

# The prevalence and contribution of histological patterns to late period renal allograft dysfunction and loss

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Internal Medicine

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**i. Declaration**

I, Sara Tracy Saffer, do hereby declare that this research is solely my own work. I am submitting this research as fulfilment for the degree of Master of Medicine, branch of Internal Medicine. I further declare that this work has not been submitted for any other examination or degree at this or any other university.

Signed,

Sara Tracy Saffer

Monday 19-11-2018

## ii. Publications and presentations arising from this research

1. Poster presentation to the World congress of Nephrology (WCN), 13-17 March 2015, Cape Town, South Africa
  - Poster details: Saffer S, Davies M, Paget G, Mosiane P, Grossberg S. Histological patterns associated with late period renal allograft dysfunction and loss.
  - Poster details: Saffer S, Davies M, Paget G, Grossberg S. Utility of serum creatinine and proteinuria in the evaluation of late period graft dysfunction
2. Poster presentation to the World Congress of Nephrology 2015 Satellite Symposium, 17-18 March 2015, Cape Town, South Africa
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4. Poster presentation to the School of Clinical Medicine Research day, 30 September 2015, Johannesburg, South Africa
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  - Poster presentation details: Saffer S, Davies M, Khan F, Paget G. Prevalence and outcome of antibody-mediated allograft injury in a historical cohort.

### **iii. Ethical Considerations**

Permission for this retrospective study was obtained from Prof G Paget (Head of Department, Department of Nephrology, Charlotte Maxeke Academic Hospital), Dr M Mofokeng (Chief Executive Officer, Charlotte Maxeke Academic Hospital), Dr A Sparaco, (Acting Head of Department, Department of Transplant Surgery, Charlotte Maxeke Academic Hospital), and the Human Research Ethics Committee of the University of the Witwatersrand (clearance number M140637).

#### iv. **Abstract**

##### **Introduction**

Late period renal allograft dysfunction and loss remains a substantial limitation to transplant longevity. The contribution of various aetiological factors resulting in late period allograft dysfunction and loss at CMJAH and their associated biochemical markers were evaluated.

##### **Methods**

The histological findings in a cohort of patients (n=242) undergoing late post-transplant period (>3 months after engraftment) renal allograft biopsy-for-cause over a 10 year period from 1/1/2004 – 31/12/2013 were retrospectively evaluated. Serum creatinine, urine white cell count, and urine protein: creatinine ratio (UPCR) at the time of biopsy together with percentage change in serum creatinine were analyzed in respect of the histological diagnosis.

##### **Results**

Immunological factors were found to be the dominant cause of graft injury accounting for the majority (50.8%) comprising cell-mediated rejection (CMR) (62%), antibody-mediated rejection (ABMR) and mixed rejection (MR) at 21.7% and 16.3% respectively. Diagnosis of ABMR was observed to increase after the introduction in 2009 of routine C4d staining, suggesting the possibility of under-diagnosis of ABMR during the 2004-2009 periods. Non-immunological causes of graft injury accounted for 49.2% of cases. Calcineurin inhibitor nephrotoxicity (16.9%) was the leading non-immunological aetiological factor identified, followed by reflux nephropathy (6.6%), recurrent / de novo glomerular disease (5.4%), thrombotic microangiopathy (1.6%), BK polyomavirus

nephropathy (0.8%), and hypertensive nephropathy (0.8%). Idiopathic interstitial nephritis / tubular atrophy (IFTA) comprised 14.7% of cases; in the remaining 2.1% no obvious cause of graft dysfunction was identified at biopsy. Graft age was lower at diagnosis ( $p=0.019$ ) and percentage change in serum creatinine was higher in cases of rejection ( $p=0.00005$ ). Discriminant analysis determined that graft age below 79 months and percentage change in serum creatinine above 23.9% were associated with rejection as a cause of graft dysfunction (OR=1.6772, 95% CI 1.006-2.965,  $p=0.0465$  and OR=2.174, 95% CI 1.2896-3.6698,  $p=0.0033$ ) respectively. Comparison of cases of CMR vs. ABMR found a higher UPCr level in ABMR ( $p=0.039$ ). Discriminant analysis determined that UPCr  $>0.20$  g/mmol was associated with ABMR (eigenvalue = 0.049, Wilk's  $\lambda = 0.952$ ,  $\chi^2 = 5.77$ ,  $df = 1$ ,  $p = 0.016$ ). Immunological patterns of injury demonstrated poorer allograft survival after diagnostic biopsy than non-immunological patterns (Cox Mantel test  $F = 2.155$ ,  $p = 0.031$ ) Furthermore, the presence of histological features consistent with antibody-mediated injury on retrospective biopsy were found to have significantly deleterious effect on post biopsy outcomes (Cox Mantel  $F = 2.16$ ,  $p = 0.031$ ).

## **Conclusion**

Immunological factors play a significant role in late period graft dysfunction and loss at CMJAH. The contribution of ABMR in limiting long-term graft survival has likely been previously under-estimated due to the lack of a histological marker such as C4d to facilitate diagnosis. Furthermore, consideration of the graft age and percentage change in creatinine from baseline may alert clinicians to the possibility of rejection as cause for late period graft dysfunction. Proteinuria arising as a result of evolving transplant glomerulopathy may indicate the presence of antibody-mediated injury. Since humoral injury is associated with poorer allograft survival; early detection through appreciation of

clinical parameters such as proteinuria may facilitate preemptive directed therapy, possibly improving renal allograft survival.

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## ix. List of abbreviations

ABMR	Antibody-mediated rejection
APC(s)	Antigen presenting cell(s)
C4d	Complement factor 4 split product
CAN	Chronic allograft nephropathy
CD	Cluster of differentiation
CI	Confidence interval
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CMR	Cell-mediated rejection
CMV	Cytomegalovirus
DSA	Donor-specific antibody
ESRD	End stage renal disease
(e)GFR	(estimated) Glomerular filtration rate
HLA	Human leukocyte antigen
IFN	Interferon
IFTA / CAN	Interstitial fibrosis tubular atrophy / chronic allograft nephropathy
IQR	Interquartile range
IL	Interleukin
LTRRT	Long term renal replacement therapy
MDRD	Modification of diet in renal disease
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MR	Mixed rejection
OR	Odds ratio
PTC	Peritubular capillary
RNA	Ribonucleic acid
SD	Standard deviation
SE	Standard error
TCR	T cell receptor
TG	Transplant glomerulopathy
Th	T-helper cell
UPCR	Urine protein : creatinine ratio
WCC	White cell count

# **1 Background**

## **1.1 Introduction**

Chronic Kidney Disease (CKD) is an ever-increasing threat to global health. A significant number of patients with CKD will progress to End Stage Renal Disease (ESRD) requiring the initiation of long-term renal replacement therapy (LRRT)[1]

Renal transplantation remains the preferred mode of LRRT in view of data demonstrating improved patient morbidity and mortality in patients undergoing transplantation compared to those remaining on dialysis [2], with a significant improvement of quality of life as well as a reduction in health care-related costs. Furthermore, transplantation offers the possibility of radical cure of ESRD and the return to adequate renal function. Cure of ESRD by transplantation is, however, only achievable if the transplanted kidney retains adequate levels of function for the remainder of the recipient's natural life span.

Although the introduction of newer, more efficacious immunosuppressant therapy has vastly improved short term outcomes, long-term transplant longevity has not been substantially increased [3, 4], and late post-transplant graft losses continue to occur at a rate of 4% per year after the first year following transplant [5]. Prolonged allograft survival is limited by a complex interplay between the recipient immune system and immunosuppression therapy: under-immunosuppression allows the recipient immune system to reject the allograft; over-immunosuppression exposes the allograft to damage from infections and drug effect. Understanding of this delicate balance requires an appreciation of the immunological reactions occurring following

transplantation, and the immunosuppressant drugs prescribed to control these reactions.

## **1.2 Transplant immunology**

Transplant immunology involves both the innate and adaptive immune response. Innate immunity is non-specific and involves the recruitment of macrophages and natural killer cells via chemokines and cytokines as well as activation of the complement cascade, which in turn promotes inflammatory cell trafficking. The innate immune system is also important in the activation of an antigen-specific adaptive immune response through recruitment of B and T cells. The relationship of the innate and adaptive immune responses is closely intertwined [4, 6-8]. For example, the innate immune system produces chemokines and cytokines that activate antigen specific T cells. This in turn leads to alloantibody production and CD8 cytotoxic cell activation. Full T cell activation is further enhanced by local tissue production of specific components of the complement cascade.[7]

T cell activation depends on antigen recognition facilitated by the interaction of the T-cell receptor (TCR) with Major Histocompatibility Complex (MHC) proteins expressed on the surface of cells. MHC proteins are derived from the expression of the polymorphic Human Leukocyte Antigen (HLA) genes located on the short arm of chromosome 6. Differences in post-transcriptional processing of ingested antigens results in their differential expression in complex with either MHC I or MHC II proteins derived from transcription of the HLA gene sequences.

MHC I complexes are produced in a process which recruits peptides derived from the processing of antigen internalised into the cytoplasm of the presenting cell. The MHC I complex presents antigen to CD-8 positive T cells (also known as cytotoxic T cells). Cytotoxic T cells in turn induce apoptosis of the infected cell or produce cytotoxins and proteases, which result in cell lysis and destruction [9]. Although MHC I complexes are present on almost all cell types there is differential expression with a high level of expression on antigen presenting cells (APCs). These include dendritic cells, macrophages, B lymphocytes and vascular endothelial cells. Due to the potent vascularity of the kidney, there is a strong presence of MHC class I on vascular endothelial cells. [7, 9]

In contrast, MHC class II complexes are derived from exogenous protein material. These proteins are degraded into peptide product within lysosomes, which together with the MHC class II complex is merged to form the final MHC II complex. Class II MHC complexes are expressed predominantly on antigen presenting cells, namely B cells (B lymphocytes), dendritic cells and macrophages, which present the antigen complex to CD-4 positive T cells. In contrast to MHC I expression, vascular endothelial and epithelial cell expression of MHC II antigen is low and only up-regulated after exposure to high levels of pro-inflammatory cytokines, particularly IFN  $\gamma$  and interleukin-2 [10].

Donor tissue primarily evokes an immune response directed against MHC-associated antigen, requiring the interaction of donor cells with recipient T cells. This allorecognition involves both MHC class I and II cellular antigenic interaction with T cells initiating a cascade of events resulting in cytokine release, cellular apoptosis and ultimately graft injury [11-13].

There are 2 modes of allorecognition in the host.

In the direct pathway donor antigen presenting cells (APCs) are recognised by recipient T cells as foreign inducing a potent alloimmune response. This is the dominant pathway involved in hyperacute or accelerated acute rejection; the contribution of this pathway diminishes with time as donor APCs are reduced.

In contrast the indirect pathway is less potent but more enduring. This pathway is activated when recipient APCs process donor antigens that are shed from the graft and then presented to recipient T cells. This leads to activation of cytokine production and co-stimulatory T cell pathways, resulting in activation of two major helper T cell pathways. The type 1 helper T- cells pathway results in the production of interleukin 2 (IL-2) and interferon gamma (IFN- $\gamma$ ) leading to a delayed type hypersensitivity response [10, 14, 15]. This pathophysiological response is generally the pathway involved in cell-mediated allograft rejection. Activation of the type 2 helper T- cell response on the other hand leads to production of IL-4, IL-5, IL-10 and IL-13 and facilitates the humoral immune response with the subsequent production of donor specific antibodies.[14, 16]

### **1.3 Immunosuppressant therapy**

Modern immunosuppression protocols are directed at suppressing the T cell as a central mediator of both the cellular and humoral response to the donor allograft. The dominant protocol in use currently at Charlotte Maxeke Academic Hospital consists

of triple therapy combination of corticosteroids, calcineurin inhibitors (chiefly tacrolimus) and antimetabolites (chiefly mycophenolate mofetil or MMF) consistent with the international Kidney Disease: Improving Global Outcomes (KDIGO) guidelines of 2009 for maintenance immunosuppression [17]. These drugs exert synergistic effects in inhibition of T cell activation.

Corticosteroids exert multiple effects on T cell proliferation and activation, through direct inhibition of lymphocyte mitotic activity as well as reduced production of activating cytokines, the latter effect mediated by increased inhibition by NF- $\kappa$ B on gene transcription, [18] leading to reduced production of IL-2.[19]

Tacrolimus, part of the calcineurin inhibitor class of drugs, is a macrolide antibiotic with potent immunosuppressive properties. The mechanism of immune suppression, similar to that of cyclosporine A, relies on the inhibition of cytokine gene transcription [20]. Tacrolimus binds to an immunophilin, FK binding protein (FKBP), that is necessary for the dephosphorylation of cytosolic nuclear factor activated T cells (NFAT), resulting in blockade of T-cell receptor signal transduction, which, together with inhibition of transcription of IL-2 RNA, inhibits T cell activation and proliferation as well as T cell dependent B-cell activation. [19, 21]

The antimetabolite class of immunosuppressants inhibit lymphocyte function via the inhibition of synthesis of guanosine monophosphate, a core component of RNA, resulting in blockade of proliferating T and B-cells and hence inhibition of cytotoxic T cell responses and antibody formation [22].

Immunosuppressant medications have significant side effect profiles, necessitating

careful balancing of the effects of toxicity with the risk of allograft loss and rejection. The development of drug protocols which would allow reduction in immunosuppression protocols whilst maintaining allograft survival is a key area of research in transplantation medicine [23]. Such research has to date not resulted in significant improvement in long-term allograft survival, and chronic allograft loss continues to be an important limitation in renal transplantation.

#### **1.4 Definition of late period allograft loss**

Late period allograft dysfunction is defined as a gradual and progressive decline in graft function after 3 months post-transplant [24] which may ultimately lead to loss of the graft. This process may be caused by a variety of insults acting independently or in concert.

#### **1.5 Challenges to graft survival**

The injury processes that limit long-term graft survival may conveniently be categorised as either immunological or non-immunological. The distinction is crucial, as treatment protocol needs to be adjusted accordingly in order to salvage graft function.

##### **1.5.1 Chronic allograft nephropathy**

Chronic allograft nephropathy (CAN) has previously been implicated as a significant contributor to late graft loss and was originally defined in the Banff '97 classification

system of renal allograft histopathological changes as a diffuse process characterised by cumulative damage to the renal allograft accompanied by chronic interstitial fibrosis, tubular atrophy, vascular occlusive changes and glomerulosclerosis [25]. Thus, CAN is not itself a diagnosis but rather a histological pattern representing prolonged and irreversible graft injury arising from a variety of underlying processes.

It used to be thought that CAN was mainly caused by calcineurin inhibitor nephrotoxicity with smaller contributions from other non-immunological processes, whilst immunological processes comprised a minority of cases of CAN [26, 27]. In the past decade however, a number of papers have instead suggested that humoral mechanisms may be the dominant factor for the development of CAN. This has resulted in a revision of the Banff classification system to include a broader classification of graft loss [28, 29].

The revised Banff 2005 criteria renamed chronic allograft nephropathy as interstitial fibrosis and tubular atrophy “without evidence of any specific aetiology” [28] reflecting growing recognition of the process as one of non-specific parenchymal injury resulting in allograft dysfunction.

### **1.5.2 Non-immunological processes leading to chronic allograft dysfunction and loss**

Non-immunological processes that are associated with the development of chronic renal allograft dysfunction include calcineurin inhibitor nephropathy, BK virus nephropathy, CMV nephropathy, reflux nephropathy and hypertensive nephropathy.

### **1.5.2.1 Calcineurin inhibitor nephropathy**

Calcineurin inhibitor toxicity may result in acute graft injury or chronic progressive dysfunction. Calcineurin inhibitor toxicity is associated with endothelial damage, which is manifested by the presence on histological evaluation of obliterative arteriopathy; tissue ischaemia resulting from this vascular injury is evidenced at biopsy by tubular epithelial cell vacuolisation and later tubular atrophy and interstitial fibrosis, and ischemic collapse and scarring of the glomeruli [21, 30-33]. Such fibrotic changes are irreversible and result in persisting allograft dysfunction with progressive reduction in glomerular filtrate rate (GFR) and eventually allograft loss [32, 34, 35].

Calcineurin inhibitor nephrotoxicity is an important contributor to late period graft dysfunction and loss. It was previously thought to have an impact on long term graft dysfunction and loss of more than 77% at 10 years post-transplant [36] however recent studies have suggested a much smaller contribution [26].

### **1.5.2.2 Polyomavirus infection**

BK virus, a member of the polyomavirus family of viruses, frequently infects the urothelium of the genitourinary tract. Whilst harmless in the general population, in immunosuppressed patients, particularly those with deficient cell mediated immunity, it is capable of mounting an ascending infection resulting in infection of the transplanted kidney. BK virus in the renal allograft recipient is clinically manifested by a slowly rising creatinine, or features of tubulointerstitial nephritis, including sterile pyuria, haematuria and/ or cellular casts consisting of inflammatory cells [37]. The

virus is readily recognised on histological examination by the presence of characteristic viral inclusions termed 'decoy cells' within tubular cells. Definitive diagnosis is confirmed by evidence of replication of BK virus in the form of circulating RNA using PCR (polymerase chain reaction).

The introduction of potent third generation immunosuppressive drug regimens has resulted in polyomavirus BK nephropathy becoming a common problem.

The presence of BK virus infection within the renal allograft is evidence of over-immune suppression of the patient; management of the infection thus includes immunosuppression drug dose reduction. At CMJAH dosage adjustment of calcineurin inhibitor is usually combined with substitution of leflunomide for antimetabolite. Leflunomide is a prodrug whose metabolite has both immunosuppressive and antiviral activity. It has been shown to cause reduction in both urine and blood viral load levels over time with a subsequent improvement in serum creatinine and graft function [38]. Available literature would suggest that allograft survival after diagnosis is poor despite such interventions, with eventual virus-induced tubulointerstitial injury leading to graft dysfunction and loss in more than 50% of cases [39]

### **1.5.2.3 CMV nephropathy**

Cytomegalovirus (CMV) is a double stranded DNA virus belonging to the family of Herpesviridae [40]. Infection with CMV generally arises on a background of potent immunosuppression (often involving T cell depletion therapy used for induction immunosuppression), either as de novo infection, as a result of activation of latent infection acquired pre-transplantation, or alternatively through the transplantation of

an infected donor organ[41]. These scenarios can result in active, virulent disease leading to allograft dysfunction and loss as well as patient mortality. CMV nephropathy is easily recognised during histological examination by characteristic intranuclear inclusions in tubular epithelial cells, the so-called “owl’s eye sign”.

#### **1.5.2.4 Reflux nephropathy**

Reflux nephropathy may arise as a result of congenital or acquired conditions present before transplant. These may be exacerbated by ureteral dysfunction caused by the surgical processes involved in attaching the transplanted ureter to the recipient bladder (important factors in this regard being ureteral denervation and the lack of the normal physiological sphincter). On histological examination the diagnosis is recognisable by the presence of dilated renal allograft tubules with intra luminal pus casts [42].

#### **1.5.2.5 Hypertensive nephropathy**

Patients with renal allografts are susceptible to diseases unique to organ transplantation as well as diseases common in the general population, such as hypertension. Idiopathic/ essential hypertension is common in the local black African community due to genetic mutations resulting in salt sensitivity and may be the underlying cause of the ESRD necessitating transplantation [43, 44].

