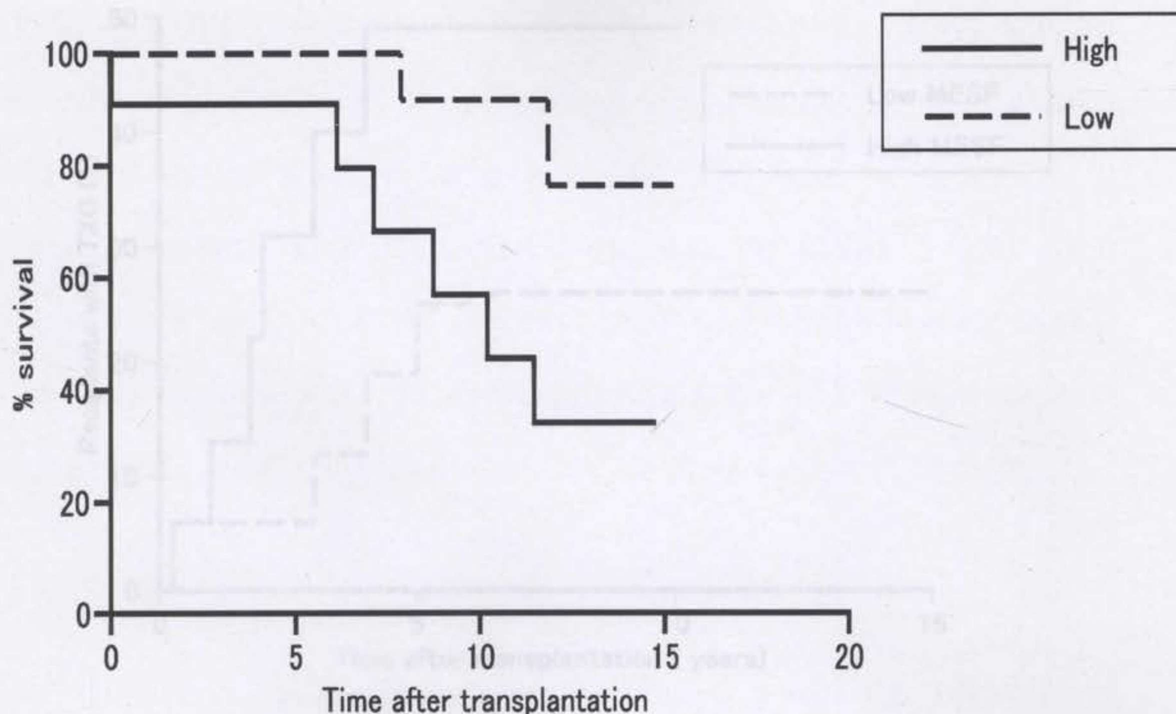


Figure 3.18: Death censored graft survival in sensitised patients with DSA (group I) according to antibody strength. Graft survival was lower in high *versus* low MESF groups (χ^2 = 6.1, P = 0.01 low *versus* high, Log Rank test).

There was more transplant glomerulopathy in patients with high MESF. The cumulative incidence of transplant glomerulopathy in the high MESF group at 5 years was 40% and at 10 years was 50% compared to 22% in the low MESF group (χ^2 = 3.5, P = 0.06 Log rank test) (Figure 3.19).

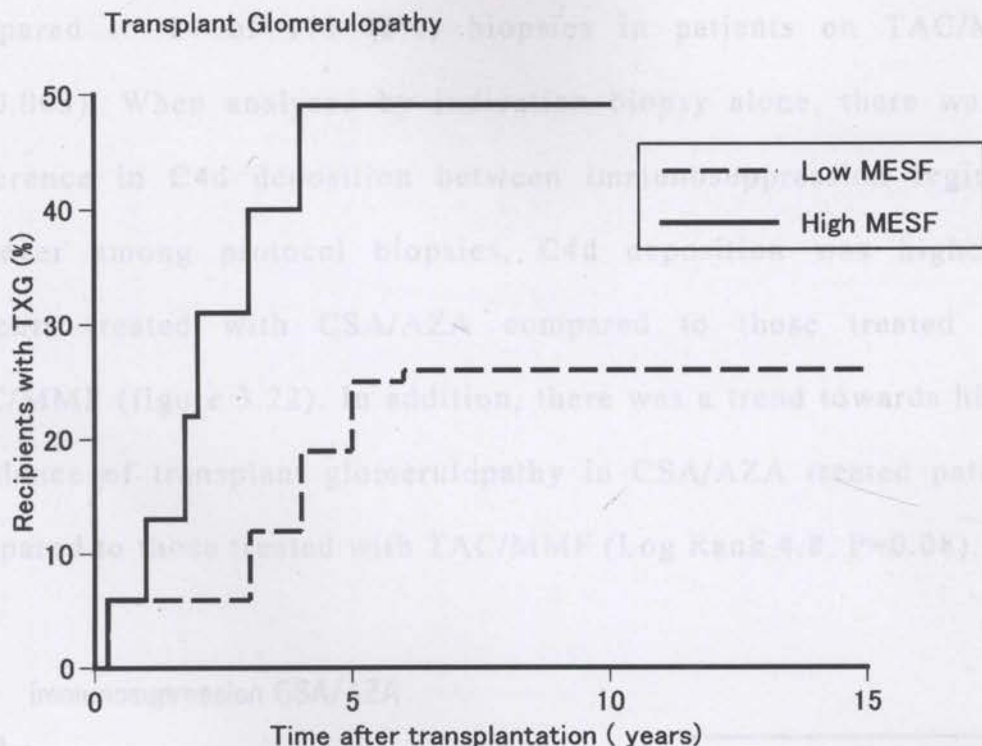


Figure 3.19: Incidence of transplant glomerulopathy in sensitised recipients with DSA according to MESF. There was a trend towards more transplant glomerulopathy in patients with high MESF (χ^2=3.5, P =0.06 log rank test).

3.3.7 Effect of Immunosuppression

GFR was lower in patients with DSA (Group I) treated with CSA/AZA, at 1, 3 and 5 years after transplant as 50±3mL/min, 36±3mL/min, 36±5 mL/min compared to 59±3mL/min, 57±5mL/min and 51±3mL/min in unsensitised patients (group III) treated with CSA/AZA (P<0.05) Figure 3.20). In the TAC/MMF group, GFR was comparable at all time-points in sensitised patients with DSA compared to unsensitised patients (Figure 3.21). Peri-tubular capillary C4d deposition in all biopsies was higher in patients treated with CSA/AZA as 41/235 (17%)

compared to 14 of 179 (8%) biopsies in patients on TAC/MMF (P=0.003). When analysed by indication biopsy alone, there was no difference in C4d deposition between immunosuppression regimens however among protocol biopsies, C4d deposition was higher in patients treated with CSA/AZA compared to those treated with TAC/MMF (figure 3.22). In addition, there was a trend towards higher incidence of transplant glomerulopathy in CSA/AZA treated patients compared to those treated with TAC/MMF (Log Rank 4.8, P=0.08).

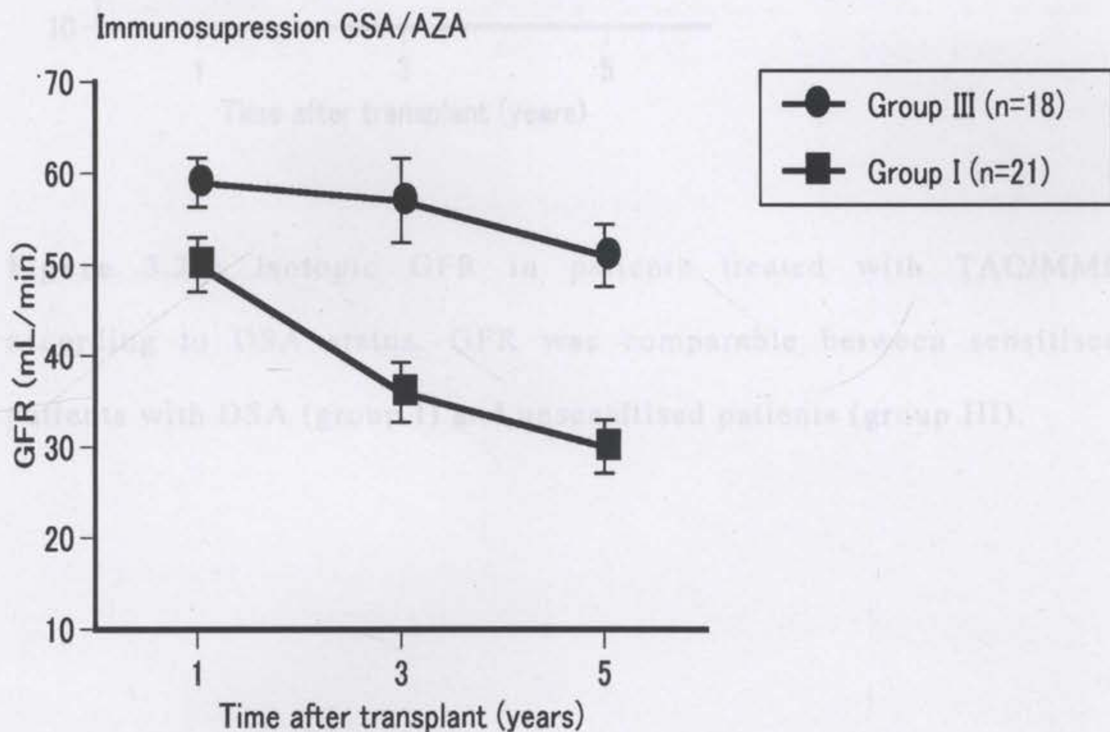


Figure 3.20: Isotopic GFR in recipients treated with CSA/AZA according to DSA status. GFR was lower in recipients sensitised with DSA (group I) compared to unsensitised recipients (group III).Group II patients excluded from this analysis due to small numbers. (Legend *= P<0.05)

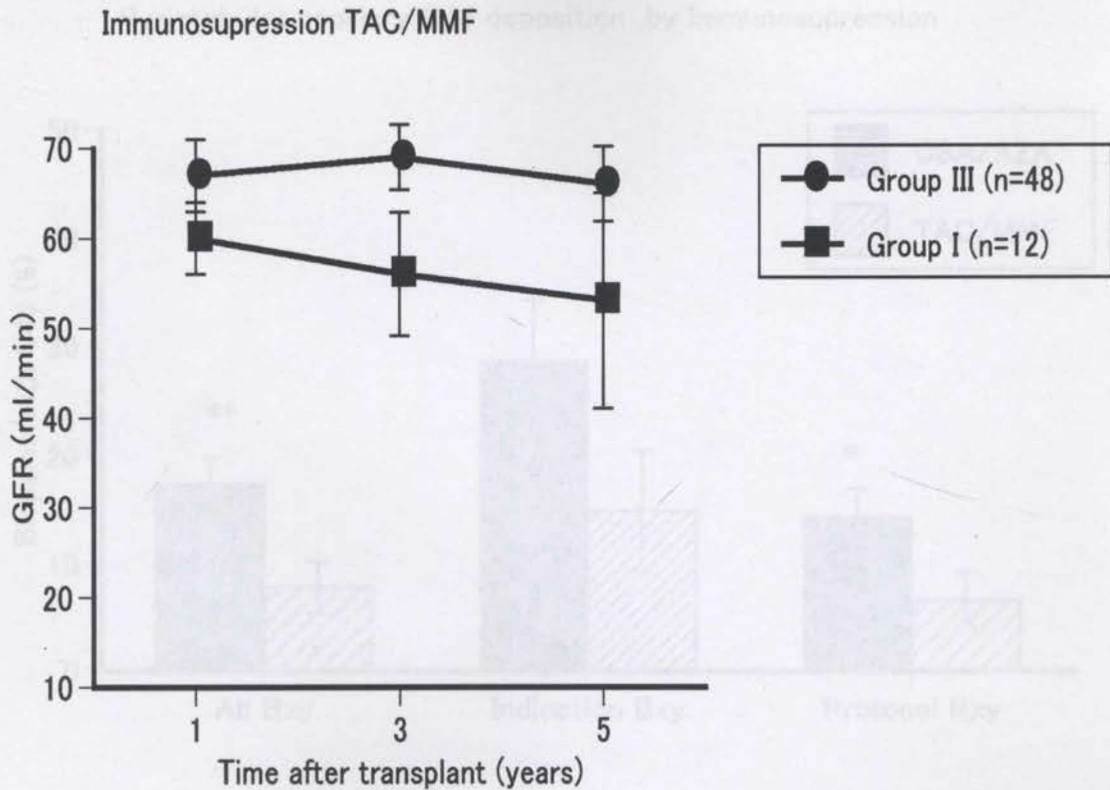


Figure 3.21: Isotopic GFR in patients treated with TAC/MMF according to DSA status. GFR was comparable between sensitised patients with DSA (group I) and unsensitised patients (group III).

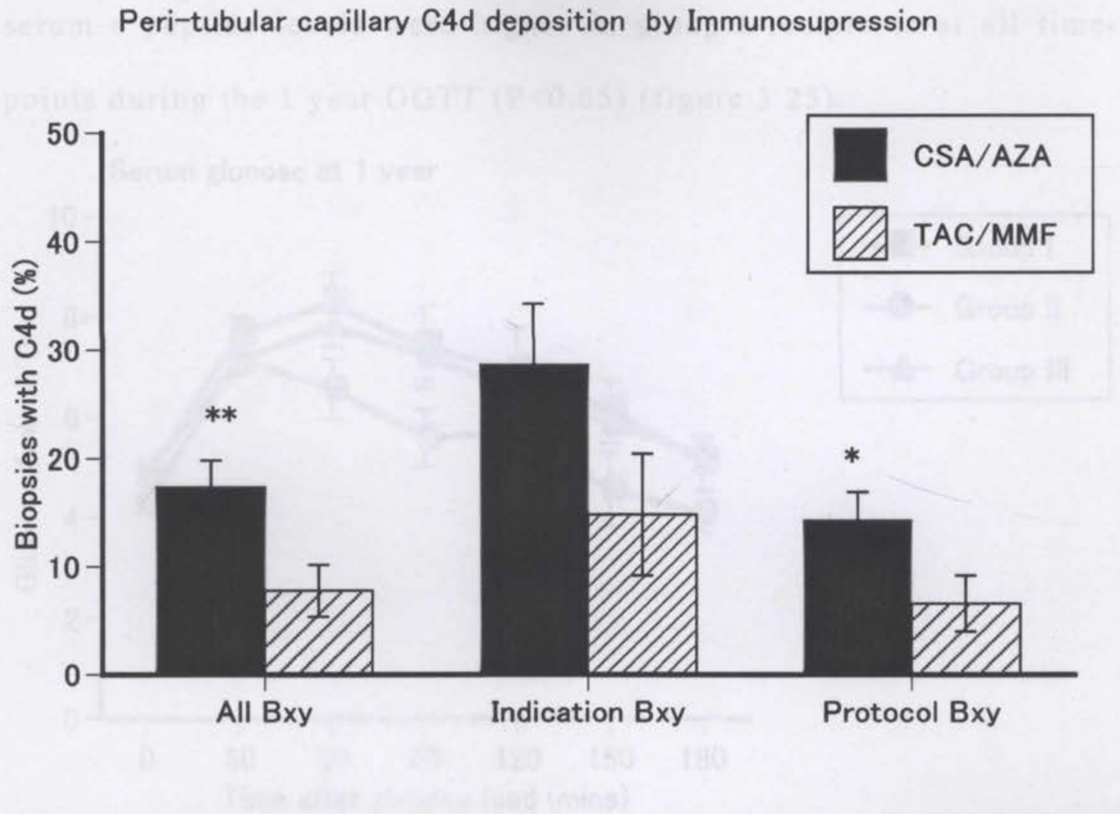


Figure 3.22: C4d deposition in peri-tubular capillaries according to immunosuppression regimen. The proportion of biopsies with C4d was higher in recipients treated with CSA/AZA compared to those treated with TAC/MMF (mean ±SE).

3.3.8 Pancreas Graft Function

OGTT

At 1 year after transplant, glucose levels in response to an oral glucose load were identical between group I and III for fasting and all time points up to 3 hours post glucose loading (P=NS). Glucose levels were lower in group II recipients although not statistically significant (P=NS) (Figure 3.23). Serum insulin levels at 30, 60, 90, and 120 minute time points were lower in group III compared to groups I and II but results were not statistically significant (figure 3.24).In contrast

serum c peptide levels were higher in group I recipients at all time-points during the 1 year OGTT (P<0.05) (figure 3.25).

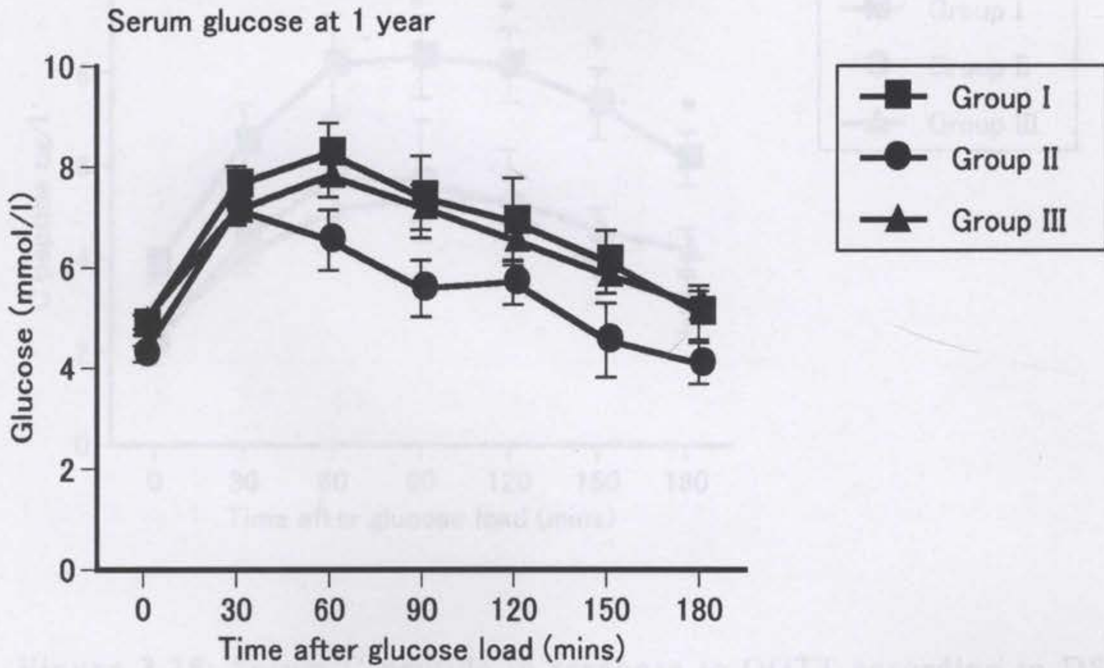


Figure 3.23: Serum glucose in response to OGTT according to DSA status at 1 year after transplantation, (mean ±SEM).

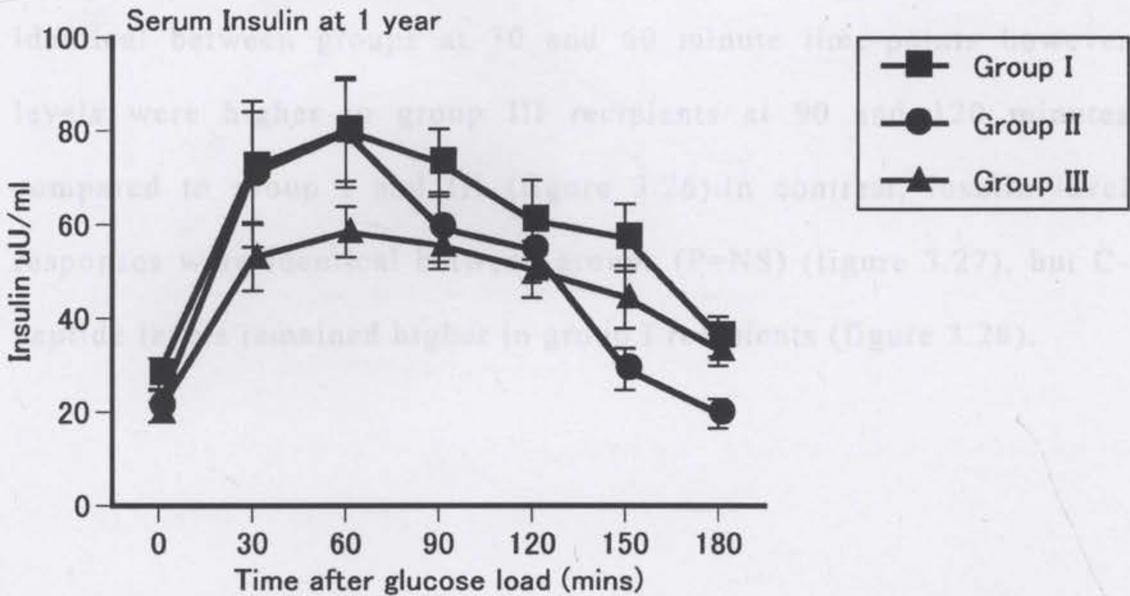


Figure 3.24: Serum insulin in response to OGTT according to DSA status at 1 year after transplantation, (mean ±SEM).

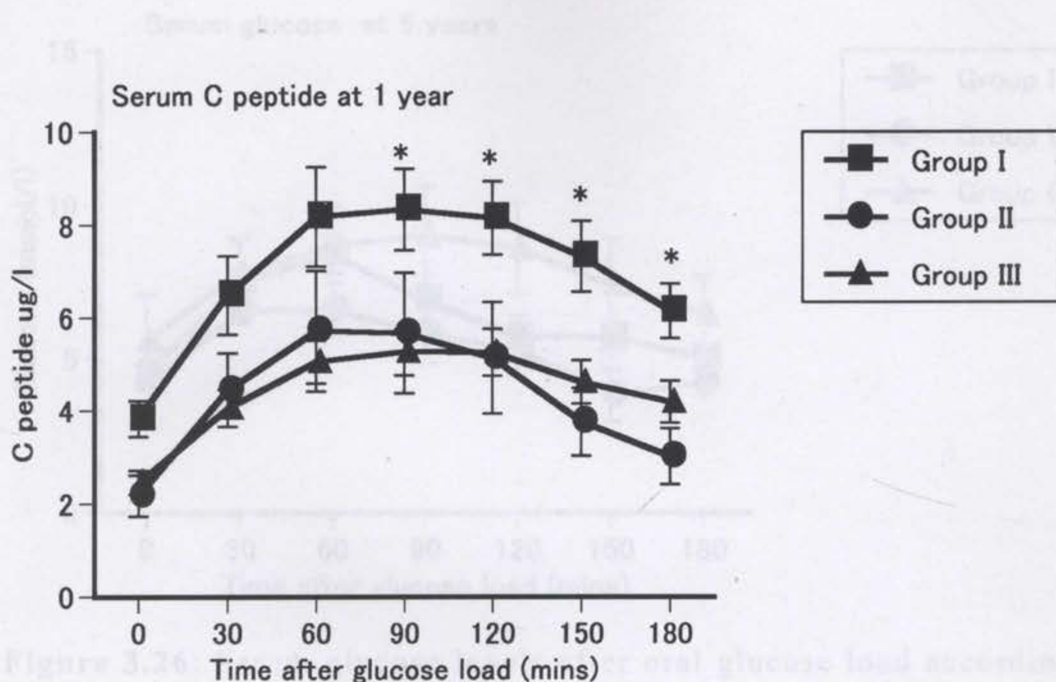


Figure 3.25: Serum C-peptide in response to OGTT according to DSA status at 1 year after transplantation, (mean ±SEM).

At 5 years, glucose levels in response to an oral glucose load were identical between groups at 30 and 60 minute time-points however levels were higher in group III recipients at 90 and 120 minutes compared to group I and III (figure 3.26).In contrast, insulin level responses were identical between groups (P=NS) (figure 3.27), but C-peptide levels remained higher in group I recipients (figure 3.28).

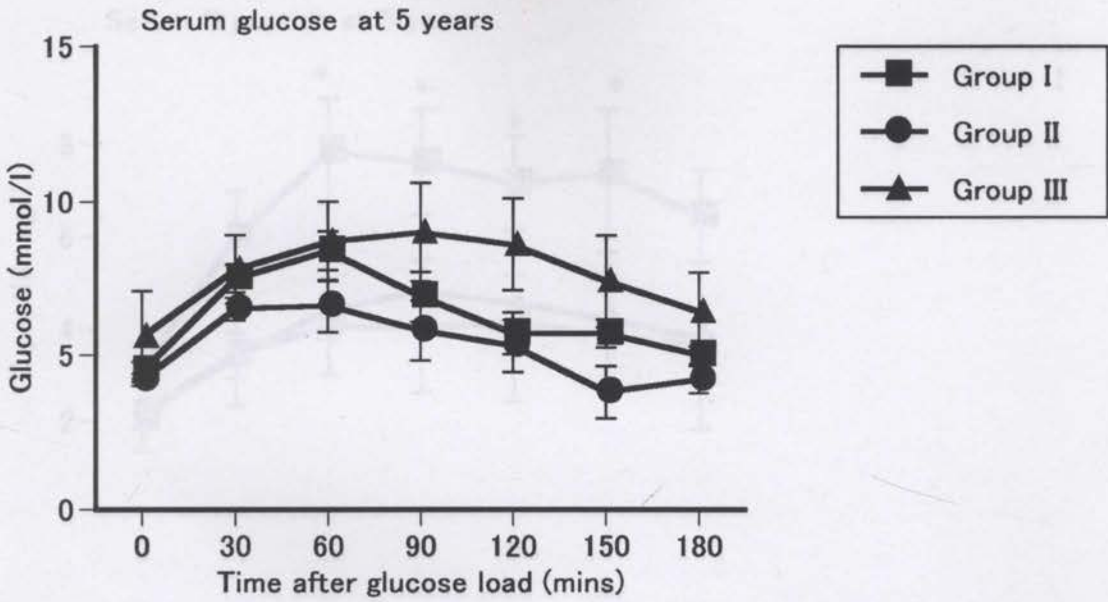


Figure 3.26: Serum glucose levels after oral glucose load according to DSA status at 5years.

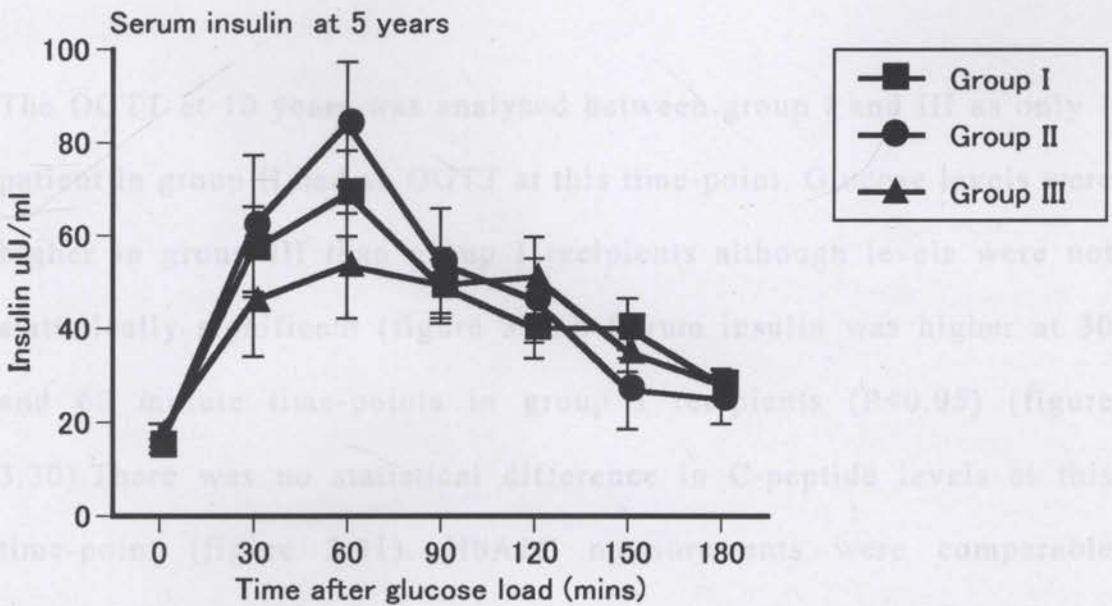


Figure 3.27: Serum insulin levels after oral glucose load according to DSA status at 5 years.

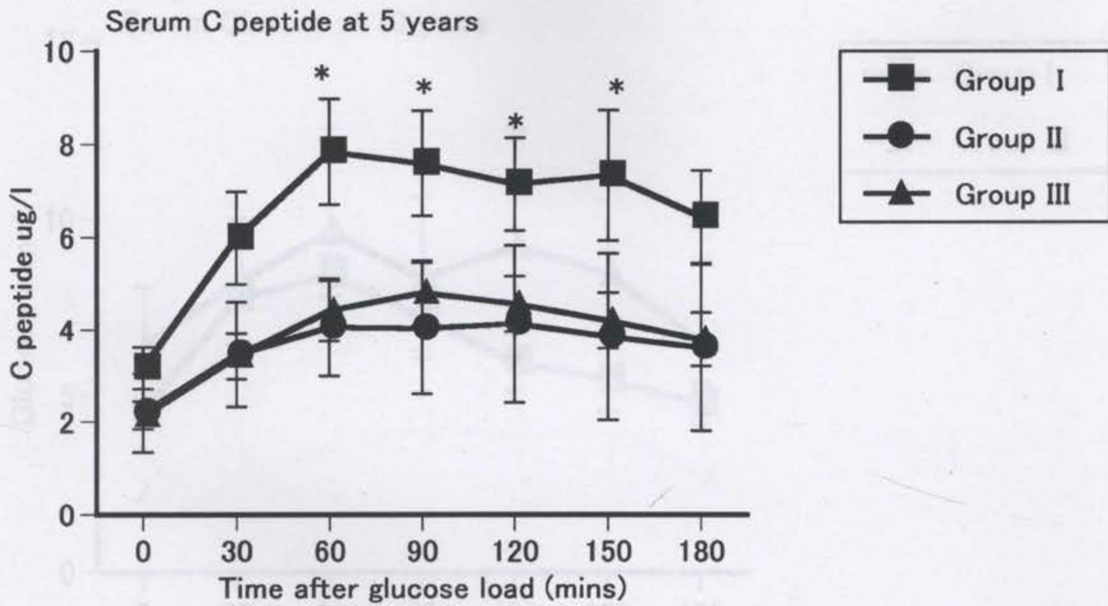


Figure 3.28: Serum C-peptide levels after oral glucose load according to DSA status at 5 years after transplantation.

The OGTT at 10 years was analysed between group I and III as only 1 patient in group II had an OGTT at this time-point. Glucose levels were higher in group III than group I recipients although levels were not statistically significant (figure 3.29). Serum insulin was higher at 30 and 60 minute time-points in group I recipients (P<0.05) (figure 3.30).There was no statistical difference in C-peptide levels at this time-point (figure 3.31). HbA1C measurements were comparable between the groups at 1, 5 and 10 year after transplant (figure 3.32).

