COMPARATIVE MODELS OF TRANSPLANT AND NON-TRANSPLANT HUMAN KIDNEY DISEASE

by

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A thesis submitted to the
University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

School of Clinical and Experimental Medicine
(Formerly Division of Medical Sciences)
The University of Birmingham
April 2012

CONTENTS

ABBREVIATIONS

ACR, albumin creatinine ratio

AH, arteriolar hyalinosis

ANOVA, analysis of variance

AP, alkaline phosphatase

Arg, arginase

ATN, acute tubular necrosis

ATP, adenosine 5'-triphosphate

CAD, chronic allograft damage

CADI, chronic allograft damage index

CA-IX, Carbonic anhydrase IX

CAN, chronic allograft nephropathy

CDI, chronic damage index

CI, confidence interval

ci, interstitial fibrosis

CIT, Cold ischaemia time

CKD, chronic kidney disease

CNI, calcinurine inhibitor

CNI-NT, calcineurin inhibitor nephrotoxicity

$Cr_{t½}$, days required to halve pre-transplant serum creatinine

CsA, cyclosporine

DBD, donation after brain death

DCD, donation after cardiac death

DGF, delayed graft function

EHVG, elastic Van Gieson

ESRF, end-stage renal failure

GFR, glomerular filtration rate

GN, glomerulonephritis

HLA, human leukocyte antigen

HRP, horseradish peroxidase

HT/ Isch, hypertensive ischaemic

i, inflammation

I/R, ischaemia reperfusion

IF, interstitial fibrosis

IF/TA, interstitial fibrosis/ tubular atrophy

IMD, interstitial macrophage density

iNOS: inducible nitric oxide

MCP-1, monocyte chemotactic protein-1

MØ, macrophage

NHANES, National Health and Nutrition Examination Survey

NO, nitric oxide

NRSOT, non renal solid organ transplant

PAMS, periodic acid methenamine silver

PAS, periodic acid schiff

PBS, phosphate buffer saline

PTECD, peri-tubular capillary endothelial cell density

RT-PCR, real time polymerase chain reaction

SCR, subclinical rejection

TA, tubular atrophy

TEC, Tubular epithelial cells

TMA, thrombotic microangiopathy

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ΔCt, change in threshold time

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CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

1.1 INTRODUCTION

Chronic allograft damage (CAD) is the most common cause of graft loss after the first year of kidney transplantation. As a result of sequential improvements in early outcomes following transplantation, the identification and management of CAD now represents the major clinical issue in human renal (kidney) transplantation. Despite this there is a lack of controlled clinical trials in this area. To some extent this reflects a lack of understanding of the pathogenesis of CAD; most of our knowledge in this area has been derived from animal models and in-vitro studies.

Most of the work presented in this thesis has focused on non-alloimmune pathways in the development of CAD. I have approached this from three perspectives. First, by analysing the role of mitochondrial complex activity in the donor organ immediately before transplantation as a marker of donor organ viability and relating this to clinical indicators of long-term outcome. Second, by studying the different patterns of kidney injury in the setting of non-renal transplantation and relating these to the patterns of injury in kidney transplants. Finally, by comparing the in situ determinants in CAD with native chronic kidney disease (CKD). I have also extended previous work on the pathogenesis of human kidney disease and assessed the molecular micro-environment in CKD.

A central hypothesis of this thesis is that the factors which promote CAD in the majority of kidney transplant recipients are largely similar to those factors that occur in non-transplant

CKD. This has important clinical implications and may direct patient management. The basis for the studies that address this is the utilisation of well characterised groups of patients with 1) CAD 2) non-renal solid organ transplants (NRSOTs) that have developed CKD and, 3) native, non-transplant CKD. I have used these three groups to make direct comparisons, but have also studied each group in detail to better understand the injury processes specific to each. In this chapter I provide the scientific background for the work presented in the experimental chapters of this thesis.

1.2 Current Status of Renal Transplantation

1.2.1 Chronic Kidney Disease

Chronic kidney disease affects 6-10% of adults in the United Kingdom; the NeoErica (New Opportunities for Early Renal Intervention by Computerised Assessment) study showed a prevalence of 5.1% of known CKD stages 3–5 in the general population, based on an analysis of single serum creatinine results held on GP computers. However, the true prevalence may be higher, as only 25% of the population had renal function testing available for inclusion (de Lusignan, Chan et al. 2005). In the US, the Third National Health and Nutrition Examination Survey (NHANES III) found that 4.7% of the population had a GFR <60 ml/min (stages 3–5), with a much higher prevalence in the elderly and in people with diabetes and hypertension (Coresh J 2003). In a follow-on study, Coresh *et al* found that the prevalence of CKD stages 1 to 4 had increased from 10% (95% CI, 9.2%-

10.9%) in 1988-1994 to 13.1% (95% CI, 12.0%-14.1%) in 1999-2004, with a prevalence ratio of 1.3 (95% CI, 1.2-1.4). (Coresh, Selvin et al. 2007).

Although most people with CKD do not reach end-stage renal failure (ESRF), a significant number progress to end-stage disease that requires treatment with renal replacement therapy (RRT). This greatly reduces the quality of life of those affected through significant morbidity and excess mortality. There were 47,525 adult patients receiving RRT in the UK on 31/12/2008, equating to a UK prevalence of 774 pmp. This represented an annual increase in prevalence of approximately 4.4%. (Renal Registry report 2009). The numbers receiving RRT is predicted to continue increasing at a rate of between 6% and 8% per annum for the next decade. This has significant cost implications: the cost of treating people with ESRF has been estimated at 1-2% of the total NHS budget, yet they comprise only 0.05% of the total population. [DH NSF Renal Services Part-1; 2004].

For many patients with ESRF kidney transplantation is the treatment of choice as it not only returns patients to a dialysis-free life and improves quality of life but can have a profound impact on overall survival (Wolfe, Ashby et al. 1999). Furthermore, kidney transplantation represents a significant cost saving compared to continuing treatment on dialysis.

1.2.2 Current Renal Transplant Activity

In the financial years 2008-9, and 2009-10, 2482 and 2679 patients respectively received a kidney transplant (including living donor transplants). Despite the slight drop in the latest year, the number of patients registered on the active transplant list at 31 March 2010 for a kidney or a kidney and pancreas transplant (7183) has risen by 46% since 2001. The number of patients waiting for a kidney transplant represents 119 patients per million population (pmp). The median (average) waiting time for a kidney transplant is 1088 days for an adult patient. [NHSBT] **(Figure 1.1)**

Figure 1.1 Renal Transplant Activity and Waiting List

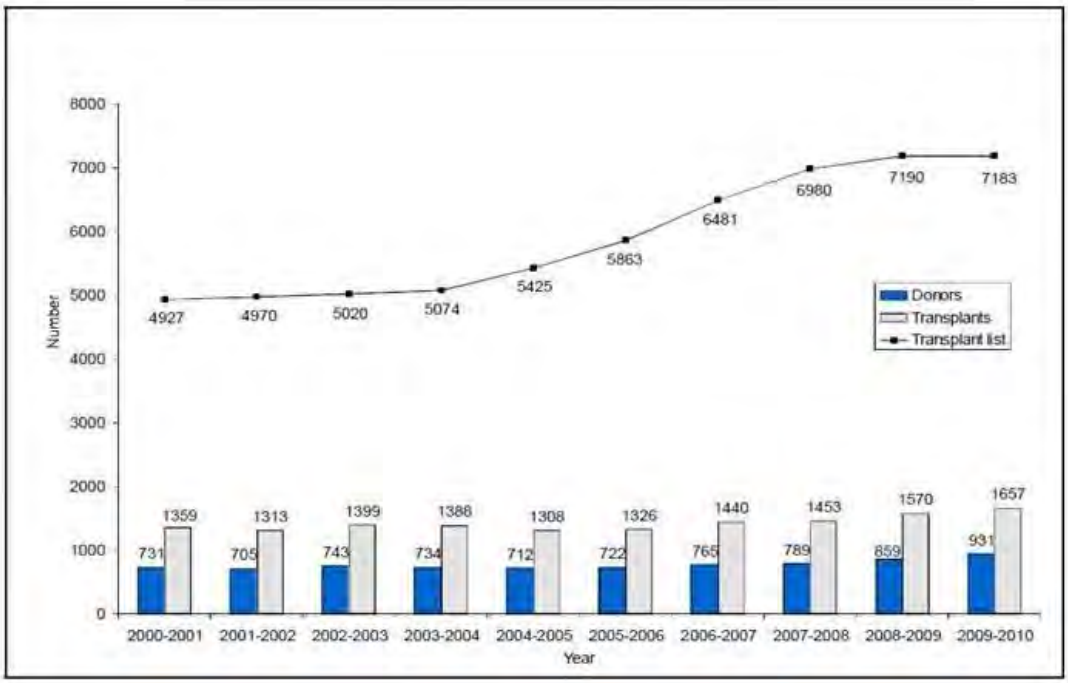


Figure 1.1: Deceased donor numbers, transplant activity and numbers on the transplant lists from 2001 - 2010 (Data supplied by NHS Blood and Transplant (**NHSBT**))

1.2.3 Outcomes following Renal Transplantation

With the introduction of newer immunosuppressive agents early graft **(Figure 1.2 and Table 1.1)** has improved over time. However it is evident that the long-term survival of transplanted kidneys which are functioning at one year post-transplant remains broadly similar with organ attrition rates unchanged the gradient of the graft survival curves are similar for all the time periods of transplantation **(Figure 1.2).** The same is applicable in the setting of donor after cardiac death transplants (DCD) **(Figure 1.3 & Table 1.2)** and living-donor transplants **(Figure 1.4 & Table 1.3)**. Although in view of th eincreasing use of extended criteria donors, the parallel curves may in fact mean an improvement in the survival outcomes.

Figure 1.2 Renal graft survival after DBD transplants

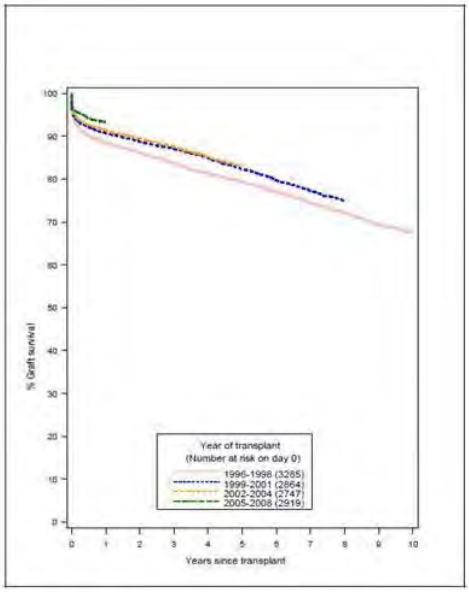


Figure 1.2: Renal graft survival following DBD transplants; note parallel graft attrition slopes (Source: NHS Blood and Transplant) DBD: Donation after brain death

Table 1.1 Renal graft survival after adult DBD transplants

Year of transplant	No. at risk on day 0	% Graft survival (95% confidence interval)							
		One year		Two year		Five year		Ten year	
1996-1998	3285	89	(87-90)	86	(85-87)	79	(78-81)	67	(66-69)
1999-2001	2864	91	(90-92)	89	(88-90)	82	(81-84)		
2002-2004	2747	91	(90-92)	90	(88-91)	83	(81-84)		
2005-2008	2919	93	(92-94)						

Table 1.1: Renal graft survival following adult DBD transplants (Source: NHS Blood and Transplant)

Figure 1.3 Renal graft survival in DCD transplants

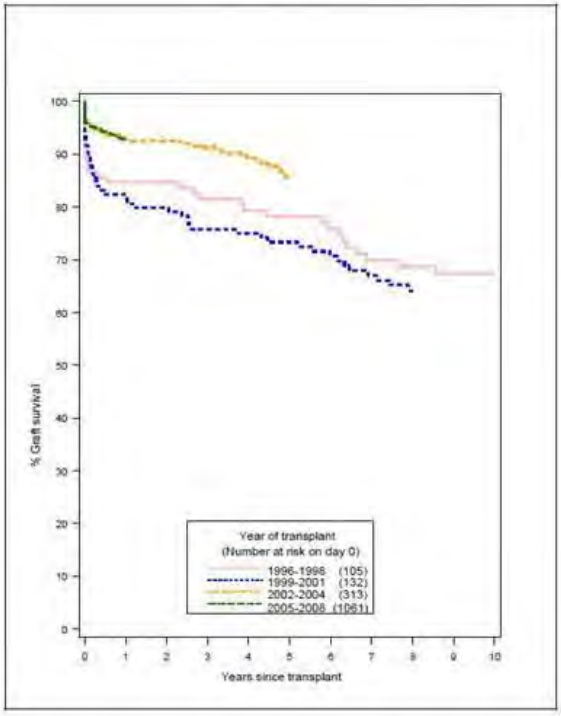


Figure 1.3: Renal graft survival following adult DCD transplants; note parallel graft attrition slopes (Source: NHS Blood and Transplant)

Table 1.2 Renal graft survival in DCD transplants

Year of transplant	No. at risk on day 0	% Graft survival (95% confidence interval)							
		One year		Two year		Five year		Ten year	
1996-1998	105	85	(76-90)	85	(76-90)	78	(69-85)	67	(57-76)
1999-2001	132	82	(75-88)	80	(72-86)	73	(65-80)		
2002-2004	313	93	(89-95)	93	(89-95)	85	(81-89)		
2005-2008	1061	93	(91-94)						