Chronic kidney disease from any aetiology may result in the development of hypertension; subsequent renovascular remodelling may result in the development of intractable secondary hypertension. Stenosis of the transplanted kidney’s renal artery

may result in post-transplant hypertension [45]. Additionally, hypertension and the metabolic syndrome is an important side effect of calcineurin inhibitors and corticosteroids [46-48].

### **1.5.3 Immunological mechanisms resulting in chronic renal allograft dysfunction and loss**

Immunological mechanisms associated with the development of allograft injury include antibody mediated rejection, cellular mediated rejection and mixed rejection. Transplant glomerulopathy (TG) was previously accepted as a defining characteristic of CAN. Recent literature supports the role for antibody mediated injury in the development of transplant glomerulopathy (TG) as evidenced by the association of TG with alloantibody and C4d deposits in the peritubular capillary (PTC) [29, 49-54].

#### **1.5.3.1 Antibody-mediated rejection**

Antibody-mediated rejection results from activation of the type 2 T helper response as outlined above. Antibodies thus generated are directed primarily against donor HLA antigens and are thus termed donor specific antibodies (DSA), either unrecognised but pre-existing prior to transplantation or generated after transplantation (so-called “de-novo DSA”). These DSAs, even at low titre, react with donor tissue antigens resulting in injury to the allograft, often culminating in late period antibody mediated rejection. During activation of the classical complement pathway by antigen-antibody complexes, complement component C4 is activated, resulting in the formation of split products including C4d. The peritubular capillaries (PTC) are the major target site for DSA [26, 55, 56]. This is partially explained by the fact that the PTC has less protective anticomplement pathways than the glomeruli; at

the PTC endothelial surface, complement deposition is largely unimpeded. Because C4d has a thiol ester bond, it binds covalently to tissue elements at the site of local activation and therefore is a resilient marker of antibody mediated injury [25]. Hence C4d positivity has the potential to provide in situ evidence for an active, on-going, humoral immune reaction, which can potentially separate late period humoral allograft rejection from other entities (e.g. Calcineurin toxicity, BK / viral nephropathy etc. as well as pure cell-mediated rejection.) [25, 57, 58]. The distinction of antibody mediated injury is vital as it arises despite potent anti-T cell pharmacological therapy, has a poorer prognosis and is historically refractory to conventional immunosuppressive therapy [57].

The diagnosis of ABMR rests upon the detection of circulating antibodies and the presence of C4d capillary deposition within the peritubular capillary network in association with other histological evidence of allograft injury and microvascular changes, including transplant glomerulopathy [59]. Transplant glomerulopathy with its cumulative damage to the microcirculation presents clinically with time-dependant development of proteinuria [26].

C4d is often conspicuously present when preformed antibodies are responsible for allograft injury; however, when de novo antibodies are implicated ABMR may be C4d negative [26, 55, 60, 61]. Explanations which have been proposed for C4d negativity include resistance at the level of the endothelium to the effects of complement mediated injury and the observation that C4d represents acute complement-dependent antibody mediated injury which may dissipate with time, disappearing 2-3 months after reduction of donor specific antibodies (DSA) levels [49].

Banff has further revised their classification system to include "chronic active antibody-mediated rejection (CAMR)" as a subset of AMR. This is characterised histologically by the presence of diffuse chronic injury to the renal parenchyma as evidenced by: peritubular capillary basement membrane multilayering, glomerular double contours, interstitial fibrosis, tubular atrophy and/ or fibrous intimal thickening of arteries; the most characteristic features being interstitial fibrosis, tubular atrophy and the presence of glomerular double contours. This diagnosis further requires the presence of positive staining for C4d as well as the presence of circulating donor specific antibodies [28, 29].

C4d-negative antibody mediated rejection as an entity has been shown to be of clinical significance [62]. In the presence of negative C4d staining a diagnosis of ABMR may nevertheless be made if histological examination detects the presence of significant endothelial damage as evidenced by glomerulitis, peritubular capillaritis, or intimal arteritis in the context of serological evidence of DSA[63].

C4d as a marker and its immunofluorescent staining has only been available at CMJAH since 2009.

### **1.5.3.2 Cell-mediated rejection**

Cell-mediated rejection arises as a result of stimulation by type 1 T helper cells of cytotoxic T cells on the background of interleukin- 2 (IL-2) and interferon gamma (IFN- $\gamma$ ), inducing a delayed-type hypersensitivity response [15, 64]. IFN- $\gamma$  activity strongly correlates with T cell activation [65]. Antigen specific effector T cells (mainly CD4, CD8 and memory effector T cells) cross the epithelial barrier and infiltrate the interstitium through interaction with donor antigen presented on macrophages. An

intense production of cytokines including IFN- $\gamma$  produces an inflammatory cascade thereby inducing damage at the site of inflammation.

In cell-mediated rejection the interstitium is particularly vulnerable with the microcirculation usually being spared. The resultant epithelial damage then triggers a wave of dedifferentiation of the tubular epithelium resulting in loss of its functional properties as a result of reduced expression of “mature” genes such as those regulating cadherence junctions and electrolyte transporters; instead of which, these cells over-express embryonic genes [66]. This injury-repair process of dedifferentiation is associated with an array of lesions including tubulitis, interstitial inflammation and fibrosis (leading to disturbance of tubular function) [26]; disruption of the renal parenchyma ultimately results in deterioration of renal function[26].

The diagnosis of cell-mediated rejection is readily made on histological examination by the presence of infiltration of lymphocytes into tubular and/or vascular epithelium, or interstitial infiltrates reflecting the pathological process outlined above [1].

Cell-mediated rejection therapy rests upon the use of therapeutic strategies to targeting the Th1 immune response, including high dose corticosteroid therapy and T cell depleting therapy (usually achieved through the use of antithymocyte globulin) [67].

## **1.6 Rationale behind this research**

It has been suggested that 98% of allograft loss has a specific cause. This in turn suggests that there is a specific physiological mechanism which can often be identified histologically in most cases of graft loss [29, 57]. Substantial evidence indicates a significant role for alloantibody in late kidney allograft loss [62] which contradicts the previously held conviction that calcineurin nephropathy is the major cause of late allograft loss [26].

Such observations have prompted a re-evaluation of the causes of late period graft loss. In the multicentre DeKaf (Deterioration of Kidney Allograft Function) study clustering methodology failed to show a consistent association between the cause of late period allograft dysfunction as described by the examining histopathologist and the type of injury observed in each cluster, suggesting an urgent need for the revisiting of the causes of late period graft loss [68] .

Local experience at CMJAH suggests a rising cumulative prevalence of ABMR in the development of late graft loss; however, the precise contribution of the various factors associated with graft loss as outlined above has not previously been investigated. Appreciation of the contribution of various factors to late post-transplant allograft dysfunction at Charlotte Maxeke Academic Hospital has the potential to alter treatment protocols currently in use to extend allograft survival which in turn will assist in ameliorating donor organ shortage and dialysis slot restrictions which remain a significant limitation to the care of patients with ESRD in the state sector.

The purpose of this study is therefore to investigate factors associated with late period allograft dysfunction and loss in the local renal allograft recipient population.

## **2 Study design**

A retrospective analysis of histological patterns and laboratory parameters in patients with renal allograft dysfunction and loss was undertaken.

### **2.1 Study Objectives**

A. This study was primarily aimed at describing the histopathological patterns of graft injury in the late post-transplant period which were analysed as two broad categories:

- Immune-mediated injury comprising antibody-mediated rejection, cell-mediated rejection and mixed antibody- and cell-mediated rejection.
- Non-immune mediated injury including calcineurin inhibitor nephropathy, recurrence of primary disease, BK virus nephropathy, glomerular disease, hypertensive nephropathy, obstructive uropathy/ reflux nephropathy, idiopathic nephropathy and thrombotic microangiopathy

Since multiple injury patterns may be detected at biopsy, the dominant pattern was assessed in respect of the management change instituted by the treating nephrologist.

B. Secondary objectives of this study comprised the evaluation of clinical parameters in identifying the underlying histological lesion with a view to improved empiric treatment for allograft dysfunction in the future and the determination of the impact of the various categories of allograft injury on survival following diagnosis.

- i. The association of clinical features with the various histological injury patterns was analysed with respect to:
  - Absolute creatinine
  - Percentage change in creatinine from baseline (calculating baseline as the mean creatinine from three laboratory results at least one month apart preceding diagnostic biopsy)
  - Proteinuria
  - Leucocyturia
  
- ii. ii. Allograft survival after diagnosis was analysed utilising Kaplan Meier survival analysis. In this regard, the following factors were analysed for their influence upon allograft survival
  - Graft age
  - UPCR
  - Creatinine
  - Percentage change in creatinine
  - Urine WCC

## **2.2 Patients and methods**

Potential patients for inclusion in this series were identified from the transplant unit biopsy database. All patients undergoing late period “for-cause” biopsy during the period 01/01/2004 – 31/12/2013 were considered for inclusion. Data used in this analysis was obtained from the patient transplant files in the transplant unit at Charlotte Maxeke Academic Hospital.

## **2.3 Study sample**

All patients with renal transplants who underwent for-cause biopsy for graft dysfunction for the period 1 January 2004 till 31 December 2013 were considered for inclusion in the study. The resultant study population size was 242 patients.

### **2.3.1 Inclusion criteria:**

- All patients undergoing for-cause biopsy in the late transplant period (> 3 months post-transplant), with a minimum of 4 and a half months of follow-up post biopsy. This follow-up period was determined from local experience (unpublished data) indicating that the average allograft survival following diagnosis with ABMR is 4 months.

### **2.3.2 Exclusion criteria:**

- Missing or incomplete file records

## **2.4 Definitions:**

### **2.4.1 Definition of late period allograft loss**

By convention this was defined as more than three months post-transplantation.

Graft loss was defined as a return to dialysis and or re-transplantation.

## 2.5 Statistical Analysis

All data in this cohort was collected on Microsoft® Excel for Mac 2011. Data analysis was then performed using Statistica™ Version 9. The Shapiro Wilk W test was used to test for normality of distribution in concert with visual inspection of the frequency plot. For variables thus determined to be normally distributed, the mean and standard deviation were used to represent the central tendency and measure of dispersion respectively; for data shown to be non-parametrically distributed the median and interquartile range (IQR) were used instead.

Analysis of continuous parametric data was undertaken using the Student t-test; for non-parametric data analysis was performed using the Mann Whitney U test. ANOVA was used for data comparison across multiple groups in the case of parametric data; for non-parametric data resort was made to the Kruskal-Wallis test. Fisher exact testing was used for categorical non-parametric variables and Chi-square testing for categorical parametric variables. Survival analysis was assessed using the Kaplan-Meyer survival estimator. A  $p < 0.05$  was considered statistically significant.

### 3 Results

#### 3.1 The epidemiology and demographics and presenting features of the cohort as a whole

Of a total of 242 patients included in this study 175 were male and 67 patients were female (72.3% and 27.7% respectively). The ratio of male to female patients was 2.61: 1.

The race of patients included in this study was heterogeneous, with a strong preponderance of patients of black African descent. The relative racial characteristics of the cohort are presented in table 3.1.1 below. The ratio of black: non-black patient was 1.52:1.

The mean age of patients at biopsy was  $37.8 \pm 12.5$  years old (range 15-64). The mean graft age (time from transplantation to diagnostic biopsy) was  $80.4 \pm 72.08$  months with the earliest biopsy being performed at 3.03 months post transplantation and the oldest graft undergoing biopsy at 337 months (28 years).

*Table 3.1.1 Sex and racial demographics of the study cohort*

	N	%
<b>Sex</b>		
Male	175	72.3
Female	67	27.7
<b>Race</b>		
Black	146	60.3
White	61	25.2
Indian	21	8.7
Mixed/ Coloured	13	5.4
Asian	1	0.4

The urine protein: creatinine ratio was recorded in 122 patients in this series (50.4%). The median UPCR was 0.09 g/mmol (IQR 0.03 – 0.31 g/mmol). The mean creatinine value at biopsy was  $252.2 \pm 160.3$   $\mu\text{mol/L}$  with a mean percentage change from baseline of  $22.9 \pm 21.9\%$ . The mean eGFR as calculated by the MDRD equation was  $32.1\text{ml/min}/1.73\text{m}^2 \pm 16.4$ . This correlates with CKD stage 3 according to the KDIGO 2014 guidelines.

The urine WCC (white cell count) was elevated in 182 patients included in this series (75.2%). The median urine white cell count measured in spot urine samples was 1000 cells/ $\text{mm}^3$  (IQR 1000 – 9000 cells/ $\text{mm}^3$ ).

The mean duration of follow-up after biopsy was  $43 \pm 37.2$  months. In 37.2% of cases allograft failure followed biopsy with median graft survival in these cases of 18.4 months (IQR 5.3 – 42.3 months); in patients with persistent graft function at the time of data collection for this study the mean duration of follow-up was  $53.2 \pm 38.8$  months (median 45.2 with IQR 18.9 – 87.2 months).

### **3.2 Ascribed causes of allograft dysfunction and loss**

The ascribed cause of injury as determined by the examining pathologist at the time of biopsy was distributed fairly evenly between immunological and non-immunological categories. Immunological injury accounted for 50.8% of cases, with cell mediated rejection (CMR) being the most commonly identified immunological pattern of injury comprising 62% of all cases of rejection. This was followed by antibody-mediated rejection (ABMR) and mixed rejection (MR) at 21.7% and 16.3% respectively (the diagnosis of ABMR was, however, observed to increase after the introduction of routine C4d staining in 2009, raising the possibility of under-diagnosis

of ABMR during the preceding 2004-2009 period of this study; this is dealt with in detail in chapter 3.2.1 below).

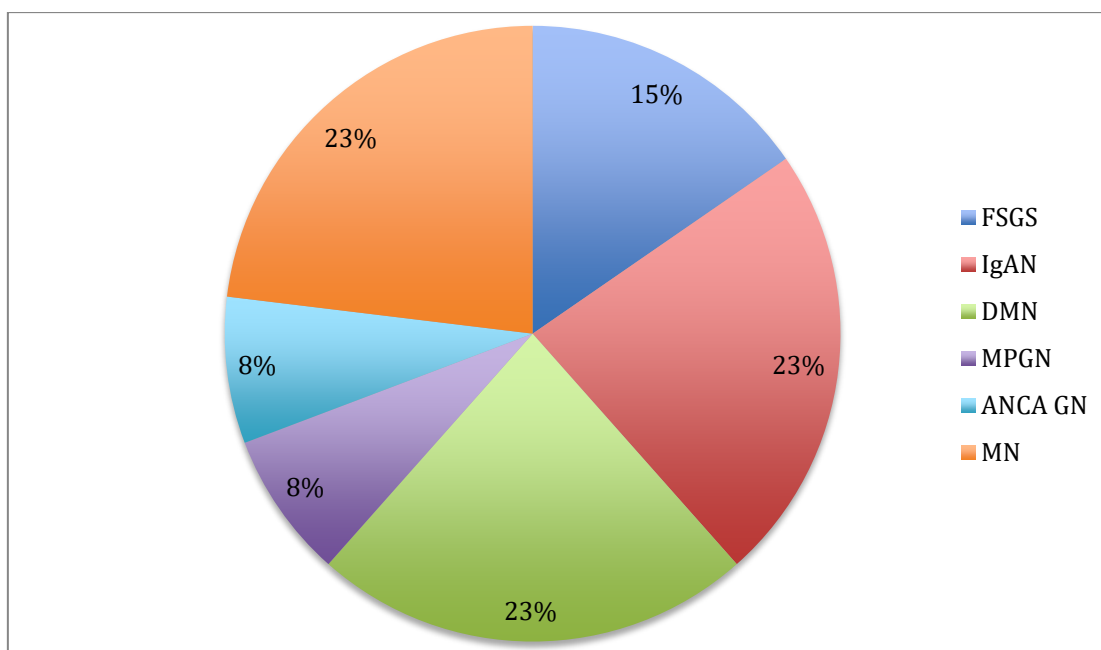
Non-immunological causes of graft injury accounted for 49.2% of cases of late period graft injury. The leading non-immunological aetiological factor identified in this series was calcineurin inhibitor nephrotoxicity (34.7% of cases of non-immunological injury), followed by non-specific IFTA / CAN (30.5%) and reflux / obstructive nephropathy (12.7%), recurrent / de novo glomerular disease (11%); thrombotic microangiopathy (3.4%), BK polyomavirus nephropathy (1.7%), and hypertensive nephropathy (1.7%) were less common non-immunological patterns of injury. In 4.2% no obvious cause of graft dysfunction was identified at biopsy. The classes of recurrent / de novo glomerular diseases identified were: IgA nephropathy (23.07%), diabetic nephropathy (23.07%) membranous nephropathy (23.07%) focal segmental glomerulosclerosis (15.38%), membranoproliferative glomerulonephritis (7.69%) and ANCA glomerulonephritis (7.69%) (See figure 3.2.1 below).