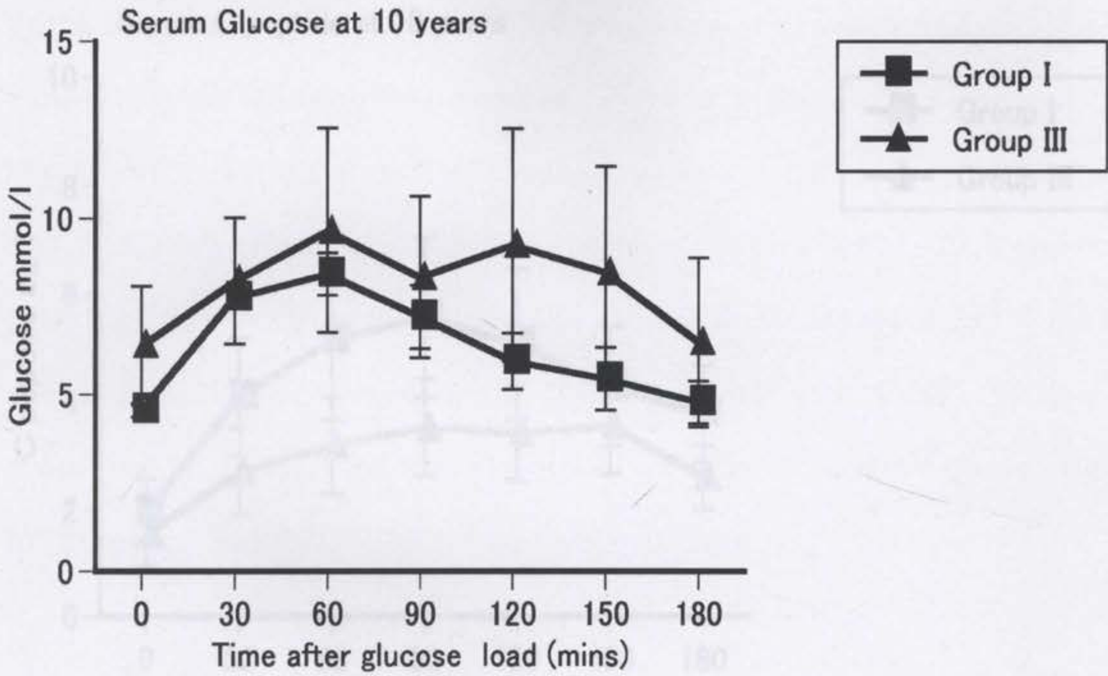


Figure 3.29: Serum glucose levels after oral glucose load according to DSA status at 10 years after transplantation.

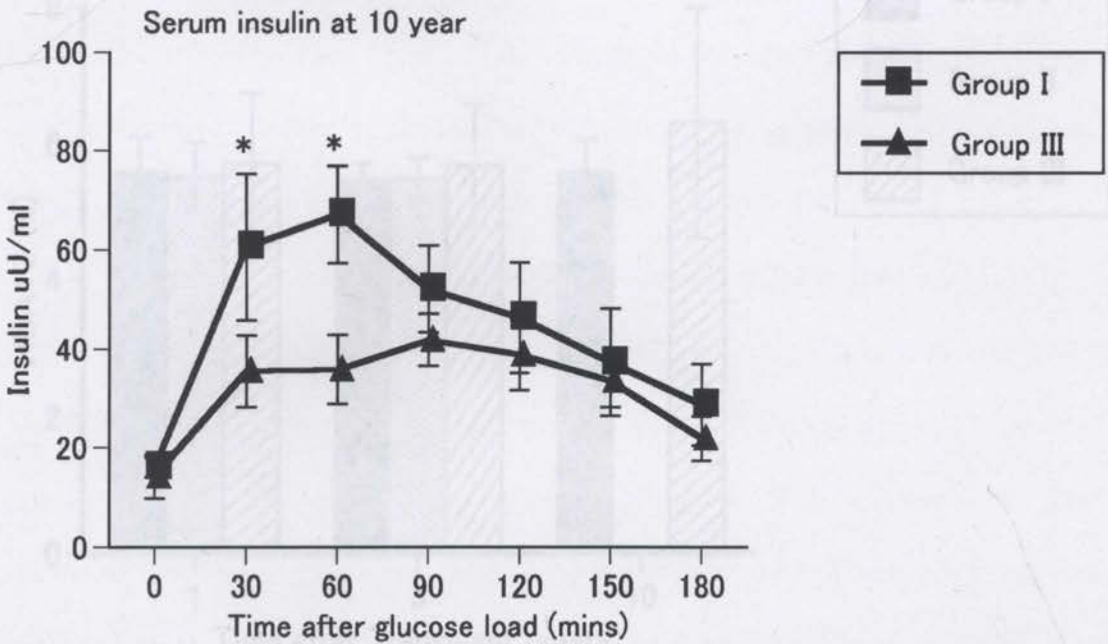


Figure 3.30: Serum insulin levels after oral glucose load according to DSA status at 10 years after transplantation.

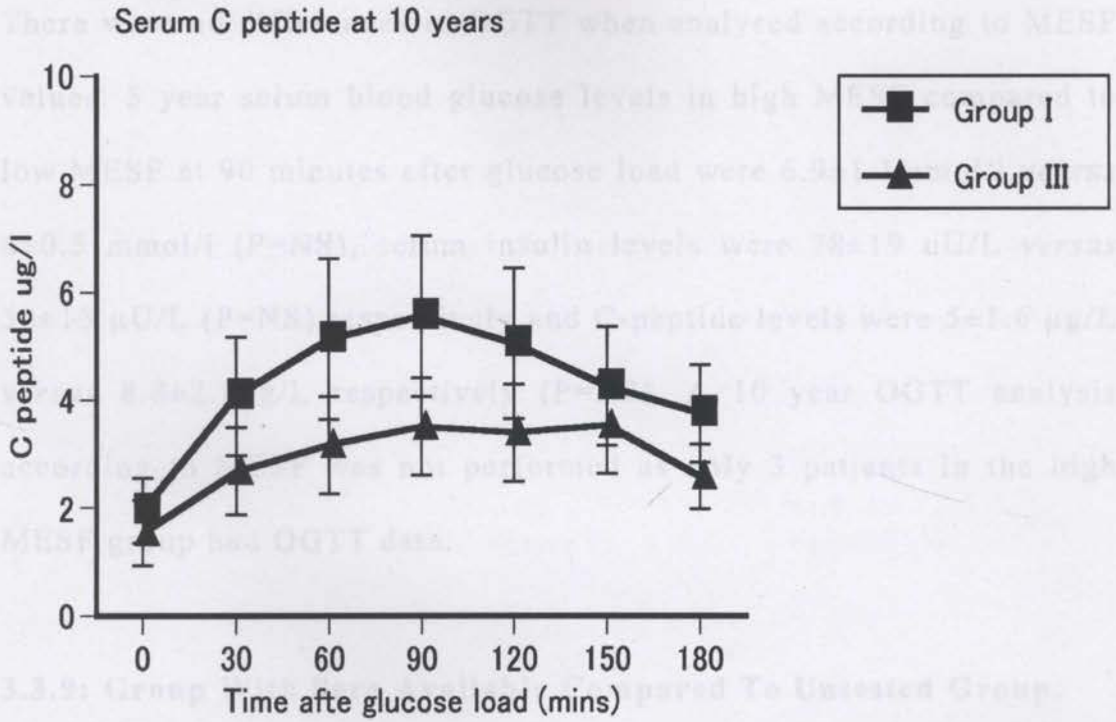


Figure 3.31: Serum C-peptide levels after oral glucose load according to DSA status at 10 years after transplantation.

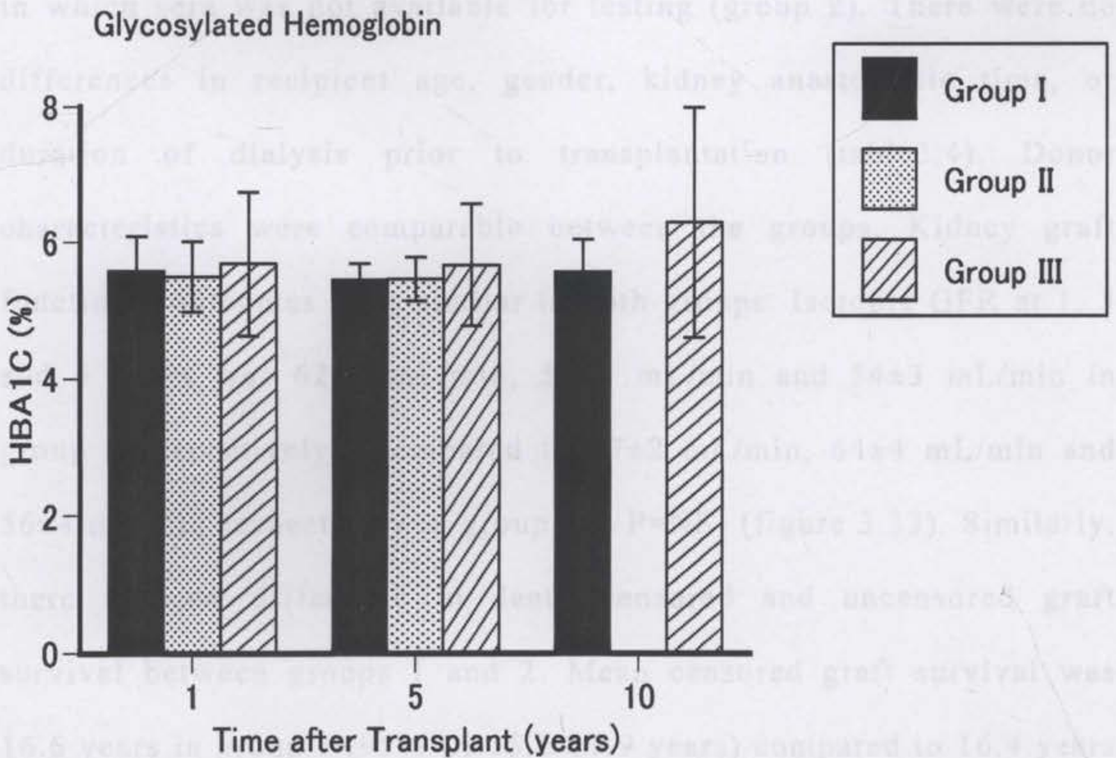


Figure 3.32: Glycosylated haemoglobin according to DSA status. Mean±SD

There were no differences in OGTT when analysed according to MESF values. 5 year serum blood glucose levels in high MESF compared to low MESF at 90 minutes after glucose load were 6.9±1.1mmol/l *versus* 6±0.5 mmol/l (P=NS), serum insulin levels were 78±19 uU/L *versus* 52±15 μU/L (P=NS) respectively and C-peptide levels were 5±1.6 μg/L *versus* 8.8±2.1μg/L respectively (P=NS). A 10 year OGTT analysis according to MESF was not performed as only 3 patients in the high MESF group had OGTT data.

3.3.9: Group With Sera Available Compared To Untested Group.

Patient demographics in the group in which stored pre-transplant sera was available for Luminex® assays (group 1) were comparable to that in which sera was not available for testing (group 2). There were no differences in recipient age, gender, kidney anastomotic time, or duration of dialysis prior to transplantation (table3.4). Donor characteristics were comparable between the groups. Kidney graft functional outcomes were similar in both groups. Isotopic GFR at 1, 3 and 5 years was 62±2 mL/min, 58±3 mL/min and 54±3 mL/min in group 1 respectively , compared to 67±2 mL/min, 64±4 mL/min and 56±4 mL/min respectively in group (all P=NS) (figure 3.33). Similarly, there was no difference in death censored and uncensored graft survival between groups 1 and 2. Mean censored graft survival was 16.6 years in group 1 (95% CI 15.2-17.9 years) compared to 16.4 years (95% CI 14.7-18.2 years) in group 2. Patient survival after transplant

was 15 years (95% CI 13.5-16 years in group 1 compared to 17.8 (95% CI 16.5 -19.2) (figures 3.34 and 3.35).

Table 3.3: Demographics characteristics of recipients were stored sera was available (group 1) compared to those were sera was not available (group 2).

	Group 1 Sera available	Group 2 Sera unavailable	P
Donor age (mean±SD)	28±10	25±8	NS
Donor gender (n, % female)	50(42)	36 (30)	NS
KAT min (mean±SD)	34±8	34±9	NS
Recipient age (mean±SD	38±9	38±8	NS
Recipient sex (n, % female)	52 (43)	55 (46)	NS
Total HLA Mismatch	199±28	185±59	NS
TAC trough 1 yr (ng/ml)	7.7±1.0	9±1	NS
Duration of dialysis (years)	2.1±2.5	2.2±1.4	NS
CSA/AZA	43 (37)	29 (27)	NS
TAC/MMF	74 (63)	80 (73)	NS

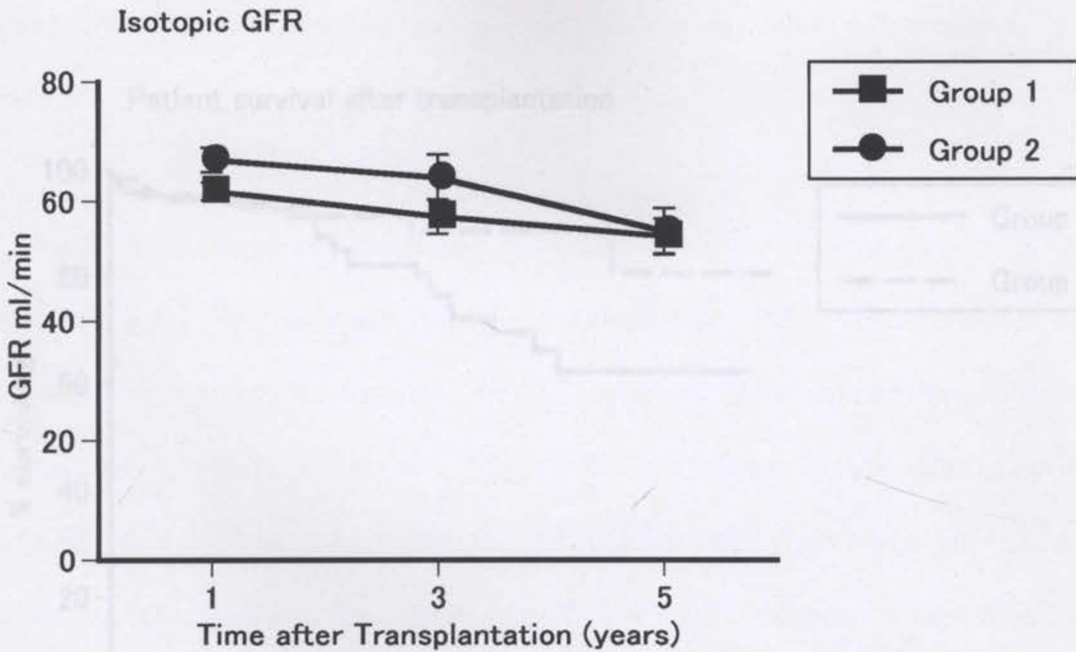


Figure 3.33: Isotopic GFR in patients with stored sera (group 1) compared to patients without stored sera (group 2). GFR was comparable between groups.

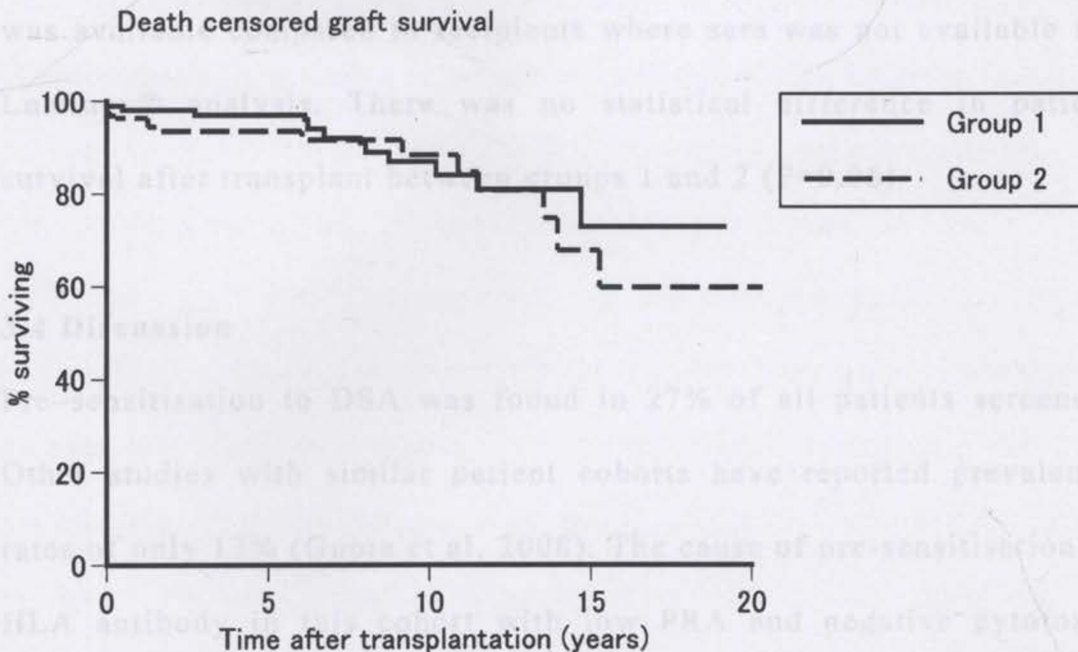


Figure 3.34: Death censored graft survival curves in recipients with stored sera (group 1) were comparable to recipients where sera was not available.

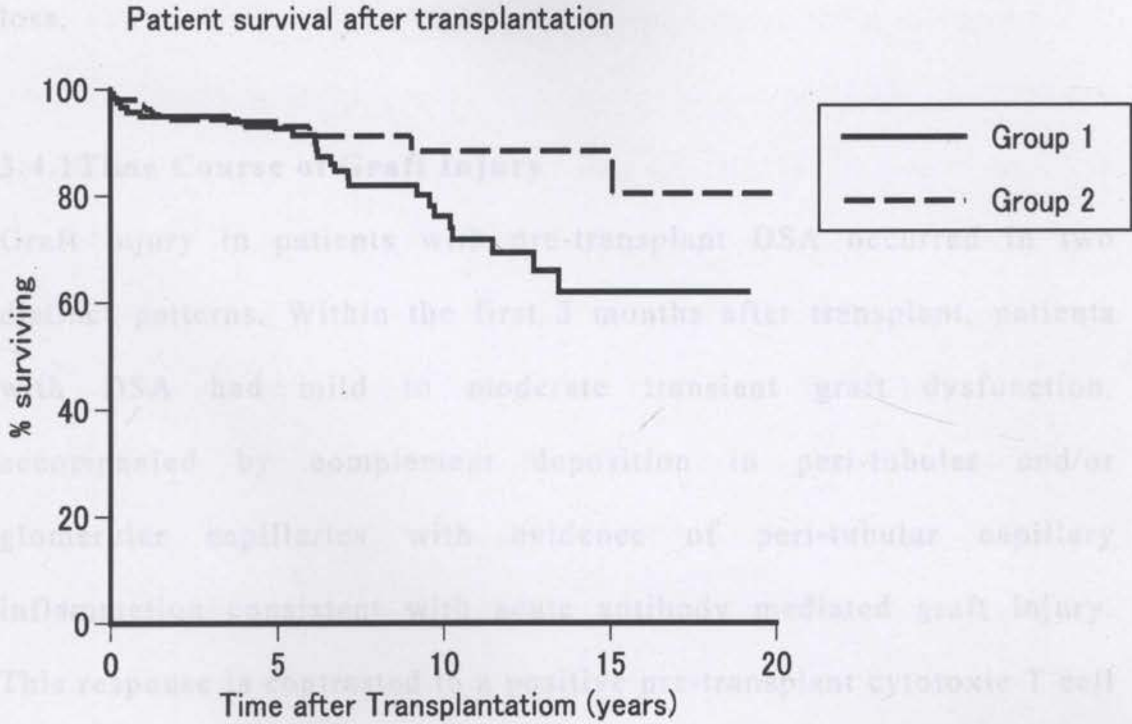


Figure 3.35: Uncensored graft survival in recipients where stored sera was available compared to recipients where sera was not available for Luminex® analysis. There was no statistical difference in patient survival after transplant between groups 1 and 2 (P=0.06)

3.4 Discussion

Pre–sensitisation to DSA was found in 27% of all patients screened. Other studies with similar patient cohorts have reported prevalence rates of only 13% (Gupta et al. 2008). The cause of pre-sensitisation to HLA antibody in this cohort with low PRA and negative cytotoxic cross-match is unclear; however, since a higher proportion of patients with DSA in this series were female this might explain high rates of pre-sensitisation. Pre-transplant DSA were associated with abnormal

graft dysfunction, pathological abnormalities, and subsequent graft loss.

3.4.1Time Course of Graft Injury

Graft injury in patients with pre-transplant DSA occurred in two distinct patterns. Within the first 3 months after transplant, patients with DSA had mild to moderate transient graft dysfunction, accompanied by complement deposition in peri-tubular and/or glomerular capillaries with evidence of peri-tubular capillary inflammation consistent with acute antibody mediated graft injury. This response is contrasted to a positive pre-transplant cytotoxic T cell cross-match (which commonly results in early and acute graft loss or primary non-function) and may reflect differing strength of antibody. The second phase of injury occurred beyond 2 years and was characterised by a slow and progressive deterioration in graft function, increasing transplant glomerulopathy, mesangial matrix expansion, and worsening interstitial fibrosis ultimately resulting in graft loss. Although graft function appeared satisfactory by serum creatinine estimation, reduced isotopic GFR at similar time-points, persistent glomerular and peri-tubular capillary C4d deposition and the appearance of transplant glomerulopathy suggests chronic subclinical antibody mediated injury. Recipients sensitised to HLA antibody but without donor specific antibody had comparable outcomes to the unsensitised patients.

Results of OGTT did not demonstrate a relationship between DSA and pancreas graft function as there was no statistical differences in blood glucose levels in response to a glucose load among the 3 recipient groups. However, serum C-peptide and insulin levels were lower in unsensitised recipients (group III) compared to sensitised recipients with DSA (group I). It is likely that this may be attributed to steroid use and body weight of recipients. However this effect was not examined due to unavailability of adequate data reflecting variation in steroid dose/body weight over time in both groups and the likelihood that recipients in group I had more steroid exposure due to higher acute rejection rates which could not be accurately quantified. On the other hand, the majority of patients in group III were treated with Tacrolimus which is reported to have increased islet cell toxicity compared to Cyclosporine. This is also likely to be a contributing factor to the observed differences.

3.4.2 Factors that Modify Graft injury

Although strong associations between circulating antibody, complement activation and inferior graft outcomes have been demonstrated in many studies (Bocrie et al. 2007; Bohmig et al. 2002; Mauiyyedi et al. 2002b; Mauiyyedi et al. 2002a; Sis et al. 2007; Worthington et al. 2007), not all patients with circulating antibody develop graft dysfunction. This suggests that other factors determine the effects of antibody. Antibody characteristics such as IgG subclass, specificity and cytotoxic potential are postulated reasons for these

observations (Bohmig et al. 2008; Kushihata et al. 2004; Sis et al. 2007). This study demonstrates that antibody strength and immunosuppression regimens influence the ability of circulating antibodies to mediate graft injury.

3.4.2.1 Antibody Strength

The fluorescent intensity (FI) of a single Luminex bead is determined by antigen density on the bead and the binding affinity of antibody. When corrected for antigen density, the FI provides a measure of antibody binding affinity and avidity. MESF (molecules of equivalent soluble fluorochromes) values, which depend on the amount of soluble fluorochrome on a bead, can be derived from bead fluorescent intensities and provide a standardised and easily transferable measure of antibody strength between different sera and laboratories. Mizutani et al demonstrated that strength of fluorescence on Luminex micro-particles correlated with antibody titre, in their study, higher MESF values predicted graft failure and dysfunction. In addition increasing MESF values correlated with worsening graft function (Mizutani et al. 2007). In our study, high MESF values identified patients at risk for early graft dysfunction and subsequent graft loss probably mediated by complement dependent mechanisms as shown by early C4d deposition in PTC. Beyond 1 year, graft injury appeared to be mediated by persisting PTC complement activation in the high MESF group. Our study supports the hypothesis that low strength antibodies could initiate a process of endothelial injury, followed by successful host-cell repair and subsequent amelioration of graft injury or graft

accommodation. This is further supported by data from *in-vitro* allograft studies which show that endothelial cell exposure to low level antibody concentrations could result in resistance to subsequent antibody and complement mediated damage (Narayanan et al. 2004), Other factors such as the varying ability of IgG subclass and iso-type to activate and bind complement may also play a role (Bartel et al. 2007). We propose that Luminex® combined with MESF, values less than 100,000 be considered low risk for poor outcomes.

It is unclear which bead to consider when assessing antibody strength especially in sera where a large proportion of beads are positive for donor specific antibodies. Mizutani (Mizutani et al. 2007) et al proposed that the strongest bead should be considered. The maximum MESF values from the most reactive bead with donor specificity were used in our analysis. These results demonstrate an adverse impact of DSA dependent on MESF on long term functional graft outcomes. On the basis of these data MESF values should be used as a measure of antibody strength when the Luminex® assay is used. The use of risk stratification by MESF values could also play a role in desensitisation protocols but would require further evaluation in this setting.

3.4.2.2 Immunosuppression

Patients with DSA treated with CSA/AZA had inferior kidney graft functional outcomes to when compared to recipients treated with

TAC/MMF. Immunosuppression protocols were determined by era without prior knowledge of antibody status with most of the benefit occurring when MMF replaced AZA. This effect may be due to potent anti-proliferative effect of MMF on B cells compared to AZA. This hypothesis is supported by studies which show lower antibody levels and reduced antibody production independent of T cell suppression in MMF treated transplant recipients (Rose et al. 2002; Smith et al. 1998; Terasaki et al. 2004). The differences in graft function are unlikely to be attributed to CNI effects as other studies demonstrate that although CNI preferentially inhibit T cell activation and production of IL-2, they have far less effect on B cell proliferation (Wicker et al. 1990). While these data are retrospective and lack the power of a randomized clinical trial, use of MMF for treatment of CAMR is a testable hypothesis

3.5 Conclusion

Pre-transplant donor HLA antibodies are common in unsensitised patients when the Luminex® assay is used. When present, they result in inferior histological and functional outcomes and chronic graft loss. Graft dysfunction occurs in two distinct patterns an early phase and a late phase. The early phase is characterised by transient graft dysfunction due to acute antibody mediated rejection and the late phase is characterised by progressive deterioration in graft function and transplant glomerulopathy. The strength of antibody binding defined by MESF values, modify these outcomes. Transplant recipients with high

MESF have inferior graft function and histological outcomes. Based on these data patients should be screened for antibody using Luminex® or other solid phase assays. In all those with antibodies detected, single antigen testing with antibody strength measured by MESF values should be undertaken to identify recipients at increased risk for adverse outcomes and thereby allow either avoidance of these mismatched antigens or pre-emptive immunosuppression modification.

CHAPTER 4

PRE-TRANSPLANT MICA ANTIBODIES

4. PRE-TRANSPLANT MICA ANTIBODIES

4.1 Introduction

This chapter examines the effect of pre-sensitisation to Major histocompatibility complex class 1 related chain A antigens (MICA) on graft function and the development of transplant glomerulopathy. The MHC class I chain-related (MIC) proteins are related to the Major histocompatibility complex (MHC) class I proteins which are ubiquitously expressed and mediate the recognition of intracellular antigens by cytotoxic T cells. The MIC genes evolved in parallel with the human class I genes and with those of most mammalian orders. The MICA gene is located near HLA-B and is by far the most divergent mammalian MHC class I chain related gene known.

(MICA) antigens are expressed on endothelial cells, dendritic cells, epithelial cells and fibroblasts but not on peripheral blood lymphocytes (PBMC) (Zwirner et al. 1999; Zwirner et al. 2000). Consequently, MICA antibodies are not detected by conventional cross-matching techniques because these rely on use of PBMC. Solid phase assays enable detection of these and many other antigens. Antibodies developing in response to endothelial MICA antigens are capable of complement mediated cytotoxicity (Zou et al. 2002), preliminary studies also show that development of post transplant MICA antibodies is associated with inferior graft outcomes; Terasaki et al demonstrated decreased survival at 1 and 4 years in patients who developed MICA antibodies after transplantation in a prospective multi-centre trial

(Terasaki et al. 2007). These findings have been confirmed in several other studies (Mizutani et al. 2006c; Mizutani et al. 2006b; Mizutani et al. 2006a; Morales-Buenrostro et al. 2006; Ozawa et al. 2006; Panigrahi et al. 2007).

Limited data is available on the effect of pre-sensitisation to MICA antibodies on graft outcomes and long term histological outcomes. Zou et al analysed pre-transplant sera from 1910 patients for both HLA and MICA antibodies using ELISA assays and Luminex® micro-spheres respectively. MICA antibodies were found in 11% of patients; pre-sensitisation due to presence of MICA antibodies was associated with renal allograft rejection and increased graft loss in the absence of HLA pre-sensitisation. The authors also examined possible causes of pre-sensitisation and concluded that cross reactivity with environmental antigens may play a role in priming the immune system to MICA antigens (Zou et al. 2007). Donor specificity of MICA antibodies was not investigated.

4.2 Methods

The study group consisted of 110 donor–recipient pairs selected from 121 SPK transplants described in section 3.2. From this group 11 patients were excluded because sera were unavailable in sufficient quantities for both Luminex® and MICA analyses. MICA antibodies were analysed on pre–transplant sera using Luminex® platform as previously described in section 2.6.2. MICA expression within the

grafts was not examined. Results were analysed against graft function measured by serum creatinine and isotopic GFR (described in section 2.7.1), histological outcomes including PTC and glomerular capillary complement deposition (described in section 2.4) and Banff light microscopy analysis described in section 2.3.

4.3 Results

4.3.1 Demographics

Of 110 patients tested, MICA antibodies were detected in 28 patients. Presence of MICA antibodies was not associated with pre-sensitisation to HLA antibodies assessed by peak and current PRA. Mean historical and current PRA were 12±3% and 2±0.8% respectively in the group with MICA antibodies compared to 15±3% and 2±0.7% in the group without. In patients with MICA antibodies 21 % of patients had a peak historical PRA of more than 25% compared to 24 % of patients without Mica abs (χ^2=0.101, P=0.5). Current peak PRA of more than 0% was found in 29% MICA$^+$ patients compared to 17% MICA$^-$ patients (P=0.141).There was no differences in the number of HLA mismatches between both groups. Mean HLA A, B, and DR mismatches were 1.6±0.1, 1.6±0.6, and 1.5±0.1 in the MICA$^+$ group compared to 1.3±0.1, 1.7±0.1, and 1.5±0.6 in the MICA$^-$ group (all P=NS).

4.3.2 Graft function

In contrast to the presence of HLA antibodies, presence of MICA antibodies had no impact on early or late graft function assessed using serum creatinine or isotopic GFR. Serum creatine was comparable in both groups through the follow-up period (Figure 4.1), mean serum creatine at 1, 3 and 5 years were 130±6μmol/l, 135±10 μmol/l and 158±24 μmol/l respectively in the group with MICA antibody compared to 118±4 μmol/l, 136±11μmol/l, 149±18 μmol/l in the group without (all P=NS).