Table 1.2: Renal graft survival following adult DCD transplants (Source: NHS Blood and Transplant)

Figure 1.4 Renal graft survival in living donor transplants

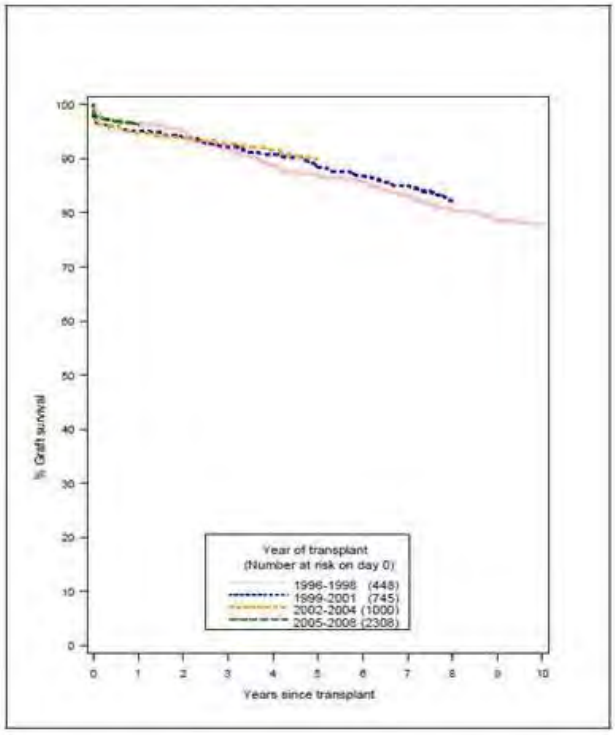


Figure 1.4: Renal graft survival following living donor adult recipient transplants; note parallel graft attrition slopes (Source: NHS Blood and Transplant)

Table 1.3 Renal graft survival in living donor transplants

Year of transplant	No. at risk on day 0	% Graft survival (95% confidence interval)							
		One year		Two year		Five year		Ten year	
1996-1998	448	96	(94-98)	95	(93-97)	87	(83-90)	78	(74-82)
1999-2001	745	95	(93-96)	94	(92-95)	89	(86-91)		
2002-2004	1000	95	(93-96)	94	(92-95)	90	(88-92)		
2005-2008	2308	96	(95-97)						

Table 1.3: Renal graft survival following recipient transplants (Source: NHS Blood and Transplant)

If the rate of renal allograft loss following the first year of transplantation could be reduced, this may contribute to less organ shortage and decrease the waiting time for patients who are on the waiting list for a transplant. This is an important consideration, since time on dialysis correlates with decreased patient survival as well as reduced graft survival following transplantation (Meier-Kriesche and Kaplan 2002) **(Figure 1.5).** However, with the current organ shortage, pre-emptive or early transplantation is not practical other than in the case of live donor transplantation. In addition, maintaining long-term graft survival has cost-savings of £25,000 per year of transplant survival. The benefit of prolonging the life of the transplanted kidney, by minimising late graft loss, is clear.

Figure 1.5: Renal graft survival and length of dialysis

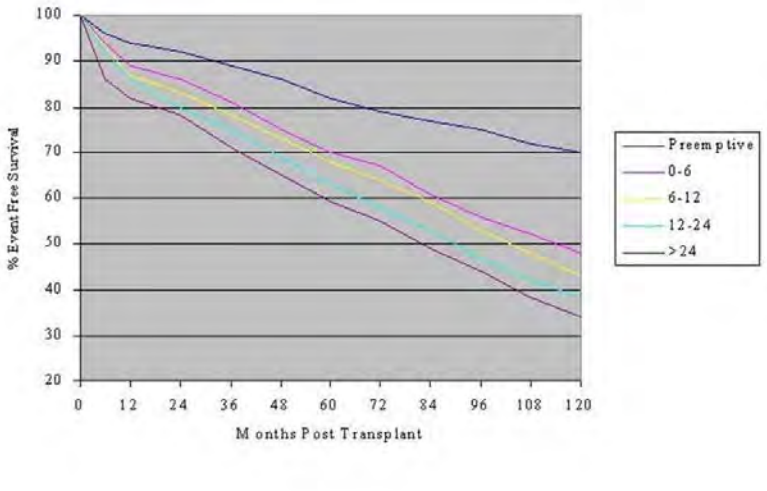


Figure 1.5: Unadjusted renal graft survival in 56,587 recipients of DBD transplants by length of dialysis treatment before transplant.

1.3 Late Renal Allograft Loss: Chronic Allograft Damage

Progressive functional failure of the majority of renal allografts has historically been thought to result from a low grade process of persistent or chronic rejection. However, this hypothesis has not been validated in the setting of human renal transplantation. The absence of sub-acute inflammatory cellular infiltration in renal transplant biopsies obtained long after transplantation has further challenged this hypothesis; a high profile study (Nankivell, Borrows et al. 2003) showed that true 'chronic rejection', defined by sequential histologic abnormalities with continuous immonologic injury, occurred only in 5.8% of recipients. Although early tubulo-interstitial injury may result from alloimmune processes, a

significant component of late damage appeared to be a result of non-alloimmune factors, particularly due to calcineurin inhibitor toxicity. This late damage is now known as 'Interstitial fibrosis and tubular atrophy (IF/TA). This is widely referred to as chronic allograft nephropathy (CAN) or chronic allograft damage (CAD); it is the most common cause of renal allograft loss after the first year of transplantation. For this thesis, I will use the term CAD to represent these changes: protocol biopsy data suggest that histological evidence of CAD was visible as early as three months after transplantation and increased in severity over time (Nankivell, Borrows et al. 2003) **(Figure 1.6).** Currently there are no pharmacological agents that can be used specifically for the treatment of CAD.

The definition of CAD/CAN as a clinical and/or histopathological entity is problematic as its indicative features are extremely heterogeneous. Originally suggested in 1991 as a more generic alternative to the then popular and misleading term ''chronic rejection'', acceptance of 'CAN' reversed the misconception that all late scarring of the graft was due to chronic rejection. The gold standard for diagnosis of CAD/CAN is based on histological evidence of interstitial fibrosis, arteriolar hyalinosis and tubular atrophy. 'Chronic rejection' remains as a term that describes an ongoing low-grade allo-specific immunological response of the host against donor tissue; it continues to represent a significant component of late graft loss, and our understanding of the processes that contribute to chronic rejection is evolving with time.

Figure 1.6: Prevalence of CAN/ CAD

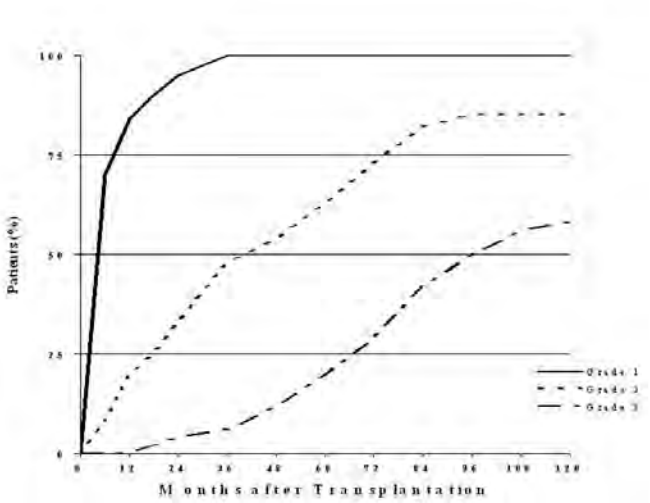


Figure 1.6: Prevalence of mild (grade 1), moderate (grade 2) and severe (grade 3) CAD/ CAN according to Banff criteria (Nankivell, Borrows et al. 2003)

A major topic discussed at the 8th Banff Conference was the elimination of the term 'chronic allograft nephropathy' (CAN) from the Banff schema for diagnosis and grading of renal allograft rejection. The rationale behind this update of the Banff schema is the misuse of 'CAN' as a generic term for all causes of chronic renal allograft dysfunction with fibrosis. It is felt that this inhibits the accurate diagnosis and appropriate therapy.

1.4 Baseline Donor Organ Function and the Development of CAD

One of the important findings from the studies of Nankivell et al is that chronic damage develops early post-transplantation (Nankivell, Borrows et al. 2003). This indicates that factors associated with these changes may be present in the peri-transplant period, both as

pre-existing scarring and inflammation within the kidney and with factors to which the kidney is exposed in the peri-operative period. It is well known that healthier donor organs provide better graft survival. However, in clinical practice organ function is measured by crude tests such as serum creatinine; and whilst there have been studies that have tried to combine measurable factors in the peri-transplant period and relate these to long-term outcomes, no widely used system has entered clinical practice to date (Asher, Wilson et al. 2005; Grossberg, Reinert et al. 2006; Irish, Wang et al. 2007; Jeldres, Cardinal et al. 2009). This is an important consideration, since if risk can be accurately defined at the time of transplantation it may influence clinical management and ultimately lead to improved clinical outcomes (Arias, Arteta et al. 2007).

One example of the uncertainty concerning peri-transplant determinants of the outcomes of kidney transplantation is the use of grafts from older donors. With age, kidneys develop serious morphological and functional alterations including decreased glomerular number and volume, intimal thickening in intraparenchymal arteries, and decreased glomerular filtration rate (Tauchi, Tsuboi et al. 1971; Kasiske and Umen 1986). Some reports have observed worsened transplant survival in grafts implanted from older donors (Feduska 1993).

However, the hypothesis that age per se could be a risk factor for histological damage has not been confirmed. In a histological study (Rossetti, Soldano et al. 2007) on grafts from older donors in which the only criterion for acceptance was based on normal serum

creatinine values at admission to the intensive care unit, mild or moderate structural alterations were present comparable to those seen in younger donor grafts, but there was no difference in long-term graft survival between the two groups. Despite this there were differences in surrogates for long-term outcomes: the recipient population with grafts from older donors had a high incidence of DGF and higher serum creatinine levels than recipients with grafts from younger donors. This may indicate that old grafts are more susceptible to functional alterations due to cold ischaemia and reperfusion injuries rather than overt microscopic damage.

One manifestation of this is the increased apoptosis present in older kidneys (Percy, Power et al. 2008). Other studies have demonstrated an age-related decline in mitochondrial respiratory chain activity. This mitochondrial respiratory chain failure has been suggested as a factor in the ageing process, and has a likely greater effect in tissues that have a high dependency on energy generated through oxidative phosphorylation (OXPHOS) and which lack a regenerative capacity (Ojaimi, Masters et al. 1999). This is a particular issue in the kidney. This idea is explored in more detail later in this chapter and is a focus of some of the experimental work presented in this thesis.

1.4.1 Peritransplant Injury and its Impact on Long-term Graft Function

In the process of graft procurement and implantation the donor organ sustains various insults. These insults have an impact on early graft function which in turn affects long-term graft function. These insults include brain stem death, cold ischaemia, warm ischaemia and

reperfusion injury. Whilst the insults in the recipients of kidneys from living donors are less prominent than in the recipients of deceased donor kidneys, most of the factors that may contribute to in situ injury are still present in this setting.

The systemic insult of brain stem death

In deceased donor transplantation, the process of brain stem death (BSD) initiates a sequence of events that is potentially damaging to the organs being retrieved, one of them is the Cushing's reflex causing catecholamine storm. Brain stem death upregulates in situ cytokine, chemokine and growth factor release along with complement and other inflammatory cascade activation to enhance solid organ immunogenicity (Takada, Nadeau et al. 1998). These local process effects may amplify the tissue damage that occurs as a consequence of ischaemia (both warm and cold) and reperfusion injury (Takada, Nadeau et al. 1998). This is well illustrated by a study comparing cadaveric donor transplantation to live donor transplantation. Cadaveric kidneys were found to express significantly higher levels of endothelial E-selectin, proximal tubular HLA-DR, and intercellular and vascular cell adhesion molecule-1(Koo, Welsh et al. 1999).

Ischaemia and Ischaemia-reperfusion injury

A significant majority of published data indicates an impact of ischaemia time on CAD/ long term clinical outcome. Some studies have suggested that prolonged ischaemia may be a risk factor for CAD (Cicciarelli, Iwaki et al. 1993). Cicciarelli et al analyzed 549 recipients of primary cadaver allografts and showed that a cold ischaemia time of less than

36 hours was associated with a significantly higher rate of graft survival. There was a significant difference between short and long cold ischaemia on both primary function and serum creatinine levels at discharge. However, the number of rejection episodes was not significantly higher in patients with prolonged cold ischaemia. In another study on 457 cadaveric renal transplant recipients, delayed graft function, acute rejection and long-term outcome correlated with the duration of ischaemia (Troppmann, Gillingham et al. 1995). Five-year graft survival was 84% when CIT was less than 24 hours and 63% when CIT was more than 24 hours.

Upon reperfusion, the reintroduction of molecular oxygen leads to formation of free oxygen radicals, which in turn activate leukocytes and endothelial cells. The pre- and early peri-transplant period are the key determinants of this response. For example, vascular endothelial cells generate free oxygen radicals as a result of prolonged ischaemia and also reperfusion (Zweier and Talukder 2006). This leads to generation of superoxide radicals, hydroxyls, hydrogen peroxide, and singlet oxygen (Wood and Gladwin 2007).