*Table 3.2.1 Ascribed causes of allograft dysfunction and loss*

	<b>N</b>	<b>%</b>
Rejection (immunological)	123	50.8
CNI nephropathy	41	16.9
Reflux / obstructive nephropathy	16	6.6
Glomerular disease	13	5.4
Idiopathic	5	2.1
TMA	4	1.6
BK nephropathy	2	0.8
Hypertensive nephropathy	2	0.8

\*Percentage of cohort as a whole

Figure 3.2.1 Recurrent / de novo glomerular disease as a cause of allograft dysfunction and loss

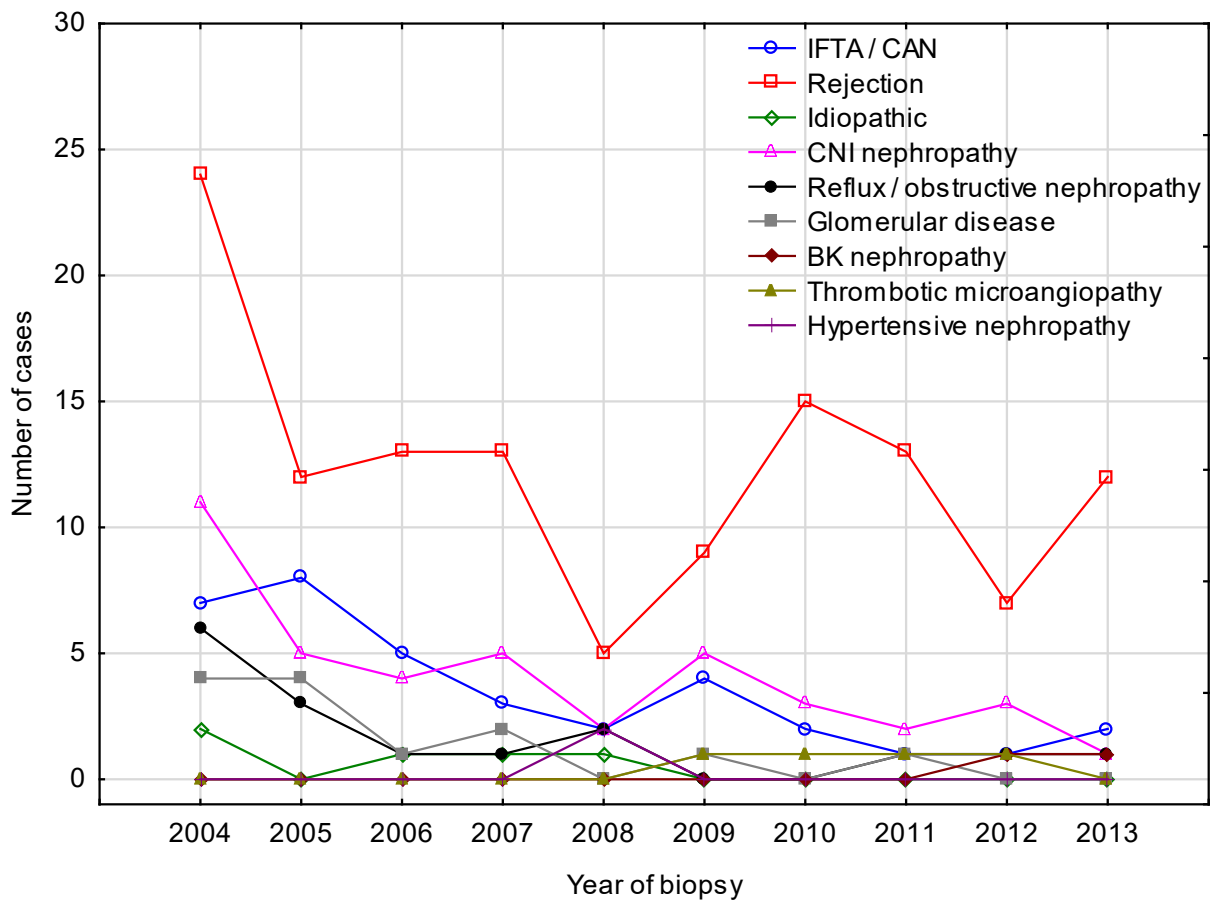


\*Percentage of number of biopsies with recurrent / de novo glomerular disease

### **3.2.1 Patterns of allograft injury over time**

The time period of this retrospective study encompasses several changes in the immunosuppression protocols employed at CMJAH and improvements in the diagnostic capabilities of the Department of Anatomical Pathology in terms of the ability to diagnose antibody-mediated rejection. Of particular importance to this study is the widespread adoption in 2010 of Tacrolimus as the primary calcineurin inhibitor in use in the Division of Nephrology, replacing Cyclosporine A (generally considered to be a more potent cause of CNJ nephropathy) and the introduction of routine C4d staining on for-cause biopsies in 2009. In order to evaluate the possible impact of these developments on the patterns of allograft injury over the course of the study, retrospective analysis was made of the trends in ascribed pathology on a year-on-year basis.

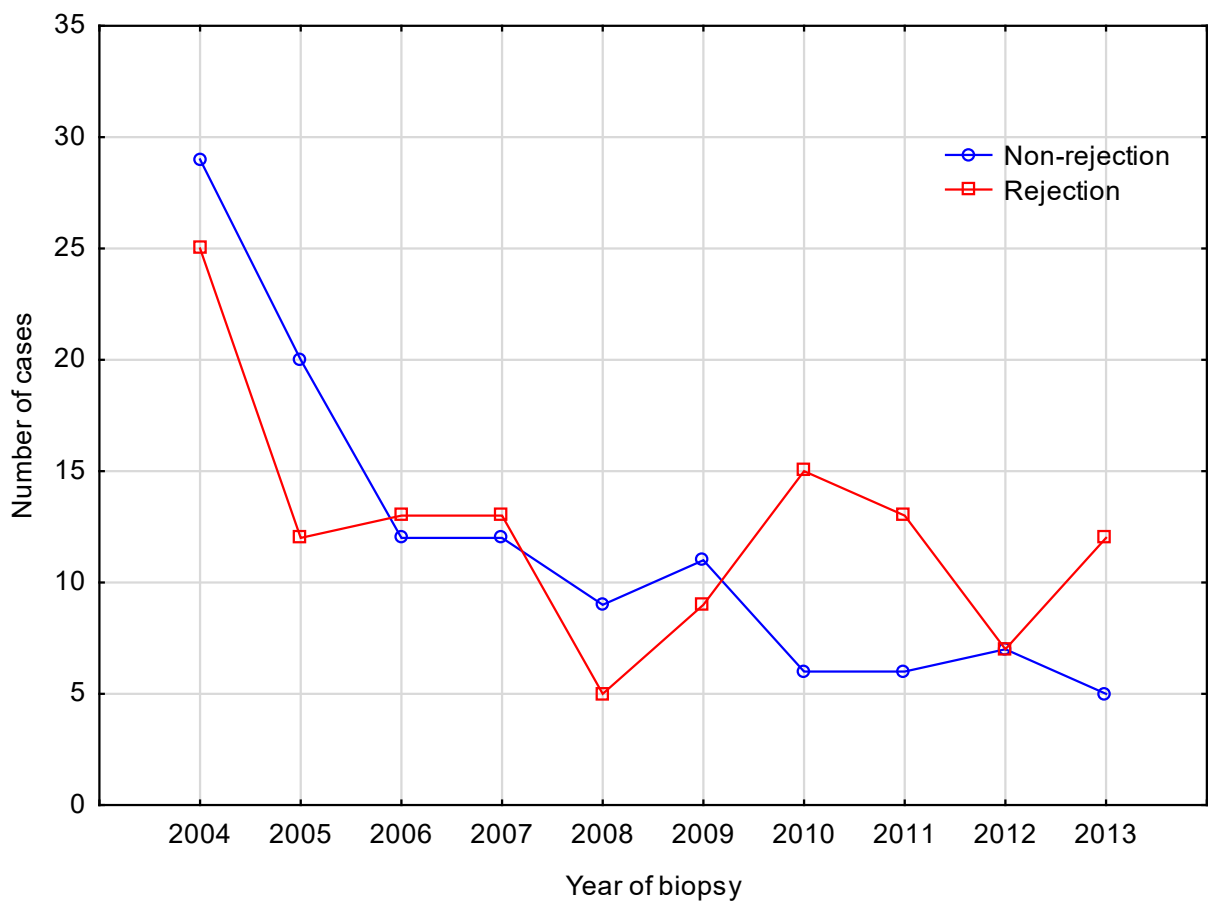
Figure 3.2.1.1 Trends in ascribed cause of allograft injury over time



Comparison of the frequency of all forms of rejection against non-immunological causes of allograft dysfunction and loss on a year-on-year basis did not show statistical significance ( $\chi^2 = 90.7$ ,  $df = 72$ ,  $p = 0.067$ ). However, as depicted in figure 3.2.1.1 above, trends were observed requiring further interrogation. In particular, a decreasing frequency of non-immunological causes against a stable background of rejection was noted.

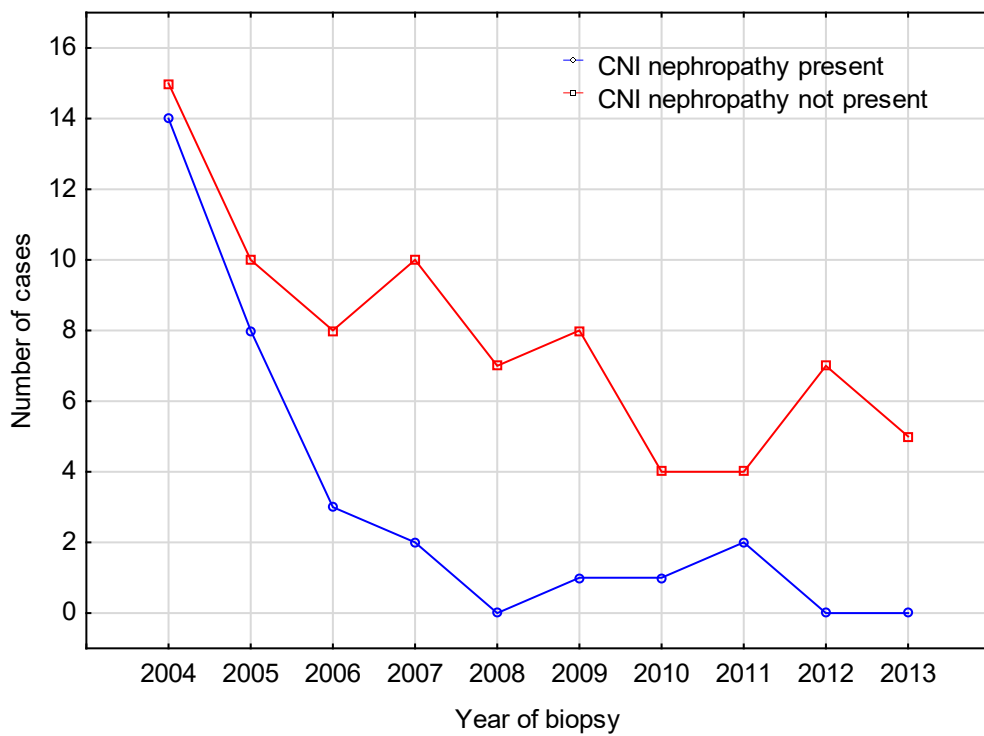
In subsequent analysis, no statistically significant difference between immunological and non-immunological causes was noted on a year-on-year basis ( $\chi^2 = 12.8$ ,  $df = 9$ ,  $p = 0.170$ , figure 3.2.1.2 below). Instead, the apparent decrease noted in figure 3.2.1.1 appears to reflect a decline in the total number of biopsies undertaken on a year-on-year basis.

*Figure 3.2.1.2 Immunological vs. non-immunological causes of allograft injury over time*



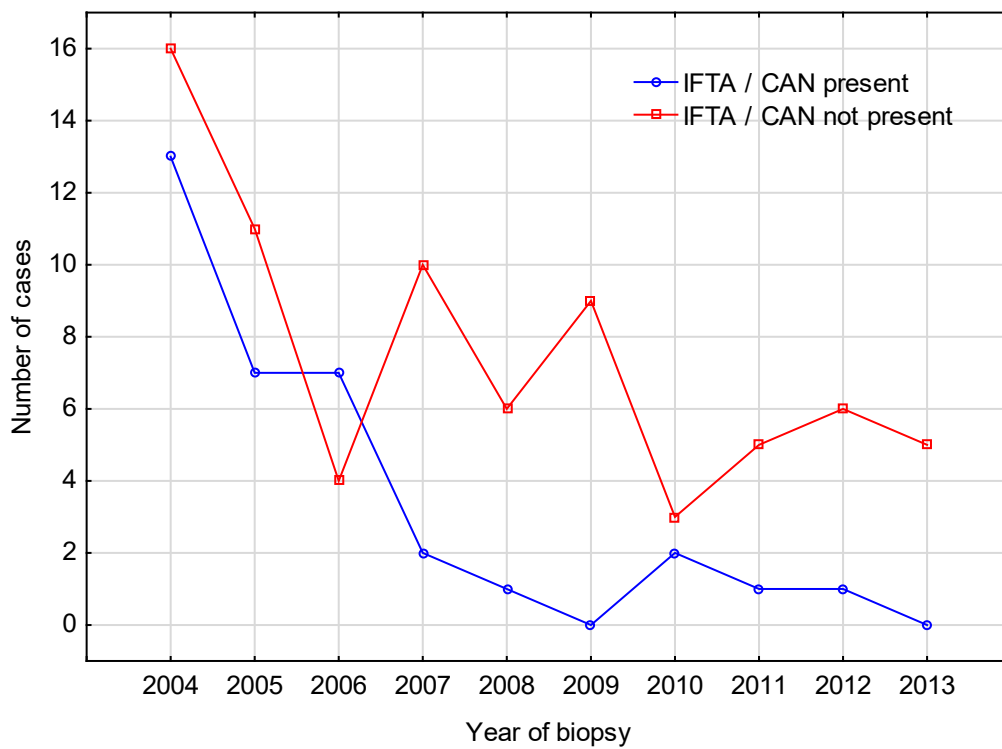
Detailed interrogation of the non-immunological injury group confirmed, however, a significant decline in the frequency of CNI nephropathy as an ascribed cause of allograft dysfunction and loss over the time period of the study ( $\chi^2 = 17.8$ ,  $df = 9$ ,  $p = 0.037$ , figure 3.2.1.3 below).

Figure 3.2.1.3 CNI nephropathy as an ascribed cause of allograft injury over time



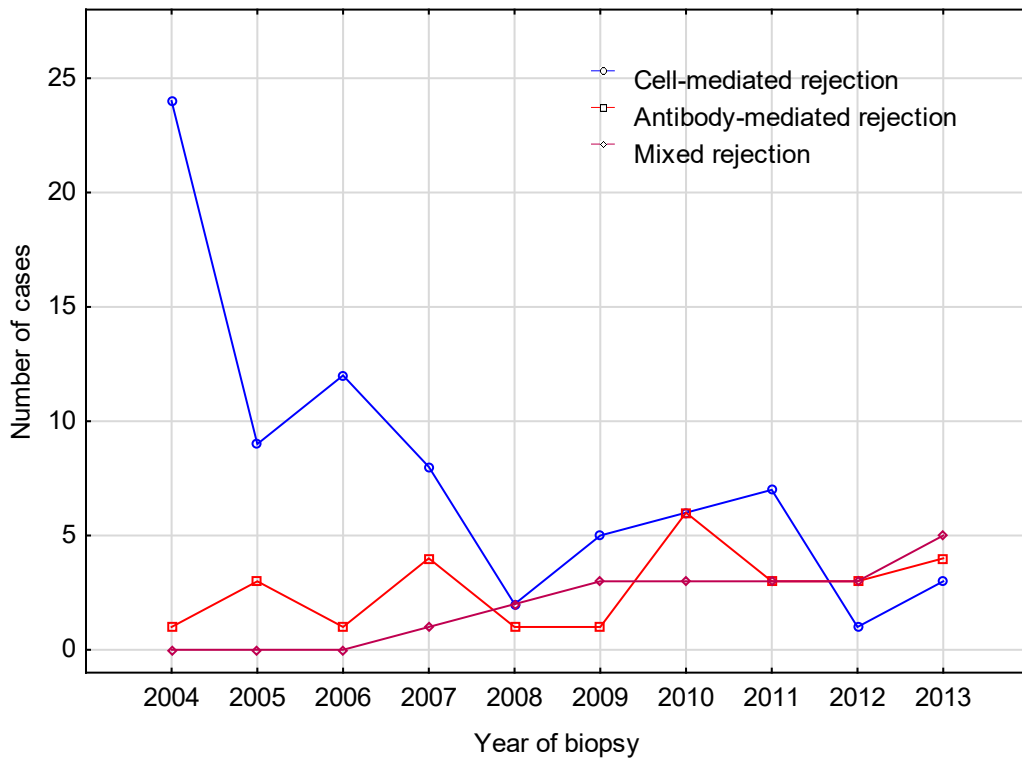
In addition, a similar declining trend for idiopathic / primary IFTA / CAN was observed over the time period of the study ( $\chi^2 = 18.6$ ,  $df = 9$ ,  $p = 0.029$ , figure 3.2.1.4 below)

Figure 3.2.1.4 IFTA / CAN as an ascribed cause of allograft injury over time



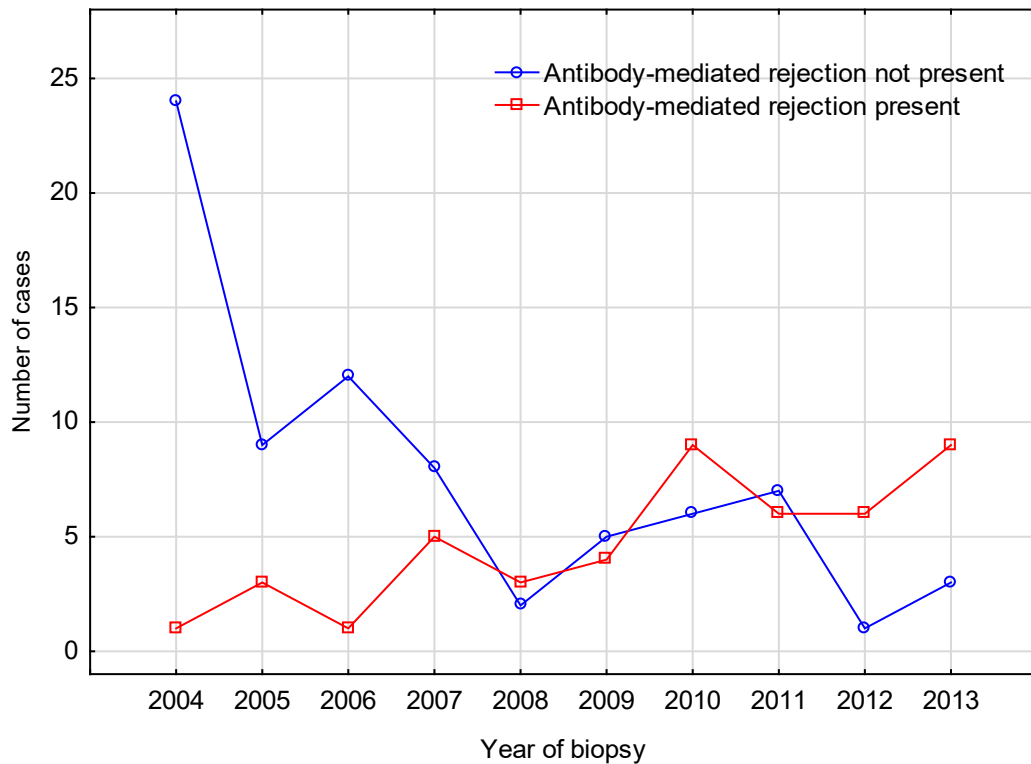
Amongst the immunological causes of allograft injury a trend was observed for a decline in cell-mediated rejection against an increasing incidence of mixed and antibody-mediated rejection over time ( $\chi^2 = 44.7$ ,  $df = 18$ ,  $p = 0.004$ , figure 3.2.1.5 below).

Figure 3.2.1.5 Ascribed rejection types over time



Repeating this analysis of trends in immunological injury over time but combining mixed and antibody-mediated rejection into a single group (i.e., those with an element of humoral injury) and comparing this groups against those with no ascribed pattern of antibody-mediated rejection present (classified for the purposes of this analysis as “pure” cell-mediated” rejection) confirmed this finding ( $\chi^2 = 26.6$ ,  $df = 9$ ,  $p = 0.0003$ , figure 3.2.1.6 below)

Figure 3.2.1.6 Presence of ascribed patterns of humoral injury over time



Analysis of the trend in the detection of humoral injury over time therefore suggests a gradual increase over the course of the study. In univariate general discriminant analysis, the presence of rejection with a humoral element at biopsy was weakly associated with a year of biopsy after 2009, and the presence of “pure” cell-mediated rejection with a year of biopsy before 2007 (eigenvalue = 0.3333, Wilk’s  $\lambda$  = 0.750,  $\chi^2$  = 34.9, df = 1,  $p < 0.0001$ ). Similarly, in univariate logistic regression, biopsy after 2009 was associated with an increased likelihood of finding humoral injury (Logit coefficient = 0.9388, SE = 0.206, Wald statistic 27.7,  $p = 0.000005$ ). Using a 2 x 2 table, the odds ratio for antibody-mediated rejection being diagnosed after 2009 was 3.17 (95% CI = 1.38 – 7.30, z-statistic = 2.73,  $p = 0.006$ ); combining mixed and antibody-mediated rejection (i.e., all forms of immunological injury featuring an alloantibody component) the odds ratio of detecting antibody mediated rejection after 2009 in this series was 6.18 (95% CI = 3.04 – 12.55, z-statistic = 5.03,  $p < 0.0001$ ).