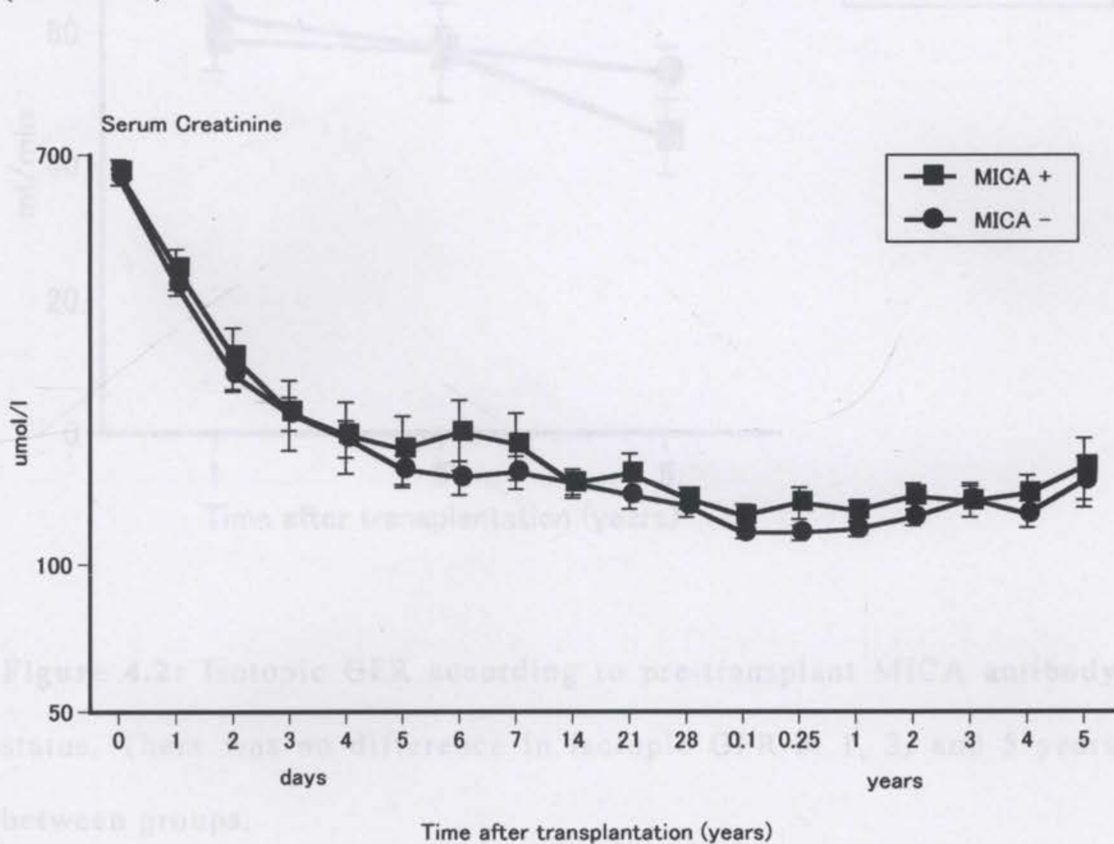


Figure 4.1: Serum Creatinine according to pre-transplant MICA antibody status. There is no difference in serum creatinine between patients with pre-transplant MICA antibodies compared to those without.

Isotopic GFR was comparable between both groups. Mean isotopic GFR at 1, 3 and 5 years was 59±4.5 mL/min, 57±7.4 mL/min, 44±5.8 mL/min in the MICA group compared to 63±1.8 mL/min, 58±3.1 mL/min, 54±3.9 mL/min in the group without MICA antibody (all P=NS) Figure 4.2.

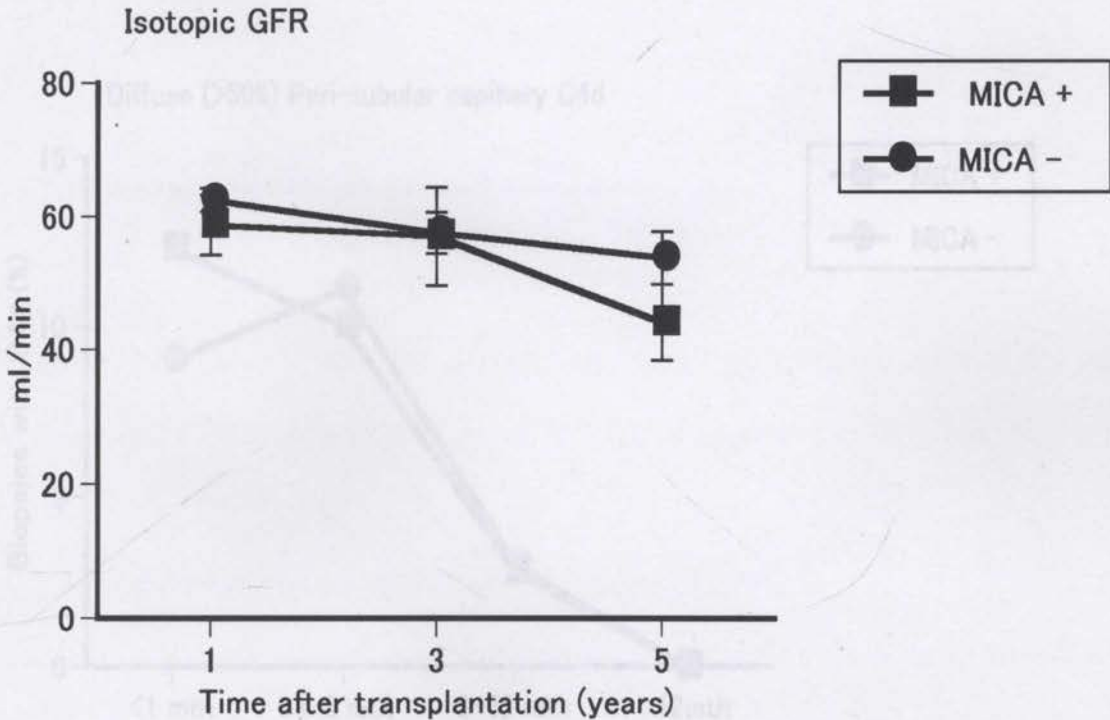


Figure 4.2: Isotopic GFR according to pre-transplant MICA antibody status. There was no difference in isotopic GFR at 1, 3, and 5 years between groups.

4.3.3 Complement deposition

There was no difference in diffuse C4d deposition between both groups. The proportion of biopsies with diffuse (>50%) C4d deposition in PTC at less than 1 month, 1-3 month and 4-12 months in the group

with MICA antibody was 12%, 10%, and 3% respectively, compared to 9%, 11% and 2.8% in the patients without MICA antibodies (all P=NS) (Figure 4.3). Glomerular C4d staining had a similar pattern in with no difference in proportion of biopsies with C4d at less than 1 month, 1-3 month, and 3-12 months in the DSA group compared to DSA negative group (Figure 4.4)

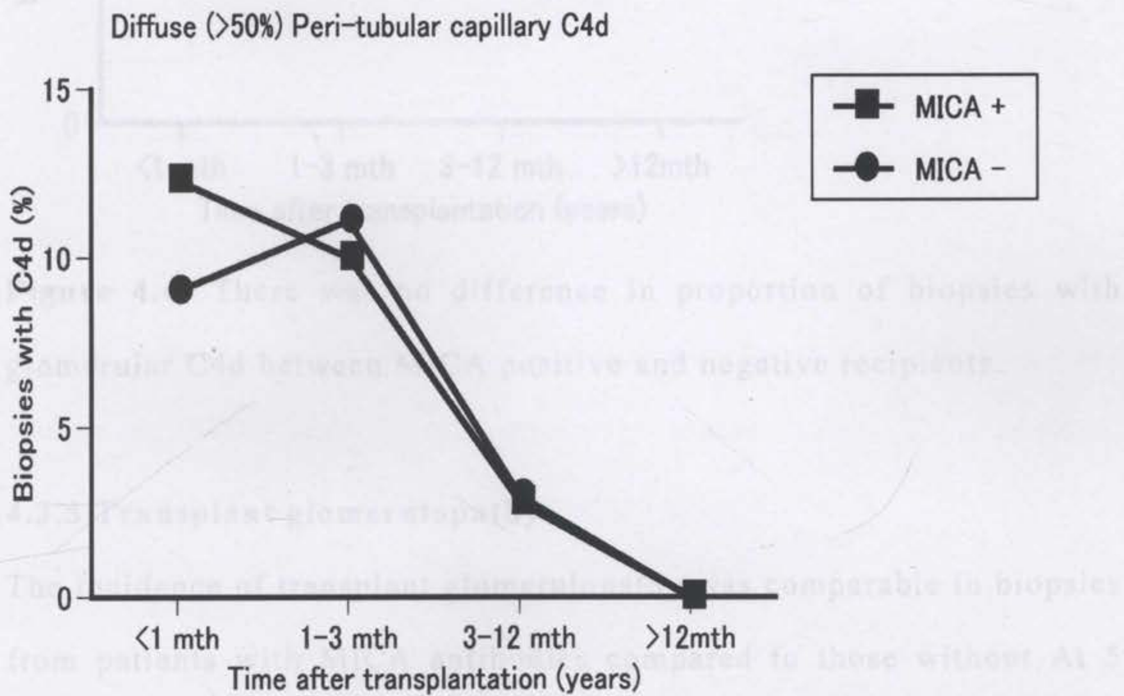


Figure 4.3: There was no difference in PTC C4d deposition between MICA positive and negative recipients

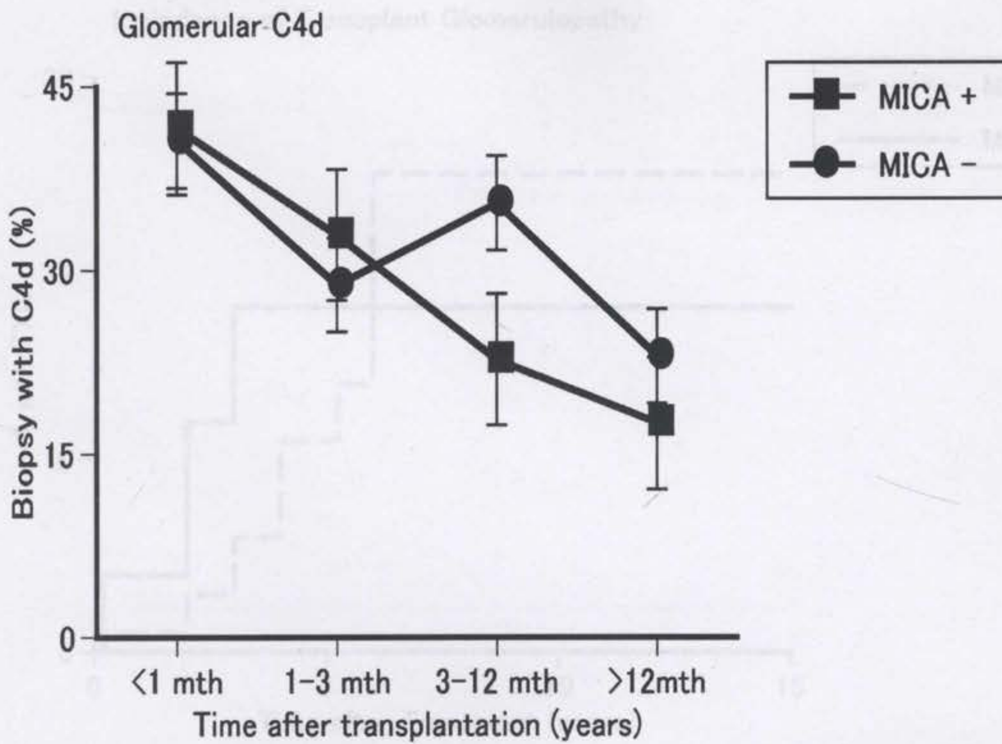


Figure 4.4: There was no difference in proportion of biopsies with glomerular C4d between MICA positive and negative recipients.

4.3.5 Transplant glomerulopathy

The incidence of transplant glomerulopathy was comparable in biopsies from patients with MICA antibodies compared to those without At 5 years following transplant, 18% of patients with MICA antibodies had transplant glomerulopathy compared to 10% of patients without antibody, by 10 years after transplant, transplant glomerulopathy had occurred in 25% of patients without MICA remaining the same in those with MICA antibody (P=NS) (Figure 4.5).

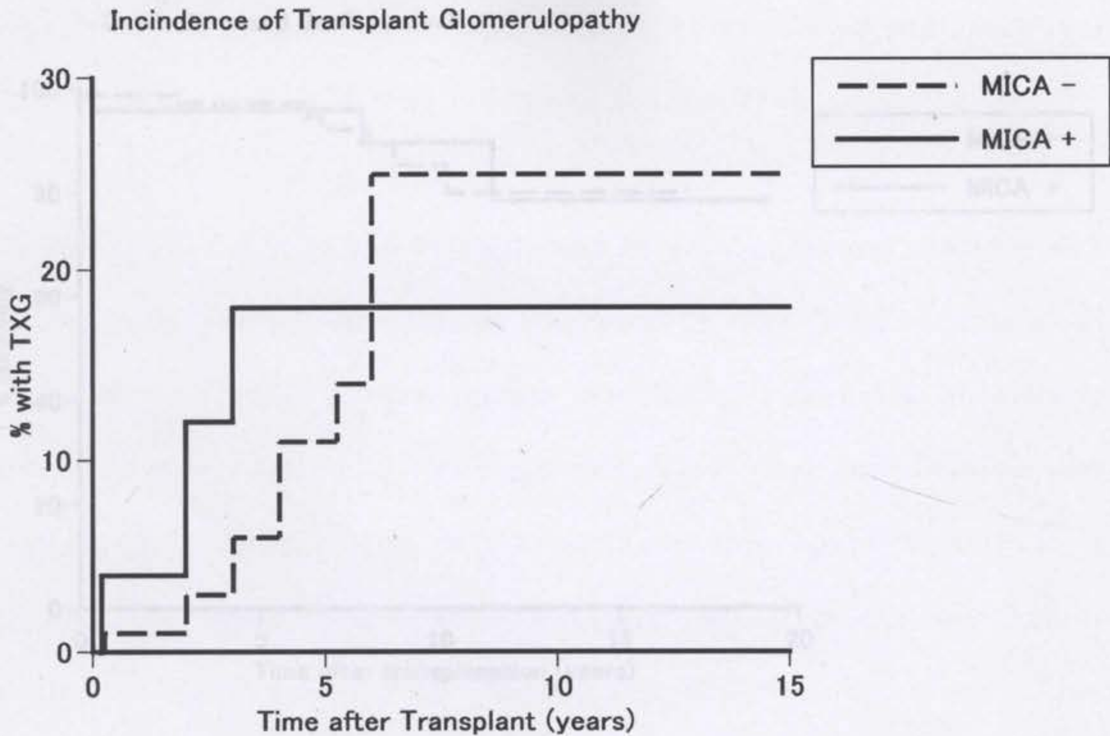


Figure 4.5: Incidence of transplant glomerulopathy according to pre–transplant MICA antibody status. Incidence rates of transplant glomerulopathy were comparable between groups.

4.3.6 Graft Survival

Pre-transplant MICA antibody status had no effect on graft survival. Graft loss occurred in 3 of 28 (11%) patients with MICA antibodies compared to 7 of 81 (9%) patients without antibody. Kaplan-meir survival curves were comparable in both groups (Figure 4.5). Mean survival was 13.9 years SE 0.7 (CI 12.5-15.3) in the patients without MICA antibody compared to 13.6 years SE 1.5 (CI 10.7-16.5) in patients with MICA antibody.

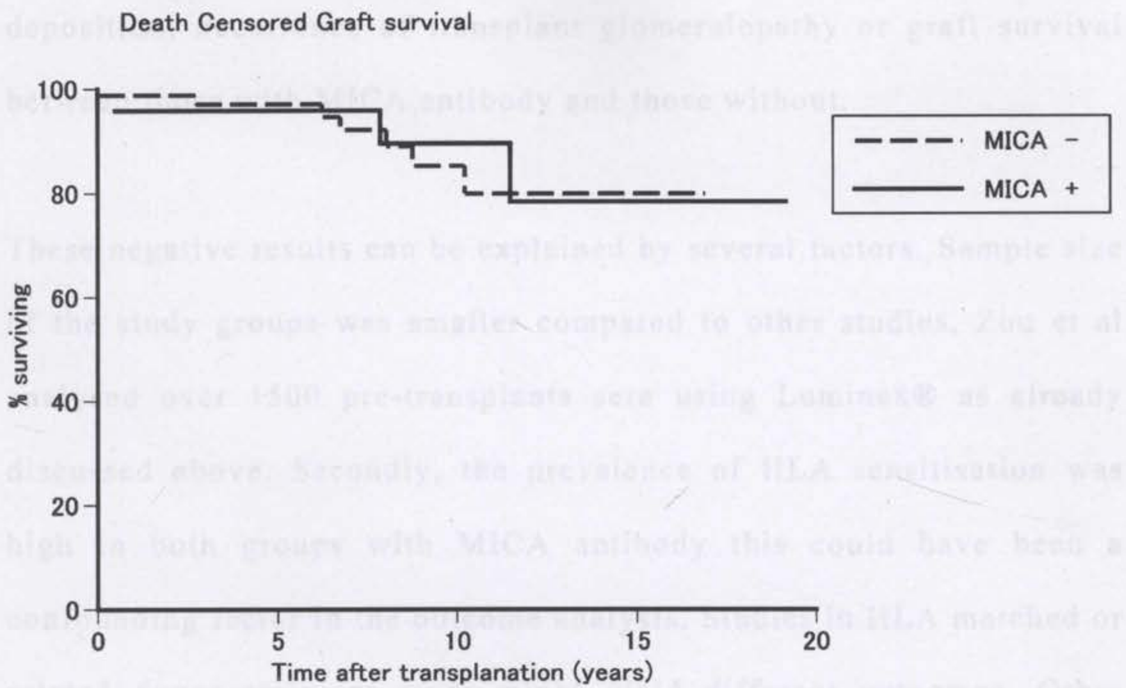


Figure 4.6: Death censored graft survival according to pre–transplant MICA antibody status. Graft loss was comparable between both groups ($\chi2=1.4$, $P=0.2$).

4.4 Discussion

This analysis of the effect of pre-sensitisation to MICA antibodies on long term graft outcomes in SPK recipients produced unexpected results on the basis of previously published data, which demonstrate an association with inferior short term graft survivals and increased rejection. This analysis does not reveal any effect on early graft function assessed by serum creatinine or late graft function assessed using isotopic GFR. Similarly, when considering chronic antibody mediated rejection, there was no difference in the rates of complement

deposition, occurrence of transplant glomerulopathy or graft survival between those with MICA antibody and those without.

These negative results can be explained by several factors. Sample size of the study groups was smaller compared to other studies, Zou et al analysed over 1500 pre-transplants sera using Luminex® as already discussed above. Secondly, the prevalence of HLA sensitisation was high in both groups with MICA antibody this could have been a confounding factor in the outcome analysis. Studies in HLA matched or related donor–recipient pairs might yield different outcomes. Other studies evaluating post transplant MICA antibodies report that presence of both HLA and MICA antibodies resulted in worse outcomes. Thirdly, assessment of donor specificity of the MICA antibodies was not performed due to unavailability of stored donor serum. Thus it is unclear whether MICA antibodies were specific to the donor, which could influence outcomes.

4.5. Conclusion

In conclusion, pre-transplant MICA antibodies appear not to have an impact on the occurrence of transplant glomerulopathy. However more studies in larger cohorts, involving related or HLA matched donors and incorporating assessment of MICA expression within grafts are required to provide data in this evolving field.

CHAPTER 5

TRANSPLANT GLOMERULOPATHY

5. EVOLUTION OF TRANSPLANT GLOMERULOPATHY:

5.1 Introduction

Evolutions of histological abnormalities in kidney allografts affected by TXG are investigated in this chapter, using electron microscopy performed in protocol biopsies. The aim of this was to identify structural markers predictive of transplant glomerulopathy.

Current guidelines for the diagnosis of TXG require demonstration of capillary loop duplication with or without mesangial matrix expansion, presence of products of complement deposition in PTC, and evidence of circulating HLA antibodies. However, this criterion is limited by the low sensitivity of conventional light microscopy, limitations in C4d detection techniques and changes in methods for antibody detection discussed in chapter 1. Consequently, the histopathological features described above may not be consistently observed in all patients with chronic antibody mediated rejection. As an example, Gloor et al found that only a small fraction of patients with transplant glomerulopathy had evidence of complement activation within the graft. In addition, there was inconsistency between circulating antibody status and presence of transplant glomerulopathy (Gloor et al. 2007). These findings could be attributed to the time of diagnosis in relation to the pathophysiology of antibody mediated graft injury. The majority of diagnoses are made in late biopsy specimens. Early diagnosis is likely to provide more consistent data and would enable interventions to be effected at a time when there is a benefit.

Electron microscopy allows identification of ultra-structural abnormalities which would predict late light microscopy abnormalities. Ivanyi et al used this technique to evaluate biopsies in patients with chronic rejection. When PTC multi-lamination was incorporated in the diagnostic criteria for TXG, they demonstrated increased sensitivity for the diagnosis of chronic rejection (Ivanyi et al. 2001). Inclusion of ultrastructure abnormalities of TXG in the diagnostic criteria for CAMR has the potential to enable early diagnosis, and to provide insight on the pathogenesis of TXG. This may result in better correlation between histopathology, complement activation and the effects of circulating antibody. Recent studies have been limited by lack of consecutive EM biopsy material making it impossible to establish a clear temporal relationship between presence of circulating antibody, C4d deposition, and appearance of structural abnormalities.

5.2 Materials and Methods

Study patients were selected from type I diabetic recipients of a successful combined kidney-pancreas transplants (spanning the era from 1987 to 1993), as previously described in section 2.1. To elucidate the sequential histopathology of glomerulopathy, 7 unambiguous cases of TXG were selected from the Westmead longitudinal histological database. Inclusion criteria were 1) presence of persistent light microscopic glomerular abnormalities (Banff cg score ≥1 observed on 2 or more protocol biopsies, confirmed by silver staining, and associated with other morphological attributes of TXG

such as mesangial matrix expansion), and 2) availability of 3 or more prior and/or subsequent protocol kidney biopsies for at least 5 years after transplantation. A contemporaneous control group (the control, n=8) was formed from patients successfully transplanted just prior to or immediately after the TXG subjects within the same immunosuppressive era. In this group, the Banff cg score was repeatedly negative on protocol biopsy assessment, but comparable degrees of allograft damage described as non-specific chronic interstitial fibrosis or tubular atrophy were observed. Immunosuppression incorporating cyclosporine (CSA; Sandimmune, Novartis, Basel, Switzerland) commenced at 10 mg/kg per day with doses adjusted to drug trough levels (150-450 ng/ml), Azathioprine (AZA) starting at 1.5 mg/kg/day, and prednisolone given at 30mg daily initially reducing to 20mg by 3 months and tapered to 10mg daily by 6 months was used. Anti-thymocyte induction was not routinely used. OKT3 therapy was used for severe and/or steroid-resistant rejection, or where rejection involved both pancreas and kidney transplants.

Ultra-structural evaluation was performed on kidney biopsies obtained from implantation up to 5 years. Detailed description of tissue preparation, data acquisition and analysis are described in section 2.5. Biosy material was analysed by transmission electron microscopy (Philips CM120 Biotwin TEM, Philips, Eindhoven, Netherlands) Images were acquired using a digital camera operating on the iTEM platform (Morada TEM digital camera, Soft Imaging Systems, Munster,

Germany), and archived as tiff files. One to two glomeruli were examined per sample for morphological changes of capillary loops (section 2.5.5.1). Glomerular basement membrane (GBM) width was estimated using the orthogonal intercept method and expressed as the harmonic mean of orthogonal intercepts as described in section 2.5.5.2. Mesangial morphometry was performed to assess matrix and mesangial cell area-fractions. The methodology is described in section 2.5.5.3. PTC ultrastructure was evaluated from 4 to 5 peri-tubular capillaries, selected from the same biopsy as the light microscopy time point. Results of all qualitative assessments were presented as proportions of the criteria of interest.

Data obtained from the ultrastructure evaluation was analysed against presence of C4d in peri-tubular and glomerular capillaries. C4d staining methods are described in section 2.4. Donor-specific antibodies (DSA) were measured using the Luminex Labscreen assay and when positive HLA specificity was confirmed by single antigen beads (Luminex, One Lambda Inc, Canoga Park, CA). The Luminex® assay is described in section 2.6.2

5.2.1 Statistical Methods

The statistical software package S-PLUS Version 6.2 (Insightful Corporation, Seattle WA, USA) was used to analyse the data. Two-tailed tests with a significance level of 5% were used throughout. The proportions of cells exhibiting particular properties of interest within

each subject over time were arcsine transformed in order to stabilise the variance prior to analysis. Linear mixed effects models were fitted to the arcsine transformed data. A continuous autoregressive model was used to account for the within patient correlation between consecutive observations. Time and subject identifier were considered as random effects and group, time and the group by time interaction were treated as fixed effects.

5.3 Results

5.3.1 Demographics

The demographics, of the study group are presented in Table 1. Overall, study recipients (n=15) were 37.6±7.1 years old and 53% were male were not highly sensitised and received organs from donors with similar demographic characteristics; test and control groups were demographically comparable. Patients were insulin independent with a mean HBA_1C of 5.8±1.8.

Table 5.1: Demographic characteristics of TXG study patients and controls. Mean ± SD.

Parameter	TXG	Controls	P
Patients (n)	7	8	NS
Recipient age (years)	36.5±7.1	38.8±7.4	NS
Recipient sex (n, % male)	2 (29)	6 (75)	NS
Transfusions (mean)	4.3±3	2.9±2.3	NS
Total HLA mismatch	5.1±0.4	4.8±1.2	NS
Peak PRA (%)	35.6±39.2	13.6±28.9	NS
Total Ischemic time (hr)	10.4±4.3	10.5±4.9	NS
Donor age (years)	33±11.3	22.2±6.1	NS
Donor sex (n, % male)	5 (71)	5 (63)	NS
Duration of dialysis (years)	1.5±1.2	1.4±1.6	NS

5.3.2 Light Microscopy

From the Westmead histological longitudinal database (n=1345 biopsies), 1079 specimens were available from the 1 month protocol time-point and beyond for analysis. Of these, 59 samples showed a Banff cg score of one or greater (5.4%), taken from 1 month to 12.3 years after transplantation (mean 4.3±3.8 years).This group was expanded to include all biopsies (n=95 from 7 patients) representing those patients with unambiguous TXG; which were compared with a contemporaneous control group (n=133 biopsies from 8 patients) for

sequential analysis. Together, these specimens (n=228) contained 14.8±9.1 glomeruli and 2.3±1.2 arteries per biopsy, and 11.4% were defined as inadequate by the Banff criteria. Screening light microscopy was scored without knowledge of the clinical details by two independent observers. The inter-observer kappa statistics for the presence of chronic allograft nephropathy; chronic interstitial fibrosis, tubular atrophy and chronic glomerulopathy were 0.61, 0.80, 0.49 and 0.35, respectively. On protocol sampling by light microscopy, the Banff cg score became abnormal for the first time in a series from a mean of 2.3 years (median 1.75, inter-quartile range 0.25-3 years) in the TXG group.

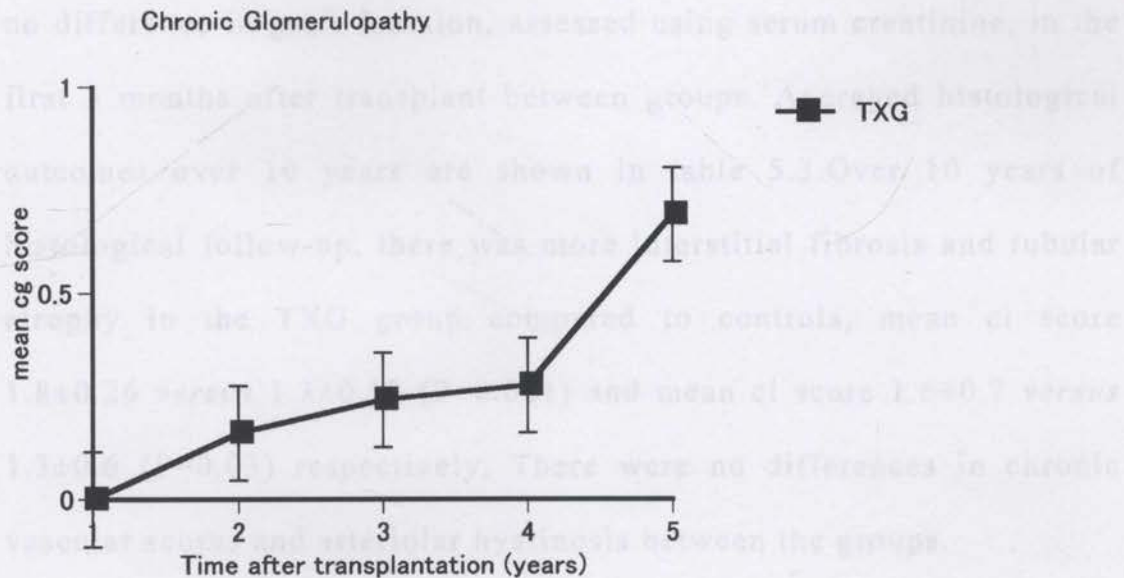


Figure 5.1: Banff mean cg scores in transplant glomerulopathy according to year of transplant inclusive of all biopsies from 1 year to 5 years.

Clinical outcomes between TXG group and control are shown in table 5.2. Outcomes were similar in both groups except for biopsies with acute tubular necrosis and the occurrence of post transplant hypertension. The numbers of acute cellular and vascular rejection episodes were 1.5±1.2 and 0.28±0.5 per patient, respectively, and the prevalence rates of subclinical rejection (including Banff "borderline") at 1, 3 and 12 months after transplantation were 71%, 73% and 46%, respectively (noting that this historical cohort has higher rates of subclinical rejection than those on current immunosuppression protocols). Acute cellular rejection, early acute humoral rejection and true chronic rejection episodes were similar in both groups. There was no difference in graft function, assessed using serum creatinine, in the first 3 months after transplant between groups. Averaged histological outcomes over 10 years are shown in table 5.3.Over 10 years of histological follow-up, there was more interstitial fibrosis and tubular atrophy in the TXG group compared to controls, mean ci score 1.8±0.26 *versus* 1.3±0.59 (P=0.001) and mean ct score 1.6±0.7 *versus* 1.3±0.6 (P=0.03) respectively. There were no differences in chronic vascular scores and arteriolar hyalinosis between the groups.

Table 5.2: Clinical outcomes of TXG study patients and controls. Mean ± Standard deviation (SD) or Number (%).

Parameter	**TXG**	**Control**	**P**
Number (patients/biopsies	7 (95)	8 (133)	
Acute tubular necrosis	3 (43%)	0 (0%)	0.05
Acute cellular rejection	1.2±1.2	1.8±1.2	NS
Early AHR < 1 month	2 (28%)	0 (0%)	NS
Total AHR	4 (58%)	2 (25%)	NS
Vascular rejection (n, %)	2 (29%)	2 (29%)	NS
Use of OKT3	5 (71%)	4 (50%)	NS
True Chronic rejection	1 (14%)	0 (0%)	NS
Late rejection	2 (29%)	0 (0%)	NS
Discharge S creatinine	225±93	222±99	NS
1 month S creatinine (μmol/L)	259±204	185±106	NS
3 month S creatinine (μmol/L)	129±41	115±23	NS
3 month CSA dose (mg/kg/d)	129±41	115±23	NS
Proteinuria	5 (71%)	3 (38%)	NS
Post-transplant hypertension	3 (43%)	7 (88%)	0.07

AHR is acute humoral rejection defined as diffuse C4d staining and PTC inflammation. Proteinuria is >0.15g/day. Post transplant hypertension defined as sustained BP>140/90.

Table 5.3: Averaged Banff histology scores in TXG *versus* controls.

Parameter	TXG	Control	P
Averaged Histology (3 months to 10 years)			
Acute glomerulitis (g)	3 (43%)	0 (0%)	0.05
Interstitial fibrosis (ci)	1.2±1.2	1.8±1.2	NS
Tubular atrophy (ct)	2 (28%)	0 (0%)	NS
Chronic vascular score (cv)	4 (58%)	2 (25%)	NS
Mesangial matrix (mm)	2 (29%)	2 (29%)	NS
Arteriolar hyalinosis (ah)	5 (71%)	4 (50%)	NS

5.3.3 Time-Course of Glomerular Capillary Loop Ultra-structural Abnormalities.

5.3.3.1 Endothelial Activation.

In grafts that developed TXG, endothelial cells exhibited early ultra-structural changes consistent with endothelial activation. These abnormalities included hypertrophy and presence of vacuoles within endothelial cytoplasm and were associated with subsequent loss of endothelial fenestration, resulting in continuous endothelium within the glomerular capillary loops (Figure 5.2).