The most extreme consequence of these events is the peroxidation of lipids that leads to the disintegration of cell membranes. However, most responses are below this level of severity, with activation of the cytokine and adhesion molecule cascade which promote in situ pro-inflammatory and pro-fibrotic changes that ultimately cause intimal proliferation and sclerosis in arterioles and small, medium, and large arteries, findings common to transplant vasculopathy.

Animal data suggests that oxygen tension is decreased in affected arterial tissue as a consequence of transplant vasculopathy. This is well illustrated during the development of arteriosclerosis, as shown by a decreased partial oxygen tension not only in tissues and capillaries behind the arterial stenosis, but also in the media of the affected artery (Bjornheden, Evaldsson et al. 1996). These hypoxic microenvironments lead to the activation of peri-vascular leukocytes and local feedback loops for the synthesis of growth factors and cytokines, which lead to local paracrine pathways for secondary cell activation and damage, including endothelial cells. Endothelial damage turns vascular flow from laminar flow into turbulent flow with subsequent increased shear stress (Davies and Tripathi 1993). However, despite the association between ischaemic–reperfusion injury and CAD in animal models, it is difficult to transfer these observations to human transplantation, where there are a large number of additional variables present (Yates and Nicholson 2006).

Delayed graft function

In clinical practice, primary non-function/ delayed graft function [DGF] is considered to be a poor initial outcome. DGF contributes decisively to worsen graft outcomes, with higher rates of acute rejection and chronic graft dysfunction, as well as worsen graft function and survival particularly in combination with acute rejection episodes. Therefore DGF has been considered a surrogate marker of poor initial outcome in renal transplantation. Many factors may contribute to the development of DGF, including the source of the organ: organs from living donors are associated with a much lower incidence of DGF, approximately 6%

compared with approximately 23% in kidneys from cadaveric donors. The cause of death in cadaveric donors also has an impact: the incidence of delayed graft function in donors who died as a result of a stroke is two times higher when compared with donors who died of a motor vehicular accident. It is estimated that after 12 hours of CIT, the rate of DGF increases by 5% for each 6 additional hours of cold ischaemia; consequently, after 24 hours of cold ischaemia, the rate of DGF is doubled compared with 12 hours of CIT (Perico, Cattaneo et al. 2004).

Delayed graft function (DGF) carries an increased risk of acute rejection, and therefore a higher prevalence and earlier onset of CAD (Troppmann, Gillingham et al. 1995) (Humar, Johnson et al. 1997) (Cosio, Pelletier et al. 1997). There is, however, contradictory data in the published literature as to whether the early onset of CAD in kidneys with DGF is independent of other factors or purely a result of the higher acute rejection rate (Shoskes and Cecka 1998) (Halloran, Aprile et al. 1988) (Sanfilippo, Vaughn et al. 1984).

1.4.2 The Need for Pre-transplantation Assessment of Organ Viability

Ischaemia-reperfusion injury is inevitable in solid organ transplantation, and is a major variable in the development of DGF. Warm ischaemia is particularly an issue in donation after cardiac death (DCD) transplantation and prolonged cold ischaemia is associated with the transportation of organs to distant centres for transplantation into a better matched recipient. Organs procured from DCD donors experience significant warm ischaemia in the donor prior to nephrectomy. This warm ischaemic damage is a key cause of DGF and of

worse early outcomes (Bilde, Dahlager et al. 1977). However, the selection of donor kidneys for transplantation on statistical grounds discards a group of grafts that, in spite of a compromising history, may not have suffered irreversible damage. This is particularly important when we know that kidneys have varying functional potential. Scoring systems utilizing donor quality parameters and measures of early allograft function have been proposed (Nyberg, Matas et al. 2003; Schold, Kaplan et al. 2005; Irish, Wang et al. 2007; Moore, Ramakrishna et al. 2009). However, their utility in clinical practice remains limited (Kubal and Bhati 2006). Therefore, an objective test that enables a distinction between viable preserved donor kidneys and permanent non-functioning kidneys prior to transplantation could lead to increased organs available for transplantation. Additionally, this may enable us to identify correctable defects at cellular level which may have an impact on graft function and survival. One focus of the experimental work that is reported in this thesis is mitochondrial function as an important early variable in kidney transplantation.

1.4.3 Role of Mitochondrial Respiratory Chain and Complex Activities in Solid Organ Transplantation

In the 1970s and early 80s, the cellular energy charge and phosphorylation potential of an organ were considered to provide an indication of post-transplantation function and studied in detail at that time (Calman 1974; Garvin, Castaneda et al. 1985; Tokunaga, Ozaki et al. 1987; Lanir, Jenkins et al. 1988). However, other studies showed that the resynthesis of adenosine 5'-triphosphate (ATP) by mitochondrial phosphorylative activity, rather than

post-ischaemic ATP levels, determines the organ's capacity to recover from ischaemic injury (Kono, Ozawa et al. 1982; Okamura, Tanaka et al. 1992) . Others showed that respiratory chain defects have been identified as early events in models for ischaemia-reperfusion (Rouslin 1983; Nohl, Koltover et al. 1993). Therefore, an objective assessment of mitochondrial respiratory chain activity may provide information directly related to graft's functional potential and/or viability.

Oxidation of nicotinamide adenine dinucleotide (NADH) is the first step of the mitochondrial respiratory chain and this is carried out by complex I. The mitochondrial $NADH/NAD^+$ ratio is determined by the relative rates of NADH oxidation through oxidative phosphorylative activity and NAD^+ reduction through intermediary metabolism. This redox state of mitochondrial NADH is associated with the respiratory state of the mitochondria. The different fluorescence properties of NADH and NAD^+ permit the measurement of NADH redox state changes. NADH fluoresces with a broad emission band (centered at ± 470 nm) when excited with ultraviolet light, whereas NAD^+ does not fluoresce. As the ultra-violet light-induced blue fluorescence from intracellular pyridine nucleotides in kidney is predominantly of mitochondrial origin, NADH fluorimetry can be used to monitor kidney cortex metabolism non-invasively (Franke, Barlow et al. 1980; Balaban and Sylvia 1981).

Changes in steady-state levels of NADH fluorescence have been associated with kidney and liver graft viability after hypothermic storage and transplantation (Thorniley, Lane et

al. 1994), with lower rates of post-ischaemic NADH fluorescence decrease observed after ischaemia/reperfusion damage in transplanted kidneys (Thorniley, Lane et al. 1994). These observations indicate that mitochondrial respiratory activity is impaired in transplanted organs. In isolated perfused rat livers, hepatic energy charge and decelerated NADH fluorescence kinetics correlated closely with survival rates after transplantation (Okamura, Tanaka et al. 1992).

Experimentally, post-ischaemic changes in mitochondrial morphology associated with falling ATP levels in rat proximal tubular cells have suggested that impairment of oxidative phosphorylation could affect cellular viability (Trump, Berezesky et al. 1982). By indirect assessment, mitochondrial Complex I activity of rat surface tubules has been shown to correlate with subsequent renal function and preservation of cellular morphology (Coremans, Van Aken et al. 2000). Cold storage of a porcine proximal tubular cell line in culture for 20h has been associated with a gradual loss of mitochondrial Complex I activity (Ahlenstiel, Burkhardt et al. 2003). Trimetazidine, a drug known to protect rat liver mitochondria from ischaemic injury, improved the survival and renal function of porcine recipients of autografts cold-stored for 48h (Hauet, Goujon et al. 2000). When another drug (trifluoperazine) with the potential to protect the inner mitochondrial membrane was added to machine perfusate of canine kidneys, immediate post-implantation blood flow through the graft was improved (Hernandez, Light et al. 1999), however no advantage was demonstrated in a similar study of machine perfused human cadaveric kidneys (Polyak, Arrington et al. 2000). In human beings, mitochondrial dysfunction in the critically ill has

been associated with a poor outcome while possession of a mitochondrial DNA haplotype (Brealey, Brand et al. 2002) associated with enhanced mitochondrial chain activity doubles the probability of survival (Baudouin, Saunders et al. 2005).

On balance, available evidence suggests that mitochondrial function could be a determinant of organs functional potential and viability. It may determine subsequent recovery of function of human renal allografts. The purpose of the study in chapter 2 is to examine this possibility.

1.5 Pathology of IF/TA [CAN]

Hume and colleagues (Hume, Merrill et al. 1955) were the first to describe the histopathological features indicative of what is now called IF/TA. They described changes affecting the vessels, tubules, glomeruli and the interstitium in the graft. The description of the histopathology has changed little since then. Studying biopsies from 128 patients 2 years after transplantation, Isoniemi et al found that even in grafts with stable function, histopathological changes consistent with those of CAD could be seen (Isoniemi, Krogerus et al. 1992). The principal histopathological features of CAD involve vascular endothelium, renal tubules, interstitium, and glomeruli. The vessels demonstrate a spectrum of changes ranging from subendothelial inflammation, through intimal hyperplasia, and ultimately atherosclerosis. These vascular changes are associated with a mononuclear cell and T-cell infiltrate (Mason and Morris 1986).

The changes seen in glomeruli are thought to be ischaemic in origin with hypocellularity and atrophy. Glomerular lesions, often described together as transplant glomerulopathy, may demonstrate mesangial cellular and matrix expansion, thickening and duplication of the basement membrane, and glomerulosclerosis. Segmental glomerulosclerosis and hyalinization with periglomerular fibrosis eventually occurs. In the tubular compartment, tubular vacoulation and basement membrane thickening are the first changes to be seen, which is followed by loss of tubular structure and tubular atrophy, which is followed by the development of interstitial fibrosis. Concentric intimal fibrosis with vascular luminal obliteration in intralobular and arcuate arteries results from smooth muscle cell replication and fibromuscular proliferation with increasing intimal thickening and medial atrophy. Increased interstitial cellular infiltrates and oedema can develop as interstitial fibrosis progresses. Finally, vessels will demonstrate a concentric proliferative intimal thickening leading to vessel occlusion and fibrosis. Variable degrees of internal elastic lamina disruption and or re-duplication may also be seen.

The injury processes associated with vascular changes, interstitial fibrosis and mesangial expansion are secondary to over-accumulation of abnormal extracellular matrix. Extracellular matrix is secreted due to a phenotypic transformation of smooth muscle cells into the endothelial media in vessels, myofibroblasts in the interstitium, and mesangial cells in the glomeruli (Furness 2001; Waller and Nicholson 2001).

1.5.1 CADI Scoring

Isoniemi et al (Isoniemi, Krogerus et al. 1992) proposed a “chronic allograft damage index” (CADI) to allow objective assessment of disease progression. The CADI is calculated as the sum of the scores of six histopathological indices: diffuse/focal inflammation, interstitial fibrosis, mesangial matrix increase, glomerular sclerosis, vascular intimal proliferation, and tubular atrophy. In a further study with six years follow up, this group demonstrated a relationship between the CADI score at 2 years and risk of developing CAD (Isoniemi, Taskinen et al. 1994). The CADI score was also found to correlate significantly with transplant function at this time point.

1.5.2 Banff Classification

The historic lack of uniformity in the diagnosis and classification of CAD led, in 1991, to a group of renal pathologists, nephrologists and transplant surgeons meeting in Banff, Canada to develop a schema for standardization of the nomenclature and criteria for the histologic diagnosis of renal allograft rejection/CAD (Solez, Axelsen et al. 1993). The schema was validated by circulating sets of slides for scoring by participant pathologists. Glomerular, interstitial, tubular and vascular lesions of acute rejection and "chronic rejection" were defined and scored 0 to 3+, to produce an acute and/or chronic numerical coding for each biopsy; arteriolar hyalinosis (an indication of cyclosporine toxicity) was also scored. The principal diagnostic categories, which could be used with or without the quantitative coding, were: normal, hyperacute rejection, borderline changes, acute rejection

(grade I to III), (5) chronic allograft nephropathy ("chronic rejection") (grade I to III), and (6) other (Fisher 1998). Freese *et al* reported that despite a low Banff score indicating better graft survival, for single components of the schema, only interstitial fibrosis was a statistically significant predictor for graft survival (Freese, Svalander et al. 2001).

Nankivell et al. (2003) reported a study on 961 renal transplant biopsies from simultaneous kidney pancreas recipients taken over a long period post-transplantation. Their data clearly demonstrated two distinct histological phases of CAD. The features of CAD within the first year after transplantation demonstrated rapidly increasing Banff scores for interstitial fibrosis and tubular atrophy. These histological appearances were found in 94.2% of biopsies at one-year post-transplant (Nankivell, Borrows et al. 2003). These early features of CAD were also reported in other studies (Freese, Svalander et al. 2001; Sijpkens, Doxiadis et al. 2003). After the first year post-transplantation, arteriolar hyalinisation, vascular narrowing and progressive glomerular sclerosis were seen with increased prevalence; for example, glomerulosclerosis was present in 37.3% of biopsies by 10 years (Nankivell, Borrows et al. 2003). The presence of two distinct phases of the histopathological appearances of CAD has led investigators to believe that these findings may represent two different aetiopathologic processes: first, an allogeneic response in the early phase (acute and/or sub-clinical rejection); second, ischaemic insults such as CNI toxicity and hypertension in the majority of cases (Nankivell, Borrows et al. 2003).

1.5.3 Histopathological Features of CNI Toxicity

Calcineurin inhibitor nephrotoxicity occurs without plasma levels of CNIs above the therapeutic range. It is, therefore, very important to characterize the morphological appearance of CNI nephrotoxicity on renal biopsy. In the acute setting, CNI nephrotoxicity is characterised by isometric vacoulation of the epithelial tubules (Furness 2001). The histological indicators of chronic CNI-induced nephrotoxicity are hyaline arteriolopathy, striped interstitial fibrosis and tubular atrophy (Mihatsch, Antonovych et al. 1994).