### 3.3 Comparison of demographic and presenting features between immunological and non-immunological patterns of injury

In order to determine whether histological pattern of injury could be predicted from demographic and presenting laboratory parameters, comparison was first made of these parameters between the broad categories of immunological and non-immunological ascribed causes of injury in order to determine statistically significant differences for further investigation.

*Table 3.3.1 Comparison of demographics and biochemical parameters between patterns of injury*

Parameter	Immunological injury N, (%)	Non-immunological injury N, (%)	p
<b>Race</b>			
Black	79 (63.7)	66 (56.4)	0.528*
White	28 (22.9)	33 (28.2)	
Indian	11 (8.9)	10 (8.5)	
Mixed/ Coloured	5 (4)	8 (6.9)	
Asian	1 (0.4)	0 (0)	
<b>Sex</b>			
Male	91 (73.4)	84 (71.8)	0.781*
Female	33 (36.6)	33 (28.2)	

\*Pearson  $\chi^2$  test

Parameter	Immunological injury		Non-immunological injury		p
	N (%)	Mean $\pm$ SD / Median (IQR)	N (%)	Mean $\pm$ SD / Median (IQR)	
Patient age (years)	122 (98.4)	37.3 $\pm$ 12.8	118 (100)	38.4 $\pm$ 12.2	0.490*
Graft age (months)	124 (100)	68.3 $\pm$ 65.5	118 (100)	93.0 $\pm$ 76.7	0.007*
Creatinine (mmol/L)	124 (100)	261.6 $\pm$ 163.3	118 (100)	242.3 $\pm$ 157.2	0.352*
% Change in creatinine	117 (94.4)	23.6 (13.8-42.0)	115 (97.5)	16.2 (5.6-27.2)	0.0001**
UPCR (g/mmol)	60 (48.4)	0.11 (0.04-0.34)	62 (52.5)	0.07 (0.02-0.19)	0.033**
Urine WCC (cells/mm <sup>3</sup> )	96 (77.4)	1000 (1000-8000)	86 (72.9)	1000 (1000-9000)	0.663**

\*Student t-test \*\*Mann-Whitney U test

Thus, grafts were older in cases where the ascribed mechanism of injury was non-immunological, but percentage change in creatinine from baseline and urine PCR were higher in cases of immunological injury.

To test the robustness of these observed differences, a model comprising graft age, urine PCR and percentage change in creatinine was generated for testing using multivariate logistic regression; year of biopsy was included in this model in view of previous findings showing a possible influence of this factor on the detection of humoral immunological injury following the advent of C4d staining. The outcome of this analysis is presented in table 3.3.2 below; only percentage change in creatinine from baseline was shown to be statistically significant, albeit with a low odd's ratio.

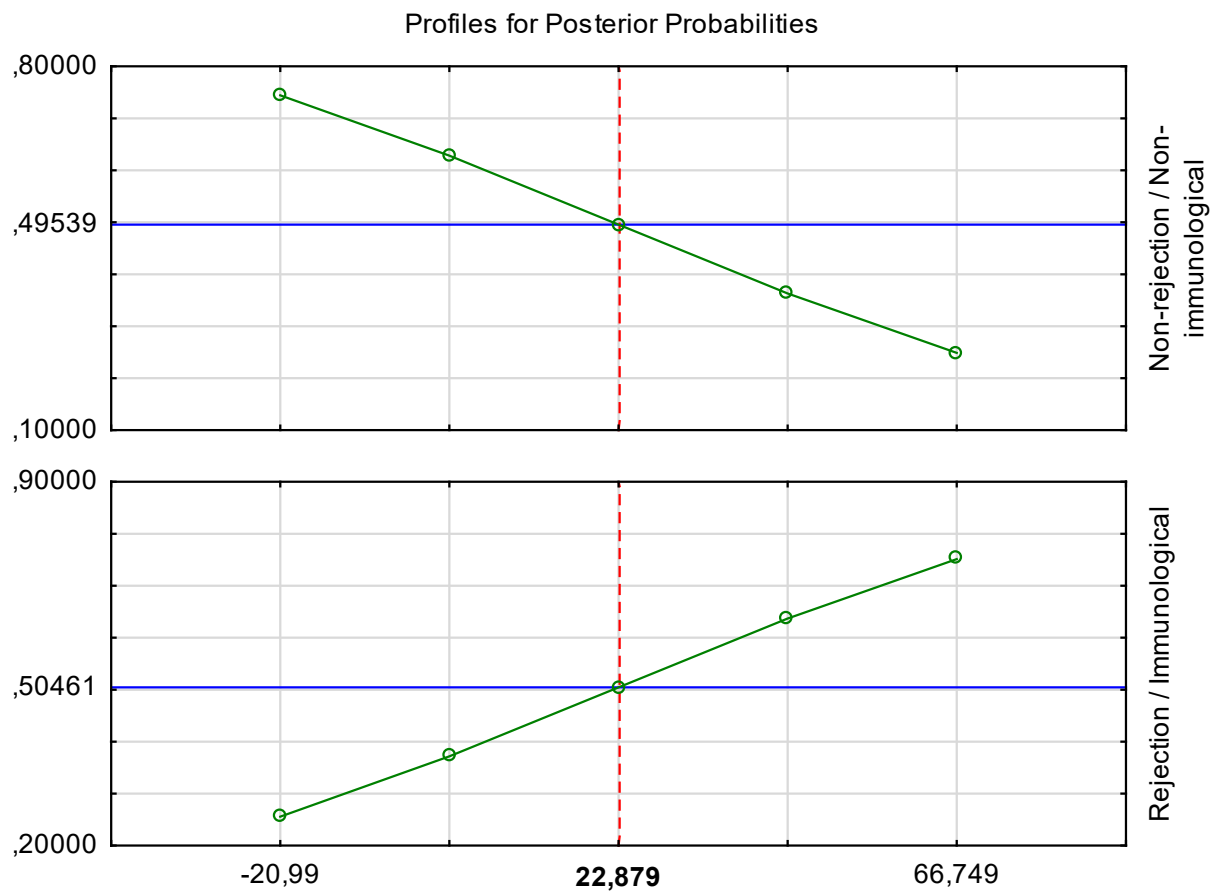
*Table 3.3.2 Logistic regression, ascribed immunological injury and presenting parameters*

<b>Parameter</b>	<b>OR</b>	<b>Logit coefficient</b>	<b>SE</b>	<b>Wald's statistic</b>	<b>95%CI</b>	<b>p</b>
<b>Graft age (months)</b>	N/S	-0.0004	0.003	0.02	-0.007 - 0.005	0.885
<b>% change in creatinine</b>	1.06	0.0270	0.010	7.17	0.007 - 0.047	0.007
<b>UPCR (g/mmol)</b>	N/S	1.209	0.819	2.18	-0.397 - 2.814	0.140
<b>Biopsy after 2009</b>	N/S	0.234	0.193	1.47	-0.144 - 0.613	0.225

In univariate analysis percentage change in creatinine from baseline remained a significant predictor of the presence of rejection as an ascribed cause for graft dysfunction (OR = 1.06, logit coefficient = 0.0262, SE = 0.007, Wald's statistic = 13.81, p = 0.0002). Using general discriminant analysis, the class mean for rejection was 28.43; analysis of the response / desirability profile graph indicated increased likelihood of rejection above a percentage change from baseline creatinine of 22.88%

(eigenvalue = 0.069, Wilk's  $\lambda = 0.935$ ,  $\chi^2 = 15.5$ ,  $df = 1$ ,  $p = 0.00008$ , see figure 3.3.1 below).

Figure 3.3.1 Response / desirability graph, percentage change in creatinine from baseline to discriminate immunological and non-immunological ascribed causes of allograft injury



### **3.4 Comparison of demographic and presenting features within respective non-immunological and immunological patterns of injury**

#### **3.4.1 Non-immunological patterns of injury**

In an attempt to reduce sampling bias, cases of BK and hypertensive nephropathy (n = 2 respectively) were excluded from subanalysis of the presentation of non-immunological injury. Results of this analysis are presented in table 3.4.1.1 below.

Table 3.4.1.1 Comparison of demographics and presenting features of ascribed non-immunological injury groups

Parameter	CNI nephropathy	IFTA / CAN	Reflux / obstructive uropathy	Glomerular disease	Idiopathic	TMA	p
<b>Race</b>							
Black	24 (58.5)*	17 (47.2)	9 (60)	7 (53.9)	4 (80)	4 (100)	0.437**
White	14 (34.2)	12 (33.3)	4 (26.6)	2 (15.4)	0 (0)	0 (0)	
Mixed/ Coloured	1 (2.4)	4 (11.1)	1 (67)	1 (7.7)	1 (20)	0 (0)	
Indian	2 (4.9)	3 (8.3)	1 (67)	3 (23)	0 (0)	0 (0)	
<b>Sex</b>							
Male	28 (68.3)	27 (75)	10 (76.7)	10 (76.9)	3 (60)	4 (100)	0.743**
Female	13 (31.7)	9 (25)	5 (33.3)	3 (23.1)	2 (40)	0 (0)	

\*Values are N (%), \*\*Pearson  $\chi^2$  test

Parameter	CNI nephropathy	IFTA / CAN	Reflux / obstructive uropathy	Glomerular disease	Idiopathic	TMA	p+
Patient age (years)	41 (100)* 39 (28-45)**	36 (100) 40.5 (27.5-46.5)	15 (100) 35 (27-44)	13 (100) 49 (25-53)	5 (100) 36 (31-37)	4 (100) 44.5 (29-56.5)	0.678
Graft age (months)	41 (100) 65.0 (21.1-140.6)	36 (100) 82.8 (53.5-159.3)	15 (100) 83.8 (35.1-153.8)	13 (100) 110.8 (66.8-131.9)	5 (100) 39.5 (23.8-58.7)	4 (100) 31.3 (20.5-60.0)	0.214
Creatinine (mmol/L)	41 (100) 189 (156-268)	3 (100) 191.5 (149-315)	15 (100) 215 (175-245)	13 (100) 173 (152-227)	5 (100) 159 (141-175)	4 (100) 217.5 (168-292)	0.646
Percentage change in creatinine	40 (97.6) 14.5 (4.8-27.7)	35 (97.2) 12.4 (5.4-25.4)	15 (100) 16.0 (9.3-22.9)	13 (100) 11.6 (3.7-31.2)	5 (100) 19.9 (17.6-21.0)	3 (75) 33.2 (14.9-52.0)	0.754
UPCR (g/mmol)	21 (51.2) 0.03 (0.02-0.1)	20 (55.6) 0.06 (0.01-0.31)	6 (40) 0.07 (0.06-0.08)	6 (46.2) 0.34 (0.19-0.51)	2 (40) 0.7 (0.21-1.19)	4 (100) 0.09 (0.04-0.13)	0.123
U WCC (cells/mm <sup>3</sup> )	31 (75.6) 1000 (1000-18000)	22 (61.1) 1000 (1000-6000)	14 (93.3) 1000 (1000-2000)	10 (76.9) 1000 (1000-1000)	4 (80) 5000 (1000-9500)	2 (50) 44000 (38000-50000)	0.254

\*N (percentage of all cases with valid data), \*\*Median (IQR), +Kruskal Wallis test

No statistically significant differences were observed between non-immunological injury patterns in terms of demographics or presenting parameters.

### 3.4.2 Immunological patterns of injury

Demographic and presenting parameters were compared across immunological patterns of injury. Statistically significant differences were detected for urine protein: creatinine ratio and graft age as shown in table 3.4.2.1 below.

*Table 3.4.2.1 Comparison of demographics and presenting features of ascribed immunological injury groups*

Parameter	Cell-mediated rejection	Antibody-mediated rejection	Mixed rejection	p**
<b>Race</b>				
Black	48 (62.3)*	17 (63.0)	14 (70)	0.974
White	18 (23.4)	6 (22.2)	4 (20)	
Mixed/ Coloured	4 (5.2)	1 (3.7)	0 (0)	
Indian	6 (7.8)	3 (11.1)	2 (10)	
Asian	1 (1.3)	0 (0)	0 (0)	
<b>Sex</b>				
Male	55 (71.4)	22 (81.5)	14 (70)	0.556
Female	22 (28.6)	5 (18.5)	6 (30)	

\*Values are N (percentage of valid cases), \*\*Chi-square test

Parameter	Cell-mediated rejection	Antibody-mediated rejection	Mixed rejection	p*
Patient age (years)	76 (98.7)* 37 (27-45)**	27 (100) 33 (29-45)	19 (95) 38 (22-56)	0.971
Graft age (months)	77 (100) 29.3 (14.5-80.4)	27 (100) 93 (50.3-156.0)	20 (100) 37.5 (25.9-88.9)	0.0004
Creatinine (mmol/L)	77 (100) 191 (164-290)	27 (100) 211 (148-343)	20 (100) 234.5 (164.5-351.5)	0.628
Percentage change in creatinine	74 (96.1) 22.9 (13.7-42.0)	25 (92.6) 22.4 (11.1-29.4)	18 (90) 37.5 (17.1-49.2)	0.931
UPCR (g/mmol)	35 (45.5) 0.07 (0.03-0.26)	16 (59.3) 0.315 (0.09-0.450)	9 (45) 0.23 (0.11-0.34)	0.033
UWCC (cells/mm <sup>3</sup> )	63 (81.8) 1000 (1000-8000)	20 (74.1) 1000 (1000-2000)	13 (65) 4000 (1000-12000)	0.680

\*Values are N (percentage of valid cases), \*\*Values are median (IQR), \*Kruskal-Wallis test

Appropriate therapy for “pure” cell- and antibody-mediated rejection rests upon the prescription of unique non-overlapping intervention strategies in a timeous manner. Treatment of antibody-mediated rejection requires the rapid depletion of anti-HLA alloantibody using plasmapheresis; whereas treatment of cell-mediated rejection requires depletion of CD8 cytotoxic T cells. Early empiric therapy whilst awaiting the result of diagnostic biopsy has the potential to limit further graft injury. Since presenting features urine protein: creatinine ratio and graft age may show difference between the various patterns of immunological injury, further analysis was undertaken using logistic regression in order to ascertain whether these parameters could be used to predict the nature of immunological injury to facilitate early empiric treatment. In order to test the utility of these predictive values, a model was generated comprising the parameters graft age and urine protein: creatinine ratio; logistic regression was then undertaken for cell- and antibody-mediated rejection against all other ascribed patterns of injury respectively. The results of this analysis are presented in table 3.4.2.2 below.

*Table 3.4.2.2 Logistic regression, presenting parameters and rejection types*

	<b>Parameter</b>	<b>Logit coefficient</b>	<b>SE</b>	<b>Wald's statistic</b>	<b>p</b>
<b>Antibody-mediated rejection</b>	1. Urine protein: creatinine ratio	2.446	1.224	3.993	0.045
	2. Graft age at diagnosis	0.008	0.004	3.523	0.061
<b>Cell-mediated rejection</b>	1. Urine protein: creatinine ratio	--2.446	1.224	3.993	0.45
	2. Graft age at diagnosis	-0.008	0.004	3.523	0.061

Since mixed rejection is initially treated, like antibody-mediated rejection, with plasmapheresis in order to limit allograft injury by alloantibody, subanalysis of the

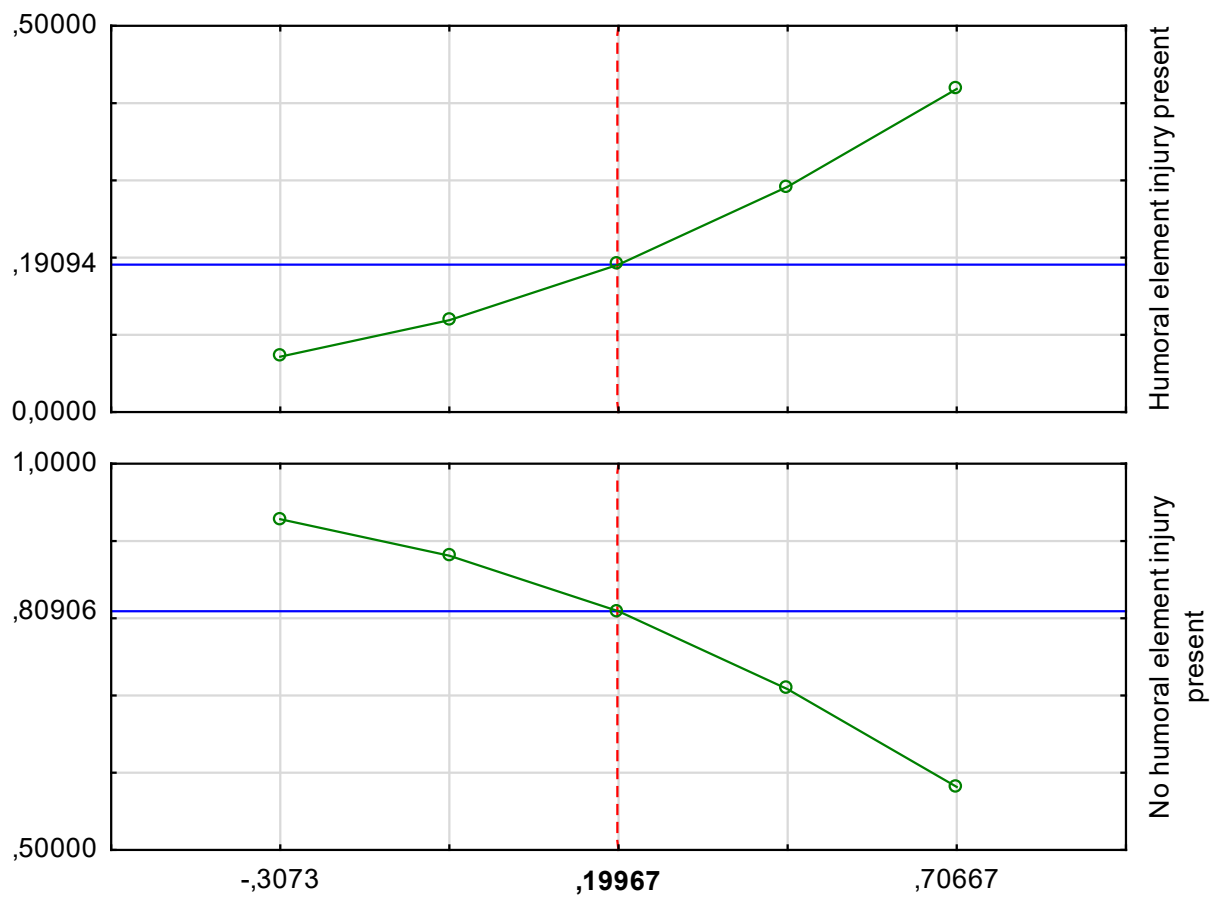
potential for these parameters to distinguish ascribed antibody-mediated injury from other patterns was also undertaken and is presented in table 3.4.2.3 below.