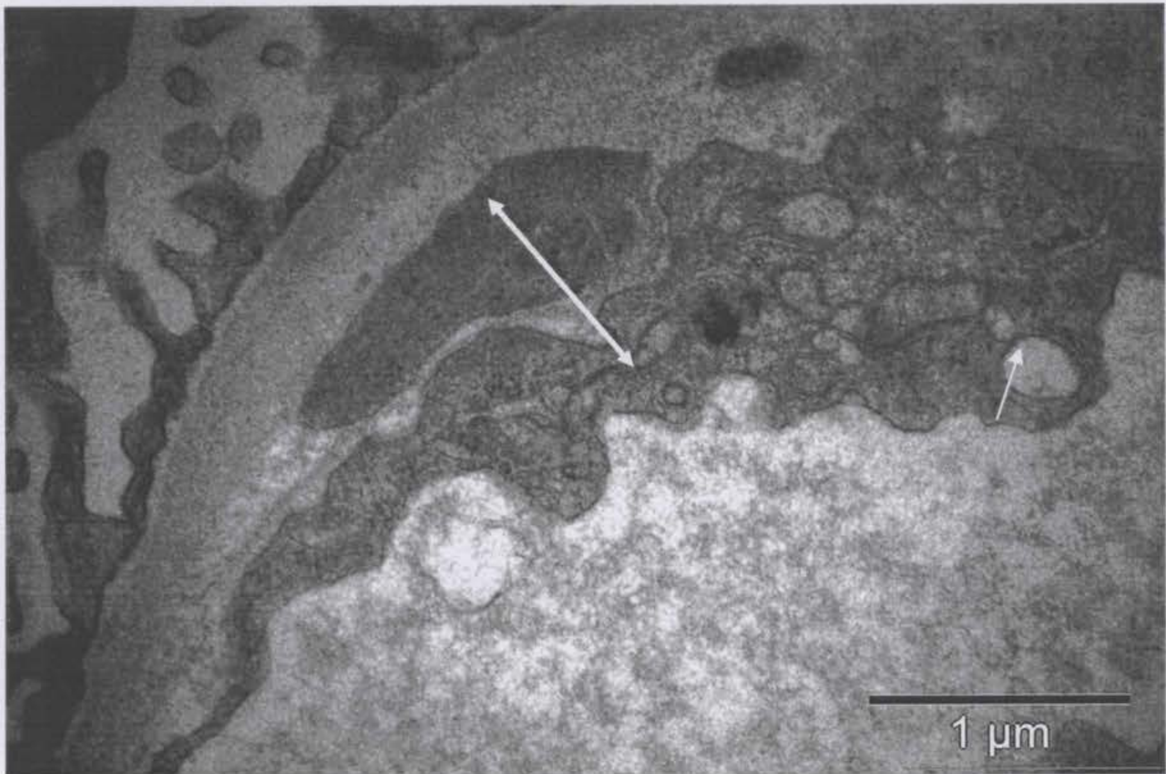


Figure 5.2: Glomerular endothelial capillary loop demonstrating thickened and hypertrophied endothelium with clear vesicular structures identified as vacuoles. Legend: double arrow = thickened endothelium, thin arrow = vacuole. Magnification 24,500X lead citrate and uranyl acetate.

Endothelial cell vacuolation and thickening were apparent from the early 1 and 3 month biopsies. The proportion of capillary loops with these abnormalities were significantly different by the one-year EM biopsy time point (both $P<0.001$ *versus* controls) (Figures 5.3 and 5.4) and the curves remained separate with time. Mean difference in proportions between TXG and controls for endothelial vacuolation was 0.40 ± 0.07 and 0.30 ± 0.07 for endothelial cell thickening (both $P<0.001$). However, there was no significant change in proportions of

either endothelial thickening or vacuolation with time between groups. Average slopes for endothelial thickening was 0.039±0.03 in TXG *versus* 0.018±0.02 in controls and for endothelial vacuolation was 0.024±0.012 in TXG *versus* -0.004±0.017 in controls both.

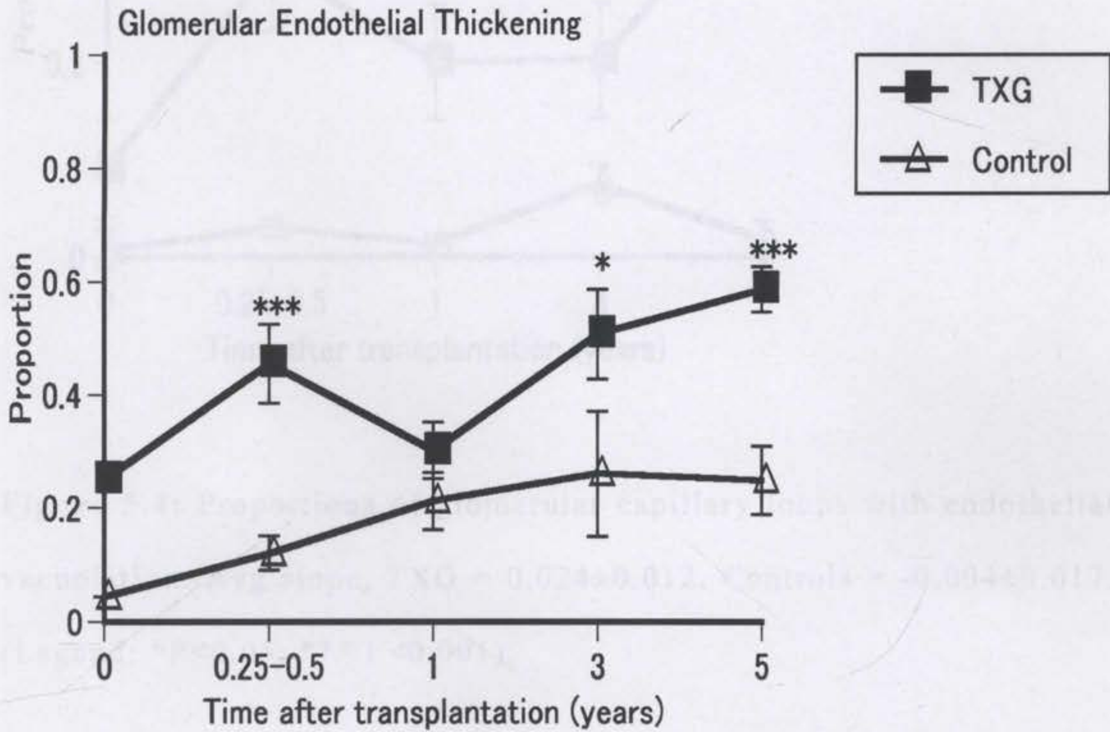


Figure 5.3: Proportions of glomerular capillary loops with endothelial thickening. Avg slope TXG = 0.039±0.018, Control = 0.018±0.02. (Legend: *P<0.05, *** P<0.001).

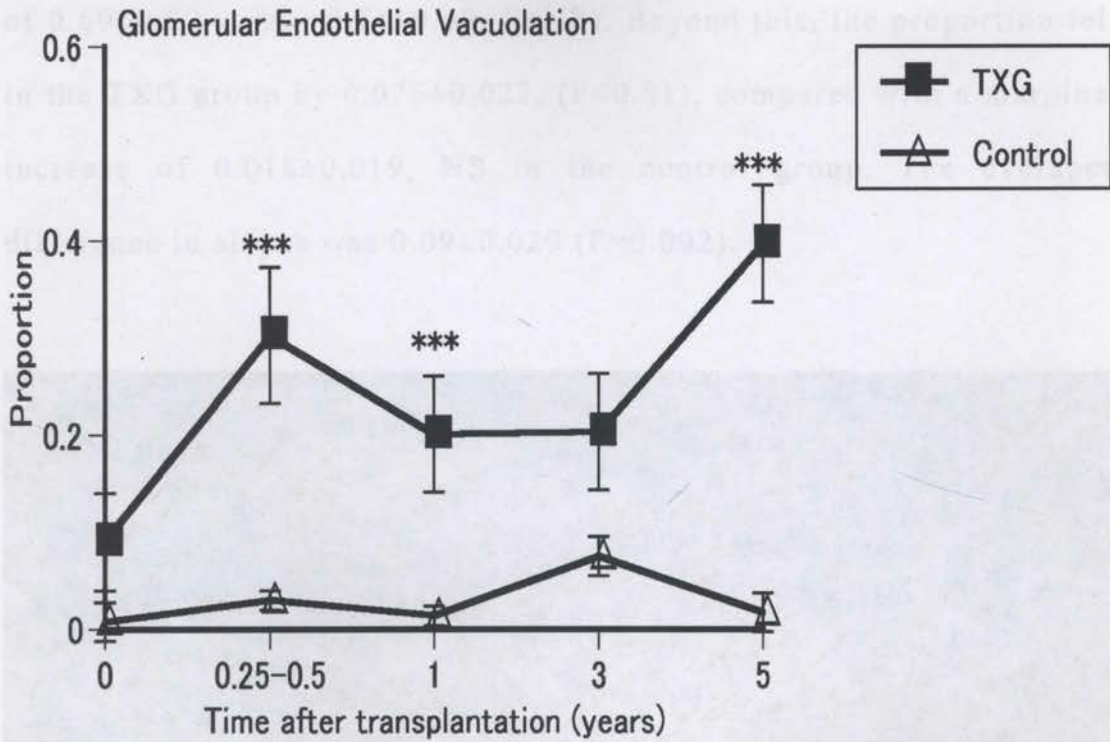


Figure 5.4: Proportions of glomerular capillary loops with endothelial vacuolation. Avg slope, TXG = 0.024±0.012, Controls = -0.004±0.017. (Legend: *P<0.05, *** P<0.001).

Glomerular capillary loops in patients with transplant glomerulopathy were characterised by loss of fenestration. (Figures 5.5 and 5.6). Fenestration of capillary loops was reduced in TXG compared to control (mean difference in proportion 0.55±0.15, P<0.01), with a trend towards further loss of fenestration with time (slope: -0.104±0.06, P=0.09) (Figure 5.7). Fenestration was maintained until late with fenestration loss occurring (and possibly consequential to) substantial structural alterations in cell morphology. By one year after transplantation, both TXG and controls demonstrated similar proportions of fenestrated endothelium in capillary loops (mean±SEM

of 0.69±0.09 *versus* 0.74±0.09, P=NS). Beyond this, the proportion fell in the TXG group by 0.076±0.022, (P<0.01), compared with a marginal increase of 0.018±0.019, NS in the control group. The averaged difference in slopes was 0.09±0.029 (P=0.002).

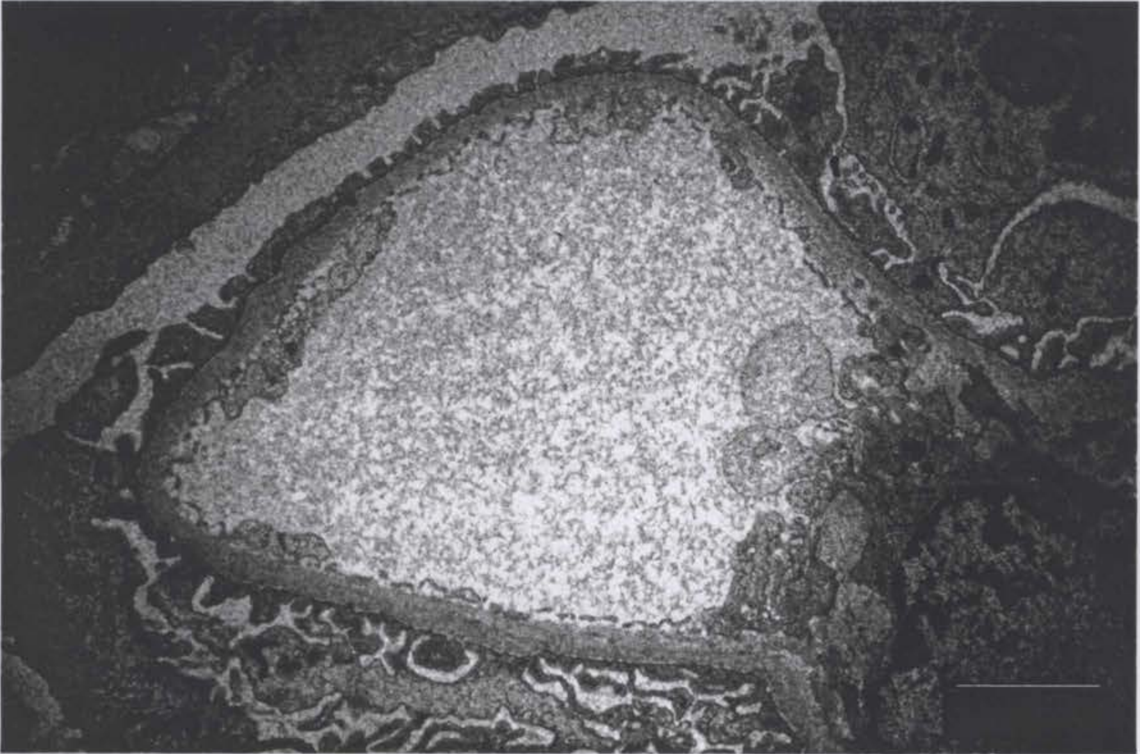
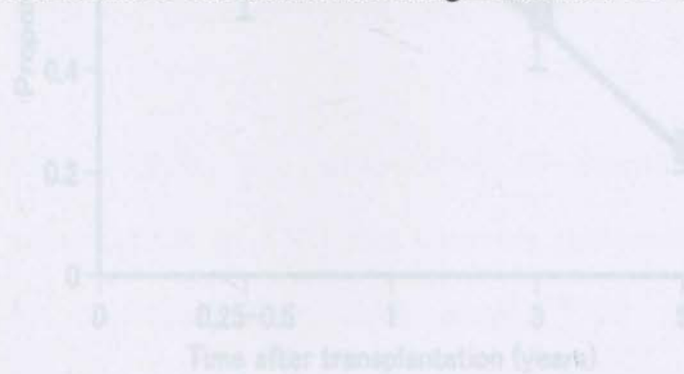


Figure 5.5: Normal Glomerular capillary loops showing normal fenestrated endothelium. Magnification X7400.



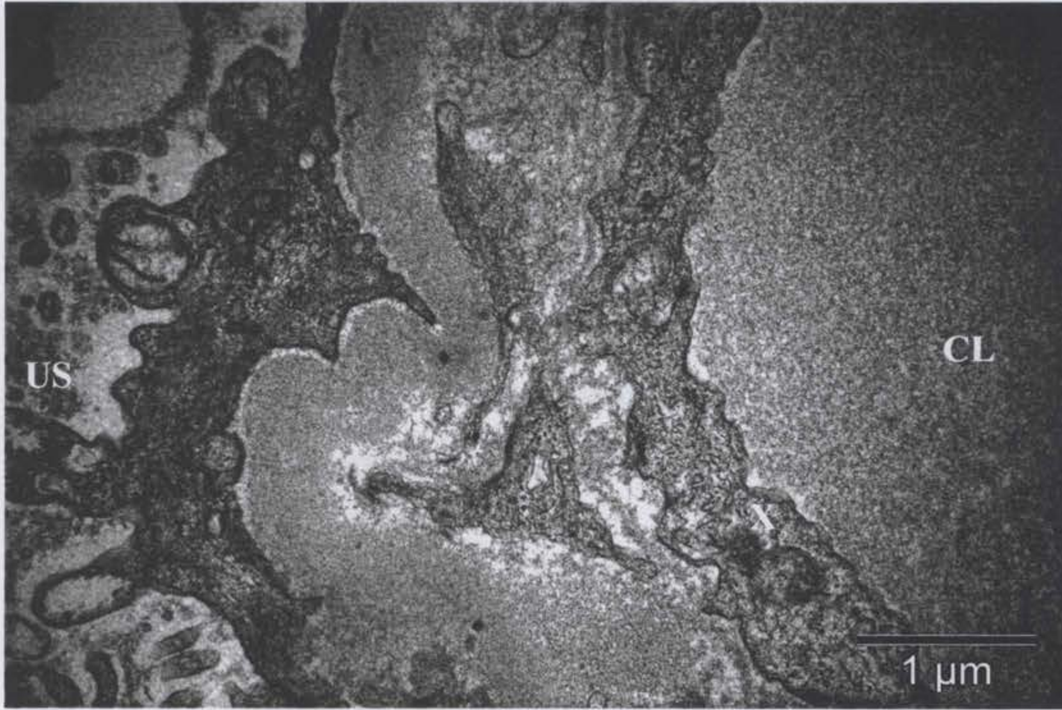


Figure 5.6: Glomerular capillary loops showing continuous non-fenestrated endothelium (X). Legend US=urinary space, CL=capillary lumen magnification 24500x Lead Citrate, uranyl acetate.

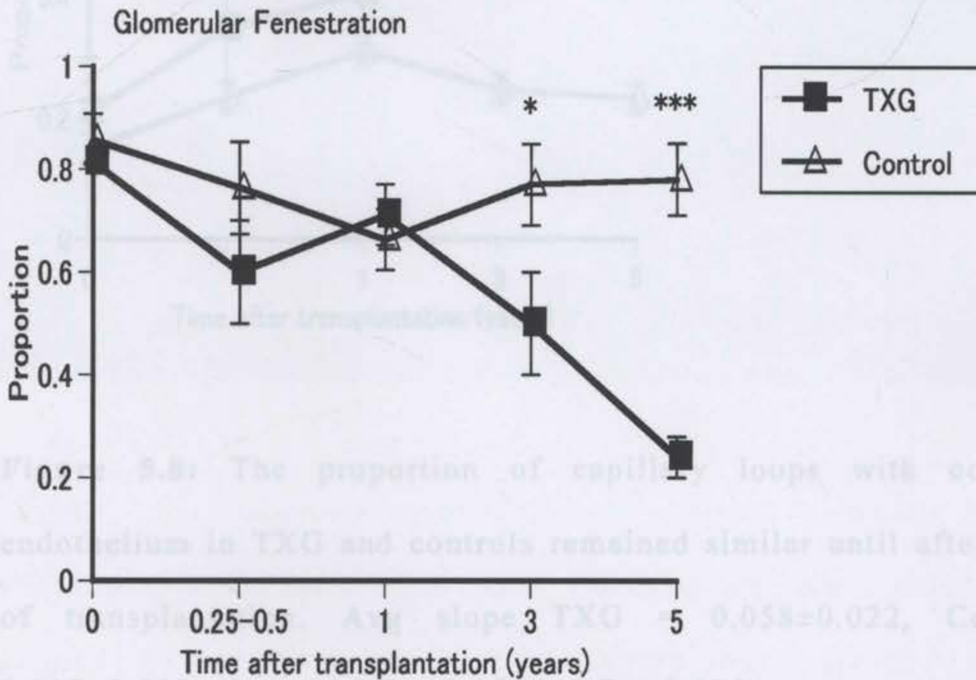


Figure 5.7: The proportion of capillary loops with fenestrated endothelium in TXG and controls was similar until 3 years after

transplantation. Avg slope TXG = -0.076±0.22, Control = 0.018 ±0.018. Legend * P=<0.05, *** P=<0.005

Loss of capillary loop fenestration was associated with increasing proportions of loops in the TXG group with a continuous endothelial layer compared to controls (Avg slope of 0.058±0.022, P<0.05 *versus* -0.015±0.019, P=NS; differences in slopes -0.073±0.03, P<0.05) (Figure 5.8).

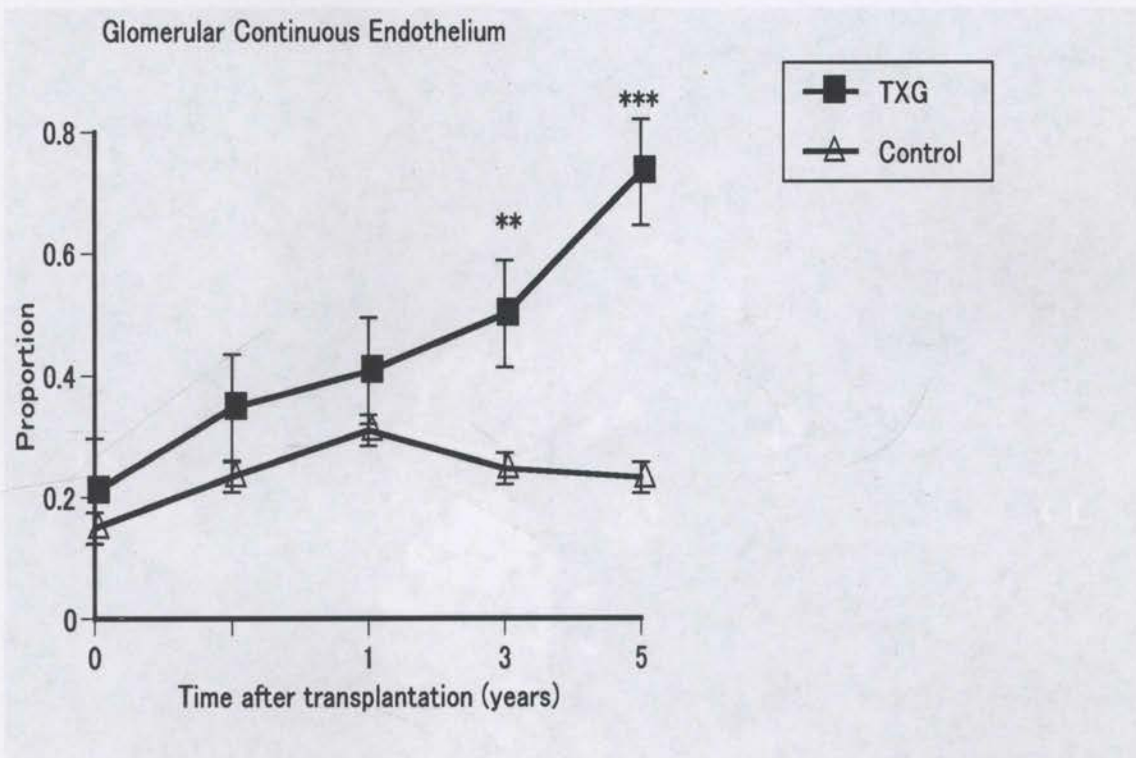


Figure 5.8: The proportion of capillary loops with continuous endothelium in TXG and controls remained similar until after 2 years of transplantation. Avg slope TXG = 0.058±0.022, Control = 0.015±0.019. (Legend * P=<0.05, *** P=<0.005).

5.3.3.2 Sub-endothelial Ultrastructure

Early endothelial injury and activation was accompanied by expansion of the subendothelial space with widening of the lamina rara interna (LRI), which was filled with floccular electron lucent material accompanied occasionally by osmiophilic inclusions. The LRI expanded inwards towards the capillary lumen, creating inter-digitations with the endothelial membrane (Figure 5.9).

Figure 5.9: Glomerular capillary loop showing expanded LRI / or widened subendothelial space with floccular electron lucent material. Legend: CL=capillary lumen, US= urinary space, arrow = expanded LRI. Magnification 24500x lead citrate and uranyl acetate.

The proportion of capillary loops with LRI expansion was greater in the TXG group compared with the control group, although once established, the proportions remained widely separate but did not change further with time after transplantation (mean differences of 0.62±0.12, P<0.001, average slopes: 0.034±0.03 *versus* 0.014±0.03, P=NS, between TXG and controls, respectively) (Figure 5.10).

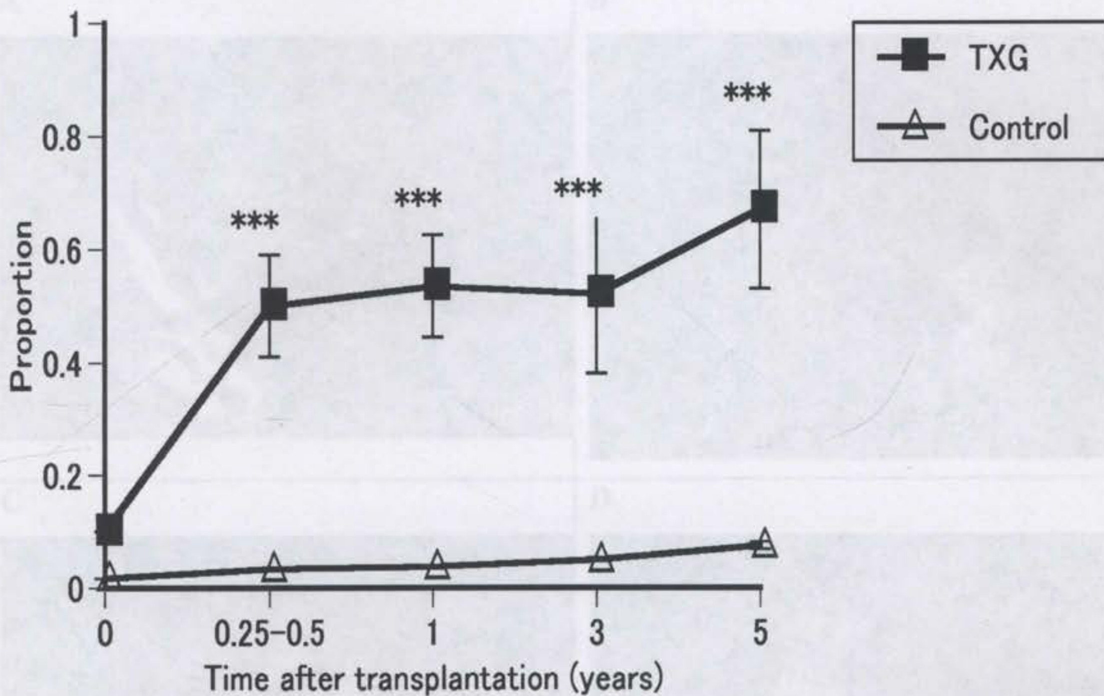


Figure 5.10: The proportion of glomerular capillary loops with widened sub-endothelial space/LRI was higher in TXG compared to controls. Avg slope, TXG = 0.034±0.03, control = 0.014±0.03. (Legend *** P= <0.005).

5.3.3.3 Serration

As the endothelial membrane and LRI expanded into each other, a serrated appearance of the capillary loop endothelial/LRI interface in the TXG group appeared (Figure 5.11). The averaged difference in proportion over time between the groups was 0.42±0.07, P<0.001. The averaged slope for serration in TXG was 0.047±0.03 (P=0.07), compared to 0.002±0.02 in the control group (P=0.94) (Figure 5.12).

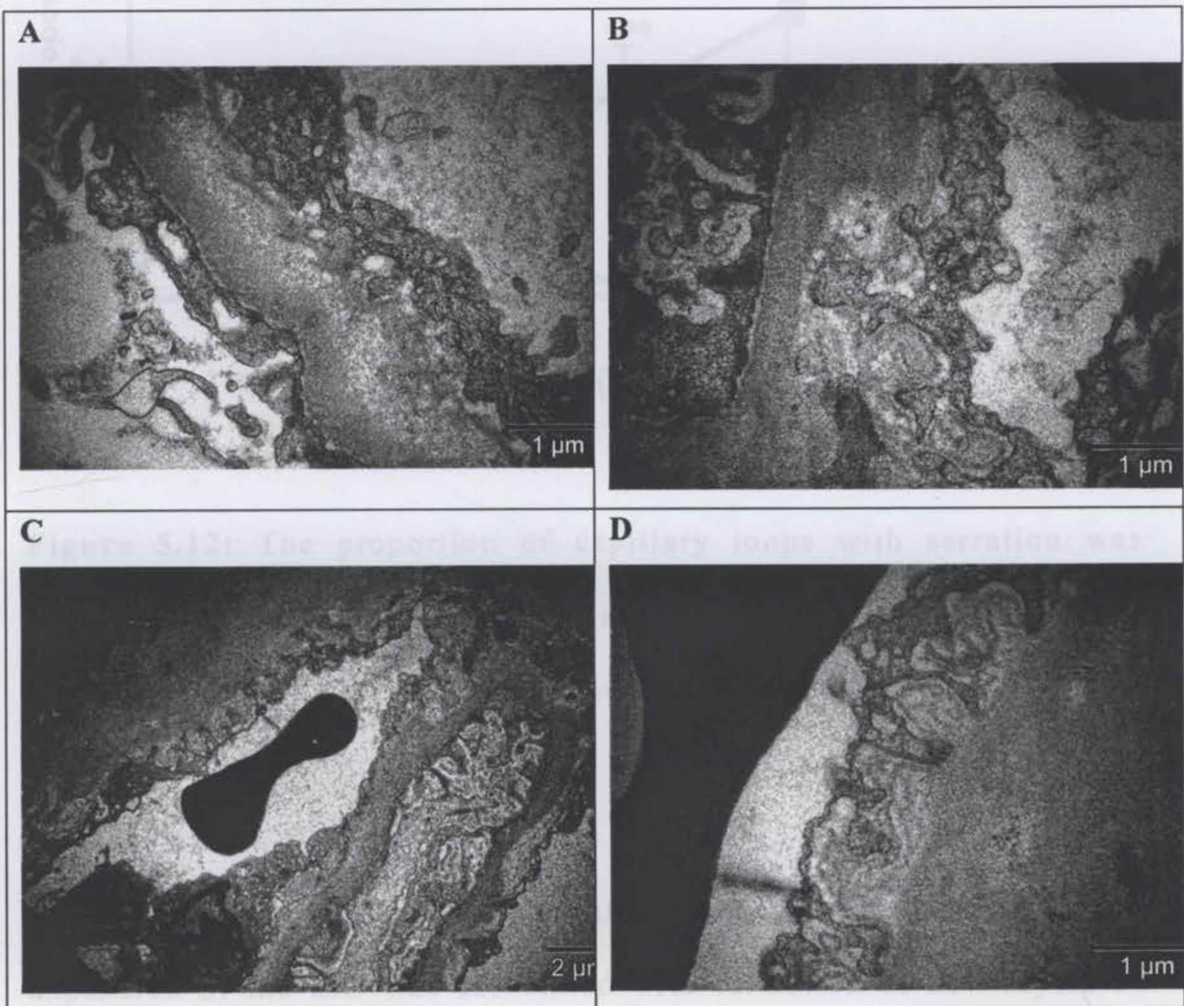


Figure 5.11: Glomerular capillary loops showing serration of subendothelial interface **A**) mild serration **B**) expansion of LRI toward capillary lumen and expansion of activated endothelium into LRI **C**)

Severe serration and **D)** discreet layers of dense linear basement membrane.

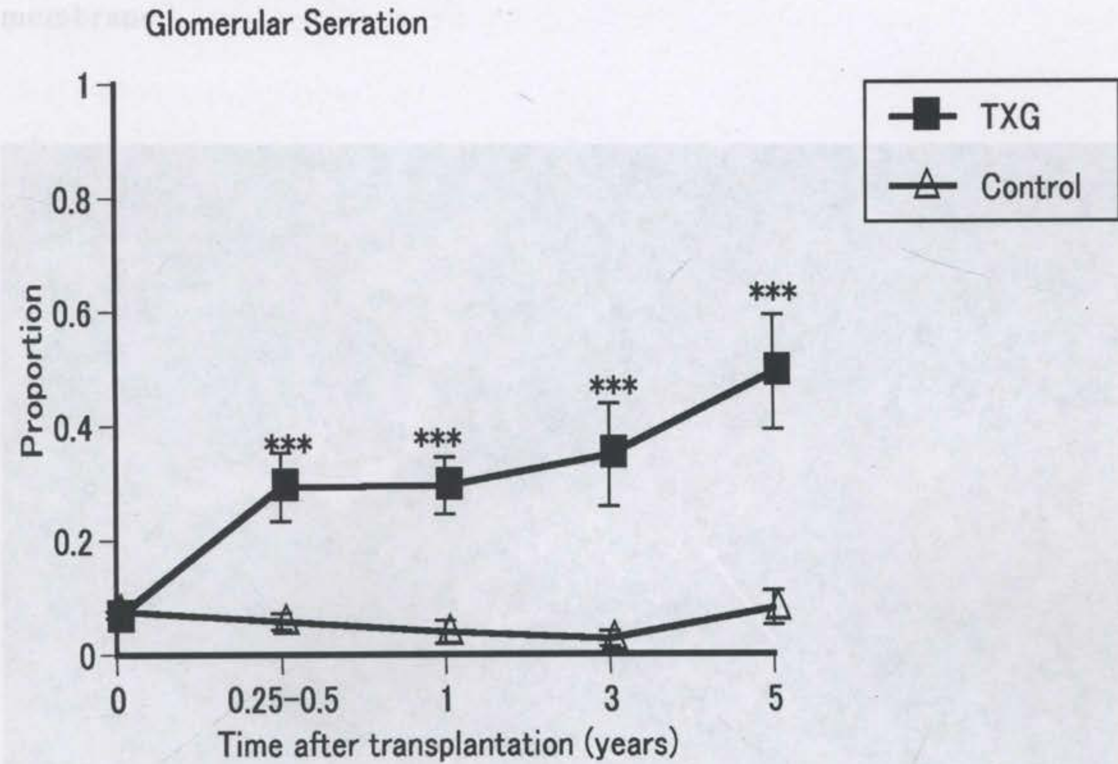


Figure 5.12: The proportion of capillary loops with serration was higher in TXG compared to controls at all time point. Avg slope TXG = 0.047±0.03, control = 0.002±0.02. (Legend *** = P<0.001).