1.5.3.1 Acute CNI-nephrotoxicity

The features of acute CNI-related nephrotoxicity can range from acute tubular necrosis (Solez, Racusen et al.) and toxic tubulopathy to vascular toxicity (Mihatsch, Antonovych et al. 1994). The morphological features include dilation and flattening of tubular profiles, loss of brush borders, necrosis of individual tubular epithelial cells, cytoplasmic basophilia and enlarged regenerative nuclei. Desquamation of tubular epithelial cells into the tubular lumen might also be present. Tubular changes are accompanied by interstitial edema and sparse lymphocytic infiltrates. In this context, the biopsy findings of CNI-induced nephrotoxicity are similar to those of post-transplantation ATN, and are thus difficult to differentiate.

1.5.3.2 Toxic Tubulopathy

Patients with CNI-induced toxic tubulopathy display acute allograft dysfunction, usually associated with an elevated serum drug level. Morphologically, isometric tubular vacuolation is observed focally, predominantly affecting the proximal straight tubules; the vacuoles are small, clear and evenly distributed throughout the cytoplasm. Ultrastructurally, the vacuoles correspond to dilations of the endoplasmic reticulum. Focal tubular calcification might also be present.

1.5.3.3 Vascular Toxicity

Drugs can have a direct toxic effect on endothelial cells, with or without the involvement of platelet aggregation. Two types of vascular toxicity have been identified: acute arteriolopathy and thrombotic microangiopathy (Busauschina, Schnuelle et al. 2004).

1.5.3.4 Acute Arteriolopathy

In patients with acute allograft dysfunction, blood level of CNI is usually elevated and the lesions are confined to afferent arteriolar vessels. There is swelling and vacuolation of endothelial cells, vacuolation, necrosis and/or apoptosis and sometimes dropout of individual myocytes, and early replacement of damaged myocytes with rounded plasma protein insudates (hyalinization) (Toki, Kyo et al. 2000). Immunohistochemically, the insudates are positive for IgM and C3. Acute arteriolopathy and toxic tubulopathy can co-exist.

1.5.3.5 Thrombotic Microangiopathy

The biopsy shows thrombotic microangiopathy (TMA), characterized by thrombi in the arteriolar lumens and glomerular capillaries (Randhawa, Tsamandas et al. 1996). Although pronounced arterial intimal changes are atypical of CNI-induced TMA, it is important to note that CNI-induced TMA cannot be differentiated from other forms of TMA on the basis of morphology alone.

1.5.3.6 Chronic CNI Nephrotoxicity

Chronic CNI nephrotoxicity was identified (in the Westmead histology study) as occurring at a mean of three years after transplantation. It worsened with time, until striped cortical fibrosis, severe circumferential and nodular arteriolar hyalinosis, and tubular calcification were present in the vast majority of patients who had used cyclosporine for 10 years. 50% had Banff criteria of grade III CAN by this time post-transplantation (Nankivell, Borrows et al. 2003). The authors showed a correlation between these features and subsequent glomerular sclerosis (Nankivell, Borrows et al. 2004), which occurred several years later. Two-thirds of the patients had significant interstitial fibrosis within the first year of transplantation. The amount of interstitial fibrosis at one year, scored using the Banff schema for interstitial fibrosis, correlated significantly with the degree of early glomerular sclerosis in that same kidney between one and four years later (Nankivell, Borrows et al. 2004). Glomerular sclerosis also correlated with the degree of arteriolar hyalinosis, further

supporting the hypothesis that microvascular injury is associated with ischaemic glomerulosclerosis (Nankivell, Borrows et al. 2004).

The morphological features of chronic CNI-nephrotoxicity are characterised by arteriolar hyalinisation, usually affecting the afferent arterioles (Davies, Bittmann et al. 2000) and characteristic "striped" interstitial fibrosis (Mihatsch, Morozumi et al. 1995; Mihatsch, Ryffel et al. 1995); these changes are neither specific nor diagnostic for CNI toxicity. Although interstitial fibrosis and mild arteriolar hyalinosis are also seen in other disease processes, such as diabetes, hypertensive and obstructive nephropathy, more severe arteriolay hyalinosis may be restricted to CNI toxicity alone.

1.5.3.7 Hyaline Arteriolopathy

An analysis of histological changes in native kidneys of heart and bone marrow recipients showed that moderate and severe arteriolar hyalinosis was observed only in patients on CNI therapy (Nizze, Mihatsch et al. 1988). Progressive or *de novo* arteriolar hyalinosis (AH) is the most pathognomonic lesion of chronic CNI nephrotoxicity (Solez, Racusen et al. 1993; Young, Burdmann et al. 1995). Morozumi et al. (Morozumi, Thiel et al. 1992) found that, in many cases, regression was seen even with severe arteriolopathy, several months after stopping or reducing the dose of cyclosporine (CsA). Arteriolar lesions consist of vacuolization of endothelial and smooth muscle cells, and focal or circular lumpy protein deposits in the arteriolar wall which usually replace necrotic smooth muscle cells which

eventually narrow the vascular lumen (Mihatsch, Thiel et al. 1988; Mihatsch, Ryffel et al. 1993). This lesion may involve the entire circumference of the vessel wall or may be noncircular (Morozumi, Thiel et al. 1992). At later stages, severe hyaline thickening with narrowing of the vascular lumen leads to hypoxia and irreversible damage such as focal segmental glomerular sclerosis, tubular atrophy and interstitial fibrosis (Mihatsch, Ryffel et al. 1995).

Hyaline arteriolopathy is probably important for the development of interstitial fibrosis and tubular atrophy (Strom, Thiel et al. 1994; Racusen, Rayner et al. 1995). Damaged smooth muscle cells are replaced by beaded hyaline deposits on the outer aspect of the media that bulge into the adventitia; beaded medial hyalinosis is focal and can be overlooked. In advanced cases, the entire wall is replaced by the hyaline material and the lumen is severely narrowed. Immunohistochemistry detects IgM and C3 at the sites of insudation. Staining occurs in a 'pearl necklace' pattern. In the Banff classification of renal allograft biopsies, arteriolar hyalinosis (AH) is quantitatively graded as mild, moderate and severe, according to the intensity and the distribution of the lesions (Racusen, Rayner et al. 1995). Reproducibility studies on AH scoring using Banff criteria showed substantial to poor inter-observer agreement (Marcussen, Olsen et al. 1995) (Gough, Rush et al. 2002) (Furness, Taub et al. 2003). This inter-observer variation is probably not only related to the method used but also may be partly explained by the focal and patchy nature of the arteriolar lesions or by the variation in severity of damage from one arteriole to the other, which makes the examination of every arteriole in the renal biopsy crucial. Strom et al.

(Strom, Thiel et al. 1994) observed that a maximum of 24.9% of arterioles showed hyaline thickening at 90 days post-transplant in a total of 557 renal biopsies.

The differential diagnosis of CNI toxicity associated arteriolopathy includes diabetes, hypertension, thrombotic microangiopathy, and age-related arteriolar hyalinosis. The changes of CNI toxicity related arteriolar hyalinosis are very similar to those that may be found in advanced cases of thrombotic microangiopathy, independent of CNI treatment. According to Mihatsch and colleagues (Morozumi, Thiel et al. 1992; Mihatsch, Ryffel et al. 1993; Mihatsch, Ryffel et al. 1995), CNI-associated arteriolar hyalinosis can be distinguished from hypertensive or diabetic hyalinosis by the presence of subendothelial deposits that are situated inside well-preserved smooth muscle cells, in comparison to the deposits in CNI-related arteriopathy which are focal or circular and replace necrotic smooth muscle cells. However, in advanced cases of hypertensive or diabetic arteriolopathy, or in late stages of CNI-associated arteriolopathy, differentiation may be difficult.

1.5.4 Interstitial Fibrosis and Tubular Atrophy

In chronic CNI-induced nephrotoxicity, the interstitium shows prominent patchy fibrosis and corresponding tubular atrophy with preferential and early involvement of the medullary rays, which results in a band-like pattern. This striped pattern is characteristic of, but not specific to, chronic CNI-induced nephrotoxicity; hypertensive kidney disease can produce a similar lesion.

1.5.5 Glomerular Changes

The effects of chronic CNI-induced toxicity on glomeruli tend to be nonspecific. Focal segmental glomerulosclerosis seems to result from hyperfiltration in response to loss of functioning nephrons, rather than being a direct effect of toxicity to podocytes and/or endothelial cells. This should be distinguished from recurrent or *de novo* idiopathic FSGS. The presence of hyaline arteriolopathy, striped fibrosis and tubular atrophy with or without isometric vacuolation of proximal straight tubules, and absence of full-blown nephrotic syndrome are helpful pointers in this setting.

1.6 Aetiopathogenesis of CAD

Chronic allograft damage is a sequential multifactorial process caused by a series of time-dependent insults **(Figure 1.7)**. In healthy adults with functioning kidneys the clinical manifestations of renal failure occur after more than 70% of nephron mass has been lost, whereas the functional reserve is much smaller in transplant recipients. Nephron loss occurs as part of generic process of senescence, and can be accelerated by any number of insults including the process of transplantation itself. The factors that promote allograft injury can be divided into two groups: allo-antigen dependant or allo-antigen independent **(Figure 1.8)**. Alloantigen independent mechanisms are now accepted as major contributory factors to graft loss; their involvement potentially accelerates natural cell senescence (Feehally, Harris et al. 1986; Nankivell and Chapman 2006). However it is important to emphasise

that both allo-antigen dependent and allo-antigen independent pathways contribute to progressive damage towards graft loss **(Figure 1.7)**.

Figure 1.7: Factors involved in the development of CAD

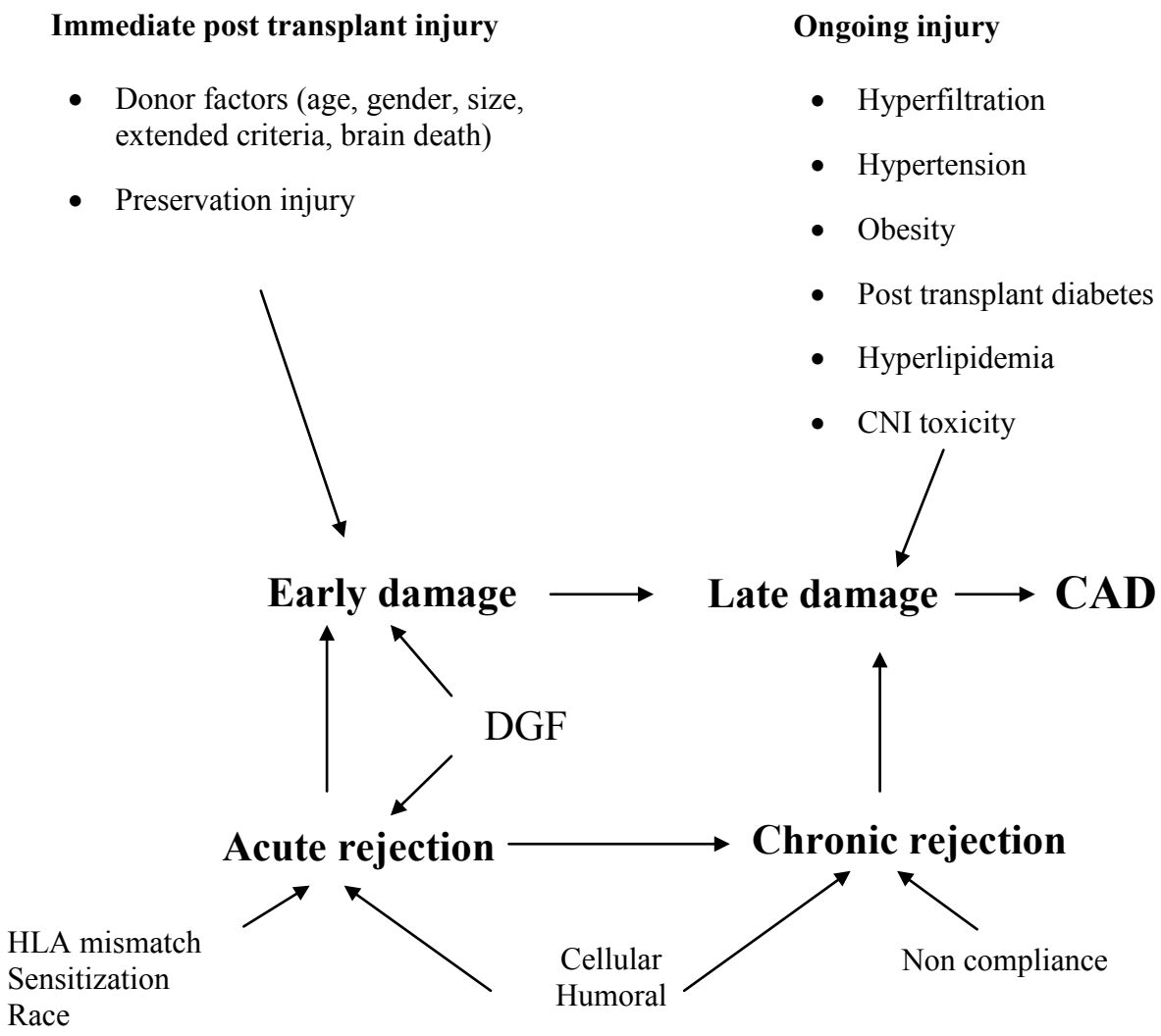


Figure 1.7: Schematic presentation of factors involved in the development of chronic allograft damage.