Table 3.4.2.3 Logistic regression, presenting parameters and antibody-mediated injury (combined antibody-mediated and mixed rejection)

	Parameter	Logit coefficient	SE	Wald's statistic	p
Antibody-mediated injury	1. Urine protein: creatinine ratio	2.168	1.118	3.765	0.052
	2. Graft age at diagnosis	0.005	0.004	1.916	0.166

In univariate analysis, urine PCR was again shown to be a significant predictor of the presence of alloantibody-mediated rejection, defined as mixed and antibody-mediated rejection ascribed causes of allograft dysfunction (Logit coefficient = 1.818, SE = 0.796, Wald's statistic = 5.22, p = 0.022). To complete this analysis, general discriminant analysis was undertaken to determine the value of urine protein: creatinine ratio that might be used to identify the presence of alloantibody injury. In this analysis, the class mean for antibody-mediated injury was 0.307 with the grand mean for the analysis being 0.199 (eigenvalue = 0.049, Wilk's  $\lambda$  = 0.952,  $\chi^2$  = 5.77, df = 1, p = 0.016, figure 3.4.2.1 below).

Figure 3.4.2.1 Response / desirability graph, urine protein: creatinine ratio and element of humoral injury as ascribed cause of allograft dysfunction



The likelihood of antibody-mediated injury as an ascribed cause of injury being present based on level of UPCR was further analysed by determination of the odds ratio using a 2 x 2 table (see table 3.4.2.4 below).

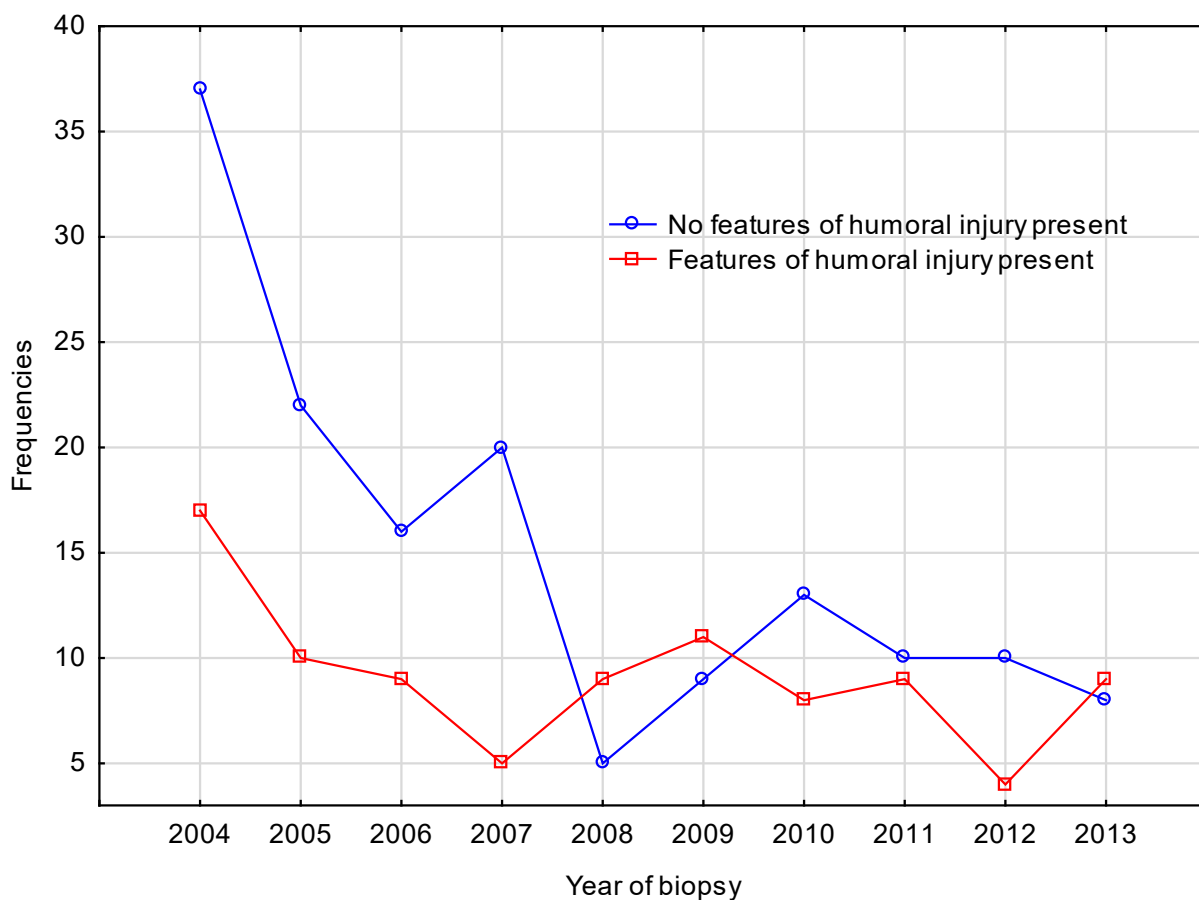
Table 3.4.2.4 Odds ratios, ascribed antibody-mediated injury by UPCR category

	<b>OR</b>	<b>95% CI</b>	<b>z-statistic</b>	<b>p</b>
UPCR > 0.307 g/mmol	3.79	1.49 – 9.62	2.804	0.005
UPCR 0.199 – 0.307 g/mmol	1.12	0.22 – 5.75	0.134	0.894
UPCR < 0.199 g/mmol	0.28	0.12 – 0.71	2.67	0.007

### **3.4.2.1 Re-evaluation of the presence of antibody-mediated injury**

Analysis of the ascribed causes of injury in this series indicated a substantial increase in the diagnosis of antibody-mediated patterns of injury after the introduction of routine C4d staining on for-cause biopsies in 2009. Since it is unlikely that antibody-mediated injury represents a novel pattern of injury in the cohort, retrospective evaluation of ascribed causes of injury was conducted for the presence of histological features associated with antibody-mediated damage. For the purposes of this analysis, biopsies were recoded as having evidence of antibody-mediated rejection if the presence of transplant glomerulopathy, peritubular capillaritis, or peritubular capillary multilayering were reported on biopsy. 241 biopsies (99.6% of cohort) had sufficient data to facilitate this re-evaluation; evidence of antibody-mediated injury was found in 91 of reviewed biopsies (37.76%). As hypothesized, the presence of antibody-mediated injury remained stable over the time course of the series (figure 3.4.2.1.1;  $\chi^2 = 14.51$ ,  $df = 9$ ,  $p = 0.105$ ).

Figure 3.4.2.1.1 Presence of features of humoral injury over time



Analysis of the presence of antibody-mediated injury across various patterns of ascribed injury is detailed in table 3.4.2.1.1 below (hypertensive nephropathy and BK nephropathy were excluded from analysis due to small numbers).

Table 3.4.2.1.1 Presence of antibody-mediated injury across ascribed patterns of injury

	Cell-mediated rejection	CNI nephropathy	IFTA / CAN	Reflux / obstructive nephropathy	Glomerular disease	Idiopathic	TMA	p**
Antibody-mediated injury present	21 (27.6)*	7 (17.1)	14 (40)	6 (37.5)	7 (53.9)	1 (20)	3 (75)	0.046
No antibody-mediated injury present	55 (72.4)	34 (82.9)	21 (60)	10 (62.5)	6 (46.1)	4 (80)	1 (25)	

\*Values are N (percentage of valid cases), \*\*Pearson  $\chi^2$  test

Exclusion of the glomerular disease and TMA injury subgroups (which may result in glomerular injury patterns similar to transplant glomerulopathy induced by antibody-mediated injury) obviated statistical significance ( $\chi^2 = 5.55$ ,  $df = 3$ ,  $p = 0.135$ ). Further exclusion from analysis of cell-mediated rejection revealed a trend towards a significantly lower presence of antibody-mediated injury in biopsies where the ascribed cause of injury was CNI nephropathy ( $\chi^2 = 5.41$ ,  $df = 2$ ,  $p = 0.066$ ). Analysis of demographic and presenting laboratory parameters is presented in table 3.4.2.1.2 below.

Table 3.4.2.1.2 Demographic and presenting parameters across antibody-mediated injury groups

Parameter	Features of antibody-mediated injury present	No features of antibody-mediated injury present	p
<b>Race</b>			
Black	48 (52.8)*	97 (64.7)	0.261**
White	26 (28.6)	35 (23.3)	
Mixed/ Coloured	7 (7.7)	6 (4)	
Indian	9 (9.9)	12 (8)	
Asian	1 (10)	0 (0)	
<b>Sex</b>			
Male	62 (68.1)	113 (75.3)	0.224 <sup>+</sup>
Female	29 (31.9)	37 (24.7)	

\*Values are N (percentage of valid cases), \*\*Pearson  $\chi^2$  test, <sup>+</sup>Fisher Exact test

Parameter	Features of antibody-mediated injury present		No features of antibody-mediated injury present		p
	N (%)	Mean $\pm$ SD / Median (IQR)	N (%)	Mean $\pm$ SD / Median (IQR)	
Patient age (years)	90 (37.7)	37.3 $\pm$ 12.7	149 (62.3)	38.1 $\pm$ 12.5	0.643*
Graft age (months)	150 (62.2)	99.4 $\pm$ 77.9	91 (37.8)	68.9 $\pm$ 66.2	0.001*
Creatinine ( $\square$ mol/L)	150 (62.2)	256 $\pm$ 154	91 (37.8)	250 $\pm$ 165	0.790*
% change in creatinine	86 (37.2)	19.2 (11.5–28.6)	145 (62.8)	23.6 (9.8–37.2)	0.778**
UPCR (g/mmol)	71 (58.7)	0.25 (0.08–0.35)	50 (41.3)	0.05 (0.02–0.21)	0.0003**
Urine WCC (cells/mm <sup>3</sup> )	69 (37.9)	2000 (1000-11000)	113 (62.1)	1000 (1000– 8000)	0.128

\*Student t-test \*\*Mann-Whitney U test

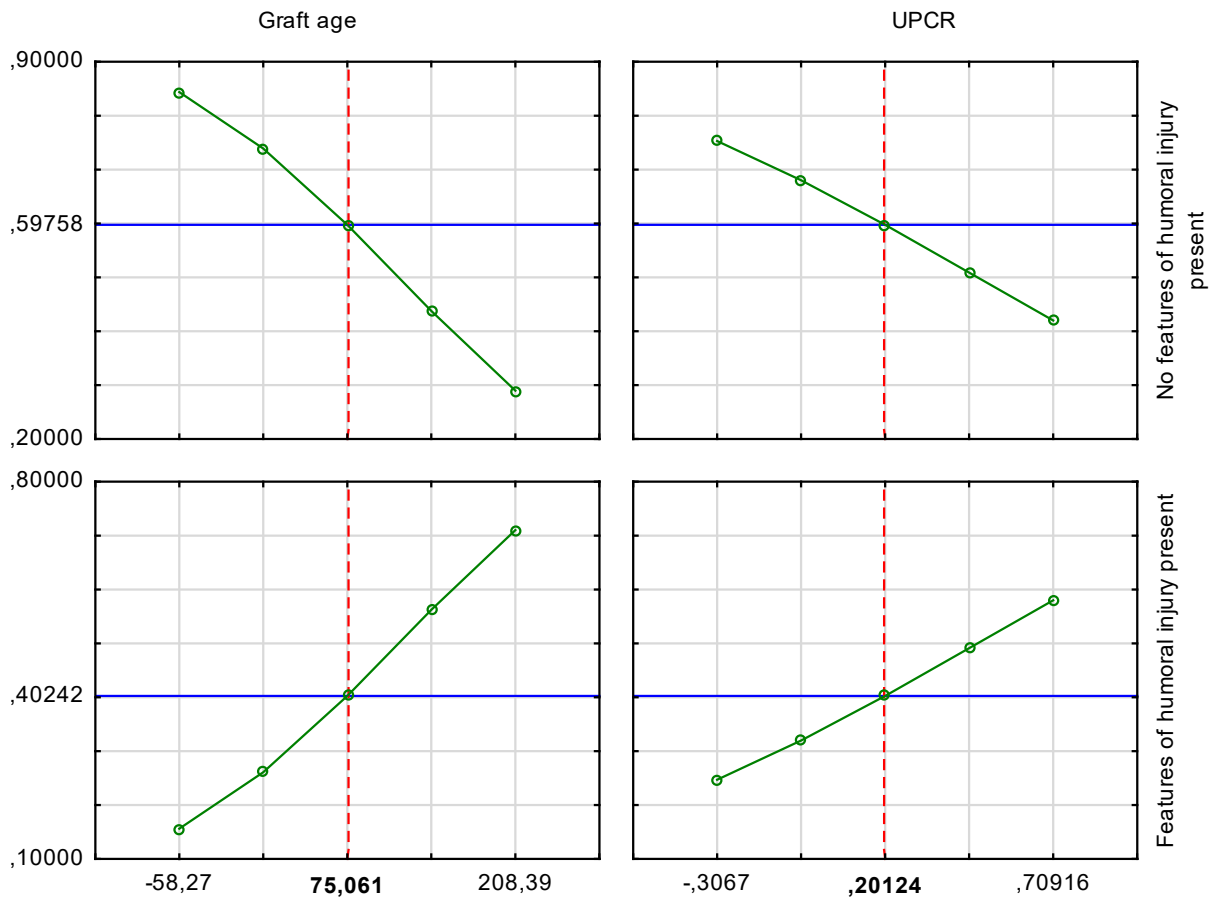
Logistic regression was undertaken in order to evaluate the predictive utility of graft age and urine protein: creatinine ratio in identifying cases of antibody-mediated injury. This analysis is presented in table 3.4.2.1.3 below.

Table 3.4.2.1.3 Logistic regression, presenting parameters and features of antibody-mediated injury on biopsy

Parameter	Logit coefficient	SE	Wald's statistic	p
Model	-1.3677	0.3528	15.02	0.0001
Graft age (months)	0.0096	0.7696	9.02	0.0027
UPCR (g/mmol)	1.3738	0.0032	3.18	0.0742

In view of the significant prediction observed for graft age, the trend towards significance observed for urine protein: creatinine ratio, and the significant predictive utility of the regression model as a whole, general discriminant analysis was performed for the generated model comprising both UPCR and graft age. In this analysis the grand mean for graft age was 75 months and that of UPCR was 0.20 g/mmol; the class mean for these parameters in the subgroup of biopsies with features of antibody-mediated injury were 98.5 months and 0.25 g/mmol respectively (eigenvalue = 0.127, Wilk's  $\lambda$  = 0.887,  $\chi^2$  = 14.1, df = 2, p = 0.0008, figure 3.4.2.1.2 below).

*Figure 3.4.2.1.2 Response / desirability graph, graft age and UPCR and presence of features of antibody-mediated injury at biopsy*



Odds ratios were calculated for the model as a whole as well as for individual parameters and are presented in table 3.4.2.1.4 below.

*Table 3.4.2.1.4 Odds ratios, urine PCR and graft age and features of antibody-mediated injury on biopsy*

Parameter	OR	95% CI	z-statistic	p
Model (graft age > 75 months and UPCR > 0.2 g/mmol)	30.9	3.9 – 243.6	3.255	0.001
Graft age > 75 months	2.4	1.4 – 4.2	3.269	0.001
UPCR > 0.2 g/mmol	2.2	1.0 – 4.8	1.995	0.046

### 3.4.2.2 Histological determinants of urine protein: creatinine ratio

To investigate the mechanism underlying the observed association between urine protein: creatinine ratio and antibody-mediated immunological injury, linear regression was performed in which a model comprising several histological parameters was generated and fitted against UPCR. The model thus generated included severity of interstitial fibrosis / tubular atrophy (IFTA), presence of transplant glomerulopathy, presence of overt antibody-mediated rejection, and presence of histological markers of antibody-mediated injury. Results of this analysis are presented in table 3.4.2.2.1 below.

*Table 3.4.2.2.1 Linear regression, UPCR and histological parameters*

Parameter	Coefficient	SE	T	p	$\beta$	SE $\beta$
Presence of transplant glomerulopathy	-0.109	0.04	-3.06	0.003	-0.366	0.120
Mild IFTA	-0.008	0.04	-0.18	0.892	0.014	0.103
Moderate IFTA	0.006	0.04	0.136	0.855	-0.019	0.105
No features of humoral injury	-0.006	0.03	-0.208	0.836	-0.02	0.119
Antibody-mediated rejection	0.043	0.03	1.418	0.160	0.144	0.101

In view of the observed significant predictive association between the presence of transplant glomerulopathy and UPCR, UPCR levels were compared between cases with evidence of transplant glomerulopathy at biopsy and those without. This analysis is presented in table 3.4.2.2.2 below.

Table 3.4.2.2 UPCR compared by presence of transplant glomerulopathy

	<b>N (%)</b>	<b>Median</b>	<b>IQR</b>	<b>p*</b>
Transplant glomerulopathy present	92 (77.9)	0.06	0.02 – 0.18	0.00004
No transplant glomerulopathy present	26 (22.1)	0.27	0.11 – 0.48	

*\*Mann Whitney U test*

Finally, the frequency of transplant glomerulopathy was compared across various categories of immunological injury, as documented in table 3.4.2.2.3 below.