5.3.3.4 Lamina Densa

Readily apparent by the first year after transplantation, widening and expansion of the LRI was associated with formation of new layers of basement membrane the lamina densa, and accompanied by cytoplasmic interposition (Figure 5.13). Subendothelial expansion and cytoplasmic interposition resulted in an increase of the measured glomerular

basement membrane diameters and apparent capillary loop thickening seen on light microscopy examination whereas the new layer of basement membrane results in apparent duplication of the basement membrane.

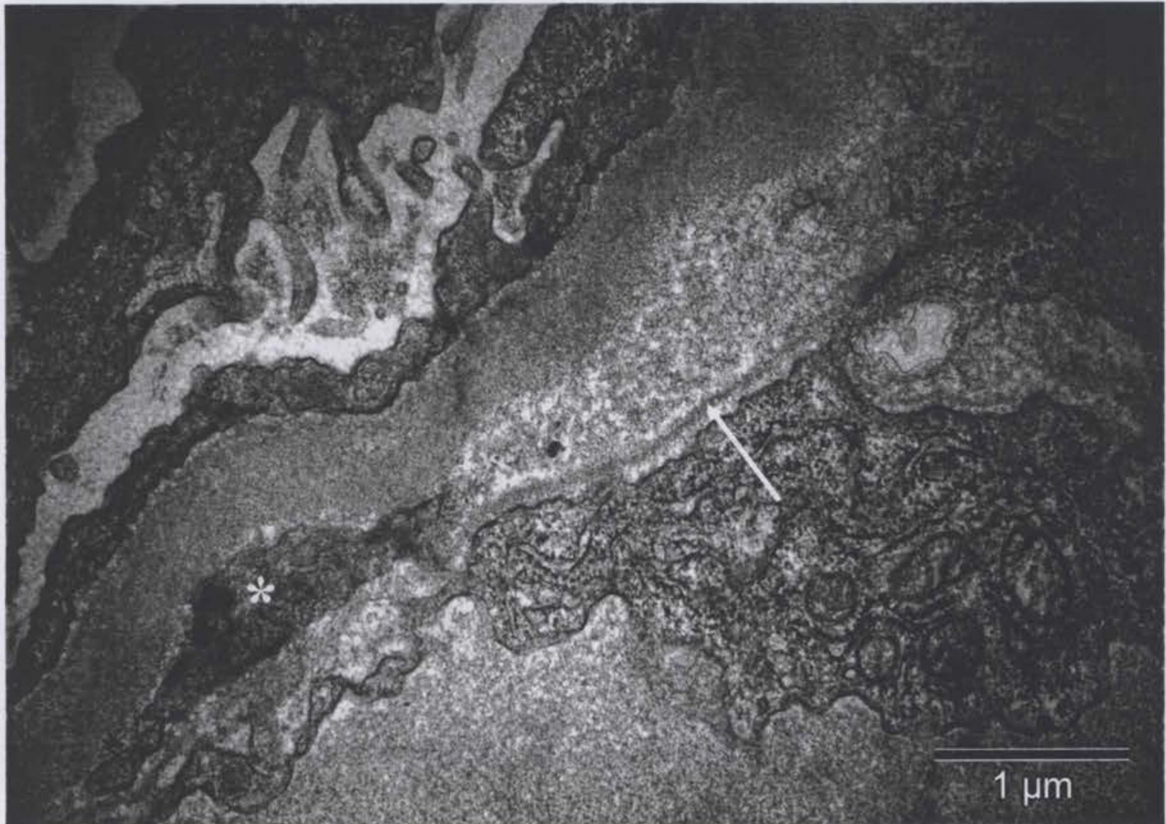


Figure 5.13: Glomerular capillary loop showing a widened sub-endothelial space with a single layer of dense basement membrane (arrow), and cytoplasmic interposition (*).

The proportion of capillary loops with new GBM basement membrane layers increased by 0.103±0.023 per year, $P<0.001$ in the TXG group compared with 0.006±0.02 in the control group, P=NS (difference in average slopes of 0.097±0.03, $P<0.01$, (Figure 5.14). Consequently, the

overall GBM width increased by a mean (±SEM) of 52.8±7.9nm/year in the TXG group (P<0.001) compared with 0.36±8.4nm/year in the control group (P=NS) (Figure 5.15). New LRI formation and expansion preceded formation of the lamina densa, the later occurring from 3 to 6 years after transplantation.

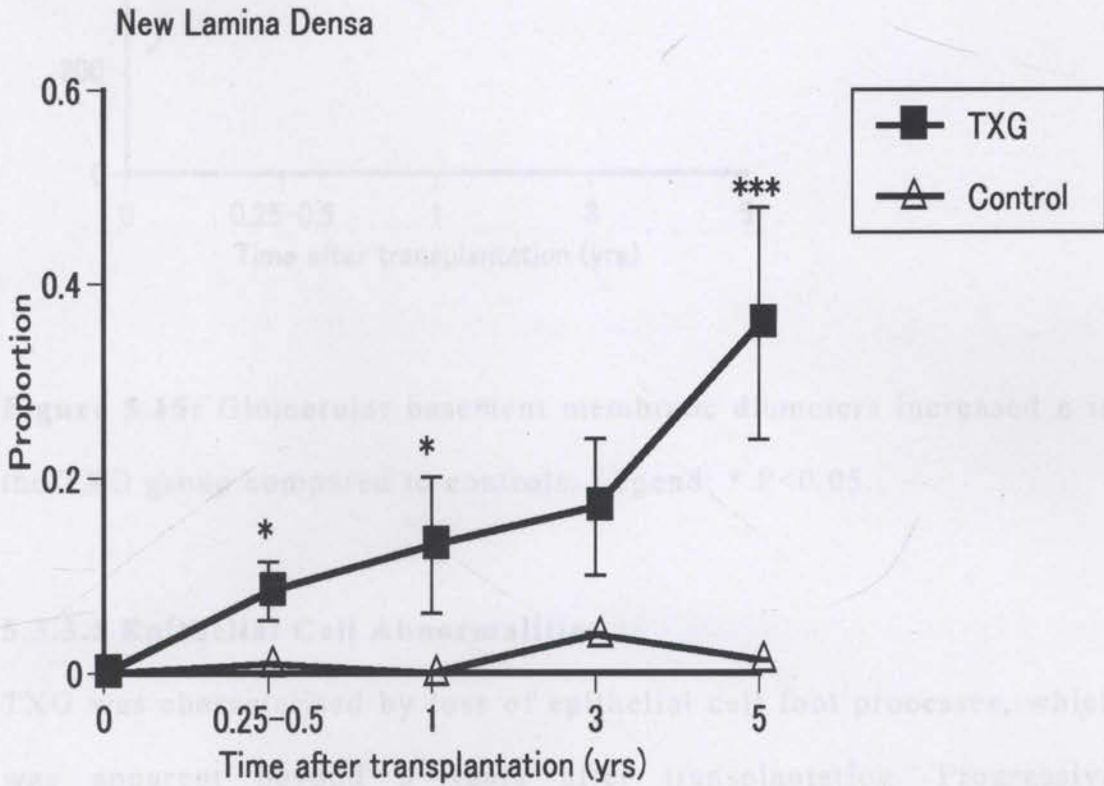


Figure 5.14: Proportion of capillary loops with new layer of lamina densa in TXG compared to controls. New Lamina densa was observed in TXG within 6 months after transplantation and persisted throughout follow up period. Avg. slope, TXG = 0.103±0.023, control = 0.006±0.02. (Legend * $P<0.05$, ** $P<0.005$).

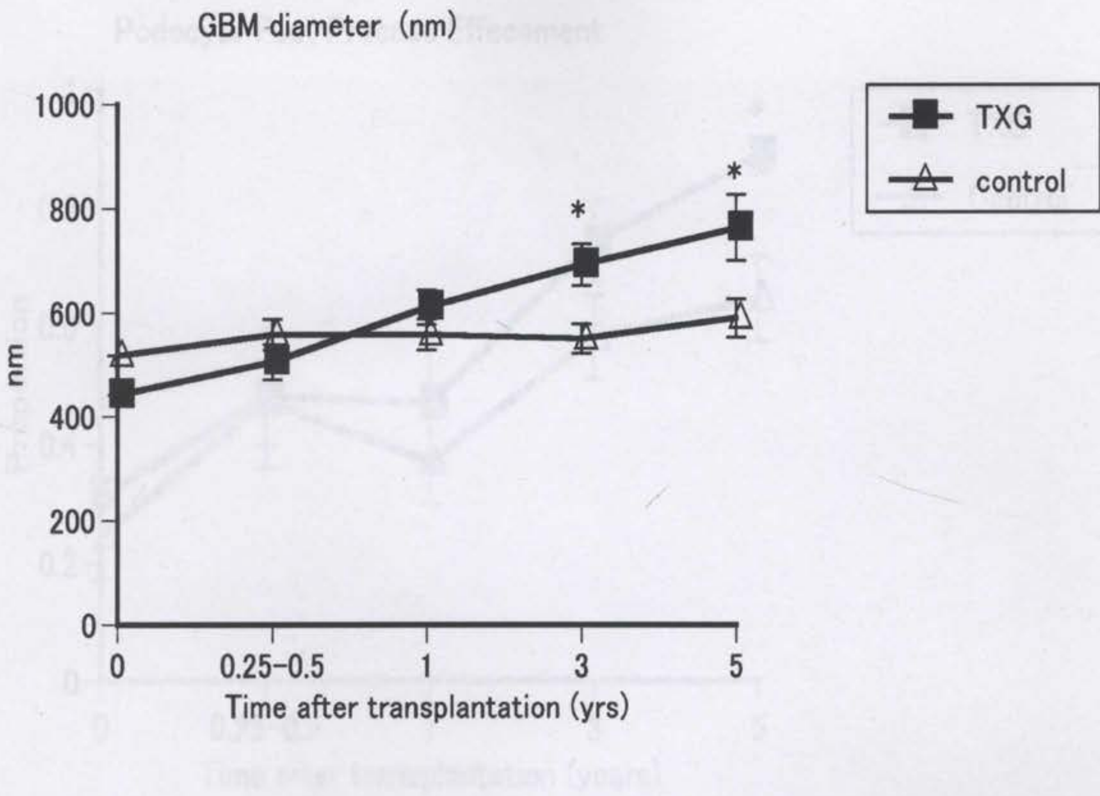


Figure 5.15: Glomerular basement membrane diameters increased n in the TXG group compared to controls. Legend: * P<0.05.

5.3.3.5 Epithelial Cell Abnormalities

TXG was characterised by loss of epithelial cell foot processes, which was apparent beyond 3 years after transplantation. Progressive glomerular epithelial cell (podocyte) fusion occurred in the TXG group (average slope 0.14±0.03, P<0.001, compared to control (0.051±0.031, P=0.11), although the slope difference was of borderline significance (0.085±0.048, P=0.11) (Figure 5.16).

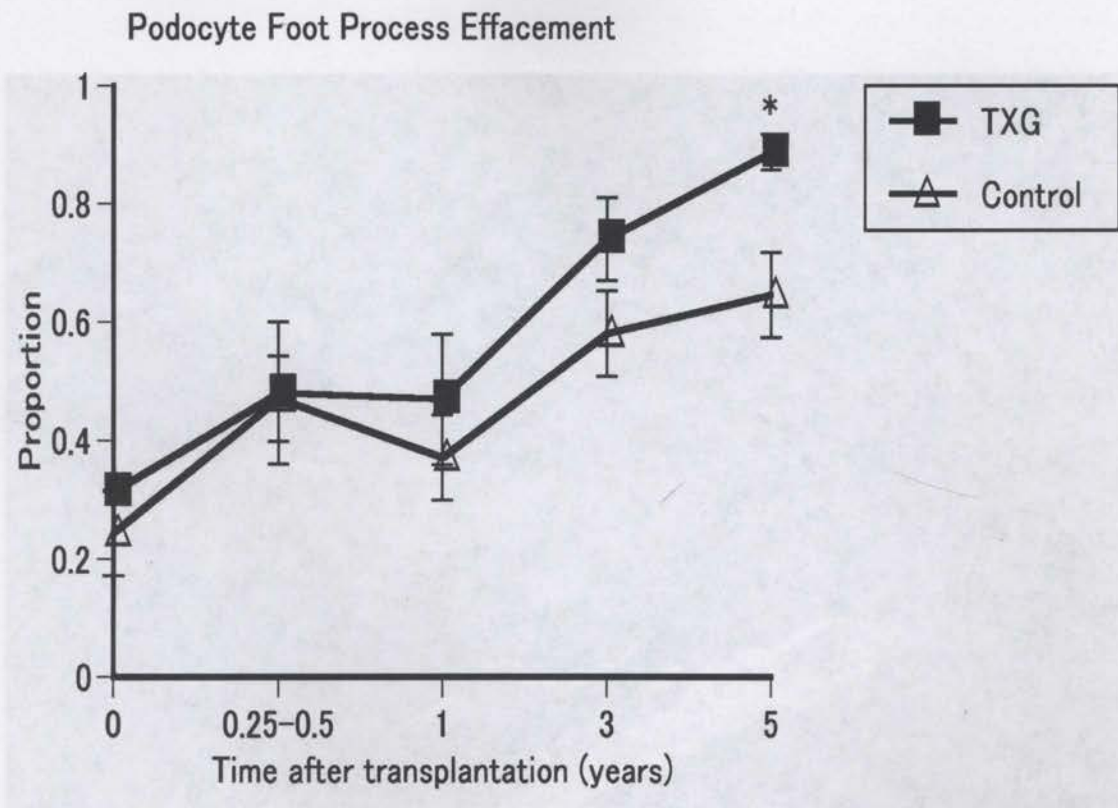


Figure 5.16: Proportion of capillary loops with podocyte fusion in TXG compared to controls. Legend: * P<0.05.

In summary, endothelial injury resulted in expansion of the sub-endothelial space, formation of a new layer of glomerular basement membrane accompanied by interposition of mesangial cells. These morphological abnormalities appeared as a thickened glomerular capillary loops with duplication of the basement membrane on light microscopy. Figure 5.17 overleaf shows a capillary loop with these features.

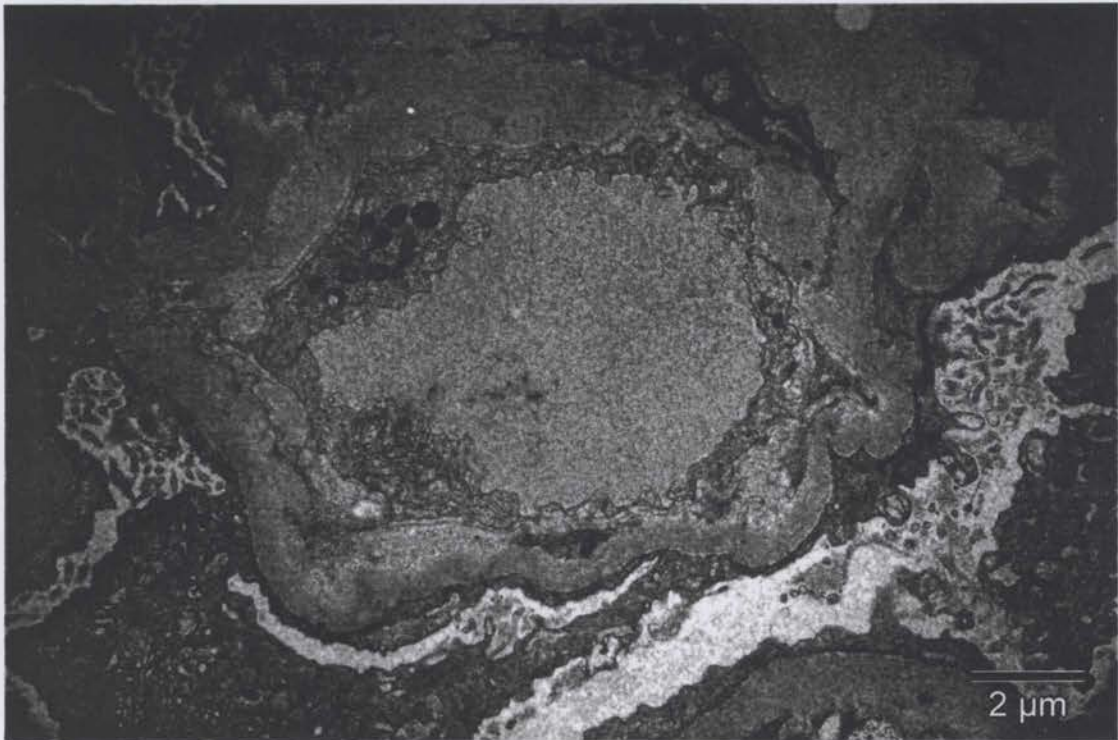


Figure 5.17: Glomerular capillary loop demonstrating endothelial thickening, new layer of basement membrane, expanded subendothelial space, and mesangial interposition resulting in thickened glomerular capillary loops.

5.3.4 Mesangial Morphometry

Mesangial matrix expansion accompanied the glomerular changes, gradually increasing in the TXG group, but much later compared with endothelial and subendothelial abnormalities. The fractional area of total mesangium was higher in TXG group ($P<0.001$), and continued to gradually increase with time compared to controls (mean difference of slopes: 0.09 ± 0.022, $P<0.01$). The total mesangial area expansion was primarily due to increased mesangial matrix accumulation, without

significant cellular proliferation. The mesangial matrix area was greater in the TXG group compared to controls (mean difference 0.015±0.007, P<0.05), and accompanied by a higher rate of matrix accumulation (average slopes: 0.029±0.01, P<0.01 *versus*. 0.005±0.007, P=NS, respectively; slope difference 0.02±0.012, P=0.055). The mesangial cellular fractions between groups were not different (mean difference 0.03±0.03, P=NS, average slope 0.017±0.008 *versus* 0.003±0.007, respectively, all P=NS) (Figures 5.18 and 5.19)

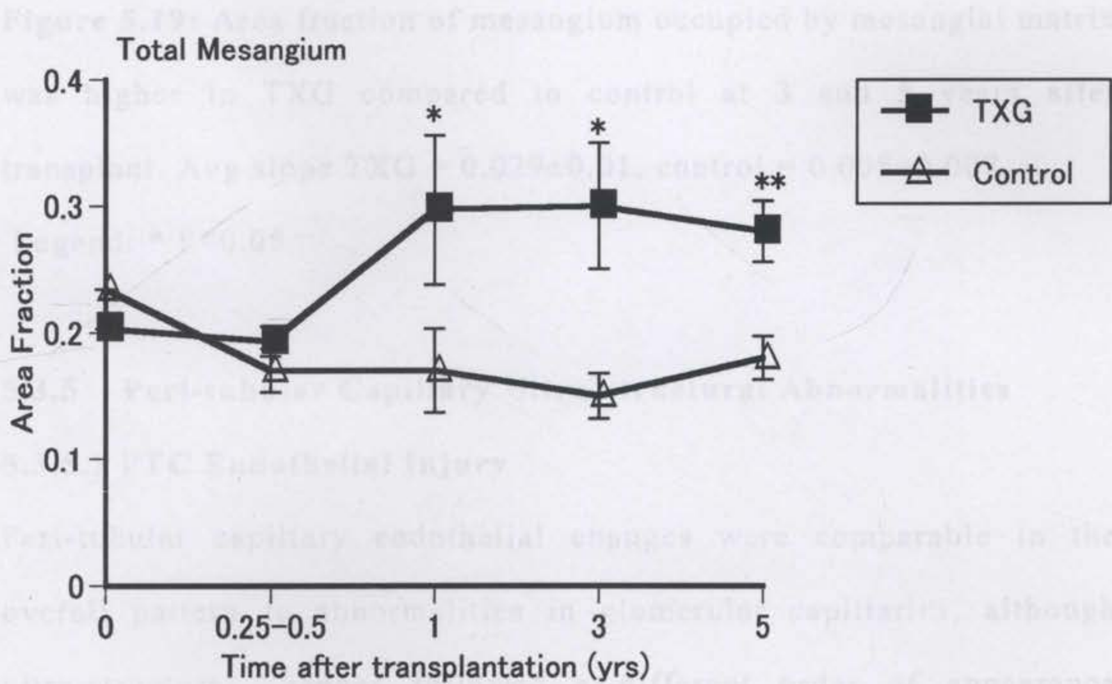


Figure 5.18: Mesangial area as a fraction of total glomerular area was higher in TXG *versus* controls at 1, 3, and 5 years after transplant. (Legend:* P<0.05, ** P< 0.01 *** P<0.001).

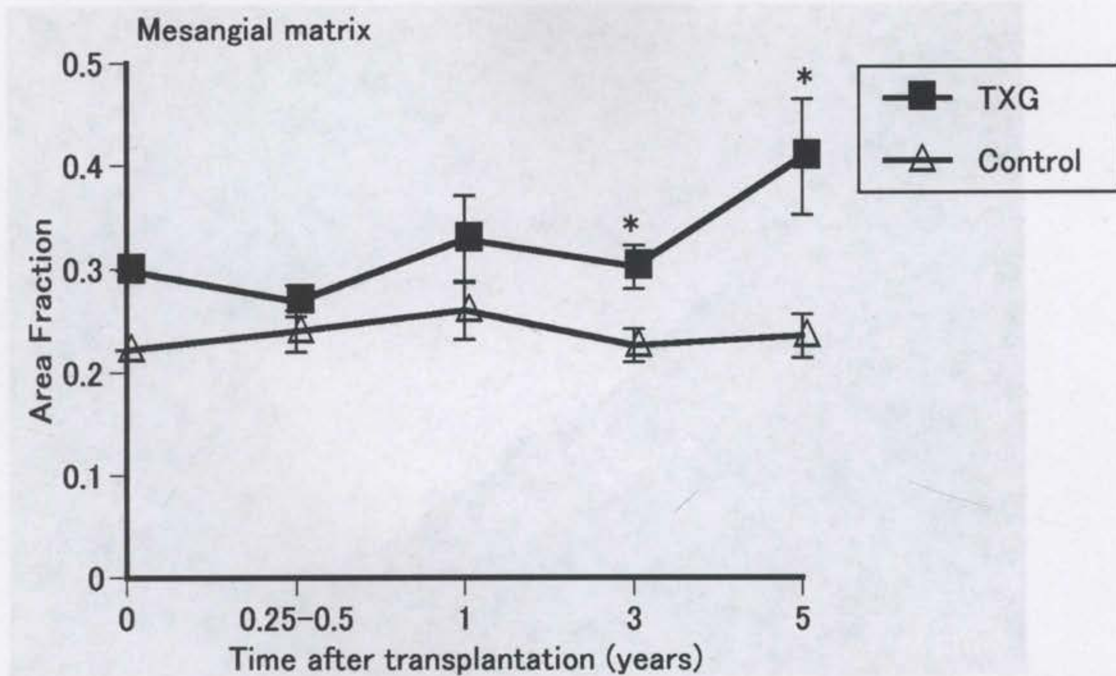


Figure 5.19: Area fraction of mesangium occupied by mesangial matrix was higher in TXG compared to control at 3 and 5 years after transplant. Avg slope TXG = 0.029±0.01, control = 0.005±0.007.

Legend: * P<0.05.

5.3.5 Peri-tubular Capillary Ultra-structural Abnormalities

5.3.5.1 PTC Endothelial Injury

Peri-tubular capillary endothelial changes were comparable in the overall pattern to abnormalities in glomerular capillaries, although ultra-structural changes followed a different order of appearance compared with glomerular endothelial changes. Thickening and loss of fenestration of the PTC endothelial cells occurred early and became more abnormal with time, followed by the onset of a serrated appearance of endothelial cell inner interface, and finally with the deposition of multiple layers of basement membrane within the PTC (Figures 5.20, 5.21, 5.22 and 5.23).

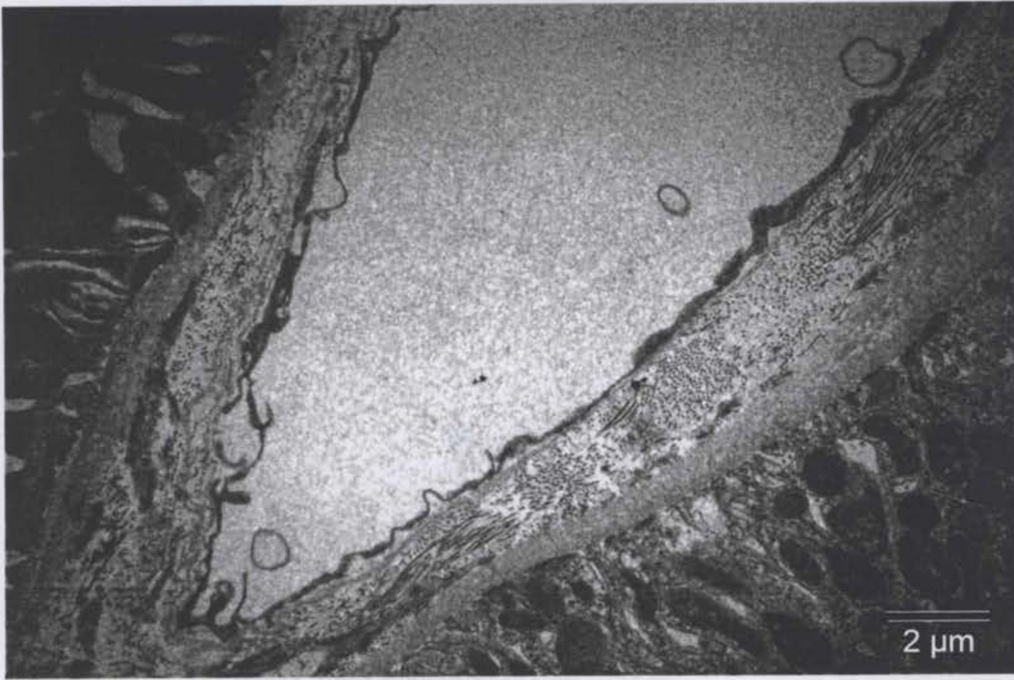


Figure 5.20: EM photomicrograph showing a normal Peri-tubular capillary.

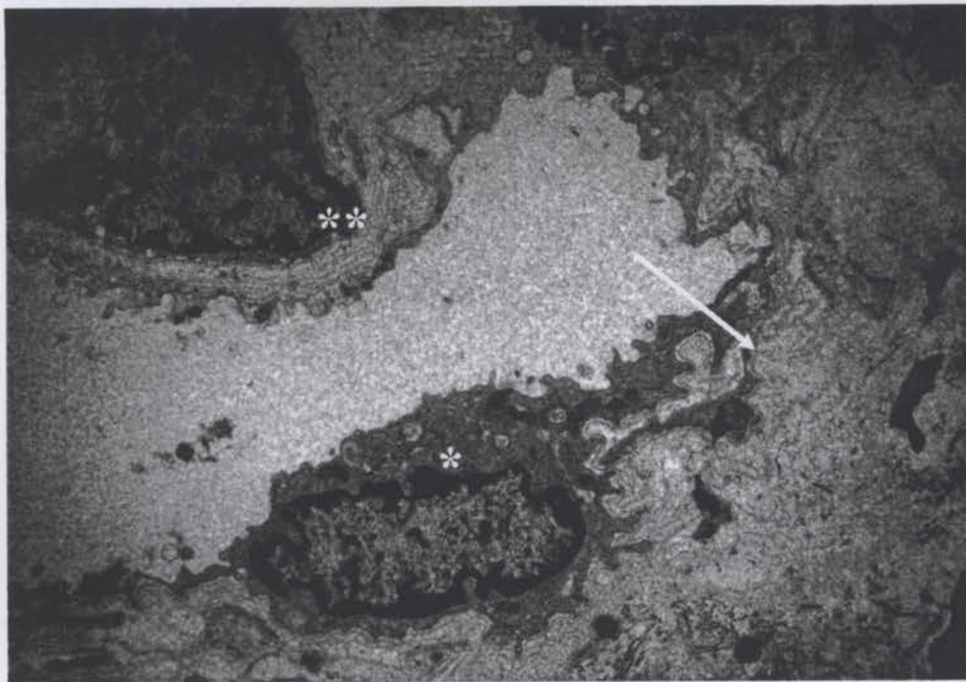


Figure 5.21: EM photomicrograph showing Peri-tubular capillary with endothelial thickening and hypertrophy (*), serration (arrow), and PTC basement membrane multi-lamination (**).

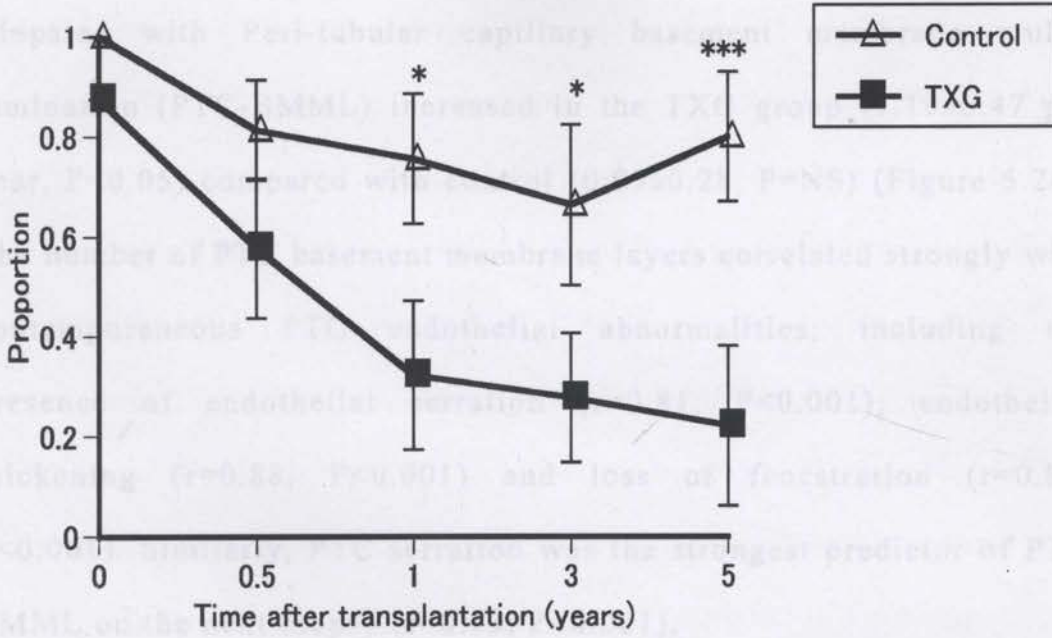


Figure 5.22: The proportion of PTC with fenestration was higher in controls compared to TXG. (Legend: * P<0.05, *** P<0.001).