Figure 1.8: Initiation and pathogenesis of Chronic Allograft Dysfunction

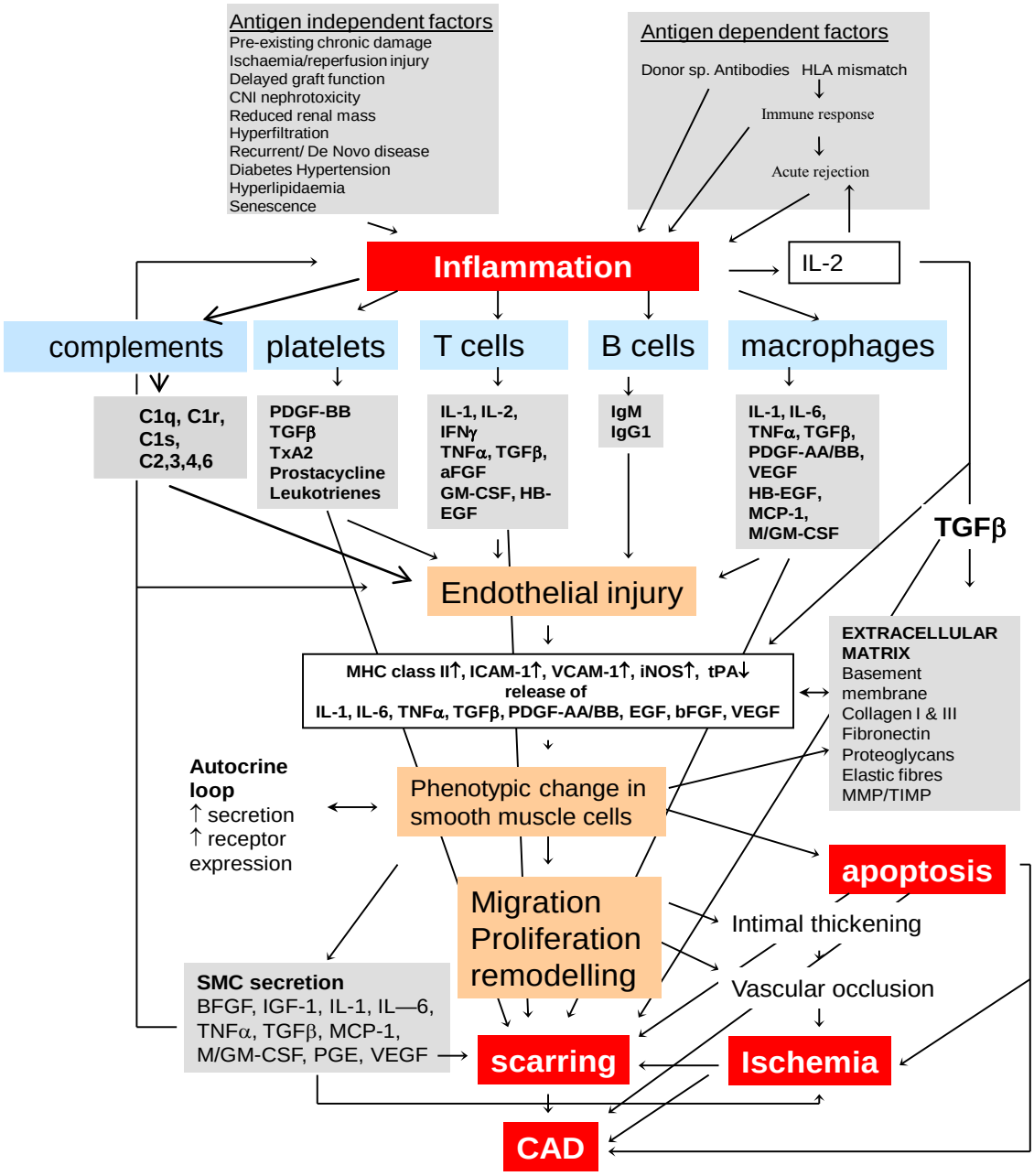


Figure 1.8: Summary of the biological factors involved in the initiation of the pathogenic process of chronic allograft dysfunction and the cellular and molecular mediators that are subsequently involved.

1.6.1 Evolution of CAD: Modes of Injury

Whilst the pathogenesis of CAD and the impact of the various aetiological risk factors in its progression is not clearly understood, most researchers agree that the primary injury occurs to the endothelium of the graft with a subsequent inflammatory response. However, the exact time scale of this injury has not been defined.

1.6.1.1 Inflammation

The presence of persistent inflammation in renal allografts that already display chronic lesions is associated with a higher probability of progression to end-stage renal disease (Moreso, Ibernon et al. 2006). A marked infiltration of inflammatory cells (predominantly macrophages and lymphocytes) is present in protocol biopsies after renal transplantation. Inflammation may be a response to injuries caused from various insults, however it may amplify the potential for rejection, or may itself provide an additional stress on the tissue which accelerates senescence and triggers fibrosis.

1.6.1.2 Chronic Ischaemia/ Hypoxia

Endothelial injury can occur during the initial post-transplant period as a result of ischaemia/reperfusion insult, acute rejection, sub-clinical rejection or host hypertension. It is hypothesized that endothelial injury and subsequent repair results in end organ ischaemia (Foegh 1990; Mihatsch, Ryffel et al. 1993). Once endothelial damage has occurred, regardless of its cause, an aberrant repair process may lead to graft arteriosclerosis, which

may lead to a significant loss of peri-tubular capillaries (Ishii, Sawada et al. 2005). Loss of peri-tubular capillaries leads to tissue hypoxia, which in turn initiates inflammation and oxidative stress. Under hypoxic or anoxic conditions cellular metabolism generates reactive oxygen species (ROS), which escalates damage. Recent studies using blood oxygen level-dependent (BOLD) MRI, have shown decreased intrarenal oxygenation in human kidney allografts with CAD (Djamali, Sadowski et al. 2007). In animal CAD models, it has been shown that tissue markers of hypoxia such as HIF-1α and HSP 27 are elevated (Djamali, Reese et al. 2005; Djamali, Reese et al. 2005).

1.6.1.3 Oxidative Stress

Oxidative stress in renal allografts may be induced by inflammation, abnormal tissue oxygenation, immunosuppressant drugs, and by comorbid clinical conditions including diabetes, hypertension, proteinuria, anemia, and dyslipidemia. Oxidative stress-induced chronic renal damage is associated with signaling pathways including inflammation, apoptosis, hypoxia, and epithelial-to-mesenchymal transition. Most of these injury pathways participate in a self-perpetuating cycle with oxidative stress. However, available data demonstrate a correlation but no causal relationship. Systemic biomarkers of oxidative stress are increased in kidney transplant recipients, similar to patients with native CKD. Reactive oxygen species (ROS) play an important role as signaling and regulatory molecules in cell proliferation, differentiation, and apoptosis (Gutierrez, Ballinger et al. 2006), and a pro-oxidant milieu can alter and denature nucleic acids, carbohydrates, lipids, and proteins, resulting in cell toxicity. The deleterious effects of oxidative stress have long

been reported in the pathophysiology of chronic kidney diseases (CKD) (Himmelfarb and McMonagle 2001) (Himmelfarb, Stenvinkel et al. 2002) (Oberg, McMenamin et al. 2004). In vitro studies have suggested that ROS play an important role in TGF-β1-induced epithelial-mesenchymal transition (Barr, Cohen et al.) of proximal tubular epithelial cells (PTECs) through activation of MAPK and Smad pathways (Rhyu, Yang et al. 2005).

Oxidative stress can contribute to tubular atrophy through apoptosis (Cho, Hunt et al. 2001; Basnakian, Kaushal et al. 2002; Allen, Harwood et al. 2003) and inflammation (Cho, Hunt et al. 2001; Vaziri and Rodriguez-Iturbe 2006). Oxidative stress can also result in activation of proinflammatory pathways including c-Jun NH_2-terminal kinase, p38-MAPK, nuclear factor-κB. The resulting inflammation, in turn, leads to ROS production by leukocytes and resident cells, which becomes a chronic self-perpetuating form of injury. Chronic hypoxia is another potential mechanism of oxidative stress-mediated chronic injury in the allograft (Nangaku 2006).

1.6.1.4 Apoptosis/ Senescence

Stress-induced accelerated aging and cellular exhaustion has been postulated as a mechanism of graft loss, supported by substantially reduced actuarial graft survival with an older donor age (Halloran P 1999). Shortened telomeres have been observed in native and transplanted older kidneys, and with human CAD (Ferlicot, Durrbach et al. 2003). In renal transplants, the presence of apoptosis is described in allograft rejection (Matsuno, Nakagawa et al. 1994) (Ito, Kasagi et al. 1995; Wever, Aten et al. 1998) as well as

cyclosporine nephrotoxicity (Ito, Kasagi et al. 1995). It is also noticed in associated with ischaemia-reperfusion injury and acute tubular necrosis (Matsuno, Nakagawa et al. 1994) (Oberbauer, Rohrmoser et al. 1999). Apoptosis is regulated by various internal mechanisms such as the expression of pro-apoptotic and anti-apoptotic proteins, such as bax and bcl-2, which are up-regulated in regenerating renal tubules following ischaemia (Basile, Liapis et al. 1997).

1.6.1.5 Tubular Epithelial Cells and Scarring

Tubular epithelial cells (TECs) contribute to renal fibrogenesis. The TECs function as a source of fibrogenic growth factors and chemokines in the initiation of fibrogenesis, contribute to tubular atrophy by undergoing apoptosis and potentially contribute through epithelial-mesenchymal transition (Barr, Cohen et al.) (Zeisberg, Strutz et al. 2001). Following tubular injury with loss of cell-to-cell adhesion, a series of orchestrated steps transforms tubular epithelial cells into myofibroblasts of mesenchymal phenotype, influenced by hypoxic injury and TGF-β1 (Liu 2004). A reduction in E-cadherin then eliminates adherens junctions formation, disturbs cell polarity and may lead to increased proliferation and transdifferentiation (Liu 2004). Interstitial fibrosis/ scarring (IF) is a hallmark of CAD and is strongly associated with tubular atrophy (TA). When established, interstitial scarring is usually accompanied by proteinuria, hypertension, impaired renal function and reduced graft survival. The increased extracellular matrix deposition that underlies the progression of scarring is a dynamic network of proteins and proteoglycans, which accumulates as a result of both increased synthesis and decreased breakdown,

mediated by factors including TGF-β1, angiotensin and immunosuppression. CNIs are also fibrogenic: for example, cyclosporine generates a pro-fibrotic cytokine profile with TGF-β 1 and TIMP-1, leading to interstitial fibrosis.

In summary, all the the above factors can cause damage independently of one another, but are nonetheless strongly interlinked. Figure 1.8 summarizes the pathways that can lead to the development of chronic allograft failure.

1.6.2 Impact of Rejection on CAD

1.6.2.1 Acute Rejection

There is a clear correlation between acute rejection and the progression of CAD (Almond, Matas et al. 1993). Acute rejection modulates tissue remodelling and causes graft damage. Using US registry data from over 80,000 renal transplants, Hariharan et al (Hariharan, Alexander et al. 1996) found that in patients experiencing one or more episodes of acute rejection the projected graft survival was reduced to almost 50% of patients remaining free of acute rejection. Multiple and late rejection episodes were particularly damaging.

Kidney damage caused by acute rejection appears to be mediated by cytokine and growth factor production by infiltrating cells, particularly macrophages (Viklicky, Matl et al. 1999). Worse outcomes are observed after multiple acute rejection episodes (Montagnino,

Tarantino et al. 1997; Viklicky, Matl et al. 1999), severe rejection, steroid unresponsive rejection and late acute rejection (Flechner, Modlin et al. 1996). Conversely, if successfully treated acute rejection where renal function returned to normal is not associated with increased rates of late graft loss (Opelz 1997; Vereerstraeten, Abramowicz et al. 1997).

Matas and colleagues reported on a series of 653 primary renal transplant recipients, where the occurrence of a single rejection episode in the first year reduced graft half life (45 +/- 11 years in those with no rejection vs. 25 +/- 8 years in those with 1 rejection episode in the first year). Multiple rejections (t1/2 = 5 +/- 11 years) and a first rejection after the first year (t1/2 = 3 +/- 1 years) have a significant effect on graft survival ($P < .05$) (Matas, Gillingham et al. 1994). Knight and Burrows found that occurrence of one or more episodes of acute rejection decreased 5 year survival in both living donor transplants and cadaveric transplants; the percentage of 5-year survival is 90% and 88% for rejection free live and cadaveric donors respectively, and 73% and 40% for those experiencing one or more episodes (Knight, Burrows et al. 2001). Many other studies have found similar evidence on the effect of acute rejection on graft outcome (Sumrani, Cacciarelli et al. 1993; Tesi, Elkhammas et al. 1993; Humar, Payne et al. 2000; Matas 2000; Messias, Eustace et al. 2001). In all these studies the effects of one or more episodes of acute rejection is associated with a poorer outcome with respect to graft survival.