Table 3.4.2.2.3 Frequency of transplant glomerulopathy considered across categories of immunological injury

	<b>Transplant glomerulopathy present</b>	<b>No transplant glomerulopathy present</b>	<b>p</b>
<b>Category of injury</b>			
Non-immunological	16 (14.5)*	94 (85.5)	0.049*
Immunological	26 (24)	16 (14.55)	
<b>Type of rejection</b>			
Cell-mediated	6 (7.8)	70 (92.1)	<0.00001 <sup>+</sup>
Antibody-mediated	15 (57.7)	11 (42.3)	
Mixed	8 (42.1)	11 (57.9)	
<b>Documented antibody-mediated injury</b>			
Mixed / ABMR	23 (51.1)	22 (48.9)	<0.00001*
Other	22 (11.8)	164 (88.2)	
<b>Features of antibody-mediated injury</b>			
Present	45 (50)	0 (0)	<0.00001 <sup>+</sup>
Not present	0 (0)	141 (100)	

*\*Values are N (percentage of valid cases), \*\*Fisher Exact test, <sup>+</sup>Pearson  $\chi^2$  test*

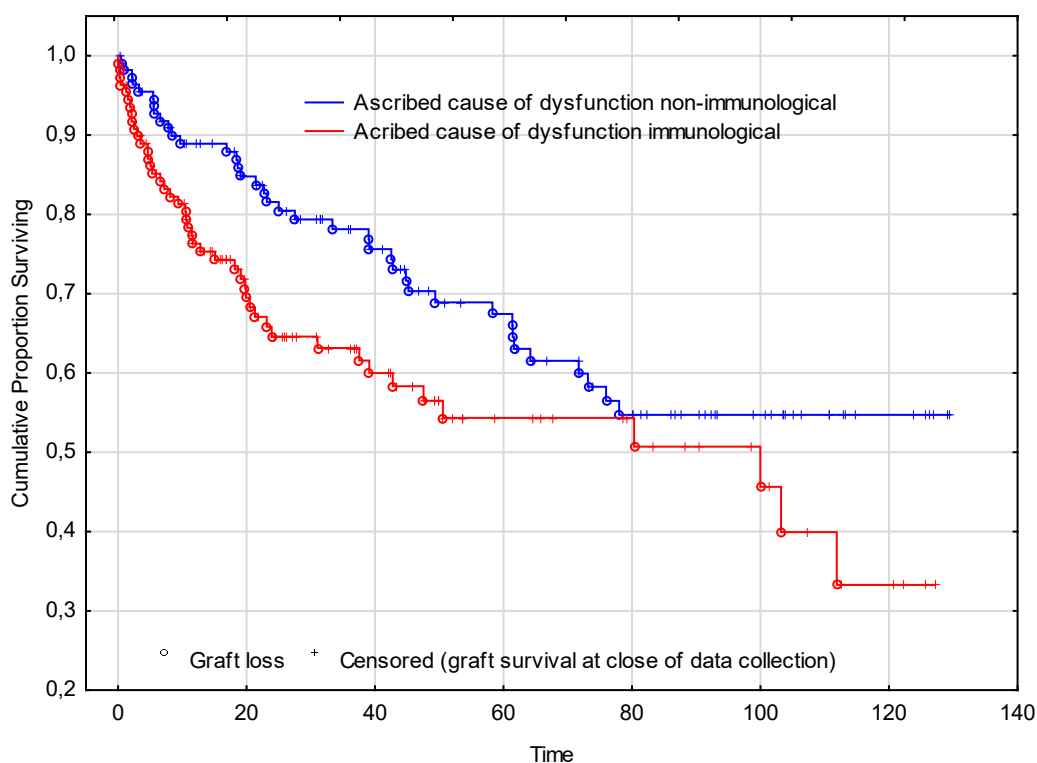
### 3.5 Allograft survival after diagnosis

#### 3.5.1 Survival according to ascribed pattern of injury

##### 3.5.1.1 Comparison of survival by mechanism of injury

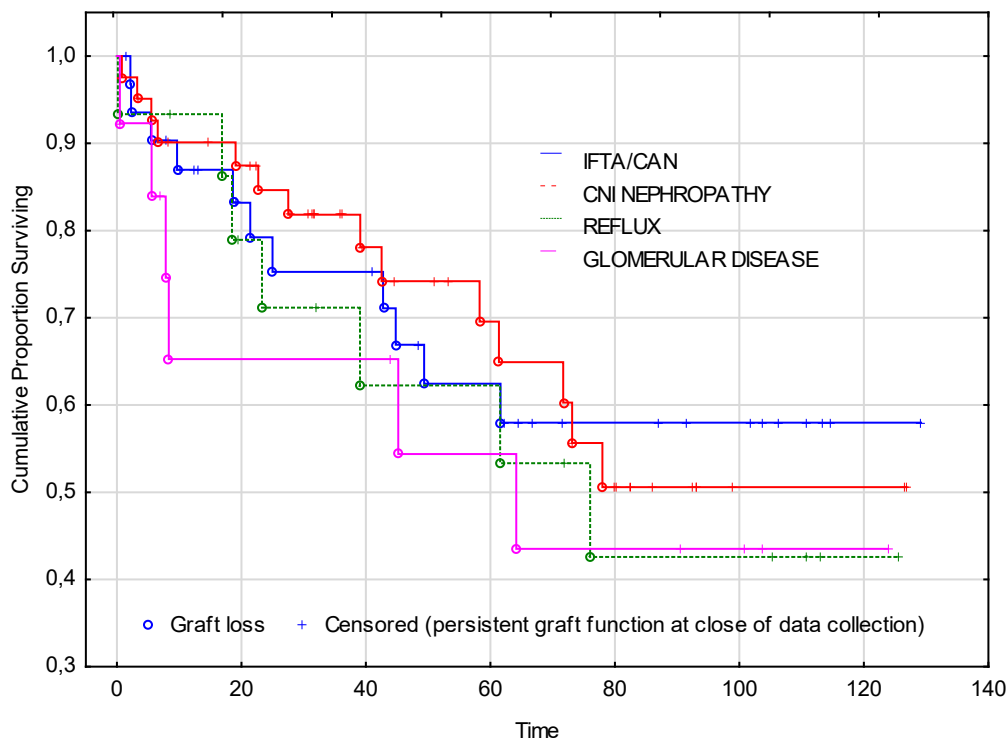
Immunological patterns of injury ascribed as cause of graft dysfunction demonstrated poorer allograft survival after diagnostic biopsy than non-immunological patterns (Cox Mantel test  $F = 2.155$ ,  $p = 0.031$ , figure 3.5.1.1.1 below).

*Figure 3.5.1.1.1 Kaplan-Meier survival curve, ascribed mechanism of allograft dysfunction*



As shown in figure 3.5.1.1.2 below, no significant difference in survival following biopsy was detected for non-immunological patterns of injury ( $\chi^2 = 1.71$ ,  $df = 3$ ,  $p = 0.656$ ; TMA, idiopathic, BK nephropathy, and hypertensive nephropathy excluded from analysis due to small numbers).

*Figure 3.5.1.1.2 Kaplan-Meier survival curve, non-immunological patterns of injury*



No significant differences were observed for ascribed pattern of rejection (figure 3.5.1.1.3 below;  $\chi^2 = 2.46$ ,  $df = 2$ ,  $p = 0.292$ ) or for those biopsies with “pure” cell-mediated rejection compared against those with an element of antibody-mediated injury (combined antibody-mediated and mixed rejection; figure 3.5.1.1.4, Cox Mantel  $F = 1.27$ ,  $p = 0.201$ ). Visual inspection of the survival plots in this latter analysis suggested a poorer survival for the combined mixed / antibody-mediated rejection subgroup for the first 120 months after diagnosis; restricting analysis to this period confirmed this impression (Cox Mantel  $F = 2.107$ ,  $p = 0.035$ ).

Figure 3.5.1.1.3 Kaplan-Meier survival curve, ascribed pattern of immunological injury

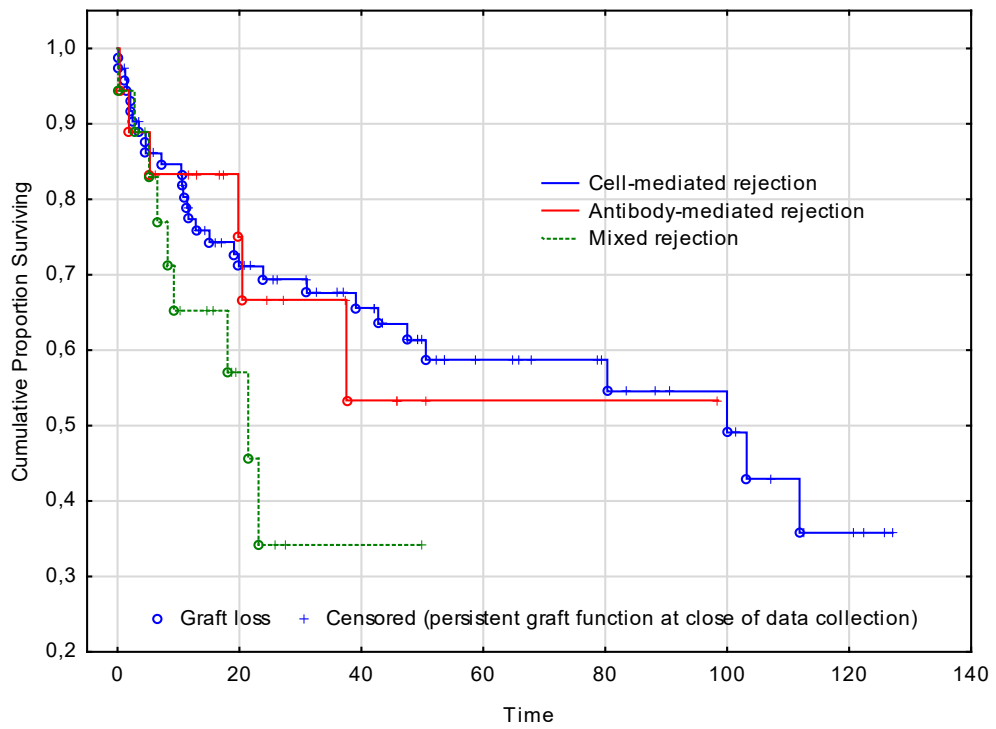
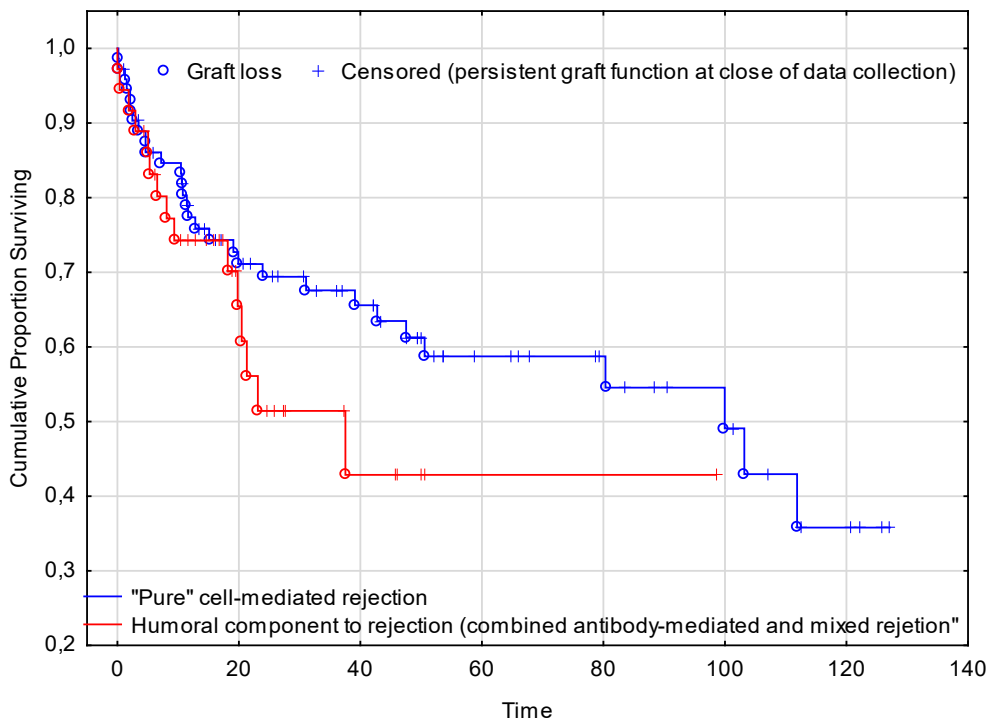


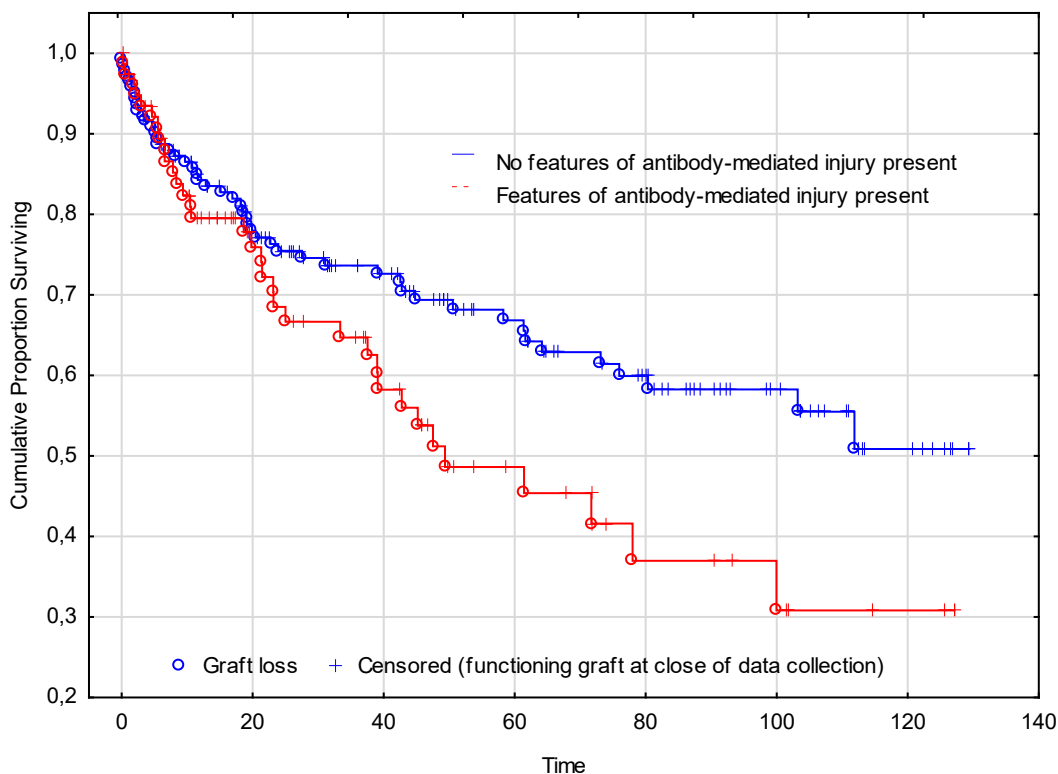
Figure 3.5.1.1.4 Kaplan-Meier survival curve, cell-mediated immunological injury compared to antibody-mediated immunological injury



### 3.5.2 Survival by retrospective determination of antibody-mediated injury

Allograft survival after diagnostic biopsy was poorer in cases in which retrospective review found features of antibody-mediated injury to have been present (Cox Mantel  $F = 2.16$ ,  $p = 0.031$ ; figure 3.5.2.1 below).

*Figure 3.5.2.1 Kaplan-Meier survival curve, features of antibody-mediated injury present compares to no features of antibody-mediated injury present*



### 3.5.3 Cox regression analysis, survival after diagnostic biopsy

Cox regression modelling was undertaken in order to evaluate the effect of presenting parameters and histological features in determining allograft survival after diagnostic biopsy. Initial analysis of these parameters is presented in table 3.5.3.1 below.

***Table 3.5.3.1 Cox regression analysis, survival after diagnostic biopsy***

<b>Parameter</b>	<b>Coefficient</b>	<b>SE</b>	<b><math>\chi^2</math></b>	<b>p</b>	<b>HR</b>	<b>95%CI</b>
Graft age	-0.0001	0.0015	0.005	0.939	0.999	0.996-1.003
UPCR	0.641	0.529	1.46	0.226	1.89	0.67-5.35
Creatinine	0.0037	0.0005	49.90	<0.0001	1.004	1.003-1.005
Percentage change in creatinine	0.002	0.005	23.17	<0.0001	1.025	1.015-1.035
Urine WCC	0.000001	0.000001	1.76	0.185	1.00	0.99-1.00
Non-immunological injury	-0.237	0.111	4.56	0.033	0.623	0.403-0.961
Cell-mediated rejection	-0.276	0.223	1.54	0.215	0.468	0.215-1.019
Antibody-mediated rejection	-0.206	0.301	0.47	0.493	0.502	0.178-1.418
Presence of CAN / IFTA	0.464	0.295	2.48	0.115	2.53	0.797-8.017
No features of humoral rejection	-0.241	0.113	4.58	0.032	0.618	0.397-0.960

Significant regressors were extracted and a regression model fitted for creatinine at presentation, percentage change in creatinine from baseline, major mechanism of injury (immunological / non-immunological) and presence of features of antibody-mediated injury. The results of this regression analysis are presented in table 3.5.1.2 below.

***Table 3.5.1.2 Cox regression analysis, allograft survival after diagnostic biopsy***

<b>Parameter</b>	<b>Coefficient</b>	<b>SE</b>	<b><math>\chi^2</math></b>	<b>p</b>	<b>HR</b>	<b>95%CI</b>
Creatinine	0.003	0.0006	26.47	< 0.000001	1.003	1,001-1.004
Percentage change in creatinine	0.011	0.005	3.37	0.066	1.011	0.999 -1.023
Non-immunological injury	-0.168	0.119	2.01	0.157	0.715	0.449 – 1.138
No features of antibody-mediated injury	-0.266	0.117	5.19	0.023	0.588	0.372-0.929

## 4 Discussion

### 4.1 The demographics of late period renal allograft dysfunction and loss

A preponderance of male patients was observed in this study. Further analysis found no statistically significant difference in gender ratios between the various categories of allograft injury. It is thus unlikely that the skewed gender ratio observed can be explained by a tendency for a specific gender towards a particular pattern of injury. Instead, it is probable that the gender ratio observed represents a bias in the rates of transplantation in the local setting. Since gender carries no weighting on the transplant waitlist it is probable that the male preponderance in this study is explained by differences in long-term renal replacement therapy access by gender; data, which this study was not designed to analyze. CKD statistics in South Africa are sparse; Stanifer et al have reported a lower prevalence of males than females in patients with CKD (41% vs. 59%) [70]; however, due to a variety of psychosocial factors, men tend to seek diagnosis and medical intervention for CKD earlier and more frequently than women, so that a preponderance of male patients accessing dialysis and transplant has been reported.

The majority of patients included in this study were of Black African origin, with Caucasians being the second largest patient groups; a minority of patients were of Coloured / Mixed descent and Indian / Asian origin. A number of factors underlie the distribution of racial demographics in this study. Firstly, they show parallels with known population parameters as described in the 2011 South African census which determined Black Africans to be the largest racial demographic (79.2% of the total South African population), followed by Caucasians (8.9%) and Mixed / Coloured (8.9%), with Indian / Asians making up a minority of the population (2.5%).[71] The

higher prevalence of Indian / Asian patients in this study is difficult to explain without additional data but may be partially influenced by a relatively higher Indian / Asian population resident in Johannesburg (comprising 4.9% of the total Johannesburg population as compared to 2.5% in the South African population as a whole). It is also possible that the disproportionate number of Indian / Asian patients in this study may be evidence of an increased risk for CKD in this population group. For example, an increased risk of CKD in South Asian descendants resident in the United Kingdom; a risk which is believed to derive from increased rates of diseases such as diabetes mellitus and IgA nephropathy, as well as from an increased genetic risk to CKD progression regardless of aetiology, with contributions [72] [73] from socioeconomic factors associated with increased risk of reduced nephron endowment.[73] Similar CKD risks may underlie the predominance of Black African patients in this study; the contribution of a genetic risk for CKD (most notably arising from the MYH9/APO1A mutation) has been extensively documented, as has the contribution of diseases associated with socioeconomic change (such as diabetes mellitus type 2) and the effect of these socioeconomic factors on nephron endowment.[74] Finally, it is probable that socioeconomic factors have contributed to the racial demographics of this study through sample bias: since this study was conducted in a state institution, population groups with lower socioeconomic standards of living resulting from South Africa's historical inequalities are more likely to be represented in this study.

The mean age of the patients in this series was  $37.8 \pm 12.5$  years (range 15-64), which correlates with the age demographic described for CKD in Sub-Saharan Africa of 20-50 years [70, 74]. This study falls within reference range noted in the CKD census described above. Some of the patients fell below the reference range stated

above, possibly explained by the majority of the patients being of African race, who are well described to have more aggressive hypertension, as well as a more aggressive form of glomerulonephritides and thus rapid progression to ESRD at a younger age [74-76].