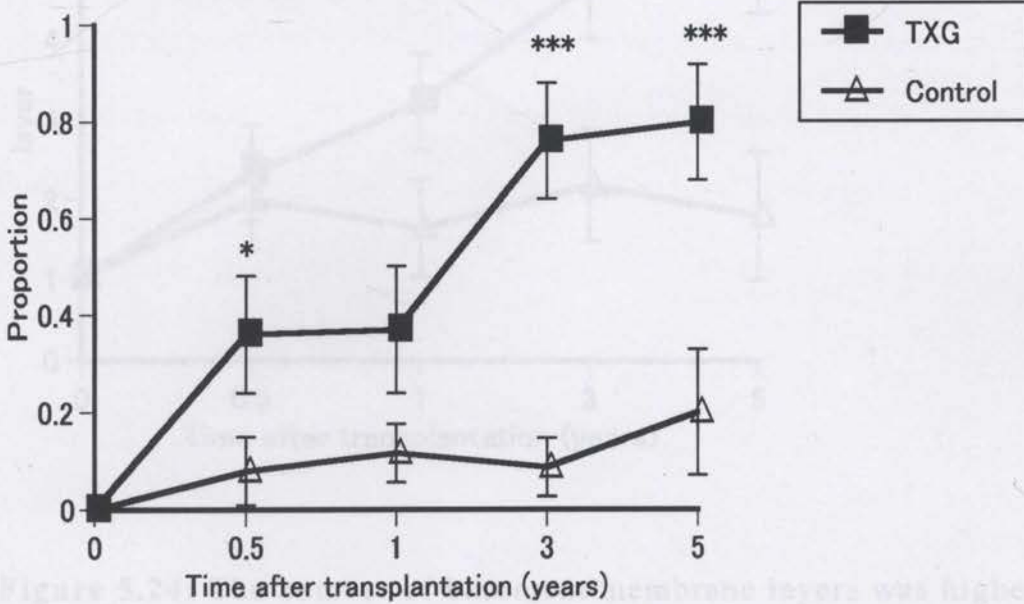


Figure 5.23: The proportion of PTC with serration at 3 and 5 years was higher in TXG compared to controls (Legend: * P<0.05, *** P<0.001).

5.3.5.2 Peri-Tubular Capillary Basement Membrane ultrastructure

Biopsies with Peri-tubular capillary basement membrane multi-lamination (PTC-BMML) increased in the TXG group (1.19±0.47 per year, P<0.05) compared with control (0.03±0.28, P=NS) (Figure 5.24). The number of PTC basement membrane layers correlated strongly with contemporaneous PTC endothelial abnormalities; including the presence of endothelial serration (r=0.81, P<0.001), endothelial thickening (r=0.88, P<0.001) and loss of fenestration (r=0.86, P<0.001). Similarly, PTC serration was the strongest predictor of PTC BMML on the next biopsy (r=0.73, P<0.001).

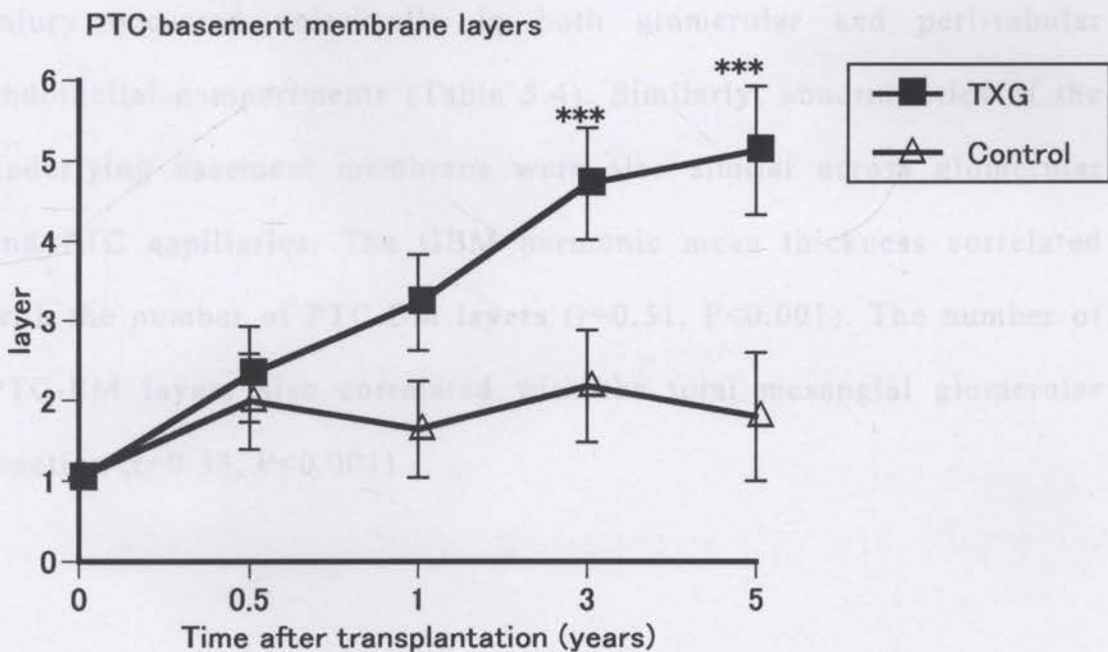


Figure 5.24: The number of basement membrane layers was higher in TXG compared to controls at 3 and 5 years after transplant. Avg slope, TXG = 1.19±0.47, control = 0.03±0.28. (Legend: * P<0.05, *** P<0.001).

5.3.6 Relationship Between Glomerular and PTC Abnormalities

Markers of endothelial injury were congruent across histological compartments: glomerular endothelial injury correlated with PTC endothelial and microvascular injury. Loss of fenestration in the glomerular capillary loops correlated with PTC abnormalities including capillary fenestration loss (r=0.46, P<0.001), increased endothelial serration (r=0.53, P<0.001) and with PTC-BMML (r=0.45, P<0.001). Other features of glomerular endothelial injury such as endothelial thickening, vacuolation, and extension of a serrated endothelium into the LRI were also significantly correlated with PTC serration, PTC fenestration loss, and PTC-BMML; suggesting that micro-vascular injury occurred universally in both glomerular and peri-tubular endothelial compartments (Table 5.4). Similarly, abnormalities of the underlying basement membrane were also similar across glomerular and PTC capillaries. The GBM harmonic mean thickness correlated with the number of PTC-BM layers (r=0.51, P<0.001). The number of PTC-BM layers also correlated with the total mesangial glomerular fraction (r=0.53, P<0.001).

Table 5.4: Correlations between glomerular (rows), and corresponding peri-tubular capillary abnormalities (columns), assessed by EM on all specimens expressed as Spearman's rho. Superscript key: a, P<0.05; b, P<0.01; c, P<0.001).

	Peri-tubular capillaries			
Glomerular capillary parameter	Fenestration	serration	Endothelial thickening	PTCB MML
Fenestration	0.46^{c}	-0.53^{c}	-0.37^{b}	-0.45
Serration	-0.37^{b}	-0.50^{c}	0.21	0.49
LRI expansion	-0.38^{b}	0.49^{c}	0.19	0.39^{b}
New Lamina densa	-0.24	0.34^{b}	0.19	0.33^{a}
GBM thickness	-0.56^{c}	0.58^{c}	0.55^{c}	0.51^{c}
Mesangial matrix	-0.30^{a}	0.31^{a}	0.13	0.30^{a}
Podocyte fusion	-0.24	0.29^{a}	0.20	0.51^{c}

5.3.7 Immunological Abnormalities

5.3.7.1 C4d Immunoperoxidase staining in PTC and Glomerular Capillaries

Of 70 protocol biopsies available for C4d staining, 11/28 (39%) were diffusely positive for C4d in the TXG group, compared to 10/42 (23%) in the control group (P= NS). Nine pre-implantation biopsies were all negative for C4d. C4d was positive in 6 of 7 patients with TXG (86%), and 5 of 8 (62.5%) with controls (P=NS) One patient with TXG had no

ultra-structural endothelial abnormalities no DSA, and was negative for C4d staining suggesting that glomerular changes observed on light microscopy were etiologically different to the other TXG patients. Hence early C4d deposition did not differentiate between grafts with TXC compared to those with CAN although at later time points (beyond 1 year) diffuse C4d staining persisted in 6 of 10(60%) TXG biopsies compared to 6 of 25 (24%) control biopsies ($P < 0.05$) (Figure 5.25).

C4d deposition was more prominent early after transplantation in glomerular and PTC capillaries, and gradually decreased in intensity and prevalence with time (both $P<0.05$). Peri-tubular capillary C4d correlated with peri-tubular capillary inflammation ($r=0.430$, $P<0.05$) and glomerular capillary loop C4d deposition ($r=0.80$, $P<0.001$). This finding suggests that parallel humoral activity occurred within all vascular compartments of the kidney. C4d deposition in PTC also correlated with loss of PTC fenestration ($r=0.45$, $P<0.01$) and PTC endothelial thickening ($r=0.35$, $P<0.05$). The correlation of PTC C4d deposition with PTC-BMML was less on contemporaneous biopsies ($r=0.23$, P=NS), however this was significantly correlated with PTC-BMML, glomerular capillary loss of fenestration ($r=-0.32$, $P<0.05$), endothelial vacuolation ($r=0.33$, $P<0.05$), LRI expansion ($r=0.48$, $P<0.001$) and mesangial matrix expansion ($r=0.58$, $P<0.001$) on the next sequential protocol biopsy ($r=0.34$, $P<0.05$) (Table 5.5).

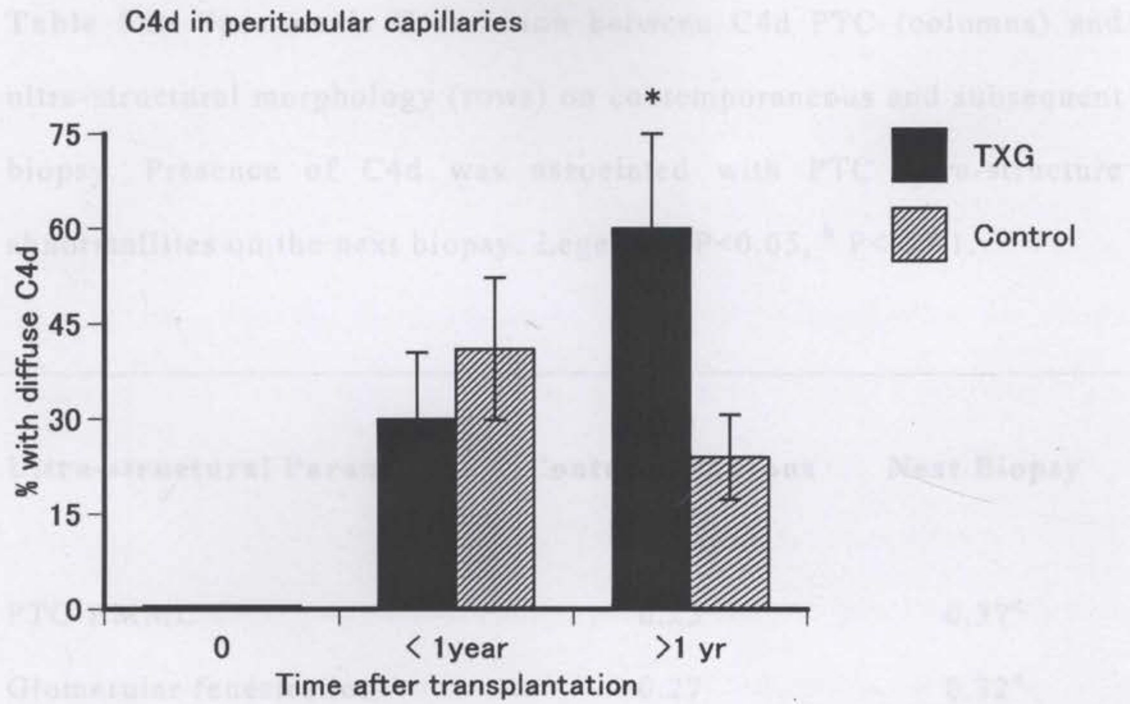


Figure 5.25: Proportion of biopsies with diffuse, circumferential peritubular capillary C4d staining (>50%) in TXG compared to controls. The proportion of positive C4d beyond 1 yr was higher in TXG group compared to controls. (Legend: * P <0.05).

Table 5.5: Spearman's Correlation between C4d PTC (columns) and ultra-structural morphology (rows) on contemporaneous and subsequent biopsy. Presence of C4d was associated with PTC ultra-structure abnormalities on the next biopsy. Legend: [a] P<0.05, [b] P< 0.01.

Ultra-structural Parameter	Contemporaneous Biopsy	Next Biopsy
PTC-BMML	0.23	0.37[a]
Glomerular fenestration	0.27	0.32[a]
LRI expansion	0.06	0.48[b]
New lamina densa	0.03	0.02
Mesangial matrix	0.12	0.58[b]
Glomerular endothelial vacuolation	0.25	0.33

In contrast, C4d immunoperoxidase staining in glomerular capillary loops was only correlated with the presence of osmophilic inclusions within the GBM (r=0.35, P<0.05); but generally was not predictive of other glomerular ultra-structural abnormalities on contemporaneous biopsies: such as loss of capillary endothelial fenestration (r=0.24, P=NS), endothelial cell serration (r=0.24, P=NS), thickening (r=0.20, P=NS) vacuolation (r=0.22, P=NS); with expansion of the LRI (r=0.06, P=NS) or new lamina densa formation (r=0.02, P=NS). Mesangial C4d

deposition did not correlate with mesangial matrix expansion (r=-0.06, P=NS).

5.3.7.2 Donor Specific Antibodies

Serum was available for assessment of anti–HLA antibodies for all 15 patients. Post transplant sera were available in 11 of 15 patients. Pre-transplant stored sera taken at transplant admission, was used in 4 patients. DSA were detected in 5 of 7 (71.4%) patients with TXG and only 2 of 8 (25%) patients with control. One patient in the CAN control developed late acute rejection from reduced medication compliance and then developed persistent subclinical cellular interstitial rejection and classical features of TXG at 14 years after transplantation, associated with *de novo* DSA. With the exclusion of this patient, DSA were associated with transplant glomerulopathy (r=0.577, P<0.05) and any occurrence of PTC C4d deposition (P<0.05).All 7 patients with DSA had C4d staining of PTC and 3 of 8 patients without detectable antibodies had C4d staining of PTC.

5.3.8 Functional Outcomes

5.3.8.1 Serum Creatine and GFR

Serum creatine between TXG and controls were comparable within the first year. At 2 years after transplant and beyond, serum creatinine was higher in the group with TXG compared to controls. The 1 and 5-year isotopic GFR measurements were 55±26 and 52±22 mls/min,

respectively. The median patient follow-up time was 18.7 years (figure 5.26).

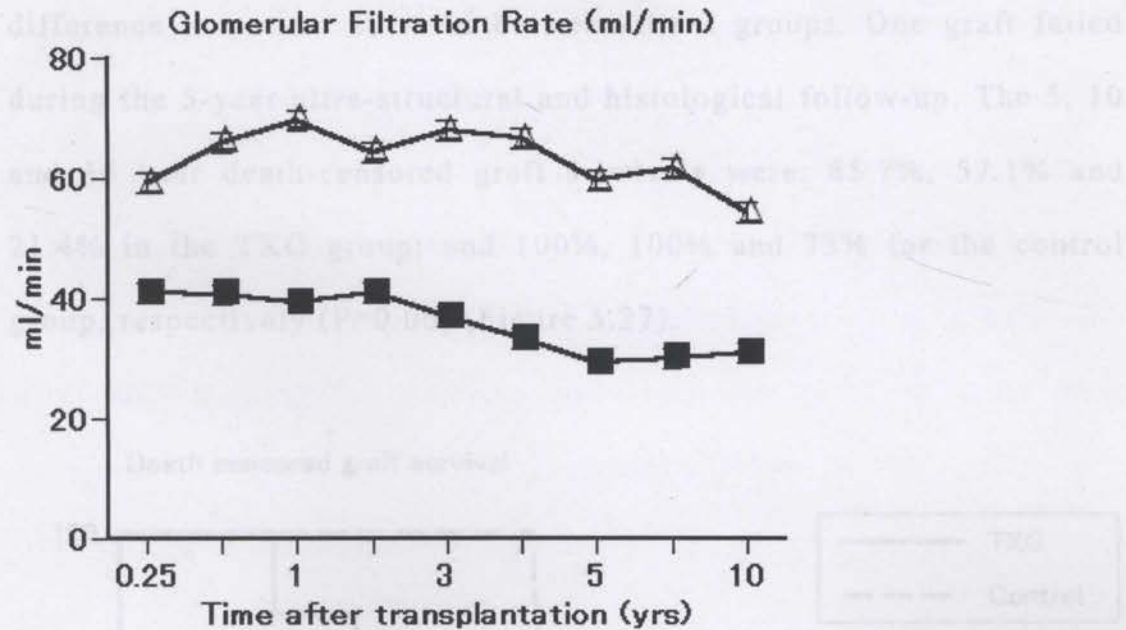


Figure 5.26: Isotopic GFR (mL/min) in recipients with TXG (■) compared to controls (Δ). GFR was reduced in patients with TXG by 3 months after transplantation compared to controls.

Proteinuria as an indicator of graft dysfunction in TXG was not examined because pancreas grafts were bladder drained leading to pancreatic secretion of proteins into the urine, consequently data on proteinuria was not available

5.3.8.2 Graft and Patient Survival

There was a trend towards shorter median survival in TXG group *versus* controls although this was not statistically significant. Median

survival in TXG was 13.6 yrs (mean 11.9years, SE 3.3, CI 8.6-15.1years) compared to 16.5 years (mean 17.6 yrs, SE 1.1 ,CI 15.4-19.8 years) in controls, P=0.07 by Peto corrected log rank) .There was no difference in patient survival between the 2 groups. One graft failed during the 5-year ultra-structural and histological follow-up. The 5, 10 and 15 year death-censored graft survivals were: 85.7%, 57.1% and 21.4% in the TXG group; and 100%, 100% and 75% for the control group, respectively (P=0.06) (Figure 5.27).

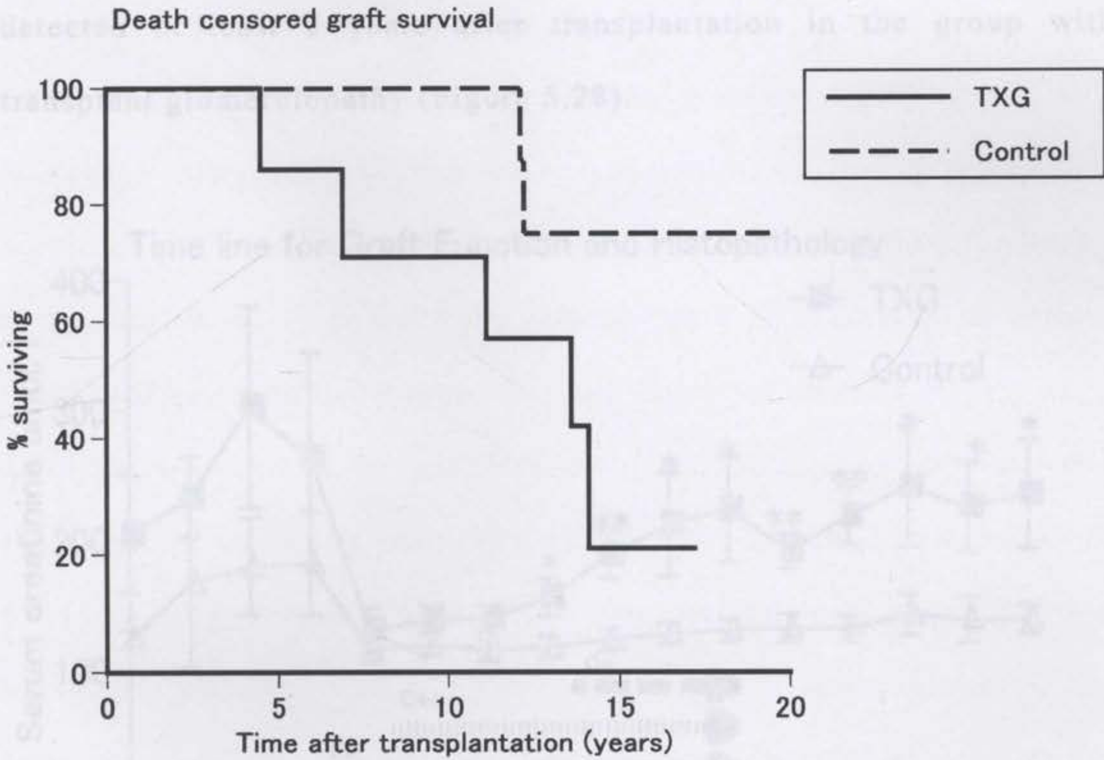


Figure 5.27: Death censored Kaplan-Meier graft survival curves after transplantation, dashed = control, TXG, solid= TXG.

5.3.9 Ultra-structure Abnormalities, C4d, and Graft Function.

Ultra-structural abnormalities were present in patients with TXG as early as 1 month after transplantation; this was associated with complement deposition in PTC and glomerular capillaries. The mean time to first biopsy with diffuse C4d deposition in PTC is 0.4 yrs median 0.3, IQR 0.02-0.6 yrs). Ultra-structural abnormalities defined as presence of LRI expansion with serration with vacuolation or thickening became abnormal mean 0.1 yrs (median 0.07, IQR 0.03 - 0.2 yrs) Graft dysfunction function and abnormal Banff cg score were detected at least 2 years after transplantation in the group with transplant glomerulopathy (Figure 5.28).

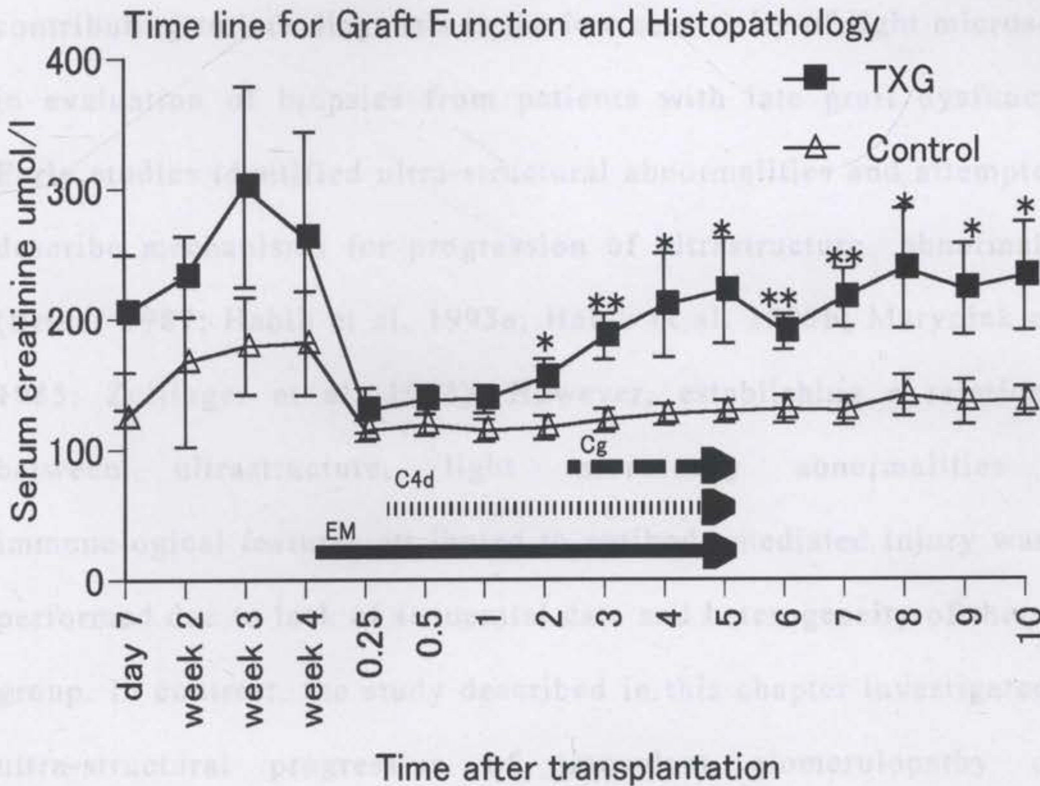


Figure 5.28: Time line for graft function, glomerular ultrastructure, diffuse C4d stain in PTC and abnormal Banff cg against time post transplant. Legend: Dashed arrow = Banff cg score, mean time to first abnormal cg 2.3 years (median 1.75 IQR 0.25-3 years). Dotted arrow= C4dPTC, mean time to first biopsy with 50% C4d is 0.4 yrs median 0.3, IQR 0.02-0.6 years). Solid Arrow= EM ultrastructure, mean 0.1 years (median 0.07, IQR 0.03 - 0.2 years). * $P<0.05$, ** $P<0.01$.

5.4 Discussion

5.4.1 Evolution of Ultrastructure abnormalities

As discussed in chapter 1, Transplant glomerulopathy is usually diagnosed many years after kidney transplantation. One of the factors contributing to late diagnosis is the low sensitivity of light microscopy in evaluation of biopsies from patients with late graft dysfunction. Early studies identified ultra-structural abnormalities and attempted to describe mechanisms for progression of ultrastructure abnormalities (Briner 1987; Habib et al. 1993a; Habib et al. 1993b; Maryniak et al. 1985; Zollinger et al. 1973). However, establishing a relationship between ultrastructure, light microscopy abnormalities and immunological features attributed to antibody mediated injury was not performed due to lack of sequential data and heterogeneity of the study group. In contrast, the study described in this chapter investigated the ultra-structural progression of transplant glomerulopathy using sequential protocol biopsies from a homogenous group of simultaneous

kidney/pancreas transplant recipients. Four significant findings into the progression of transplant glomerulopathy are derived from this study.

Firstly, abnormalities are detectable very early (from one month) in patients destined for subsequent TXG, long before the classical light microscopy pathology is evident or graft dysfunction occurs. In this study, light microscopic changes diagnostic of TXG were detected by routine protocol biopsy two to three years after transplantation and in other indication biopsy studies at 4 to 8 years (Regele et al. 2002; Sijpkens et al. 2004; Vongwiwatana et al. 2004). This implies that, subclinical endothelial injury and cellular activation begin soon after transplantation, prior to secondary alterations in the subendothelial space and reformation of the GBM and suggests, as expected, that electron microscopy examination is more sensitive than light microscopy and offers superior diagnostic recognition of TXG.

Secondly, the early time-sequence of injury begins with phenotypic changes in glomerular endothelial cells accompanied by subendothelial space expansion, and formation of new lamina densa. Endothelial cell activation is typified by vacuolation and hypertrophy, interdigitation of the endothelial cell processes within the subendothelial space surrounded by secreted fibrillary material of new lamina rara interna. Later ultra-structural changes include transition from fenestrated to continuous endothelium, further expansion of the subendothelial space, thickening of the GBM, mesangial matrix expansion, and finally late

podocyte fusion. Parallel changes of endothelial cell hypertrophy with activation occur in the PTC followed by basement membrane multi-lamination. Histological similarity between new layers of subendothelial lamina densa and PTC lamination, in addition to presence of endothelial activation in glomerular and peri-tubular capillaries, suggests a common microvascular patho-physiological response mediated by circulating factors. This conclusion is consistent with findings from other studies (Vongwiwatana et al. 2004).

Thirdly, this study shows that the pathogenesis of TXG occurs over time with ongoing regeneration and repair of the endothelial membrane. This is demonstrated by lack of progression in involved capillaries after the first year following transplantation. It would have been interesting to know not only the proportion of involved glomerular capillary loops but also how the severity of the individual changes evolved with time but this was not possible because endothelial parameters such as fenestration, thickening, vacuolation and serration were collected as qualitative data due to variation at each time-point.

Fourthly, these data give insight into the mechanisms through which TXG occurs. The likely aetiology of TXG has been postulated as sub-lethal endothelial cell injury with subsequent repair. However, in this study sequential ultra-structural data demonstrated endothelial cell activation, rather than complete cell lysis. Necrosis and/or apoptosis of

the endothelial layer, which is seen in acute antibody-mediated injury associated with high titre DSA, diffuse C4d deposition, and acute graft dysfunction, were not observed. Damaged endothelial cells are unlikely to be capable of secreting subendothelial fibrillary material typical of TXG (and observed in this study) or synthesizing the multiple laminae of the PTC basement membrane. Morphometric analysis of TXG which demonstrated abundant mitochondria, ribosomes, and golgi apparatus, associated vacuolation, serration of the subendothelial interface, and the synthesis of new lamina rara interna support the hypothesis of endothelial activation. St John et al (St John et al. 2001) demonstrated sustained and high level laminin synthesis by endothelial cells during development of the glomerular basement membrane. This study, and that of Walker et al which showed reduced clearance of fibrillary material from the sub-endothelial space (Walker 1973) suggest that subendothelial space expansion in TXG may be a consequence of endothelial activation and accumulation of fibrillary material in the subendothelial space

Finally, data from the PTC ultrastructure study, suggests that capillary injury occurs in discrete waves. The multiplication of PTC basement membrane layers (like tree rings) and even the linear patterns of subendothelial GBM indicate discrete waves of injury, due to either varying circulating antibody levels (a hypothesis supported by varying and intermittent C4d staining in TXG) or variable intrinsic properties within the endothelium which may facilitate resistance to injury. In

earlier studies, moderate (5 to 6 layers) or severe (≥7 layer) lamination were associated with "chronic rejection".(Drachenberg et al. 1997; Mauiyyedi et al. 2001; Monga et al. 1990; Monga et al. 1992; Regele et al. 2002; Vongwiwatana et al. 2004). The significance of milder lesions (2 to 3) layers was considered less specific due to its presence in native renal disease and in dysfunctional transplanted kidneys with other types of glomerular diseases (Drachenberg et al. 1997; Gough et al. 2001; Ivanyi et al. 2000). These data indicate that in surveillance protocol biopsies lower levels of multi-lamination (i.e. 3 or 4 layers), are predictors of TXG. Therefore, 3 or 4 layers of PTC-BMML should be considered abnormal in kidney transplantation, and patients with PTC multi-lamination should be reviewed for evidence of CAMR by DSA and C4d staining.

5.4.2 Evolution of immunological abnormalities

Endothelial C4d deposition is an established marker of acute humoral allo-reactivity (Feucht 2003; Feucht et al. 2005). Prevalence rates range from 91% in biopsies with indication-driven transplant glomerulopathy, or 61% with "chronic rejection" to 2% in well functioning protocol biopsies (Colvin et al. 2005; Mengel et al. 2005; Sund et al. 2003). In the cohort of biopsies described in this thesis, only 39% of biopsies demonstrated C4d staining. The low prevalence may be due to a milder form of disease in lesions detected by protocol biopsies.

5.4.2.1 Peri-tubular Capillary C4d

C4d in peri-tubular capillaries was detected in 36% of biopsies with TXG and it was correlated with PTC inflammation and ultra-structural abnormalities. This finding suggests that C4d may have a pathogenic role in the evolution of TXG. However, because C4d was incompletely expressed across time in the verified TXG group and it was detected in the control group without glomerular endothelial injury, its reliability as a diagnostic marker for CAMR requires verification.

Alternatively, this finding may be explained by "accommodation", a phenomenon where graft injury is initiates responses, which ameliorate subsequent injury (Platt, 2002). Such responses may include increased expression cyto-protective molecules discussed in section 1.2.5.7.1 or regulation of the complement pathways which results in sub-lytic levels of the MAC (Colvin et al. 2005) discussed in section 1.2.5.6.1.

Several studies support this hypothesis (Haas et al. 2006; Mengel et al. 2005; Nickeleit et al. 2003). Nickeleit et al (Nickeleit et al. 2003) reported C4d deposition in 14% of diagnostic biopsies that did not have morphological evidence of rejection.