1.6.2.2 Subclinical Rejection

If a protocol biopsy is performed in all patients following a transplant, histological features of rejection are present in some patients despite no evidence of renal dysfunction. This has

been labelled subclinical rejection (SCR) and the incidence varies between studies. Nankivell and colleagues reported the incidence on serial renal protocol biopsies and detected SCR rates of 60.8% at 1 month post transplantation to 25.5% at 1 year (Nankivell, Borrows et al. 2004). In a Canadian series, incidence of SCR on protocol biopsy is between 15 and 43% depending upon the immunosuppressive regime (Nickerson, Jeffery et al. 1999) with the majority of cases being mild according to the Banff classification (Rush, Jeffery et al. 1995). Roberts et al reported lower rates on protocol biopsy at day seven in recipients with stable renal function: 13% showed acute rejection and 12% showed borderline changes, and at day 28, 8% showed acute rejection and 16% showed borderline changes, (Roberts, Reddy et al. 2004). Shapiro et al reported the incidence of subclinical borderline or frank tubulitis is 46% in patients without DGF and improving graft function on week one protocol biopsies (Shapiro, Randhawa et al. 2001).

Shishedo and colleagues studied patients without acute rejection on protocol biopsies compared with those with histological evidence of SCR: they reported increased CAD and lower creatinine clearance at three and five years post-transplant (75 vs 63 at three years and 70 vs 56 at five years), as well as worse actuarial graft survival at five and ten years (5 year survival: 100 vs 75%; 10 year survival, 93 vs 51%) (Shishido, Asanuma et al. 2003). The detrimental effects of SCR are also supported by reports of successful treatment. Rush et al (Rush, Jeffery et al. 1995) found that treatment of SCR with pulsed steroids resulted in superior outcomes at 1 year than a control group of non-biopsied patients (87% vs 72% graft survival).

1.6.2.3 Chronic Rejection (chronic antibody mediated rejection)

Chronic rejection due to allo-immunity in the allograft may be cell- or antibody mediated. Protocol biopsies, performed at predefined times after transplantation, regardless of graft function at the time of biopsy, have contributed significantly to our understanding of the mechanisms and the clinical course of chronic rejection. Specific pathological features can be identified to make a definitive diagnosis of chronic rejection (Solez, Colvin et al. 2007). Nankivell and colleagues proposed that true cell-mediated chronic rejection was SCR persisting for at least 2 years (Nankivell, Borrows et al. 2003). Lymphocytic tubulitis or arteritis signals alloimmune injury, whilst chronic transplant glomerulopathy, formation of neo-media and neo-intima inside the vessel are reliable signs of chronic rejection. Microvascular injury is typical of antibody-mediated processes (Racusen, Colvin et al. 2003). Both chronic transplant glomerulopathy (CTG) and peri-tubular capillary basement membrane multilayering are associated with positive staining for C4d in peri-tubular capillaries (Regele, Bohmig et al. 2002; Sis, Campbell et al. 2007). Significant progress has been made in the field of renal transplantation since Kissmeyer et al's pioneering observation of the deleterious impact of allo-antibodies in renal grafts in the 1960s (Kissmeyer-Nielsen, Olsen et al. 1966). In 1992, Halloran and colleagues described rejection episodes following renal transplantation related to the presence of anti-HLA donor-specific antibodies (DSA) (Halloran, Schlaut et al. 1992). Many studies linking the presence of circulating anti-human lymphocyte antigen (HLA) antibodies to impaired long-term outcome indeed support the concept of chronic antibody mediated rejection (CAMR) (Kerman, Orosz et

al. 1997; Piazza, Poggi et al. 2001; Worthington, Martin et al. 2003; Terasaki and Ozawa 2005; Piazza, Poggi et al. 2006). Other studies investigating transplant biopsies for the concurrent presence of alloantibody and CTG (the morphological hallmark of chronic rejection) have also supported an association between alloantibody and chronic rejection (Mauiyyedi, Pelle et al. 2001; Regele, Bohmig et al. 2002; Cosio, Gloor et al. 2008).

1.6.3 Calcineurin Inhibitor Nephrotoxicity

In 1983, a new class of immunosuppressive agents - calcineurin inhibitors (CNIs) – were introduced, which dramatically improved renal allograft survival rates in the first year following transplantation. The two agents in this class, cyclosporine and tacrolimus, work by inhibiting calcineurin via different effector molecules, cyclophylin and Tacrolimus binding protein (FK-BP) respectively. Calcineurin inhibition blocks up regulation and activation of T cells mediated mainly by IL-2, but also by IL-4, tumour necrosis factor alpha (TNFα) and gamma interferon (γIFN). Although the exact mechanism of nephrotoxicity is not fully understood, several factors have been implicated in the pathogenesis of CNI-induced nephrotoxicity. Renal and systemic and intra-renal (afferent arteriolar) vasoconstriction, increased release of endothelin-1, decreased production of nitric oxide and increased expression of TGF-beta are the major adverse pathophysiologic abnormalities of these agents.

Cyclosporine was first used at a dose of 25 mg/kg/day in pilot human renal transplant recipients, based upon large animal experimental data (Calne, White et al. 1978), but was rapidly found to be both nephrotoxic and to have a range of non-nephrotoxic side effects, such as hypertension, hyperlipidemia, glucose intolerance, hirsutism and gingival hypertrophy, so the dose was then reduced to 10 mg/kg/day. At this dose, however, monotherapy was found to provide inadequate immunosuppression (Calne, Rolles et al. 1979) and a starting dose of 17.5 mg/kg/day was thus used in the early phase II/III clinical studies (Morris, Chapman et al. 1987). This early experience changed the clinical practice of transplantation, since acute rejection became less common and a differential diagnosis of nephrotoxicity had to be considered for renal dysfunction. The functional impact of acute CNI nephrotoxicity was well described in the early formal randomized clinical trials (Morris, Chapman et al. 1987; Hall, Tiller et al. 1988) with impaired tubular function, higher serum urate levels and altered potassium handling. Hypertension and a reduction in GFR were seen in these cases, but rapid recovery to baseline renal fuction levels was observed after cessation of the drug. Synergisitic toxicity with non-steroidal anti-inflammatory agents indicated the vascular nature of the functional insult caused by CNIs.

1.6.3.1 Acute CNI Nephrotoxicity

Studies in animal models show that in the acute setting CNIs can cause afferent arteriolar vasoconstriction, leading to a drop in glomerular filtration rate and elevation of serum creatinine level in the absence of structural abnormalities in the biopsy specimen. This functional toxicity is often reversed by lowering the dose of the drug. Acute CNI-related

nephrotoxicity can take the form of functional toxicity that can delay the recovery from post-transplantation acute tubular necrosis (Solez, Racusen et al.).

Calcineurin inhibitors can also have a direct toxic effect on endothelial cells, causing an acute arteriolopathy or a thrombotic microangiopathy. Patients with acute arteriolopathy present with acute allograft dysfunction in the setting of elevated plasma drug levels. The clinical outcomes of CNI-induced acute arteriolopathy can be variable. Some patients recover transplant function after lowering the CNI dose, while in others renal damage is irreversible.

Thrombotic microangiopathy (TMA) is a rare manifestion of CNI-related vascular nephrotoxicity. The clinical picture resembles hemolytic–uremic syndrome. CNI-induced TMA cannot be differentiated from other forms of TMA on the basis of morphology alone. Therefore, a set of differential diagnoses, including acute humoral rejection (C4d positivity), bacterial or viral infection, recurrent hemolytic–uremic syndrome and anticardiolipin antibody-induced TMA should be considered.

Toxic tubulopathy is observed with acute allograft dysfunction and elevated serum drug levels. Due to the lowering of maintenance CNI doses over the past decade, the incidence of tubulopathy has fallen significantly.

1.6.3.2 Chronic CNI Nephrotoxocity

The histological picture of chronic evolving CNI nephrotoxicity was defined both in transplanted and native kidneys; the widely agreed hallmarks are striped or diffuse interstitial fibrosis, nodular arteriolar hyalinosis and tubular calcification (Mihatsch, Thiel et al. 1988). The concern that chronic CNI nephrotoxicity was a major long-term problem was reinforced in the mid-1990s through follow-up trials of CNI treated patients (Hollander, van Saase et al. 1995). Shorter-term histopathological studies, such as that derived from a randomized trial of cyclosporine and tacrolimus, also showed that 60–70% of patients in both groups had chronic histological damage at 2 years after transplantation (Solez, Vincenti et al. 1998). Definitive evidence that CNI nephrotoxicity impacted graft survival has, however, been elusive, but two analyses of Australian data, both registry and long-term follow-up of an early randomised controlled study, clearly showed worse long-term outcomes in those maintained on CNI compared with those maintained without CNI [Mathew TH, McDonald SP et al.2001(ANZDATA registry report)(Gallagher, Hall et al. 2004)]. Nankivell et al reported (Nankivell, Borrows et al. 2003; Nankivell, Borrows et al. 2004) on a group of simultaneous kidney-pancreas recipients who underwent protocol renal transplant biopsies over an extended period of time, which further characterised the extent of CNI nephrotoxicity and fuelled the debate over how to manage CAN in general and in particular the CNI component of CAN (Chapman, O'Connell et al. 2005). It is now clear that renal damage is grossly underestimated by normal serum creatinine early on, and the damage becomes largely irreversible by the time the serum creatinine starts to climb above the baseline. In the absence of a transplant biopsy it remains extremely difficult to establish

the diagnosis of chronic CNI nephrotoxicity. The main clinical conditions to be differentiated from chronic CNI-induced nephrotoxicity are chronic rejection, *de novo* or recurrent glomerulonephritis, and polyoma virus nephropathy.

Certainly CNIs have played an important role in improving graft survival, mainly due to a reduction in acute rejection episodes, but this has been at the expense of CNI nephrotoxicity. Calcineurin inhibitor nephrotoxicity is a very important cause of CAD, especially after the first year post-transplant (Nankivell, Borrows et al. 2003; Nankivell, Borrows et al. 2004). The balance between preventing immunologic allograft loss and the management of CNI-related nephrotoxicity is still an issue in renal transplantation. Historical reports from the early CsA era showed that conversion from CsA to azathioprine (AZA) at 1 year after renal transplantation resulted in improvement in both blood pressure control and renal allograft function, and was not associated with significant adverse effects on long-term patient or graft survival, despite an increased incidence of acute rejection within the first few months after conversion (MacPhee, Bradley et al. 1998).

1.7 Management of CAD

Early detection is important for preventing the progression of CAD, so accurate diagnosis and subsequent treatment is vital. For diagnosis it is important to note that events in the development of CAD occur earlier than changes in serum creatinine or GFR. One suggestion is the use of "protocol" transplant biopsies, which are performed at routine intervals, regardless of renal function, at pre-defined times after transplantation. These

biopsies monitor subclinical acute rejection or the initial presence of chronic damage to the allograft before it becomes clinically apparent, by a rise in serum creatinine or proteinuria. However, protocol biopsies have not been systematically evaluated and there remain concerns about the known complications of biopsy routinely applied to all patients. Urinary markers for early chronic damage are being evaluated, but none of the putative markers have been validated to date.

Thus, due to the multiple factors that contribute to the pathogenesis and the lack of definitive diagnostic techniques, a pragmatic approach to the management of CAD is followed in kidney transplant centres.

The importance of minimizing the risk of acute rejection within the first year has been established and adequate immunosuppression with CNIs as a part of the regimen remains a mainstay of treatment. However, newer regimens may allow CNI withdrawal, minimization or substitution on a routine basis in the future. Long-term immunosuppression has been the subject of intense study and although no consensus regimen has been developed, those that include mycophenolate mofetil or sirolimus in place of CNIs after the first year have been associated with decreased incidence of biopsy-proven arteriopathy and higher creatinine clearance, without significantly increased risk of acute rejection (Weir, Anderson et al. 1997; Dudley, Pohanka et al. 2005). Beyond the institution of appropriate immunosuppressive therapy, emphasis on patient adherence should not be overlooked. Additionally, to minimize immune-mediated allograft failure, avoidance of sensitization

and a careful pretransplant immunologic assessment (particularly to avoid donor-specific antibodies in patients with a second transplant) are necessary.

Aggressive control of blood pressure is considered universally important in the management of allografts and recent evidence suggests that, in selected patients, the judicious use of ACE-I/ARB was associated with a better creatinine clearance and decreased incidence of death and allograft failure (Lin, Valeri et al. 2002; Zaltzman, Nash et al. 2004). Although studied in a limited population this class of antihypertensive agents may be beneficial in kidney transplant recipients: a multicentre randomised controlled trial to address this is a priority.

Other risk factors that can be controlled include management of dyslipidemia, diabetes, and other comorbid conditions. Finally, early identification of decline in kidney function and a prompt evaluation of the cause of the dysfunction should be accomplished to institute appropriate management to slow and perhaps halt the progression of CAN.
Medications such as statins have been advocated to treat CAD, but most other therapeutic agents had very limited actions with limitations on their impact on overall outcome.

To conclude, late allograft loss after renal transplantation is a major problem. The work presented in this thesis focuses on non immune mechanisms responsible for long term renal graft losses. In the experimental work reported in this thesis, I have focused on in situ

determinants of injury present in the kidney. I have used the following specific models to address this:

1. In chapter 2, I hypothesized that regardless of the immune or non immune injuries; each donor kidney has a pre determined functional potential, and this may play a role in graft survival. To test this hypothesis I measured mitochondrial complex levels in the donor kidneys prior to implantation. I have correlated mitochondrial function in the donor kidneys with early clinical outcomes and medium term function of the transplant.
2. In chapter 3, I have focused on the relative contribution of CNI nephrotoxicity in renal damage. To assess this I have used a model of CKD in NRSOT, I have performed a detailed analysis of the renal histology in NRSOT, to ascertain patterns of injury specific for CNI toxicity and to correlate to clinical outcomes.
3. In chapter 4, I hypothesized that final pathway of renal injury is common to CKD, CAD and NRSOT; and particular injury processes may be specific to the individual clinical setting. To to identify predominant mechaninsm in each group, I have made direct comparisons of the injury processes across the study groups.
4. In chapter 5, I have performed molecular analysis of the renal micro-environment and have correlated the injury processes identified on the renal biopsies with various gene expressions in native chronic kidney disease.
5. A final discussion is presented in Chapter 6.