The mean graft age at diagnosis in this study was 80.4 months (6.7 years) and the mean duration of allograft survival following biopsy was 43 months (3.6 years); the mean duration of allograft life expectancy in this study was therefore 123.4 months or 10.3 years, consistent with international experience [5].

#### **4.2 Ascribed patterns of allograft injury**

A small majority of immunological injury was observed over the course of this study, with the dominant pattern of immunological injury described over the full period of the study being cell-mediated rejection.

Increasing evidence suggests that immunological injury is the dominant cause of late period allograft dysfunction and loss, supported by an appreciation of the prevalence and significance of chronic progressive damage by antibody. Rejection as a whole has been suggested to account for up to 64% of graft failures[77]. In this series, “pure” cell-mediated injury was the dominant reported form of immunological injury (62%) with antibody-mediated injury (mixed and “pure” antibody-mediated rejection) accounting for 38% of cases of rejection, and 19.4% of biopsies overall. Previous studies have consistently described an antibody-mediated rejection incidence of 5–10% in conventional kidney transplant recipients based on biopsies performed to investigate allograft dysfunction [52, 53, 78-80]; Sellares et al reported a prevalence of antibody-mediated rejection overall of 18% [85]. Following the advent of C4d

staining to facilitate the diagnosis of antibody-mediated rejection, this injury pattern has been reported as a more common cause of graft injury and loss. Mauiyyedi et al, for example, have reported a prevalence of 61% for chronic antibody mediated rejection in allografts biopsied for late period dysfunction, a finding that has been supported by other studies [58, 77, 81, 82]. Cell-mediated rejection has been found to be an important cause of graft loss within the first year of engraftment, becoming progressively less common thereafter, and virtually non-existent after 10 years of allograft survival; it is likely that cell-mediated rejection as an ascribed cause of allograft injury in this cohort with a mean graft age of 10 years at diagnostic biopsy represents a misdiagnosis of “pure” antibody-mediated or mixed injury which can be difficult to distinguish using traditional light microscopy in the absence of robust markers such as C4d staining.[77] [83] This is considered in further detail below.

Amongst non-immunological causes of graft injury, the most common ascribed causes were CNI nephropathy, reflux nephropathy, and recurrent / de novo glomerular disease.

Histological patterns consistent with calcineurin inhibitor nephrotoxicity were found in 16.9% of patients. Sis et al and Moruzumi et al have reported a prevalence of 25.8% and 43.2% respectively of calcineurin inhibitor nephrotoxicity in biopsies undertaken for allograft dysfunction [62]; Khambhan et al reported a prevalence of 22% for CNI nephropathy in their review of indication biopsies in their paediatric recipients [84]. The lower frequency of CNI nephropathy in the Khambhan and CMJAH series than that reported by previous series is of note and may partially be explained by the timing of the studies concerned. Khambhan et al reviewed biopsies taken between 2004-2014 whereas the former reviewed biopsies from 1997-2005. These distinct time periods span the introduction and uptake of CNI minimization strategies, which may account for the reduction in CNI nephropathy seen in the Khambhan and CMJAH

series, consistent with the findings of Chow et al who showed that CNI minimization reduced the development of interstitial fibrosis and tubular atrophy [85]. These factors are considered in more detail below.

Reflux nephropathy accounted for 6.6% of all allograft injury in this study. Whereas symptomatic reflux (in the form of recurrent urinary tract infections or allograft pyelonephritis) occurs in less than 1% of renal transplants, asymptomatic (infection-free) reflux is likely to be an underestimated contributor to allograft injury [86]. Some degree of vesicoureteric reflux is not uncommon in the transplant recipient, arising from the nature of the vesicoureteric anastomosis and the denervated transplanted ureter; the prevalence of vesicoureteric reflux in adult transplant recipients has been documented to be between 24% and 86%, and the presence of reflux is associated with increased risk of allograft loss regardless of the occurrence of documented allograft pyelonephritis.[87, 88] The lower rate of reflux nephropathy observed in the CMJAH cohort likely originates in the reliance in this study on histological parameters; vesicoureteric reflux may be as common in recipients in this hospital as in other studies but not represented in this study since the presence of such reflux does not necessarily translate into histological injury.

Glomerular disease was found to be the cause of dysfunction in 5.2% of patients. The reported prevalence of recurrent glomerular disease as a cause of transplant dysfunction is variable, ranging from 8.4 to 21% [62, 82, 89]. Since the recurrence of glomerular disease following transplant may depend on an underlying genetic predisposition towards autoimmunity, it may be that the lower rate of glomerular disease observed in the CMJAH cohort reflects a population in whom ESKD is more

commonly caused by non-immunological disease processes such as hypertension, diabetes mellitus, and reduced nephron endowment.

The most commonly reported glomerulonephritides in the CMJAH cohort were IgA and membranous nephropathy (23% of glomerulonephritides each); diabetic nephropathy comprised a further 23% of cases. IgA nephropathy (IgAN) is one of the most common causes of glomerular injury worldwide, and thus a not-infrequent cause of ESKD[90] [91]; since IgAN affects younger patients with fewer comorbidities many of these patients access transplantation as their preferred method of renal replacement therapy. IgAN has a recurrence rate of 60% post-transplant, and the prevalence of IgAN recurrence increases with graft age; recurrence is twice as common in males compared to females[90, 91]. The contribution of IgAN to ESKD in the local context has not been well documented; available data suggests that, whilst rare in black patients,[92] it is not uncommon in the South African Caucasian and Indian populations.[93] It is thus possible that the frequency of IgAN in the CMJAH cohort was influenced by the number of Caucasians and Indians / Asians and the high proportion of males included in this study. The documented prevalence of IgAN as a cause of allograft dysfunction is highly variable; El Zoghby et al reported a rate of 9% in a North American cohort comprising 90% Caucasians of whom 60% were male; in contrast, Briganti et al reported a prevalence of 28.8% in an Australian cohort which was 68% male (racial demographics not reported).[94]

Membranous nephropathy is the most common nephropathy in adult Caucasians;[95] approximately one-third of affected patients develop ESKD requiring renal transplant[96]. About 42% of these patients develop recurrent membranous nephropathy after transplant and de novo membranous nephropathy occurs in about

2% of transplant recipients.[97, 98] The prevalence of membranous nephropathy in the local context has been reported as 18.5% (Cape Town)[92] and 19.5% (Johannesburg) [99]. The prevalence of post-transplant membranous nephropathy in the Briganti series was low at 9.6%; in the El Zoghby series the prevalence was recorded at 13%. The unusually high frequency of post-transplant membranous nephropathy in the CMJAH cohort is difficult to explain, given the low prevalence of the disorder in native kidney biopsies in the local context and the relatively low rate of recurrence and de novo disease post-transplant. Membranous nephropathy may occur as a feature of rejection [100]; it is thus possible that the higher rate seen in this study is a reflection of the prevalence of immunological injury in this cohort.

Diabetic nephropathy is a not-uncommon cause of allograft injury post-transplant, and is estimated to occur in up to 40% of diabetics undergoing renal transplant [101]; the prevalence of diabetic nephropathy is increased by NODAT which may occur in up to 20 - 30% of patients post-transplant which can be complicated by nephropathy [102, 103]. Diabetes mellitus is an important cause of ESKD in black African and Indian / Asian patients, and the risk of NODAT is increased in the black African population[102]. Thus, the prevalence of diabetic nephropathy in this study may reflect the demographics of the included patient cohort.

Focal segmental glomerulosclerosis was surprisingly a relatively uncommon nephropathy in the CMJAH cohort. FSGS is the most common nephropathy in adults of black African descent and recurs in 20 – 30% of recipients following transplant; a higher rate of FSGS in this cohort composed in the majority by black African patients might therefore have been expected. [104] Indeed, both El-Zoghby et al Briganti et al have reported higher rates of post-transplant FSGS in their cohorts composed

primarily of Caucasian patients (57.6% and 30.7% respectively).[89, 94] A number of factors may underlie the low prevalence of FSGS in the CMJAH cohort. Firstly, FSGS recurrence post-transplant is more likely in white than in non-white recipients [105]; secondly, recurrence may be more common with living donor kidneys than is the case with cadaveric allografts. [105, 106] This latter finding may be explained by the lack of a genetic risk for FSGS (e.g., MHY9/APOA1 mutation) in the transplanted kidney, especially in the local setting where the donor pool is primarily non-black. Thus, although a primarily black African population may be expected to have a high prevalence of ESKD secondary to FSGS, this does not necessarily translate into a high rate of recurrence post-transplant. Furthermore, FSGS recurrence post-transplant follows two broad categories of presentation: a fulminant course with significant proteinuria developing in the early post-transplant period and a more insidious, less clinically overt course with gradual progression during late follow-up; of the two, the fulminant course is more common[100, 107]. It is therefore possible that the lower number of FSGS cases reported in this study was influenced by the restriction of this analysis to the late post-transplant period.

#### **4.2.1 Patterns of injury over time**

Although statistically non-significant, an apparent decline in the frequency of non-immunological patterns of injury against a relatively stable prevalence of immunological injury was observed. Subanalysis found a significant decline in the frequency of two of the major categories of non-immunological injury (CNI nephropathy and “idiopathic” CAN / IFTA) and a significant increase in the presence of antibody-mediated injury.

It has previously been suggested by Nankivell et al that CNI nephropathy is a phenomenon of progressively accumulating histological injury such that the presence

of elements of CNI nephropathy is almost universal in allografts reaching 10 years of survival.[108] Such observations prompted a revision of immunosuppression protocols favouring a calcineurin inhibitor minimization strategy; adoption of such protocols by the CMJAH transplant unit may have led to the apparent decrease in CNI nephropathy observed. It has, however, also been suggested that chronic progressive CNI nephropathy is an over-diagnosed entity without an adequately formulated histological definition [109]. Thus, advances in local pathological diagnostic ability, facilitated by improvements in the Banff system, may have resulted in assignation of biopsies, which might otherwise have been diagnosed with CNI nephropathy to other categories of injury. It is probable that similar considerations underlie the observed decline in the frequency of “idiopathic” IFTA / CAN.

In the DeKaf study, CAN / IFTA was the primary or secondary injury diagnosis in 48% of biopsies and CNI nephropathy in 30%[68]. In a subsequent paper from this group, biopsies were reclassified using cluster analysis on the basis of acute and chronic injury scores; in those clusters with the highest frequencies of ascribed CNI nephropathy and / or CAN / IFTA, the frequency of the presence of underlying antibody-mediated immunological injury was found to be 30 – 60% [57]. In reviewing Nankivell’s data, these authors suggest that the majority of cases described as having CNI nephropathy have evidence of subclinical rejection[109].

It is thus likely that the decline in CNI nephropathy and “idiopathic” CAN / IFTA cases detected during the course of the biopsy results in part from a reduction in injury (for example, following from the implementation of CNI minimization strategies) as well as from a reappraisal of the significance of antibody-mediated injury in this cohort. This latter hypothesis is somewhat substantiated by the observed significant increase in both forms of rejection with a humoral component (mixed and “pure” antibody-mediated rejection) over the course of the series, and by the identification using

discriminant analysis of 2009 (marking the advent of routine C4d staining) as the time point at which antibody-mediated injury became more frequently diagnosed. These findings are best interpreted as evidence of improved appreciation for the significance of antibody-mediated injury in the local context, since analysis of the presence of histological markers of antibody-mediated injury showed no significant difference over the course of the series.

#### **4.2.2 Presenting parameters and patterns of allograft injury**

Comparison of the major patterns of injury (immunological and non-immunological) in this series appeared to show that a younger graft age, higher proteinuria, and higher percentage change in creatinine from baseline were associated with the cause of allograft injury being ascribed to rejection. In logistic regression analysis, only percentage change in creatinine was shown to be a weak predictor of the presence of immunological injury.

It is likely that the association of a lower graft age with the reported presence of rejection reflects the high frequency of reported cases of cell-mediated rejection in this series. In subsequent analysis, a statistically significant difference in graft age was observed for the cell-mediated and antibody-mediated rejection groups (27 months vs. 93 months); cell-mediated rejection has been reported to be more common in the first year of allograft life, whereas antibody-mediated rejection is more frequently observed at late follow-up [83]. The association of proteinuria with rejection in this analysis likely arose as a result of the significant association of proteinuria with antibody-mediated rejection (*vide infra*). It is conceivable that the failure of these parameters to maintain significance in logistic regression analysis reflects the lack of association of these parameters with rejection *per se* but rather with rejection subgroups.

General discriminant analysis suggested that a percentage change in creatinine above 22.9% was associated with the presence of rejection in this cohort. This likely reflects local biopsy practices and selection bias arising from the methodology of this study. It has been general practice at CMJAH to consider for-cause biopsy in the event of a persistent rise in creatinine of at least 25% from baseline. Rejection (particularly acute cell-mediated rejection as opposed to chronic antibody-mediated rejection) is more likely to result in a more rapid deterioration in graft function compared to non-immunological mechanisms of injury. For the purposes of this study, percentage change of creatinine from baseline was defined as the difference between the creatinine preceding biopsy and the average creatinine of 3 preceding measurements divided by this average creatinine measurement. Thus, a more rapid rise in creatinine compared to the antecedent 3 measurements towards a 25% change from this baseline would be more likely to trigger a for-cause biopsy which in turn would be more likely to be associated with a rapidly evolving injury such as rejection.

No statistically significant difference was noted in presenting parameters between non-immunological injury groups, although some interesting trends were observed. Glomerular disease as an ascribed pattern of injury was associated with advanced graft age and with nephrotic range proteinuria.

The timing of glomerular disease recurrence following transplant is highly variable depending upon the type of glomerular disease involved; numerous other recipient factors in addition influence the aggressiveness of the glomerulopathy and hence the timing of recurrence. IgAN, for example, presents with clinical recurrence usually within the third year of transplantation [110]; idiopathic membranous nephropathy usually recurs two to three years after engraftment, but later recurrences are also well described [111]. The mean time to diagnosis of diabetic nephropathy post-

transplant in recipients both with pre-transplant diabetes as well as those developing NODAT has been reported as 5.9 years [112]. The mean graft age at diagnosis of glomerular disease in this study was 110.8 months (9.2 years) which is considerably older than might be expected given the dominance of IgAN, membranous nephropathy, and diabetic nephropathy in this cohort. It is possible that the discrepancy between this study and reported patterns of recurrence from other studies is unclear but may relate to the relatively small number of cases of glomerular disease (which may have led to bias) and to local biopsy practices. Since for-cause biopsy protocols at CMJAH emphasise deterioration in allograft creatinine over developing proteinuria as an indication, it may be that diagnosis of glomerular disease in this cohort is delayed.

The elevated level of proteinuria seen in the glomerular disease cohort is to be expected given the preponderance of podocytopathy patterns of injury (specifically, membranous and diabetic nephropathy). Nephrotic range proteinuria is not uncommon in IgAN, occurring in up to 39% of cases in one study of native kidney IgAN [113]; thus the presence of a significant proportion of IgAN cases in this cohort may also have contributed to the levels of proteinuria observed. Statistical significance was not achieved in this analysis, likely due to the high level of proteinuria observed in the “idiopathic” group. The small number of biopsies in this group (n = 4) is likely to have resulted in a sampling bias.

A markedly elevated creatinine and percentage change in creatinine from baseline were observed for the thrombotic microangiopathy group, although statistical significance was not reached for this analysis. Although this group was also of small size, raising the possibility of sampling bias, significant renal dysfunction of rapid evolution is a noted presentation of thrombotic microangiopathy in both pre- and post-transplant presentations [114]. Notable causes of post-transplant thrombotic

microangiopathy (TMA) include recurrent atypical haemolytic uraemic syndrome, calcineurin inhibitor drug effect, and humoral rejection; all of these are likely to result in significant levels of allograft dysfunction [114].

Comparison of immunological patterns of injury found significant differences in graft age and urine protein: creatinine ratio.

Using the Kruskal Wallis test, median graft age was highest in the “pure” antibody-mediated rejection group (93 months, 7.75 years) and lowest in the “pure” cell-mediated rejection group (29.3 months, 2.44 years), with mixed rejection having an intermediate age of 37.5 months (3.13 years) at diagnosis. The tendency for cell-mediated rejection to occur at an earlier graft age has been previously reported[83]. In contrast, two distinct periods are observed in which antibody-mediated rejection may occur. Firstly, early period hyperacute or accelerated acute ABMR may occur in the first hours to days after engraftment, due to the presence of pre-formed donor-specific antibodies[115]. A second, late-onset form of ABMR is also recognised, arising from the development of de novo donor-specific antibodies developing after engraftment [115]. The development of de novo donor-specific antibodies has been detected in 11% of transplant recipients after the first year of transplant, rising to 20% by 4 years after engraftment; following the evolution of these antibodies, 24% of affected grafts fail within 3 years [116]. For reasons that are at present poorly elucidated, late-onset ABMR has a poorer response to anti-rejection therapy than either early period ABMR or cell-mediated rejection, resulting in poor allograft outcomes[117, 118]. The design of this study excluded cases of hyperacute and accelerated acute ABMR and optimised detection of late-period ABMR; the median graft age at diagnosis of ABMR in this study of late period allograft injury and failure (7.75 months) is neatly consistent with the described evolution of late-period, de novo ABMR, in which the likelihood of onset of allograft dysfunction and failure increases

after the 7<sup>th</sup> year of engraftment[116].

Proteinuria was found to be significantly higher in cases diagnosed with antibody-mediated rejection; using general discriminant analysis, a UPCR above 0.307 was strongly predictive of ABMR whilst a UPCR below 0.199 was strongly protective against this diagnosis. This latter value is of note: at CMJAH, a spot urine protein:creatinine ratio of 2.4 g/mmol equates to nephrotic range proteinuria (in standard definition, more than 3.5g of protein per 24 hours). Analysis of the CMJAH cohort therefore suggests that nephrotic range proteinuria is predictive of ABMR whilst subnephrotic proteinuria is protective against this finding.

Proteinuria has recently been reported to show association with recurrent / de novo glomerular disease, transplant glomerulopathy, and microcirculation injury[119]. Both transplant glomerulopathy and microcirculation injury are known histological markers of antibody-mediated injury in ABMR; limited data further supports a direct association between ABMR and significantly increased levels of proteinuria [120].

In the present study, regression analysis by histological pattern of injury found transplant glomerulopathy to be the sole significant determinant of UPCR level. Further analysis confirmed a significantly higher level of proteinuria in those cases with transplant glomerulopathy compared to those without. In subsequent analysis, significantly higher frequencies of transplant glomerulopathy were observed in those biopsies with immunological injury compared to those with non-immunological injury; in the immunological injury subgroup, transplant glomerulopathy was more frequently detected in those with ascribed antibody-mediated injury.

Thus, significant levels of proteinuria occur in the setting of antibody-mediated rejection due to the development of transplant glomerulopathy in this setting. Transplant glomerulopathy characteristically assumes the histological features of

membranoproliferative glomerulonephritis[29]: this pattern of histological injury is associated with podocyte injury resulting in significant proteinuria.