5.4.2.2 Glomerular C4d

In contrast, to most classical studies of TXG and C4d deposition (Nadasdy et al. 2005; Troxell et al. 2006) which used immunofluorescence techniques, immunoperoxidase techniques enabled assessment of glomerular capillary loops and mesangium. Glomerular C4d staining correlated poorly with histology and ultrastructure

abnormalities compared to PTC C4d staining. However, absence of glomerular C4d in implantation biopsies confirms that C4d deposition occurring after transplantation, is a graft specific event (Nickeleit et al. 2002). Further evaluation of glomerular C4d immuno-electron microscopy may yield results that are more conclusive.

5.4.2.3 DSA, C4d and glomerular ultrastructure

Although this study was limited by sample size and lack of contemporaneous sera, an association between DSA with C4d deposition and TXG, could easily be demonstrated. This is consistent with other reports (Sijpkens et al. 2004; Worthington et al. 2007). The widespread correlations of endothelial abnormalities across anatomically distinct compartments (glomerular and PTC), supports a circulating (humoral) factor mediating the pathogenic response. Endothelial cells present a variety of antigenic determinants which are potential targets for complement-fixing alloantibody, where endothelial cell binding may result either activation or endothelial dysfunction (Glotz et al. 2006; Rifle et al. 2006). Cases of early antibody-mediated rejection which clinically present as a result of localised endothelial necrosis, activation of coagulation and local complement pathways, and finally macrophage and neutrophil recruitment (Colvin et al. 2005; Halloran et al. 1992) are likely to represent a more severe response to alloantibody injury mediated by high strength antibody as alluded to in chapter 3. TXG is likely to be etiologically similar, but characterised

by a more indolent course with lower antibody levels, and expressed with chronic pathophysiology typified by endothelial activation and increased protein synthesis, rather than complement-mediated lysis.

C4d staining was observed in some biopsies in the absence of DSA. C4d deposition without DSA has been reported in 3 of 24 patients with a negative post-transplant flow cytometry cross match (Bohmig et al. 2002), and in 9 of 14 patients with diffuse C4d staining but absent *de novo* HLA antibodies (Worthington et al. 2007). These findings could be attributed to C4d deposition consequent upon non–HLA complement activating antibodies (such as anti-endothelial antibodies (Collins et al. 2006), low or fluctuating antibody titres below the detection limit of the assay and antibody adsorption onto the graft (Martin et al. 2003).

5.5 Conclusion:

The main conclusion from this study is that TXG can be predicted in the early transplant period by a distinctive set of ultra-structural changes that represent glomerular endothelial activation with sub-endothelial expansion and formation of new basement membrane long before they can be detected by light microscopy. These changes occur in the first year after transplantation, are predictive of subsequent TXG, and correlate with presence of DSA and persistence of C4d deposition beyond 1 year. Accompanying changes to the PTC which including multi-lamination suggest that these changes occur along the whole capillary bed, in discrete waves of injury and are not restricted

to the glomerulus. The effects of antibody could be referred to as a "pan-capillaritis". Thus electron microscopy is a sensitive method for the diagnosis of TXG, and its incorporation in the evaluation of a failing allograft along with screening for HLA antibodies and C4d deposition should be considered.

CHAPTER 6

MMF ALTERS EXPRESSION OF CHRONIC HISTOLOGY

6. MMF ALTERS EXPRESSION OF CHRONIC HISTOLOGY

6.1 Introduction

In this chapter, the effects of mycophenolate mofetil on the histological features of chronic allograft dysfunction including those due to chronic antibody mediated injury were evaluated. Biopsies in MMF treated patients were compared with a historical control group of azathioprine (AZA) using light microscopy and electron microscopy techniques.

Mycophenolate mofetil (MMF) is the pro-drug of mycophenolic acid (MPA), a selective, non-competitive inhibitor of inosine 5-monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme of guanosine triphosphate synthesis. Inhibition of de novo purine synthesis and guanosine nucleotide depletion reduces T- and B-lymphocyte proliferation, and primary (but not secondary) humoral and cellular immune responses (Allison et al. 2000). MPA induces apoptosis of activated T-lymphocytes with clonal elimination, suppresses glycosylation of adhesion molecules limiting recruitment of lymphocytes and depletes tetrahydrobiopterin reducing inducible nitric oxide synthase activity, nitric oxide production and peroxynitrite-induced tissue damage (Allison et al. 2000). In prospective trials, induction therapy with MMF reduced acute rejection rates in kidney transplantation and improved graft survival in retrospective analyses (Ojo et al. 2000).

MMF use in established chronic tubulo-interstitial disease or in cyclosporine nephrotoxicity in order to support dose reduction or elimination of calcineurin inhibitor therapy results in improvement of transplant renal function, blood pressure and lipid levels (Francois et al. 2003; Schnuelle et al. 2002) (Ducloux et al. 2002). Intriguingly, addition of MMF alone without alteration of the calcineurin inhibitor dose also improves or stabilizes renal function in both human and experimental studies independent of cyclosporine (CSA) levels. Furthermore, the improved graft survival with MMF use from retrospective analysis cannot not be simply accounted for by immunological factors, and the presence of an additional mechanism was postulated (Ojo et al. 2000).

Mycophenolic acid exerts potent anti-proliferative effects in vitro of smooth muscle, mesangial, vascular endothelial and renal tubular cell cultures (Badid et al. 2000; Dubus et al. 2003; Hauser et al. 1999; Heinz et al. 2002; Mohacsi et al. 1997; Morath et al. 2003). MMF also reduces mesangial matrix deposition, glomerulosclerosis, regenerative proliferation, myofibroblast infiltration and interstitial fibrosis and collagen formation in models of glomerulonephritis, chronic rejection, cyclosporine nephrotoxicity and several models of non-immune renal disease (Azuma et al. 1995; Badid et al. 2000; Bayazit et al. 2003; Raisanen-Sokolowski et al. 1995; Ryuzo et al. 2001; Shihab et al. 2003; Tapia et al. 2003; Van den Branden et al. 2003; Yu et al. 2001; Zhang et al. 2003). These data imply that MPA exerts an anti-

proliferative effect capable of modifying the response to injury, additional to its immunosuppressive properties. Histological data in human renal transplantation are lacking.

6.2 Methods.

6.2.1 Study Population and Design

The study group consisted of consecutive type I diabetic recipients of a successful combined kidney-pancreas transplant (n=50) selected from 241 recipients of a simultaneous kidney pancreas transplant described in section 2. Inclusion criteria for Banff light microscopy analysis was the availability of 3 or more protocol kidney biopsies. Donor recipient pairs had negative CDC T cell cross-match prior to transplantation, the Luminex® assay was not performed on this cohort. Patients were excluded when baseline therapy was substantially altered as with conversion to Tacrolimus from Cyclosporine because of early acute rejection (n=2 patients) or MMF pulmonary toxicity (n=1), or because of pancreas thrombosis and recurrence of diabetes (n=9) or calcineurin-mediated haemolytic uremic syndrome (n=1). Study group patients were divided into two age matched and gender matched cohorts based on therapy with either azathioprine (n=25) or Mycophenolate mofetil (n=25). Kidney biopsies were undertaken at implantation, 1 and 2 weeks, 1, 3, 6 and 12 months and then yearly until 10 years. Biopsies were evaluated for light microscopy abnormalities according to Banff criteria described in section 2.3. Electron microscopic evaluation was performed to determine the extent of total mesangium and mesangial

matrix abnormalities in 10 patients treated with azathioprine, and 11 patients treated with Mycophenolate Mofetil based therapy. Electron microscopy techniques are described in section 2.5.5.3.

6.2.2 Statistical analysis

An unpaired Student's t-test or a Wilcoxon test was used for nominal data and a conditional binomial test examined categorical data. Cox regression was used for survival analysis and a generalized estimating equation was used for analysis of repeated measurements. Multivariate analyses corrected for repeated measures were used to adjust for differences in initial group demographics and increased initial use of oil-based cyclosporine compared with the micro-emulsion formulation in the AZA group. Differences in histology associated with MMF treatment were evaluated for their relationship with immunological factors by subset analyses of patients without acute rejection. Data are expressed as mean±SD unless otherwise stated. A probability of less than 0.05 was considered significant.

6.3 Results

6.3.1 Clinical results and protocol histology

Demographic parameters are shown in tables 6.1. Overall, study recipients were 37.6±7.3 years old and 56% were male. Recipients had comparable mismatch scores and total ischemic times. Kidney-pancreas recipients experienced sustained euglycemia with a mean HBA1C of 5.4±0.9%.

Table 6.1: Clinical outcome according to the use of mycophenolate mofetil and azathioprine (Mean ± SD or Number (%).

	AZA (n=25)	MMF (n=25)	P
Recipient age (years)	37.9±8.2	37.4±6.3	NS
Recipient sex (n, % male)	12 (48%)	16 (64%)	NS
Recipient weight (kg)	63.8±8.9	71.6±14.6	NS
Total HLA mismatch	4.1±1.4	4.3±1.4	NS
Ischemic time (hours)	12.9±2.8	11.2±3.4	NS
Donor age (years)	22.5±8.9	22.3±7.6	NS
Duration of dialysis (years)	1.3±0.8	1.4±0.7	NS

Of 360 study biopsies analysed (296 were collected as per protocol and 64 were clinically indicated performed at protocol times), 13.6±7.9 glomeruli and 2.2±1.0 arteries were present per biopsy. Inadequate samples, defined as less than 7 glomeruli or no artery, occurred in 16.6% of biopsy specimens. The inter-observer kappa statistics were good to excellent for the presence of chronic allograft nephropathy; chronic interstitial fibrosis, tubular atrophy, arteriolar hyalinosis and chronic glomerulopathy were 0.61, 0.80, 0.49, 0.51 and 0.35, respectively.

The numbers of acute cellular and vascular rejection episodes were 1.0±0.9 and 0.10±0.3 per patient, respectively, and the prevalence rates of sub-clinical rejection (including Banff "borderline") in the study group overall at 1, 3 and 12 months after transplantation were 61.3%, 28.0% and 7.9%, respectively.

Histological and functional outcomes according to immunosuppression therapy are shown in table 6.2.The AZA group experienced more acute cellular and vascular rejection episodes and OKT3 use, compared with MMF ($P<0.05$,) but did not experience more sub-clinical or chronic rejection. The 5 year isotopic GFR was 48.8±19.9 ml/min in the AZA group and 56.2±25.5 ml/min in the MMF group. Serum creatinine at 3 months after transplantation was 124±25 μmol/L in the AZA group and 117±21 μmol/L in the MMF group. The mean patient follow-up was 7 years and biopsy follow up was 3.8±2.5years. No grafts failed during the 5-year histological follow-up. One patient each in both groups required post-transplant dialysis.

AZA-treated patients (72%) were initiated on the oil-based CSA formulation before subsequent universal conversion to the micro-emulsion formulation (at 1.6±0.9 years after transplantation), which was then continued in all patients. The MMF-treated group was treated entirely with micro-emulsion CSA.

Table 6.2: Histological and functional outcomes in MMF treated patients compared to AZA treated patients.

Parameter	**AZA**	**MMF**	**P**
Number (patients/biopsies	25 (207)	25 (153)	
Acute cellular rejection/patient	1.3±0.9	0.7±0.9	0.05
Vascular rejection/patient	0.2±0.4	0	NS
Use of OKT3	17 (68%)	8 (32%)	NS
Persistent SCR	6 (24%)	7 (28%)	NS
Duration of SCR	0.54±1.7	0.42±0.79	NS
True Chronic rejection	2 (8%)	3 (12%)	NS
Clinical cyclosporine nephrotoxicity	7 (28%)	2 (8%)	NS
Post transplant hypertension	15 (60%)	17 (68%)	NS
S creatinine (μmol/L)			
Discharge	151±43	139±38	NS
3 month	124±25	117±21	NS
5 year Isotopic GFR(mL/min)	48.8±19	56.2±25.5	NS

SCR=subclinical rejection, AZA = azathioprine, MMF = mycophenolate mofetil

6.3.2 Time-course of tubulointerstitial damage

Tubulointerstitial damage was reduced in the MMF group for Banff chronic interstitial fibrosis (Coefficient from 1 to 5 years was -0.42±0.15, P<0.01) and tubular atrophy scores (Coefficient=-0.32±0.14, P<0.05), compared with AZA (Figures 6.1 and 6.2).

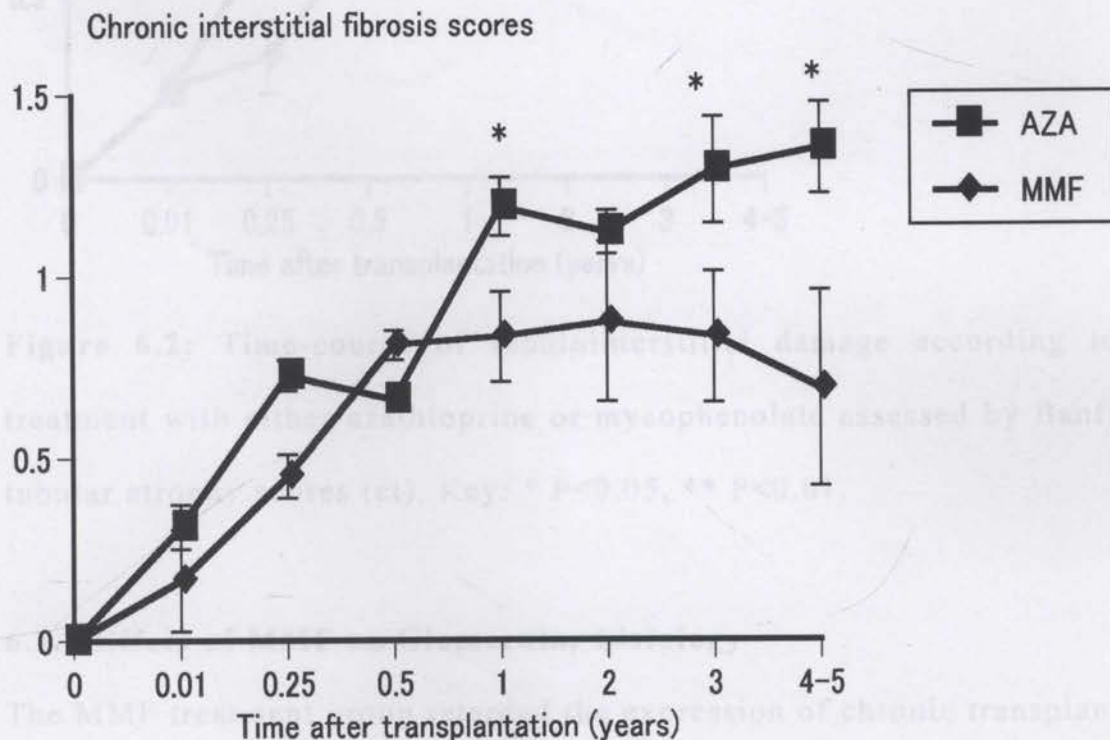


Figure 6.1: Time-course of tubulointerstitial damage according to treatment with either azathioprine or mycophenolate assessed by Banff chronic interstitial fibrosis scores (ci) Key: * P<0.05, ** P<0.01.

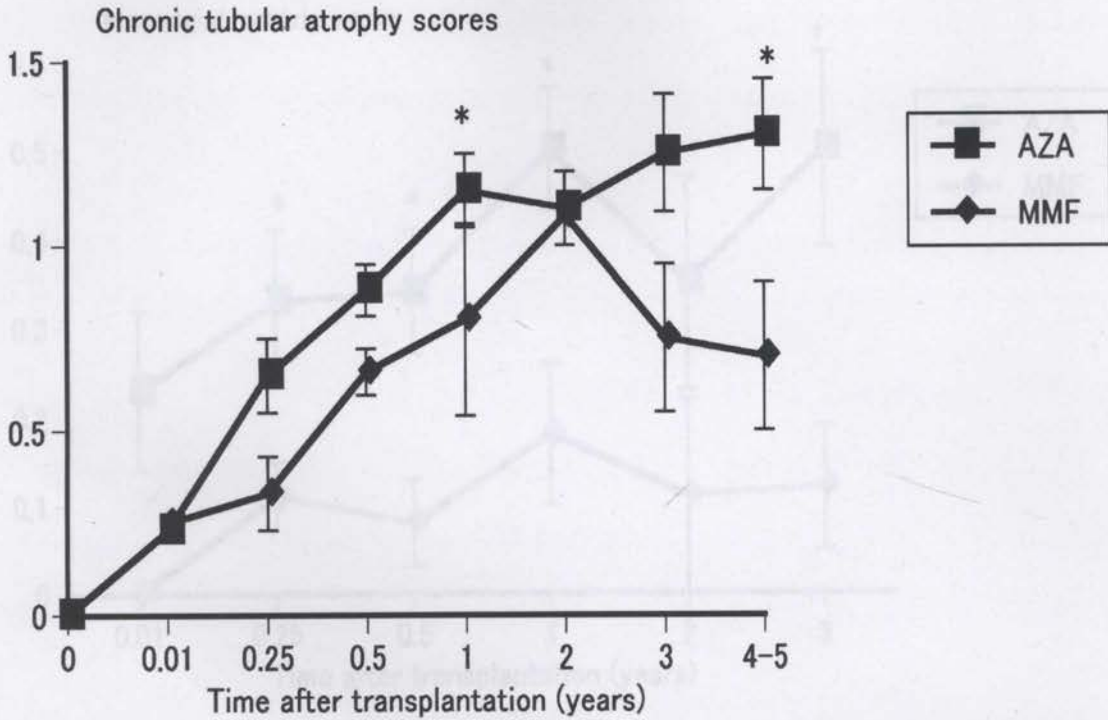


Figure 6.2: Time-course of tubulointerstitial damage according to treatment with either azathioprine or mycophenolate assessed by Banff tubular atrophy scores (ct). Key: * P<0.05, ** P<0.01.

6.3.3 Effect of MMF on Glomerular histology

The MMF treatment group retarded the expression of chronic transplant glomerulopathy and glomerulosclerosis. Banff mesangial matrix scores increased rapidly from one month after transplantation until one year, and then rates stabilized in both groups. MMF treatment substantially reduced mesangial matrix scores and prevented the early increase within the first year after transplantation (Coefficient=-0.21±0.08, P<0.01 *versus* AZA, (Figure 6.3).

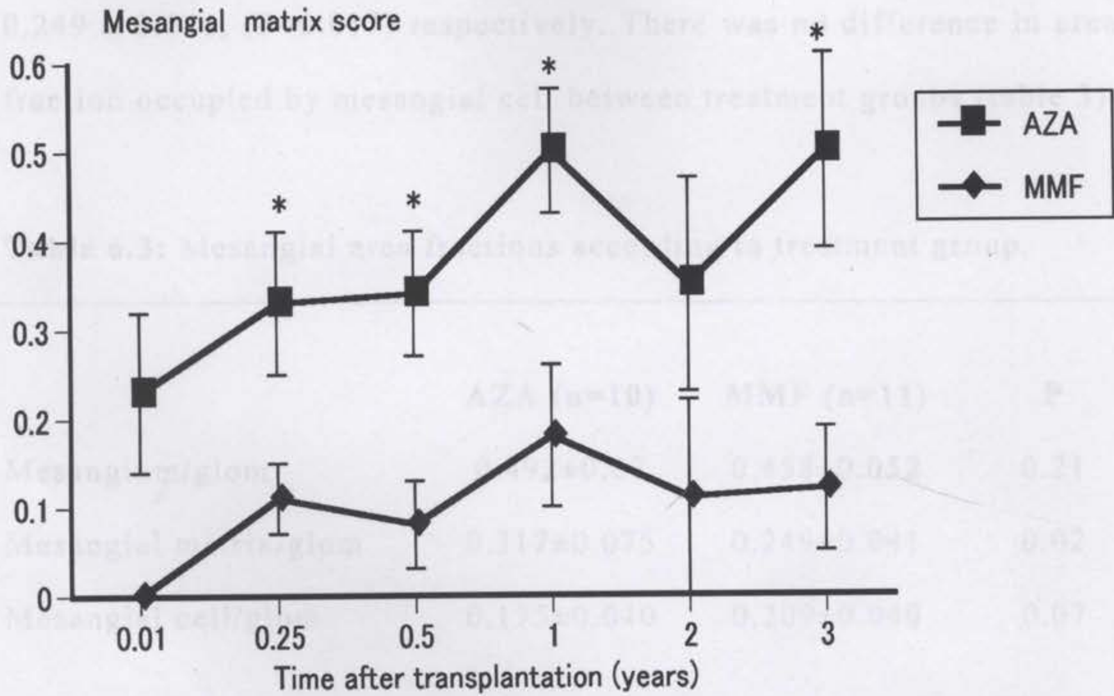


Figure 6.3: Differences in Banff mesangial matrix scores according to time after transplantation and treatment group. Key: * P<0.05, ***P<0.001.

Mesangial matrix scores correlated with chronic interstitial fibrosis (r=0.26, P<0.001), tubular atrophy (r=0.31, P<0.001), chronic glomerulopathy (r=0.21, P<0.001) and arteriolar hyalinosis scores (r=0.27, P<0.001). By generalized estimating equation, mesangial matrix accumulation adjusted for time after transplantation was associated with chronic interstitial score (P<0.001) and use of mycophenolate was protective (P<0.001).

The mesangial matrix area fractions per glomeruli was higher in AZA treated patients compared to MMF treated patients 0.317± 0.075 *versus*

0.249 ± 0.041, (P=0.017) respectively. There was no difference in area fraction occupied by mesangial cell between treatment groups (table 3).

Table 6.3: Mesangial area fractions according to treatment group.

	AZA (n=10)	MMF (n=11)	P
Mesangium/glom	0.492±0.07	0.458±0.052	0.21
Mesangial matrix/glom	0.317±0.075	0.249±0.041	0.02
Mesangial cell/glom	0.175±0.040	0.209±0.040	0.07

AZA= Azathioprine, MMF = Mycophenolate Mofetil

Overall, chronic glomerulopathy scores (Banff cg) remained at relatively low levels by 5 years after transplantation in both groups (mean combined cg scores were 0.08±0.27 from one month until 5 years inclusive), although there was a trend to reduced chronic glomerulopathy scores in the MMF group (coefficient=-0.04±0.02, P=0.09 *versus* AZA) with differences detectable at 3 month when chronic glomerulopathy grades were less with MMF treatment compared with AZA (0.48±0.43 *versus* 0.78±0.38, respectively, P<0.05). The extent of global glomerulosclerosis was minimal until one year after transplantation when progressive increases occurred. By 5 years after transplantation, glomerulosclerosis was less in the MMF-treated group compared with AZA (12±6% *versus* 32±22% sclerosed glomeruli, respectively, P<0.05) (Figure 6.4).

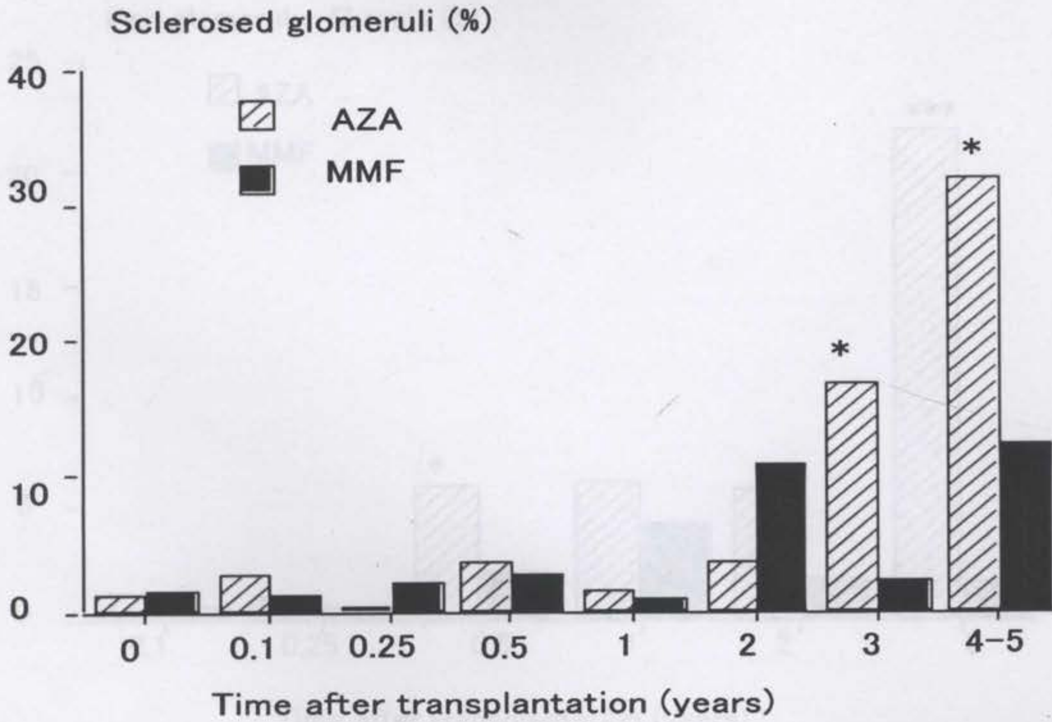


Figure 6.4: The extent of sclerosed glomeruli according to time after transplantation and treatment group means±SEM. Hatched bar is azathioprine-treated group and solid bar represents mycophenolate-treated biopsies. Key: * P<0.05.

Of significance, there was also a marked reduction in peri-glomerular fibrosis with MMF treatment compared with AZA (2.7±5.3% of glomeruli affected *versus* 9.1±12.3% respectively, P<0.001) in biopsies taken from 1 to 5 years after transplantation, inclusive. The difference was greatest by 3 years after transplantation, but its presence as a distinct entity was reduced at 5 years by the onset of substantial global glomerulosclerosis (Figure 6.5).

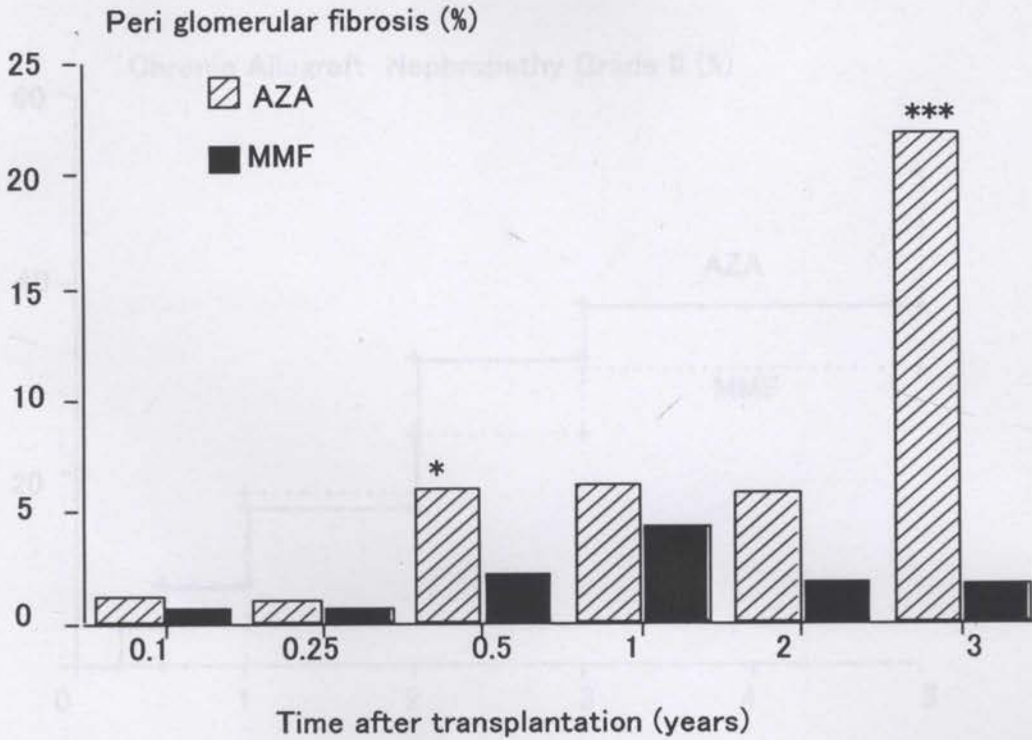


Figure 6.5: The extent of peri-glomerular fibrosis according to time after transplantation and treatment. Group means±SEM. Hatched bar is azathioprine-treated group and solid bar represents mycophenolate treated biopsies. Legend: * P<0.05, ***P<0.001.

By survival analysis, significant CAN (defined as CAN grade II or more) was greater in the AZA group compared with MMF, although this did not reach significance (P=0.15). See Figure 6.6 overleaf.

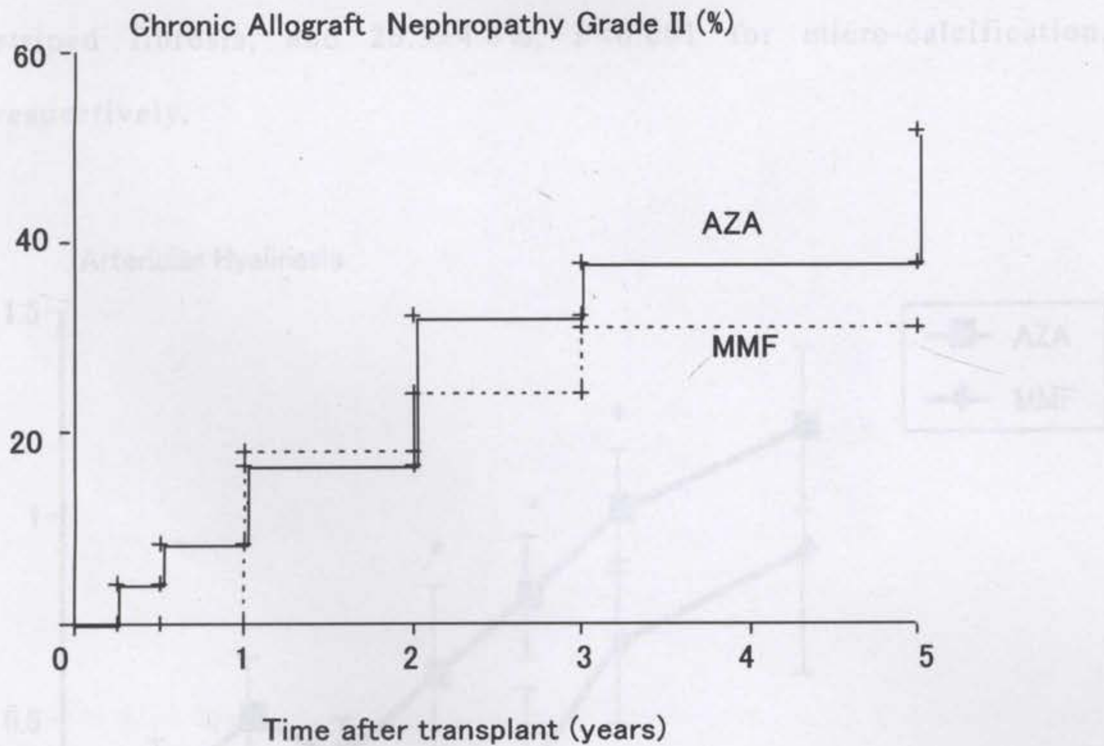


Figure 6.6: Kaplan Meier curves for the onset of moderate (grade II) chronic allograft nephropathy defined by the Banff criteria and based on its occurrence on sequential biopsies (P=NS between curves).

6.3.4 Microvascular injury and the expression of cyclosporine nephrotoxicity

The histological expression of CSA nephrotoxicity was ameliorated by MMF treatment. This was demonstrated by reduced Banff arteriolar hyalinosis scores (Coefficient=-0.35±0.16, P<0.05), (Figure 6.7), and lower prevalence of striped fibrosis (Coefficient=-0.15±0.05, P<0.01, Figure 6.8) and significantly reduced micro-calcification (Coefficient=-0.21±0.05, P<0.001, Figure 6.9) compared with AZA

treatment. The relative mean reductions attributable to MMF were 38.2±14.9%, P<0.05 for arteriolar hyalinosis, 17.6±6%, P<0.01 for striped fibrosis, and 26.5±4.8%, P<0.001 for micro-calcification, respectively.