CHAPTER 2

ASSESSMENT OF BASELINE DONOR ORGAN VIABILITY: QUANTITATIVE ANALYSIS OF MITOCHONDRIAL FUNCTION IN PRE-IMPLANTATION BIOPSIES

2.1 INTRODUCTION

It is well known that the quality of the donor organ has an impact on post-transplantation outcome. However, organs from marginal donors have been increasingly used with encouraging results. There have been no attempts to date to measure the functional potential of donor organs, which is an important issue, since functional reserve of the kidney may be variable and not apparent with a crude marker such as serum creatinine. Additionally, it is well known that the damage of allografts derived from ischaemia/reperfusion (I/R) during transplantation may impact short- and long-term graft function and outcome. With one or more insults, different kidneys will behave differently depending on their functional reserve: for instance, a kidney from a young donor who died of a motor vehicle accident may have better functional reserves when subjected to an insult of ischaemia-reperfusion injury than a kidney from a marginal donor who died of a stroke. At a cellular/molecular level, ischaemia is associated with a decrease in ATP, which leads to ischaemic injury and no reflow after reperfusion. It is known that different organs from an individual have varying mitochondrial activity. It is also known that mitochondrial activity deteriorates with age and ischaemia-reperfusion injury. Mitochondrial transition permeability is associated with the development of calcium-dependent pores in the mitochondrial wall, resulting in depolarization of the mitochondria, uncoupling of oxidative phosphorylation and mitochondrial swelling with consequent necrosis or apoptosis.

In this chapter, I evaluate the relationship between mitochondrial complex activities and outcome in the first year post-transplantation.

2.1.1 Mitochondrial Complexes and Measurement of Their Activities (Saraste 1999)

Mitochondria have two compartments: the matrix confined with the inner mitochondrial membrane and the intermembrane space surrounded by the outer membrane. Mitochondria generate the energy required for the cellular metabolism in the form of adenosine triphosphate (ATP). This occurs mainly through oxidative phosphorylation, which involves electrons passing along a series of protein complexes situated in the inner mitochondrial membrane and which form the electron transport chain (ETC). Electrons generated from reduced nicotinamide adenine dinucleotide (NADH) are collected by complex I (NADH dehydrogenase) and from succinate by complex II (succinate dehydrogenase). Subsequently, electrons are passed to complex III (cytochrome bc_1) through coenzyme Q (CoQ) and to complex VI [cytochrome c oxidase, (COX)] using cytochrome c as a carrier **(Figure 2.1)**. The energy produced from the electron flow is utilized to drive out protons (H^+) at the level of complexes I, III, and VI. As the inner mitochondrial membrane is impermeable, an electrochemical gradient is formed and is used to reintroduce protons through the proton channel of complex V (ATP synthase). The proton flow drives the condensation of adenosine diphosphate (ADP) and inorganic phosphate to form ATP. The ATP is exchanged with cytosolic ADP by adenine nucleotide translocator (ANT) **(Figure 2.1)**. Thus, the maintenance of the electrochemical gradient is important for O_2

consumption by the ETC, which is coupled with ADP phosphorylation by complex V (ATP synthase).

Figure 2.1 Mitochondrial Complexes

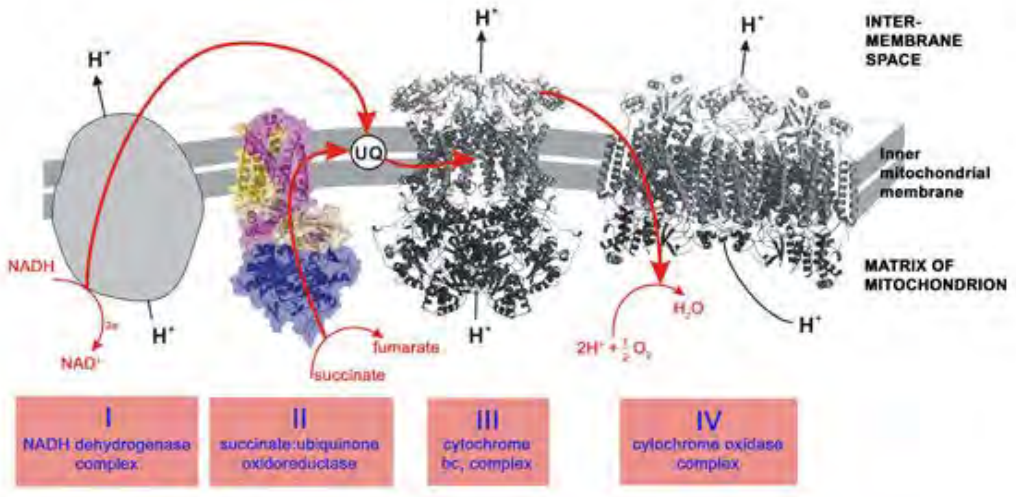


Figure 2.1: All four complexes are located in the inner mitochondrial membrane (Sun et al, Cell 2005)

2.1.1.1 Complex I (Kussmaul and Hirst 2006)

NADH: ubiquinone oxidoreductase (complex I) is a major source of reactive oxygen species in mitochondria and a significant contributor to cellular oxidative stress. Complex I provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle. The complex couples the oxidation of NADH and the reduction of ubiquinone to the generation of a proton gradient which is then used for ATP synthesis

(Figure 2.2). Mutations in this complex are associated with many diseases, including Leber Hereditary optic neuropathy, MELAS syndrome and Altzeimer's disease.

NADH: ubiquinone oxidoreductase catalyzes the oxidation of NADH, the reduction of ubiquinone, and the transfer of $4H^+$/NADH across the coupling membrane.

$$NADH + H^+ + Q + 4H^+_N <==> NAD^+ + QH_2 + 4H^+_P$$

Neither the pathway of electron transfer, nor the mechanisms of proton transport are known with any certainty. The redox centers are well characterized through spectroscopy and redox potentiometry.

Figure 2.2 Complex I

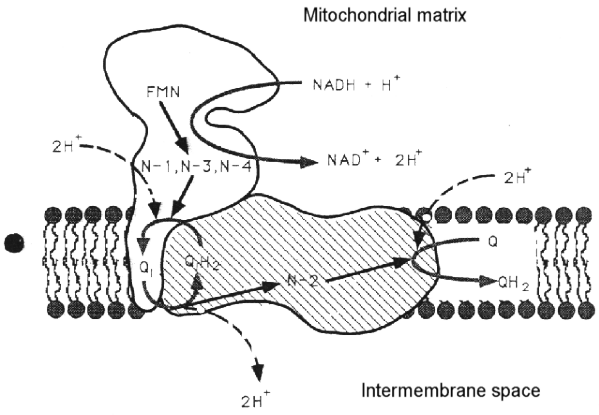


Figure 2.2: Boot shaped structure of Complex I

2.1.1.2 Complex II (Rustin, Munnich et al. 2002)

Complex II is also called succinate dehydrogenase, and is the only membrane bound enzyme of the Krebs cycle. The dehydrogenation of succinate has too small a gradient for any H^+ pumping, so this Complex only generates 1 UQH_2 per succinate oxidised, and pumps no protons. It gets its electrons from the oxidation of succinate only and feeds them *via* flavin and an iron-sulfur cluster into the UQH_2 pool. Complex II (EC 1.3.5.1) is not a proton pump; rather, it serves to funnel additional electrons into the quinone pool (Q) by removing electrons from succinate and transferring them to Q. Complex II consists of four protein subunits: SDHA, SDHB, SDHC, and SDHD. Other electron donors (e.g., fatty acids and glycerol 3-phosphate) also funnel electrons into Q, again without producing a proton gradient.

2.1.1.3 Complex III (Cadenas, Boveris et al. 1977)

Complex III is also called cytochrome reductase. It pumps 4 H+ per UQH2, and produces 2 cyt-cRED (reduced cytochrome-c) per UQH2 oxidised. The iron in the haem groups of b and c cytochromes goes from Fe3+ to Fe2+. The complex manages to pump 4 protons through a biochemical pathway called the Q-cycle, which delivers the two electrons from one UQH_2 to two cyt-c molecules, which only carry one electron each. Complex III removes, in a stepwise fashion, two electrons from QH_2 and transfers them to two molecules of cytochrome *c*, (a water-soluble electron carrier located within the intermembrane space). At the same time, it moves two protons across the membrane,

producing a proton gradient (in total 4 protons: 2 protons are translocated and 2 protons are released from ubiquinol). When electron transfer is hindered (by a high membrane potential, point mutations or respiratory inhibitors such as antimycin A), Complex III may leak electrons to oxygen, resulting in the formation of superoxide, a highly-toxic species, which is thought to contribute to the pathology of a number of diseases, including aging. In 1981, Stumpf and Parks et al described a method to measure the middle portion of mitochondrial respiratory chain, i.e. from FAD linked succinate dehydrogenase through coenzyme Q to cytochrome *C* (Complex II/III). This assay measures mitochondrial inner membrane electron transfer chain (ETC) activity (Stumpf and Parks 1981).

2.1.1.4 Complex IV (Siegbahn and Blomberg 2008)

Complex IV is more commonly termed 'cytochrome oxidase'. It pumps 2 H^+ per 2 cyt-c_{RED}, and produces 1 H_2O per 2 cyt-c_{RED} oxidised. Complex IV receives its electrons from cytochrome-c, which is a small, mobile protein that diffuses from Complex III to Complex IV. The electrons are passed through a number of cytochrome-a and copper ion centres. Cu_B and cyt-a_3 actually perform the reduction of oxygen to water. Each NADH originally oxidised yields 2 electrons, and these are enough to reduce half an O_2 molecule to H_2O (*i.e.* four electrons - two NADH - are required to reduce a whole *molecule* of dioxygen). Cytochrome *c* oxidases; it removes four electrons from four molecules of cytochrome *c* and transfers them to molecular oxygen (O_2), producing two molecules of water (H_2O). At the same time, it moves four protons across the membrane, producing a proton gradient.

The aim of the studies reported in this chapter was to analyse mitochondrial complex activity pre-transplantation, as a baseline measurement of cellular potential in the donor organ. These results were then related to clinical outcomes and surrogates for clinical outcomes in the first year post-transplantation.

2.2 METHODS

2.2.1 Ethical Review

The study protocol was reviewed and approved by the South Birmingham Research Ethics Committee (reference number: 04/Q2707/268). The study was then conducted following institutional approval by the Research and Development Department of Queen Elizabeth Hospital, Birmingham, UK.

2.2.2 Patients

Subject to written informed consent provided by the intended recipient of a cadaveric renal transplant, a single superficial cortical sample of kidney tissue weighing approximately 10 mg was obtained at the end of the cold ischaemic period using a sterile spring-loaded biopsy needle (14 gauge *Achieve* needle) held tangentially to the lateral surface of the donor kidney. One biopsy was obtained from each of 52 consecutive cadaveric grafts. Each biopsy was transferred to a cryo-vial and was snap frozen in liquid nitrogen and transported to a refrigerator. The biopsies were then stored at -80º until thawed for analysis.

Recipient's demographic details were obtained. It was noted whether transplantation preceded or followed onset of dialysis. They were also classified according to whether the graft that they received was from a brain dead donor (DBD) or non – donor after cardiac death (DCD), and whether they received a single-organ transplant following prior dialysis or not. Cold ischaemia time (CIT, h) was recorded. Time (days) required to halve pre-transplant serum creatinine ($Cr_{t½}$) was recorded, and used as an indicator of graft recovery. Delayed graft function was defined as a requirement for dialysis in the first week following transplantation and recipients were classified into those with and those without DGF. Graft function at 6 months and at one year was assessed by serum creatinine values.

2.2.3 Donor kidneys

Kidneys were harvested in a standard fashion and perfused with soltran Marshall's solution (*Soltran*®, Baxter Healthcare Ltd) at 4º Celsius before removal from the donor and subsequent cold storage.

2.2.4 Biopsies

After thawing, each biopsy was subjected to ultrasonification to rupture mitochondrial membranes in order that the activity of mitochondrial complexes could be measured. The following methods were employed to measure activity of mitochondrial complexes.

2.2.4.1 Complex I

Complex I was determined by a modification of the method of Hatefi (Hatefi 1978) where 0.1 mM NADH in 10mM Tris buffer (pH=7.50), 300 mM mannitol, 4mM $MgSO_4$, 10 mM KH_2PO_4, 10mM KCL and 0.2mM EDTA were incubated in a cuvette for 5 minutes at 37ºC and measured at a wavelength of 340nm. To every 1ml of this solution, 100µl of freshly thawed mitochondrial suspension was added. NaN_3 was added to inhibit further reaction and stabilise Complex I. The reaction was inhibited by rotenone (10mM). Results were expressed as nM NADH oxidized/min/mg protein.