#### **4.2.2.1 Re-evaluation of the presence of antibody-mediated injury**

Prior analysis of this cohort demonstrated an increase in the diagnosis of antibody-mediated injury after the advent of routine C4d staining in 2009. Since it is likely that this increase reflects greater appreciation of the presence of this pattern of injury rather than an actual increase in ABMR, re-analysis of the series was undertaken for the presence of histological patterns consistent with antibody-mediated injury.

In this analysis, no significant difference in the presence of antibody-mediated injury was discernible on a year-on-year basis, confirming the effect of improved diagnostics in the form of C4d staining on the frequency of ABMR diagnosis.

Analysis of the frequency of antibody-mediated injury by ascribed diagnostic categories showed a high frequency thereof in the TMA and glomerular disease categories. Thrombotic microangiopathy is known to occur as a manifestation of humoral rejection[121]; the retrospective detection of features of antibody-mediated injury in this histological group is therefore not surprising. Similarly, membranoproliferative glomerulonephritis (presenting as transplant glomerulonephritis) and membranous nephropathy are known glomerular injury patterns seen in association with ABMR[29, 122, 123].

Analysis of the presenting features of antibody-mediated injury again demonstrated association of this pattern of injury with advanced graft age and elevated levels of proteinuria, the mechanisms underlying these findings have been discussed previously.

### **4.3 Allograft survival after diagnosis**

Immunological categories of injury in this study were observed to have a poorer survival after diagnosis than non-immunological categories. In subgroup analysis, no significant difference in survival was found amongst non-immunological patterns of injury, although visual inspection of the survival plot suggested that glomerular disease and reflux nephropathy have a non-significant poorer survival than either CNI nephropathy or idiopathic IFTA / CAN.

Allograft survival after diagnosis with glomerular disease is variable and depends on the particular type of glomerular disease diagnosed and therapeutic interventions administered. Both de novo and recurrent forms of glomerular disease have poorer survival compared to allografts without this diagnosis[124, 125]; however, IgAN (which comprised 23% of glomerular disease in this study) is generally associated with a low risk of allograft loss of 3 – 5% and 10 year survival rates have been shown to be similar to allografts unaffected by IgAN [126-128]. In contrast, membranous nephropathy is associated with poor allograft survival after diagnosis (allograft loss has been documented in 50% of cases), possibly reflecting the association of this histological pattern of injury with antibody-mediated rejection.[126] The natural history of diabetic nephropathy after transplant has been poorly studied but is believed to follow a course analogous to native disease in type 1 diabetics with graft failure supervening in the second decade after engraftment,[129] although rapid progression with graft loss within 8 years has also been described[130]. In this study it is conceivable that allograft survival after diagnosis with diabetic nephropathy was reduced due to late diagnosis with already advanced stage disease (the biopsies in this series having been undertaken “for-cause”, i.e., with already clinically significant renal function aberration and / or proteinuria. FSGS, relatively rare in the CMJAH cohort at 15% of cases of glomerular disease, is associated with a two-fold increased

risk of graft loss compared to other glomerular diseases[94]; allograft survival after diagnosis is poor with 84% of adults with recurrent FSGS developing graft loss within 32 months of diagnosis[131]. Membranoproliferative glomerulonephritis, rare in this cohort at 8% of cases, is associated with a 43% risk of rapid allograft failure, the median time to which has been documented at 6.5 months[123]. Recurrent ANCA small vessel vasculitis has been found in small size studies to have a reasonable prognosis with 68% of such cases achieving remission using a standard treatment protocol comprising pulse cyclophosphamide and methylprednisone[132]; however, 30 – 50% of such patients who achieve remission will suffer a further relapse within 3 – 5 years[133]. Since IgAN, which was a common pattern of injury in this study, carries a reasonable prognosis when detected in the renal allograft, it is likely that the non-significantly poorer survival in the glomerular disease subgroup of the cohort arises as a result of the poor outcomes of membranous nephropathy and FSGS, with a possible contribution from advanced diabetic nephropathy.

Reflux nephropathy has been associated with non-significantly poorer allograft survival than non-reflux controls[134]. However, the allograft survival rate in cases of reflux nephropathy has been reported as 82% at 5 years,[134] considerably higher than that observed in this series. It is possible that the poorer survival documented in this series reflects selection bias, since recruitment of cases with established allograft dysfunction may have resulted in preferential inclusion of allografts with more severe established injury.

It has long been held that chronic calcineurin inhibitor mediated injury has the potential to limit long-term allograft survival, a view supported by the development of significant and lasting native renal dysfunction in non-renal solid organ transplant recipients treated with CNIs[135]. The development of end stage native kidney disease in these recipients depends partly on the type of organ transplanted and

varies from 1% of heart transplant recipients at 4 years after engraftment to 20.3% at 10 years[136]. In the transplanted kidney, dose reduction of calcineurin inhibitor results in a rapid and sustained improvement in graft function, [137] which may explain the non-significant comparatively better survival in this group.

CAN / IFTA has previously been associated with poor long-term outcomes[138], with the average allograft survival after diagnosis being 38% at 10 years; however, survival after diagnosis is strongly influenced by the severity of the histological injury observed as well as therapeutic interventions undertaken in response to diagnosis (such as alteration in the prescribed immunosuppression protocol). Thus, mild histological lesions result in a 63% 10 year survival with modification of immunosuppressants; in contrast, the 10 year survival in cases with severe histological lesions in which immunosuppressants are not modified is 16%[139]. The effect of immunosuppressant modification in other studies on allograft survival reflects the contribution of CNI nephropathy to the CAN / IFTA injury subgroup. Since CMJAH practices CNI minimization, the relatively better survival of the CAN / IFTA injury subgroup in the CMJAH cohort (approximately 60% at 10 years) may reflect improved survival in a mild injury cohort in the context of immunosuppression modification.

Recent data suggests that rejection may play an important and hitherto underappreciated role in the development of CAN / IFTA which may account for the poor long-term outcomes of allografts manifesting this pattern of injury. The significant effect of immunological injury in comparison to non-immunological injuries in limiting long-term allograft survival is well demonstrated by the present study.

In subgroup analysis, no significant difference was found in survival following diagnosis of immunological injury by rejection type although visual inspection of the

survival plot suggested poor outcomes for mixed rejection in comparison to both antibody- and cell-mediated rejection. Subsequent analysis comparing cases of rejection featuring an element of antibody-mediated injury (via, “pure” humoral and mixed rejection) to “pure” cell-mediated rejection similarly failed to show a statistically significant difference in survival; however, significantly poorer survival for the combined antibody-mediated injury subgroup was confirmed for the first 120 months (10 years) following diagnosis. In further analysis, a significantly poorer survival after diagnosis was observed in cases in which histological evidence of antibody-mediated rejection was retrospectively noted.

Poor outcomes after diagnosis with mixed rejection in comparison to other types have been previously reported in small studies[140]. This effect is believed to reflect the combined injury delivered to the allograft through simultaneous activation of both arms of the immune response.

Late period antibody-mediated rejection is known to carry a poor prognosis with significant risk of allograft loss. It has been suggested that early- and late-period ABMR are separate clinical entities: whereas early period ABMR is associated with an evanescent decline in allograft function due to preformed antibody which demonstrates rapid response to therapeutic intervention, late period ABMR is typically associated with a progressive decline in renal function in association with the development of proteinuria due to the elucidation of de novo antibody which responds poorly to therapy[117, 118]. The pathophysiological mechanism underlying this discrepancy remains unclear. Interestingly, a tendency for late period ABMR to be associated with an inflammatory cell infiltrate has been reported[141, 142]. It is as yet unclear whether this infiltrate is best interpreted as evidence of concomitant mixed rejection (albeit not meeting full Banff criteria for the diagnosis of a cellular rejection component); or whether the infiltrate instead should be viewed as a

component of “pure” ABMR, and if so, whether it represents an effector response to antibody presence or instead acts as a source for antibody elucidation[115]. Regardless of the pathophysiological effect of the infiltrate, cluster analysis from the DeKaf cohort has confirmed a role for the i-score (a measurement of the volume of interstitial inflammatory infiltrate present) in predicting allograft survival; significantly, this study further confirms the presence of undiagnosed antibody-mediated injury across multiple non-immunological ascribed categories of injury[57].

Cox regression analysis was undertaken in order to evaluate the effect of histological injury patterns and clinical surrogates of the severity of this injury on allograft survival. Serum creatinine at diagnosis was found to exert a weak effect on graft survival (HR 1.003); absence of antibody-mediated injury was found to exert a moderate protective effect on this survival (HR 0.588, indicating risk reduction of 41.2%).

Serum creatinine has previously been shown to predict allograft outcomes; creatinine level (and change thereof from baseline) at one year following engraftment has been shown in a number of studies to predict long-term survival[143-145] and has been validated as a survival predictor (with the development of donor specific antibody) in the recently formulated adjustable predictive score (AdGFS) of long-term renal graft failure[146]. Serum creatinine represents allograft filtration capability; thus, creatinine predicts survival as a surrogate for severity of allograft injury. The hazard ratio for creatinine in this study, although statistically significant, is not particularly impressive being marginally above 1.0 (the level of no effect). It is likely that this is due to graft loss being a function of multiple factors including the nature of the insult delivered to the allograft, rather than the severity of this insult alone. This hypothesis is supported by the finding that the type of insult (immunological vs. non-immunological, and the presence of evidence of antibody-mediated rejection) also appears to have an effect

in determining graft survival. In addition, spot creatinine level may not correlate fully with the severity of the particular injury, which resulted in an indication for biopsy. It is, for example, conceivable that an allograft may have pre-existing levels of dysfunction (arising for example from antecedent injury such as peri-engraftment ischaemia), which would manifest in a higher baseline creatinine which would in turn result in a higher creatinine at diagnosis from the current injury. In support of this hypothesis, initial multivariate analysis found a higher hazard ratio (1.025) for percentage change of serum creatinine from baseline.

It is also noteworthy that multivariate analysis failed to find a significant effect for histological markers of established injury (IFTA / CAN), suggesting that acute injury is a more important factor in allograft outcomes. Of note, type of rejection (“pure” cell-mediated versus “pure” ABMR) did not appear to affect allograft survival. Interestingly in this regard, the presence of histological features suggestive of antibody-mediated injury was found to play a role in determining allograft survival in both the initial multivariate model as well as the subsequent restricted model. In the context of the observation that 27.6% of cases reported as “pure” cell-mediated rejection had some evidence of antibody-mediated injury, it may be that the failure of type of rejection to demonstrate an effect on allograft survival is due to the admixture of undiagnosed antibody-mediated rejection within the “pure” cell-mediated rejection subgroup. In this regard, the observation that an absence of retrospective evidence of antibody-mediated rejection at biopsy was associated with a 41% reduction in the risk of allograft loss may be construed as evidence for a significant effect on the part of antibody in determining allograft survival.

## 5 Conclusions

Advances in renal transplantation have increased the numbers of transplanted kidneys surviving the early post engraftment period but have not resulted in increased long-term allograft longevity. Increasing long-term allograft survival is dependent upon developing an understanding of the factors, which lead to late-period allograft injury and loss. Whereas progress is being made in this regard in other centres, local data is lacking. This study was therefore undertaken with the aim of describing the local experience of aetiologies underlying late-period allograft dysfunction and loss. The relative contribution of each of these aetiologies to allograft loss was further quantified by survival analysis, and the effect of each aetiology on allograft dysfunction was assayed through comparison of markers such as serum creatinine and proteinuria.

A preponderance of males of black African descent was included in this study. This most likely reflects local population demographics of the ESKD population accessing transplantation as a renal replacement therapy. A relatively higher-than-expected number of Indian / Asian patients was also observed, possibly reflecting an increased risk of ESKD in this group. The mean graft lifespan in this cohort of late-period injury in this cohort was 10.3 years, which is neatly consistent with the reported average duration of allograft survival after engraftment in the international literature of 10 – 12 years.

Rejection constituted a slim majority of cases of late-period allograft injury. Such immunological injury was diagnosed in slightly younger allografts (68.3 vs. 93 months) and was associated with a more significant decline in renal function and heavier proteinuria (23.6% change vs. 16.2% and 0.11 g/mmol vs. 0.07 g/mmol). These differences are believed to have resulted from the tendency of cell-mediated rejection to occur at an earlier stage post-engraftment and from the tendency for

rejection to be associated with a more rapid decline in renal function; the heavier proteinuria detected is believed to have been a result of the presence of antibody-mediated injury in the rejection subgroup. Regression analysis determined percentage change in creatinine to be the only significant, albeit weak, predictor of rejection (OR 1.06).

The most commonly encountered non-immunological patterns of injury in descending order were CNI nephropathy, primary IFTA / CAN, reflux nephropathy, and glomerular disease. Idiopathic dysfunction, TMA and BK nephropathy were uncommon in this cohort. Presenting parameters were not able to distinguish the various categories of non-immunological injury. CNI Nephropathy and IFTA / CAN incidence were observed to decline over the course of the study period, possibly reflecting changes in CNI prescription and improved diagnostic capabilities.

Cell-mediated rejection was the most common type of rejection reported by the examining pathologist at the time of biopsy at 62% of such cases. Cell-mediated rejection was observed to occur earlier in the allograft lifespan (29.3 months vs. 37.5 months for mixed and 93 months for antibody-mediated rejection) and was associated with a lower urine protein: creatinine ratio. In regression analysis, urine protein: creatinine ratio above 0.307 was found to predict the presence of ABMR (OR 3.79); graft age was not found to be a useful predictor. Further analysis demonstrated the association of UPCR with ABMR to be caused by the development of glomerular injury in the form of transplant glomerulopathy in this rejection type. Incident diagnosis of cell-mediated rejection was observed to decline over the course of the study period whilst that of mixed and “pure” antibody mediated rejection increased. Discriminant analysis identified 2009, the year of the introduction of routine C4d staining on for-cause biopsies, as the break point for this increasing incidence of antibody-mediated injury diagnosis. Retrospective review of biopsies for

evidence of antibody-mediated injury found relevant changes in between 17 and 40% of contemporaneously assigned categories of injury; 54% of glomerular disease diagnoses and 75% of TMA cases had histological injury patterns which could be ascribed to antibody, chiefly due to the presence of transplant glomerulopathy-like changes in these groups. The incidence of antibody-mediated injury did not change over the course of the study, confirming the hypothesis that many such cases were likely to have been missed prior to the advent of C4d staining. Regression analysis indicated UPCr above 0.2g/mmol and graft age above 75 months to be predictive of the presence of this injury pattern (OR 2.2 and 2.4 respectively).

Survival analysis indicated that immunological injury was associated with poorer allograft survival after diagnosis. No significant difference in survival was observed amongst the non-immunological patterns of injury although visual inspection suggested that glomerular disease and reflux nephropathy and poorer survival than CNI nephropathy or idiopathic IFTA / CAN. It is hypothesized that this is due to the potential presence of allograft survival limiting diagnoses such as recurrent membranous or mesangiocapillary glomerulonephritis within the glomerular disease subgroup, and the relatively better outcome for CNI nephropathy in the context of CNI minimization.

Amongst immunological injury categories, no significant difference in survival was observed, although visual inspection of the survival plot indicated relatively poorer outcomes for mixed and antibody-mediated rejection, consistent with data from other institutions. Comparison of the retrospectively validated cohort of patients with evidence of antibody-mediated injury confirmed significantly poorer survival for this group compared to those without this pattern of injury. In Cox regression analysis, the absence of antibody-mediated injury was further shown to be protective against allograft loss (a 41% risk reduction being observed).

In conclusion, the present study relates to the first attempted description of the causes of late period allograft dysfunction and loss in the local setting. Immunological injury appears to be the most common cause of allograft loss; whilst cell-mediated rejection, CNI nephropathy and idiopathic IFTA / CAN appear to be decreasing in incidence there is increasing detection of antibody-mediated injury; probably due to improved surveillance for this pattern of injury. Graft age, change in creatinine from baseline, and UPCR may be able to allow presumptive diagnosis of the most probable pattern of injury for emergent initiation of empiric therapy, which may attenuate the significant risk of graft loss in patients with antibody-mediated injury.

## **6 Limitations**

This study is subject to two major limitations. Firstly, being a retrospective study, the diagnosis of the cause of ascribed allograft dysfunction is dependent on the opinion of the examining pathologist at the time of diagnostic biopsy. Banff criteria have advanced considerably over the period of study with new categories (most notably that of ABMR) having been introduced. The effect of these changes is most appreciable in the analysis related to the presence of histological patterns consistent with antibody-mediated injury, in which between 17 and 75% of various ascribed categories of injury have the potential to be reassigned to the ABMR category.

The second major limitation also relates to this analysis: in strict definition, the diagnosis of antibody-mediated injury requires the demonstration of serum alloantibody; the retrospective nature of this study makes this impossible.

Additional limitations include the small number of cases in some subgroups (for example, BK nephropathy), and missing presenting data in some cases that reduced the number of cases available for analysis. Attempts were made to adjust for these factors by utilization of non-parametric statistical tests.

## 7 Appendix

### 7.1 Banff classification for renal allograft biopsies

#### 1. Normal

#### 2. Antibody mediated changes

This is due to documentation of circulating donor antibody, and C4D or allograft pathology.

##### C4d deposition without morphologic evidence of active rejection

C4d+, presence of circulating antidonor antibodies, no signs of acute or chronic TCMR or ABMR. Cases with simultaneous borderline changes or ATN are considered as intermediate.

##### Chronic active antibody-mediated rejection

C4d+, presence of circulating antidonor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries.

#### 3. Borderline changes

#### 4. T Cell mediated rejection

##### Acute T cell mediated rejection

##### Chronic active T cell mediated rejection

'Chronic allograft arteriopathy' (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)

#### 5. Interstitial fibrosis and tubular atrophy, no evidence of any specific aetiology

#### 6. Other; Changes not considered to be due to rejection, these include

i post transplant lymphoproliferative disorder

- ii non specific changes
- iii acute tubular necrosis
- iv acute interstitial nephritis
- v cyclosporine or FK506- associated changes, acute or chronic
- vi subcapsular injury
- vii pretransplant acute endothelial
- viii papillary necrosis
- ix de novo glomerulonephritis
- x recurrent disease
  - immune complex glomerulonephritis
  - focal segmental glomerulosclerosis
  - diabetes
  - haemolytic uraemic syndrome
  - other
- xi pre-existing disease
- xii viral infection
- xiii obstruction/reflux, urine leak

On further definition of the interstitial fibrosis and tubular atrophy category, it is classified according to the percentage of renal parenchyma involved.

As per Banff '97 criteria[147]

*Quantitative criteria for interstitial fibrosis (“ci”)*

Score	Grade	% cortical area involved
ci0	No interstitial fibrosis	<5
ci1	I/Mild	6-25
ci2	II/Moderate	26-50
ci3	III/Severe	>50

*Quantitative criteria for tubular atrophy (“ct”)*

Score	Grade	% cortical tubules involved
ct0	No tubular atrophy	
ct1	I/Mild	<25
ct2	II/Moderate	26-50
ct3	II/Severe	>50

This significance of classifying the grade of IFTA (interstitial fibrosis and tubular atrophy) is the standardisation of the findings on biopsy that quantify the extent and severity of allograft injury.

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