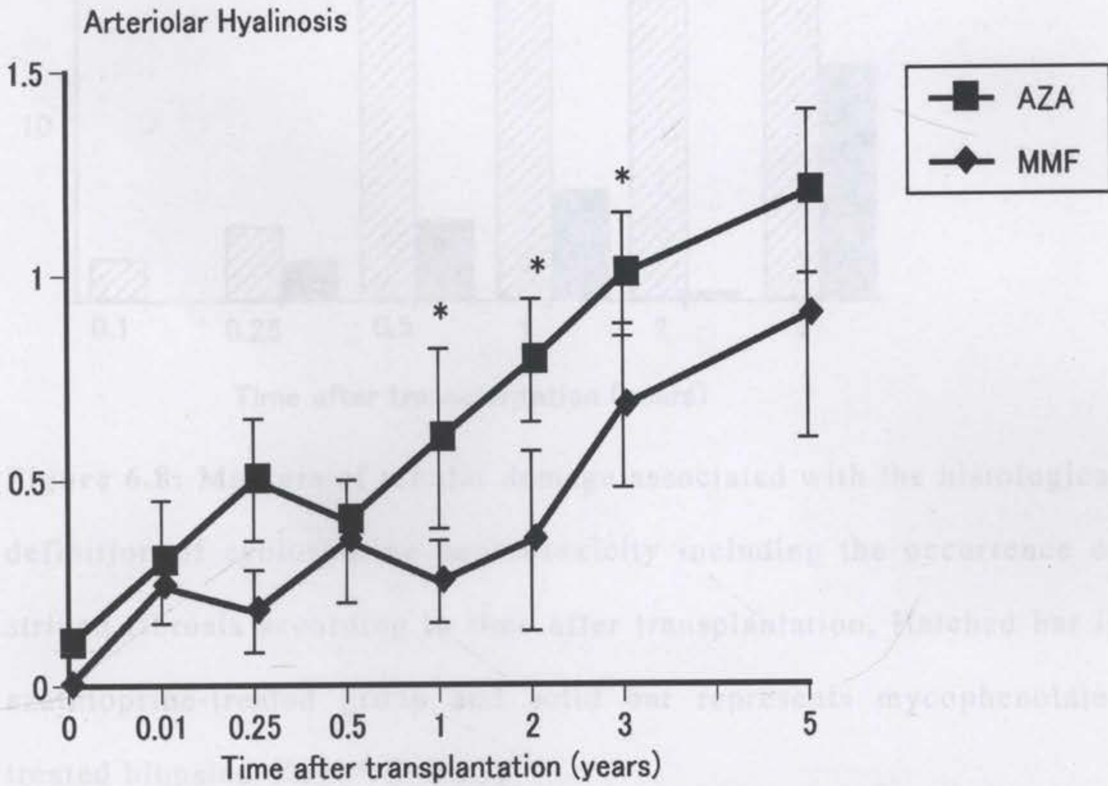


Figure 6.7: Arteriolar hyalinosis scored by the Banff schema is reduced by mycophenolate treatment. Data displayed by time after transplantation. Mean±SEM. Key: * P<0.05, ***P<0.001.

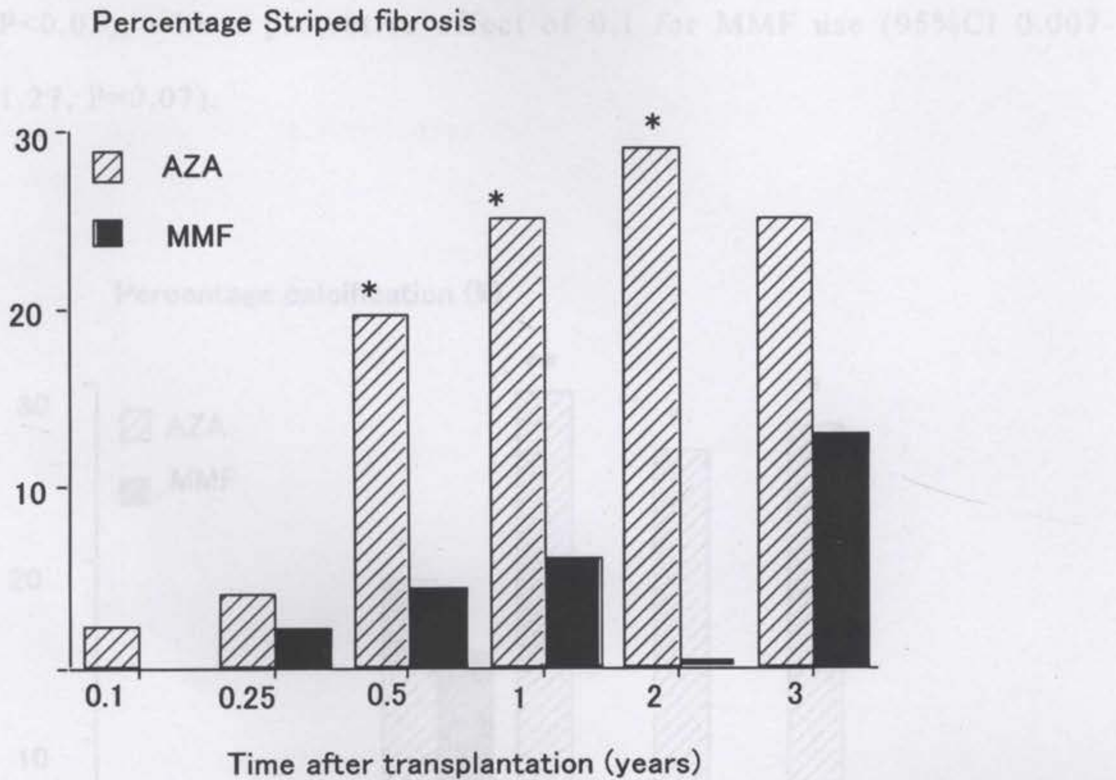


Figure 6.8: Markers of tubular damage associated with the histological definition of cyclosporine nephrotoxicity including the occurrence of striped fibrosis according to time after transplantation. Hatched bar is azathioprine-treated group and solid bar represents mycophenolate-treated biopsies. Key: * P<0.05)

MMF reduced the prevalence of total micro-calcification and CSA-related micro-calcification (defined as micro-calcification that could not be attributed to earlier acute tubular necrosis on sequential biopsies). By Cox proportional hazard modelling, the predictors of micro-calcification were complex, with adjusted hazard ratios of 37.5 (95%CI 1.5-952.0, P<0.05) for post-transplant renal dysfunction (defined as serum creatinine failing to fall below 150 umol/l by day 8), 43.7 for use of micro-emulsion CSA (Neoral ®, 95%CI 1.35-1414,

P<0.05), with a protective effect of 0.1 for MMF use (95%CI 0.007-1.27, P=0.07).

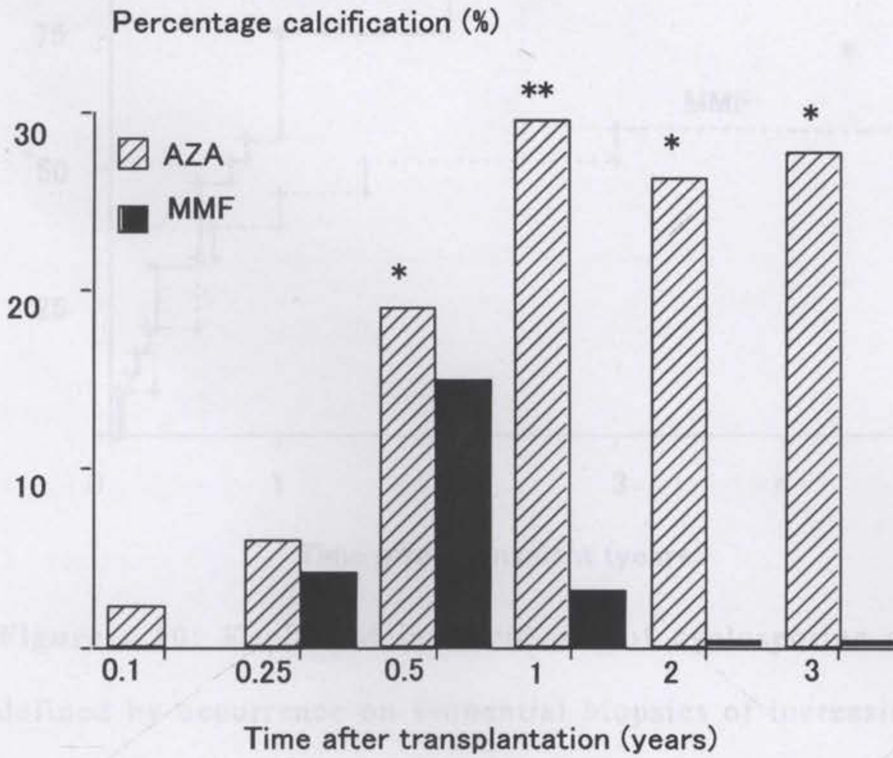


Figure 6.9: Markers of tubular damage associated with the histological definition of cyclosporine nephrotoxicity including tubular micro-calcification (not explained by prior acute tubular necrosis, top panel) Hatched bar is azathioprine-treated group and solid bar represents mycophenolate-treated biopsies. Legend: * P<0.05, **P<0.01).

By Kaplan Meier analysis, MMF treatment retarded the appearance of CSA nephrotoxicity on sequential histology (P<0.05), although by 5 years after transplantation the curves had almost rejoined (Figure 6.10).

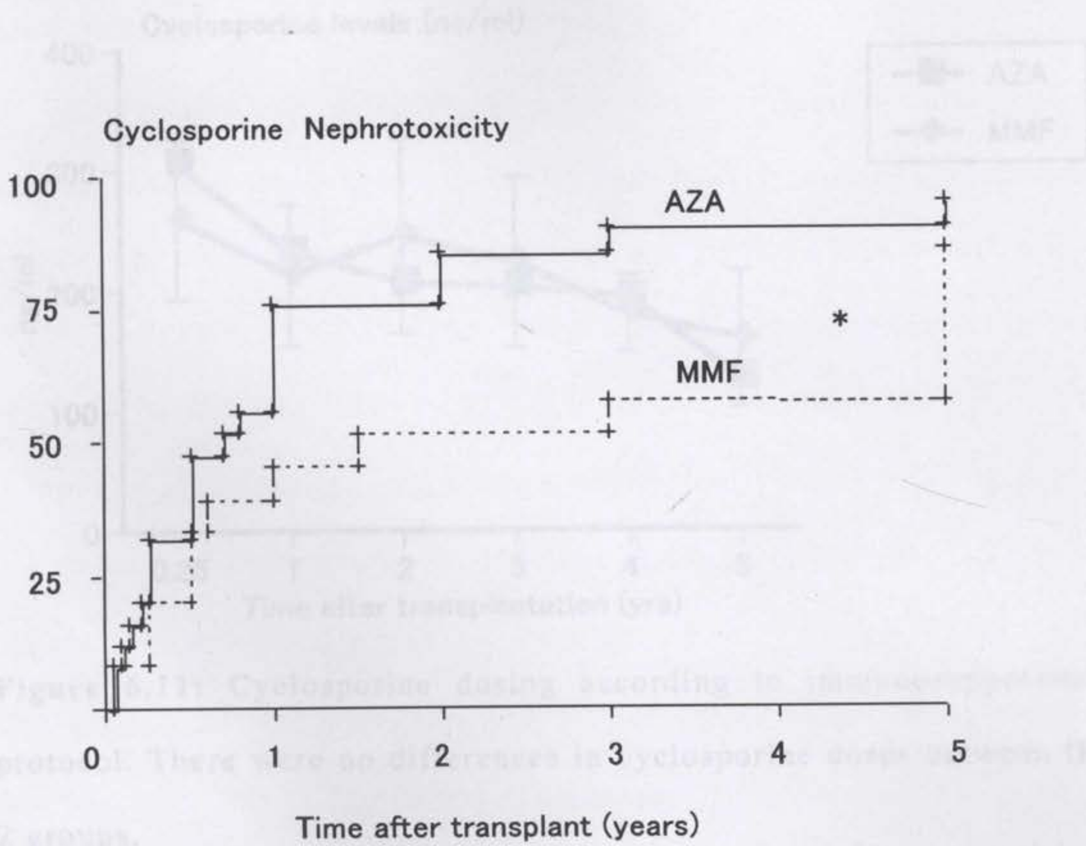


Figure 6.10: Kaplan Meier incidence of cyclosporine nephrotoxicity defined by occurrence on sequential biopsies of increasing or de novo arteriolar hyalinosis and/or striped fibrosis ($P<0.05$ between curves, bottom panel).

There were no differences between CSA doses and levels between groups, and no effect when adjusted for the use of the micro-emulsion formulation of CSA (Figures 6.11 and 6.12).

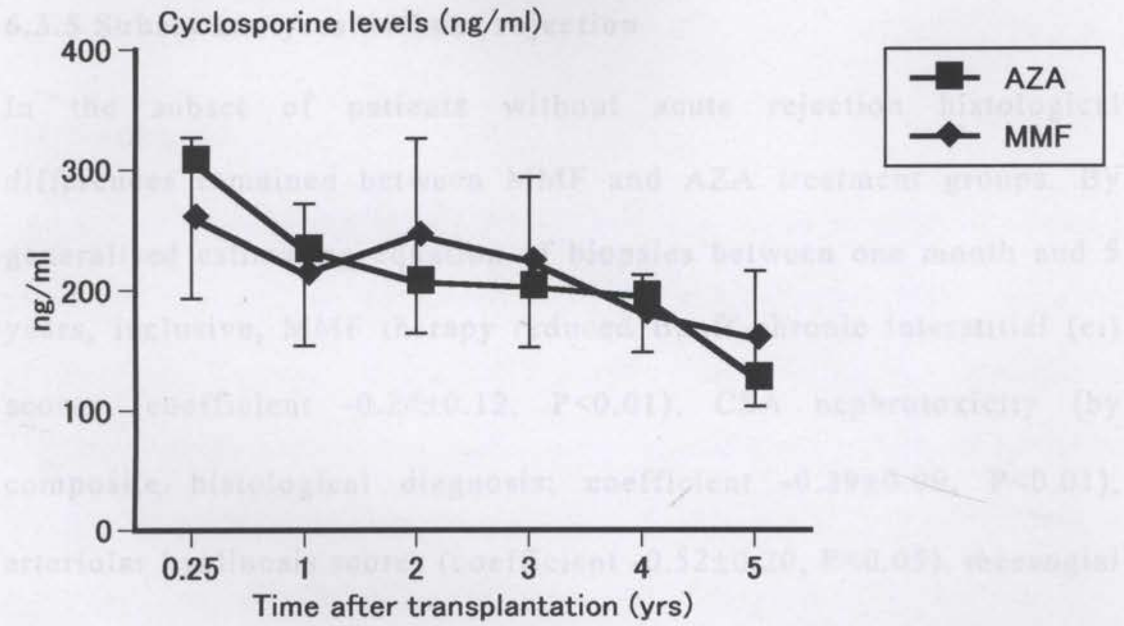


Figure 6.11: Cyclosporine dosing according to immunosuppressant protocol. There were no differences in cyclosporine doses between the 2 groups.

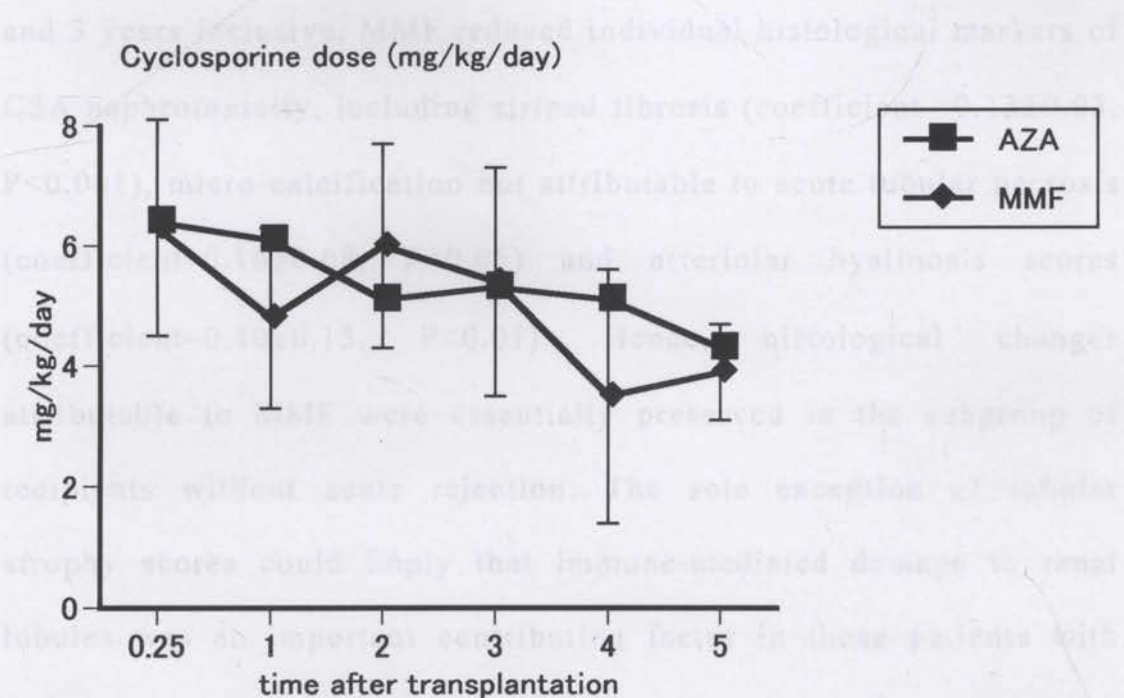


Figure 6.12: Cyclosporine levels in patients treated with azathioprine compared to MMF. Cyclosporine levels were comparable between both groups.

6.3.5 Subset analyses without rejection

In the subset of patients without acute rejection histological differences remained between MMF and AZA treatment groups. By generalised estimating equation of biopsies between one month and 5 years, inclusive, MMF therapy reduced Banff chronic interstitial (ci) scores (coefficient -0.24 ± 0.12, $P<0.01$), CSA nephrotoxicity (by composite histological diagnosis; coefficient -0.29 ± 0.09, $P<0.01$), arteriolar hyalinosis scores (coefficient -0.52 ± 0.20, $P<0.05$), mesangial matrix scores (coefficient -0.19 ± 0.05, $P<0.001$), peri-glomerular fibrosis (coefficient -4.5 ± 1.6, $P<0.01$), percentage sclerosed glomeruli (coefficient -6.83 ± 2.5, $P<0.01$) and but not for chronic tubular atrophy scores (coefficient -0.14 ± 0.11, P=NS). In biopsies between one month and 3 years inclusive, MMF reduced individual histological markers of CSA nephrotoxicity, including striped fibrosis (coefficient -0.13 ± 0.03, $P<0.001$), micro-calcification not attributable to acute tubular necrosis (coefficient-0.16 ± 0.08, $P<0.05$) and arteriolar hyalinosis scores (coefficient-0.40 ± 0.13, $P<0.01$). Hence, histological changes attributable to MMF were essentially preserved in the subgroup of recipients without acute rejection. The sole exception of tubular atrophy scores could imply that immune-mediated damage to renal tubules was an important contributing factor in those patients with rejection.

6.4 Discussion

These data show that MMF alters the histological expression of chronic allograft dysfunction compared with AZA treatment. MMF reduced glomerular injury, mesangial matrix accumulation, transplant glomerulopathy scores, and subsequent glomerulosclerosis in addition to, limiting the development of chronic interstitial fibrosis, tubular atrophy, peri-glomerular fibrosis, and delaying the histological presentation of arteriolar hyalinosis, striped fibrosis and tubular micro-calcification which are characteristic features of CSA nephrotoxicity. The histological modification of chronic histology was independent of acute rejection suggesting a non-immune mechanism, which I hypothesize, is from its direct anti-proliferative and anti-fibrotic properties.

Experimental data supports the hypothesis that MPA suppresses not only proliferating lymphocytes but a wide variety of other cells at therapeutic concentrations used in human transplantation (Deierhoi et al. 1993; Morath et al. 2003). Most cell types constitutively express the type I isoform of IMPDH, inhibited by MPA and use both IMPDH-dependent de novo purine synthesis and the MPA-independent salvage pathways (Allison et al. 2000). Occurrence of gastrointestinal intolerance and hemopoietic suppression (Mathew 1998) at conventional doses indicate that other cells are affected . The data

presented here show the effects of MMF on glomerular, microvascular and tubulointerstitial morphology.

6.4.1 Effect of MMF on Chronic Glomerular Injury

MMF reduced markers of glomerular injury including mesangial matrix deposition and transplant glomerulopathy scores, (which are features of CAMR) as discussed in chapter 1, in addition, MMF was associated with reducing peri-glomerular fibrosis and early glomerulosclerosis, compared with AZA treatment. Mesangial matrix accumulation was ameliorated by MMF, independent of acute rejection status. Several in vitro studies show that the human mesangial cell is sensitive to MPA through direct inhibition of cell proliferation in a dose-dependent manner. Mesangial cellular turnover, apoptosis, and mitogen-induced proliferation are rapidly and substantially inhibited by MPA at low and clinically relevant drug concentrations (Deierhoi et al. 1993; Hauser et al. 1999)

Transplant glomerulopathy scores were lower in MMF treated patients. In experimental models of glomerulonephritis, MMF limited glomerular hypercellularity and hypertrophy, expression of α-smooth muscle actin and extracellular matrix deposition (Yu et al. 2001; Ziswiler et al. 1998). Post-transplant glomerulosclerosis is likely exacerbated from ischemia from arteriolar and vascular insufficiency, mesangial matrix synthesis and from tubular destruction. MMF may reduce glomerulosclerosis by limiting arteriolar hyalinosis and glomerular

ischemia, mesangial matrix deposition, direct immune-mediated tubular destruction and the chronic interstitial fibrogenic healing response to injury.

6.4.2 Effect of MMF on Chronic Tubulo-interstitial Injury

MMF reduced chronic interstitial fibrosis and tubular atrophy compared with AZA therapy in this study and limited early tubulointerstitial damage. While reduced tubular atrophy scores were independent of the presence of acute, rejection, consistent with direct immune-mediated tubular destruction, chronic interstitial fibrosis generation was reduced in both rejecting and non-rejecting recipients, suggesting an additional non-immune mechanism. Peri-glomerular and striped fibrosis were reduced in MMF treated patients. Because these fibrotic responses are etiologically disparate and occur within differing structural compartments of the transplanted kidney, MMF probably operates by modifying a broad pathological response to injury – irrespective of its originating cause.

Anti-fibrotic effects of MMF have been observed in experimental glomerulonephritis (Ziswiler et al. 1998), models of true chronic transplant rejection and CSA nephrotoxicity (Azuma et al. 1995; Jolicoeur et al. 2003; Viklicky et al. 2000), and in vitro fibroblast and mesangial cell cultures (Ziswiler et al. 1998). Chronic rejection models in Fischer to Lewis rats, showed substantially reduced chronic tubulointerstitial damage, fibro-intimal hyperplasia and

glomerulosclerosis in MMF treated animals (Azuma et al. 1995). Interestingly, FK778, the active Leflunomide metabolite and de novo pyrimidine synthesis inhibitor, also reduced chronic histological changes such as transplant glomerulopathy and glomerulosclerosis in the same experimental chronic rejection model in addition to decreasing serum allo-specific antibody production (Pan et al. 2003). This suggests that inhibition of de novo nucleotide synthesis through either of the pathways is effective in ameliorating damage from chronic rejection. Reduction of tubulo-interstitial damage has also been demonstrated in non-immune models of renal failure, with improved renal function being accompanied by less early tubulointerstitial damage, glomerulosclerosis and reduced pro-fibrotic factor expression (Badid et al. 2000; Bayazit et al. 2003; Fujihara et al. 1998; Van den Branden et al. 2003; Zhang et al. 2003).

6.4.3 Effect of MMF on Expression of CSA Nephrotoxicity

The delay in histological expression of CSA nephrotoxicity in this study defined by reduced arteriolar hyalinosis, striped fibrosis and tubular micro-calcification is consistent with human (Ducloux et al. 2002; Francois et al. 2003; Weir et al. 2001) and experimental studies (Jolicoeur et al. 2003; Shihab et al. 2003). CSA elimination or dose reduction using adjunctive MMF therapy improved renal transplant dysfunction (Weir et al. 2001); especially when CSA nephrotoxicity was the primary cause of impairment (Ducloux et al. 2002), but also stabilized the decline and sometimes improved renal function,

independent of the CSA dose or levels (Gonzalez Molina et al. 2004; Henne et al. 2003) with the greatest benefit in the least damaged grafts.

Differences due to transplantation era were potential confounders in this study; however, patients in this cohort were demographically comparable at entry except for the initiation with oil-based CSA in some AZA-treated patients, prior to subsequent universal conversion to the micro-emulsion formulation. However, histological differences persisted after statistical adjustment; oil-based CSA formulation exposure was limited in duration, and CSA doses and trough levels were comparable between groups. So observed differences cannot be attributed to increased absorption of micro-emulsion CSA. Thus, MMF therapy may have a potential adjunctive role in mitigating the response to arteriolar injury from cyclosporine, although this protective effect was not sustained.

6.5 Conclusion

MMF therapy exerts widespread and profound effects on the initiation and progression of damage to the transplant kidney. MMF reduces tubulointerstitial, striped and peri-glomerular fibrosis; the evolution of glomerular abnormalities including transplant glomerulopathy, mesangial matrix deposition and glomerulosclerosis, and delays histological presentation of progressive arteriolar hyalinosis and the lesions of cyclosporine nephrotoxicity. These data suggest that MMF has a beneficial role as a long-term immunosuppressive agent that is

capable of limiting the pathogenic fibrotic process characteristic of chronic glomerular and tubulo-interstitial injury.

CHAPTER 7
SUMMARY AND CONCLUSIONS

7. SUMMARY AND CONCLUSIONS

This chapter summarizes the major findings, limitations of the data and outlines directions for further research. In chapter 1, chronic allograft dysfunction, its pathology, and pathogenesis with reference to the role of HLA antibodies were reviewed. Several unresolved issues were identified; these include uncertainty about clinical relevance of pre-transplant antibody detected using sensitive solid phase assays, inconsistencies in patterns of chronic antibody mediated injury in renal allografts and lack of specific therapeutic interventions for chronic antibody mediated rejection.

The aims of the studies described in this thesis were:

1) to examine the effects of pre-transplant antibodies detected using the Luminex® assay on histological and functional outcomes in SPK transplant recipients,

2) to identify early histopathological features predictive of transplant glomerulopathy, and

3) to investigate therapeutic options for chronic allograft dysfunction.

The clinical and histological relevance of antibody detected using sensitive assays was examined with a view to describing the time-course of graft injury and the factors that influence outcomes. The early diagnosis of chronic antibody mediated rejection was examined by identifying ultra-structural abnormalities suggestive of TXG using electron microscopy techniques. The role of MMF in amelioration of chronic transplant histology was investigated by comparing light and

electron microscopy histopathology in transplant recipients treated with Mycophenolate mofetil and Azathioprine immunosuppression therapy.

Pre–transplant DSA was detected in large number of unsensitised transplant recipients as determined by a CDC assay when the Luminex® assay was used. Presence of DSA was associated with early graft dysfunction due to acute antibody mediated rejection and late graft dysfunction due to chronic antibody mediated rejection. However, in comparison to pre-transplant antibody detected by the CDC cross-match, early graft dysfunction was transient and graft loss occurred later in the post transplant period. Antibody strength measured by MESF determined outcomes in recipients with DSA. High MESF values were associated with inferior outcomes. Immunosuppression regimens in recipients with DSA also had an impact on functional and histological outcomes. These results suggest that renal allografts in recipients with pre-transplant DSA detected using Luminex® will initially function but subsequently fail depending in part on antibody characteristics and measures taken to mitigate their effects such as immunosuppression therapy. Other factors such as antibody class (whose effect was not evident in this study due to distribution of antibody) could also play a role in determining outcomes. Therefore, the Luminex® single antigen assay should be used in pre-transplant screening to identify covert sensitisation and antibody strength measured using MESF values should be performed to identify patients

likely to develop graft dysfunction. This would enable avoidance of donor recipient combinations likely to have poor outcomes and also identify recipients for closer post-transplant monitoring.

Pre-transplant MICA antibodies were not associated with graft dysfunction or inferior histological abnormalities in contrast to recently published data. Studies in this area with determination of donor MICA status are required.

Ultrastructure abnormalities in recipients with DSA who developed morphological characteristics of chronic antibody mediated rejection were examined. From this study, endothelial abnormalities that predict late transplant glomerulopathy were identified. Sub-endothelial injury and activation occur early in the post transplant period, long before graft dysfunction and light microscopy abnormalities are detected using conventional techniques. These changes are associated with early and persistent complement activation within the graft. Graft injury occurs in discrete waves as shown by peri-tubular capillary morphology, resulting in endothelial activation and extracellular matrix secretion, with subsequent glomerulosclerosis. Transplant recipients with DSA and evidence of complement deposition, should have early protocol electron microscopy performed to identify patients with ultra-structural abnormalities indicative of continued antibody mediated injury.

Interventions for chronic antibody mediated graft injury in recipients with DSA are still limited and rely on preventative measures such as avoidance of donor-recipient combinations with a positive pre-transplant and desensitisation protocols. Findings from chapter 6 show that MMF has a potential role in altering the expression of chronic renal injury when compared to azathioprine. This is attributed to its anti-proliferative effect on B cells, which are involved in antibody production, but is also due to amelioration of other known causes of chronic renal injury such as immunosuppression toxicity and persistent subclinical cellular rejection. Although the exact role of MMF in therapy for chronic antibody mediated injury still requires investigation maintenance immunosuppression in patients with DSA should incorporate a potent anti–proliferative agent such as MMF.

Interpretation and applicability of these data is limited by:

1) Lack of sera contemporaneous with histopathology and functional time points. Although histology and functional studies were collected prospectively, DSA analysis was performed retrospectively. Contemporaneous DSA would answer the question whether fluctuation in antibody titre influence patterns of complement deposition and histological outcomes. Post transplant monitoring in patients with DSA is required to provide more data in this area.

2) A standard CDC T cell cross-match was used at time of transplant to identify presence of donor HLA antibody. It is therefore unknown how many of these donor-recipient pairs would have been avoided if augmentation with anti-human globulin or flow cytometry cross-match were used. However, the study described in chapter 3 was not designed to compare the Luminex® single antigen test to different cross-match techniques as predictors of post transplant outcome, although this would provide interesting comparative information. Indeed, the data allows the testable hypothesis that DSA testing using Luminex® SAG might replace cross-matching.

3) Lack of validation of MESF values in different population groups to determine thresholds. In addition, validation by an independent test such as flow cytometry if positive would resolve the issue whether low MESF are true DSA. This is necessary before results can be applied to different patient populations.

4) Prospective randomised control studies are still required to identify therapeutic options for patients with chronic antibody mediated rejection.

In conclusion, these results support the hypothesis that DSA are common in otherwise unsensitised patients if the Luminex® single antigen assay is used as a screening tool. Patients with DSA are at risk for adverse long term outcomes; however, stratification using antibody

strength will identify those at risk. Use of EM techniques enables identification of recipients destined to develop histological abnormalities due to chronic antibody mediated injury early, thus facilitating rationalisation of immunosuppression therapy.

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Figure 1: Distribution of DSA in pre-transplant sera from SPK recipients tested using the Luminex assay.