2.2.4.2 Complex II/III

The method of Stumpf and Parks (Stumpf and Parks 1981) was employed where the reaction was followed over 5 min at 30ºC measuring the reduction of 0.1mM cytochrome c in 50mM KH_2PO_4 buffer (pH=7.7), 2.5 mM NaN_3 and 0.1µM rotenone. Freshly thawed mitochondrial suspension (100µl) was added and the reaction started using 10mM sodium succinate. The reaction was inhibited (>90%) by the addition of 0.01µM antimycin.

2.2.4.3 Complex IV

This assay was based on the method described by Miro *et al*(Miro, Cardellach et al. 1998) which follows the oxidation of 10µM ferrocytochrome c in 10mM KH_2PO_4 buffer (pH=6.5) and 0.3mM sucrose. The mitochondrial suspension was preincubated with laurylmaltoside (2.5mM) for 5 min at 37ºC at a wavelength of 550 nm. To every 1ml of this buffer solution,

100 μM of freshly thawed mitochondrial suspension was added (to a concentration of 2mg/ml). To prevent the oxidation of NADH, 0.9mM NaN_3 was added to the reaction mixture. This reaction was fully inhibited by 0.2mM KCN. Results were expressed as nM ferrocytochrome c oxidized/min/mg protein.

2.2.4.4 Citrate Synthase

The activity of this enzyme was measured by a method (Kuznetsov, Strobl et al. 2002) briefly, tissue homogenate was incubated with 101μM 5,5'-dithiobis(2-nitrobenzoic acid), 500μM oxaloacetic acid, 305μM acetyl CoA, 0.25% Triton X-100 and 50μM EDTA in a final volume of 200μM 0.1M Tris-HCl buffer adjusted to pH 8.1 at 30º Celsius. The reaction was started by the addition of the homogenate (20μl) and changes in the absorbance at 412nM followed for 100s.

2.2.4.5 Determination of Activity of Each Complex

In all these reactions, there is a linear change in absorption with time, the gradient of which permits determination of the activity of the preparation. This activity was indexed according to the corresponding citrate synthase activity in each biopsy. Each biopsy may have a verying volume of mitochondria and citrate synthase levels represent the volume of mitochondria and therefore complex activity controlled for citrate synthase provides better estimate of that complex activity per biopsy.

2.2.5 Statistical Methods

Data distributions were compared using Student's *t*-test when distributions were Gaussian by Kolmogorov-Smirnov test and homoscedastic by F-test. The Mann-Whitney U-test was employed when distributions were not Gaussian or were heteroscedastic. Contingency table analysis was undertaken employing Fisher's χ^2-test or Fisher's exact test, as appropriate, to compare the frequency of DGF in different sub-groups. Linear correlations were attempted between continuous variables employing the method of least squares. Probability values <0.05 were considered to be statistically significant.

2.3 RESULTS

2.3.1 Donor Kidneys

From September 2005 to April 2007, pre-implantation biopsies from 54 cadaveric kidneys were included in the study. Kidneys that were to be transplanted into a recipient with high risk for or from bleeding (e.g. Jehovah's Witness, coagulation disorder) were excluded from recruitment. Also donor kidneys with abnormal anatomy (e.g. requiring complex arterial reconstruction) were excluded from the study to avoid a further risk factor of early complications. Thirty-two donors (69%) were males and 22 (31%) were females. Median donor age was 47 (13-67). Eight (17.4%) donors were DCD and the remaining DBD. Four (7.4%) donors had suffered anoxic death.

2.3.2 Donor Kidney Function by Conventional Methods

The most recent serum creatinine is conventionally used as a surrogate marker for donor renal function. It is considered along with other parameters such as urine output in the hours prior to cardiac death. Mean serum creatinine in the cohort was 79.4 ± 0.4 μmol/l.

2.3.3 Mitochondrial Complex I-IV Activities

Each biopsy was assessed for Complex I, II/III, IV and citrate synthase levels. Results were obtained in duplicates and where variability was noted the experiments were repeated. An average of the two readouts was used for analysis. Individual complex activities were controlled for the amount of citrate synthase present in the biopsy and the ratio was called 'indexed Complex activity'.

2.3.3.1 Measured Complex Activities and Intra-Cohort Variations

The three Complex activities were found to have variations within the cohort **(Figure 2.3)**. Mean Complex I activity was 317 ± 8.4 per min/mg protein, with a range from 136.08 to 398.54. Mean Complex II/III activity was 81.33 ± 6.69 per min/mg protein, with a range of 36.2 to 304.43. And mean Complex IV activity was 347.8 ± 12.79 per min/mg protein, with a range from 37.37 to 559.19. Mean Citrate synthase present in the biopsies was found to be 245.14 ±7.7 per min/mg protein with a range from 59.51 to 360.03.

It was clear that there was wide variation in each of the Complex activities and also in the citrate synthase values in the biopsies within the cohort **(Figure 2.3)**. It would have been possible that some biopsies contained more and/or a better quality of mitochondria than others. To control for this confounding factor the index values for each of the complexes were obtained by dividing the average complex activity by citrate synthase value for that biopsy.

The indexed values for each of the complexes were then called as Index I through to Index IV for the corresponding complexes. Mean Index I activity was 1.34 ± 0.04, with a range from 0.61 to 2.35, mean Index II/III was 0.33 ± 0.03, with a range from 0.15 to 1.16, whereas mean Index IV was 1.44 ± 0.54 with a range of 0.63 to 2.76. Thus, similar variations were also observed in indexed values. This supported the observation that mitochondrial function was variable between grafts.

Figure 2.3 Variability in the Complex Activities

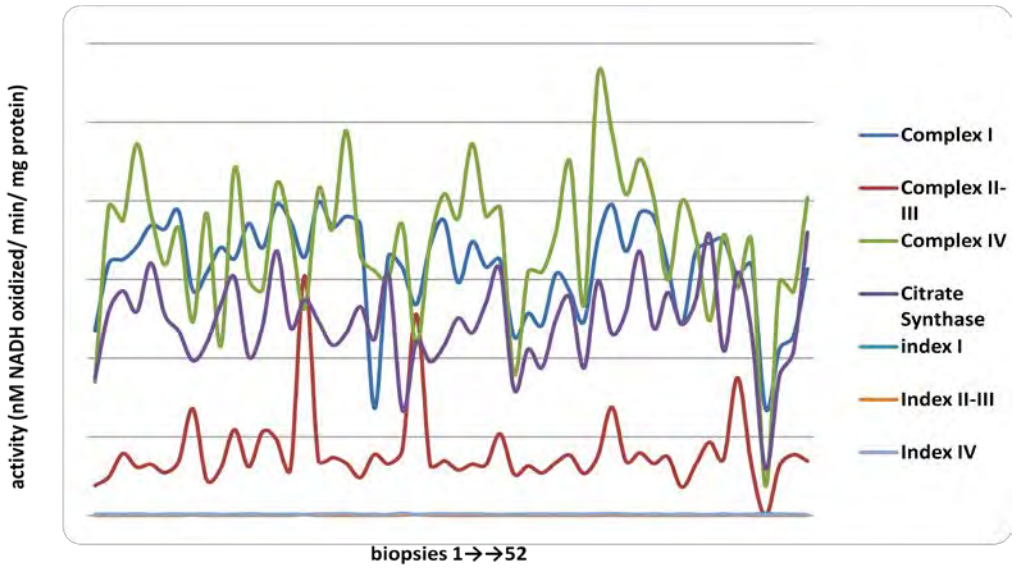


Figure 2.3: Complex I, II-III, IV and citrate synthase activities for all biopsies, note inter and intra- biopsy variability of activities of complexes.

Figure 2.4 Complexes I and IV

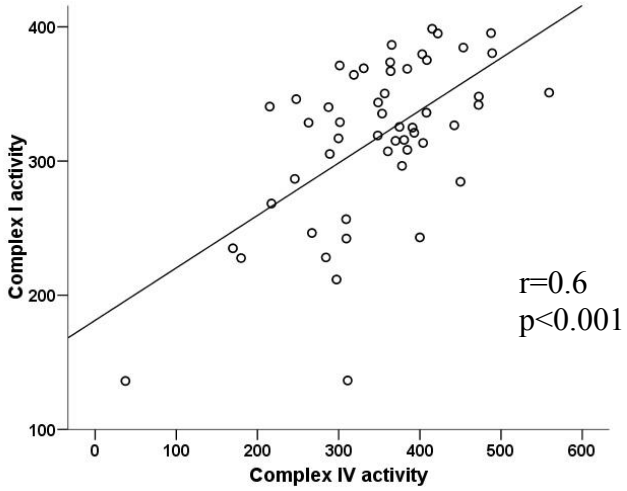


Figure 2.4: Note positive correlation between Complex I and Complex IV activities. Complex II/III activity did not correlate with the other two complex activities as well as citrate synthase **(Table 2.1).**

The correlation between Complex I and IV was maintained for the indexed activities of these Complexes **(Figure 2.4 & 2.5)**. Again Index II/III did not share correlation with either Index I or Index IV **(Table 2.2)**.

Figure 2.5 Indices I and IV

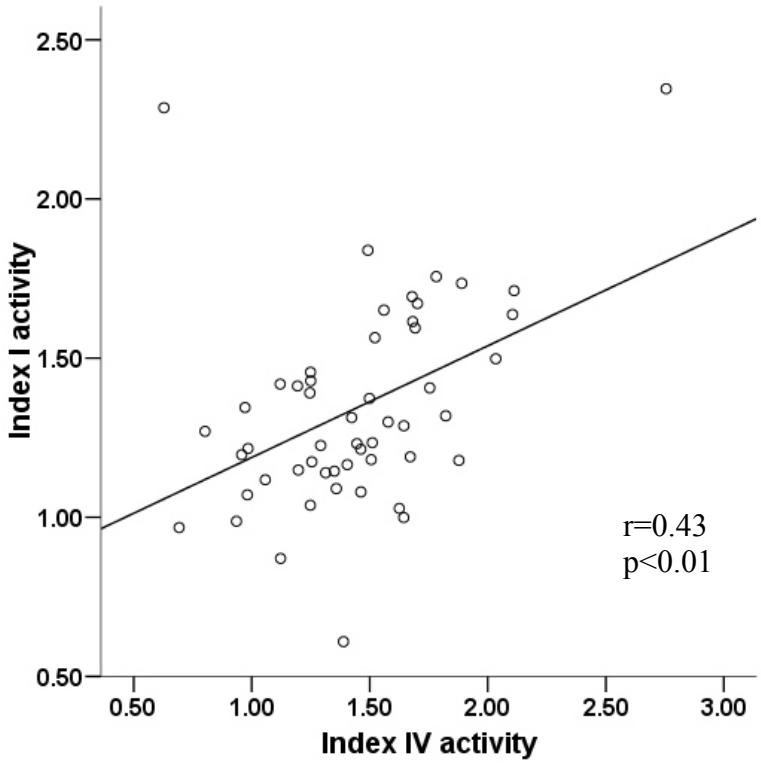


Figure 2.5: Note positive correlation between indexed Complex I and IV activities. (r: correlation coefficient, p: significance)

Table 2.1 Correlations across Complexes I-IV

		Complex I	Complex II-III	Complex IV	Citrate Synthase
Complex I	r	1	0.020	**0.595**	**0.511**
	p		0.887	**0.000**	**0.000**
	n		51	52	52
Complex II/III	r		1	-0.200	0.148
	p			0.160	0.299
	n			51	51
Complex IV	r			1	**0.476**
	p				**0.000**
	n				52
Citrate Synthase	r				1
	p				
	n				

Table 2.1: Note positive correlations between Complex I and Complex IV activities; correlation is significant at the 0.001 level (2-tailed). (r: correlation coefficient, p: significance, n: number of cases)

Table 2.2 Correlations across Indices I-IV

		Index I	Index II/III	Index IV
Index I	r	1	0. 130	**0.425**
	p		0.364	**0.002**
	n		51	**52**
Index II/III	r		1	-0.075
	p			0.60
	n			51
Index IV	r			1
	p			
	n			

Table 2.2: Note positive correlations between Index I and Index IV activities; correlation is significant at the 0.01 level (2-tailed). (r: correlation coefficient, p: significance, n: number of cases)

2.3.3.2 Effect of Donor Factors on Mitochondrial Function

Donor Age

Median donor age was 45 [13-67]. Donor age correlated negatively with Index IV [weak correlation with r = -0.283] only **(Table 2.3)**. Donor age did not correlate with any other indexed activities.

Donor Renal Function

Donor renal function before organ procurement was assessed by Serum Creatinine levels. Medianan Serum Creatinine at the time of hispitalization was 75 [46-200]. None of the Complexes or the indices of Complex activities correlated with donor serum creatinine (**Table 2.3**).

Table 2.3 Indices and Donor Age and Creatinine

		Donor Age	Donor Cr.
Index I	r	-0.044	0.088
	p	0.761	0.540
	n	51	51
Index II-III	r	0.104	0.107
	p	0.473	0.459
	n	50	50
Index IV	r	**-0.283**	-.022
	p	**0.044**	0.876
	n	**51**	51
Donor Age	r	1	0.078
	p		0.584
	n		51

Table 2.3: Negative correlation between donor age and Indexed activity for Complex IV [weak correlation r=-0.283]. (r: correlation coefficient, p: significance, n: number of cases)

Other Donor Characteristics

None of these characteristics such as sex, cardiac death prior to harvest, anoxic cause of death had any influence on the indices for mitochondrial Complexes I-IV **(Figure 2.6).**

Figure 2.6 Indices and Donor Variables

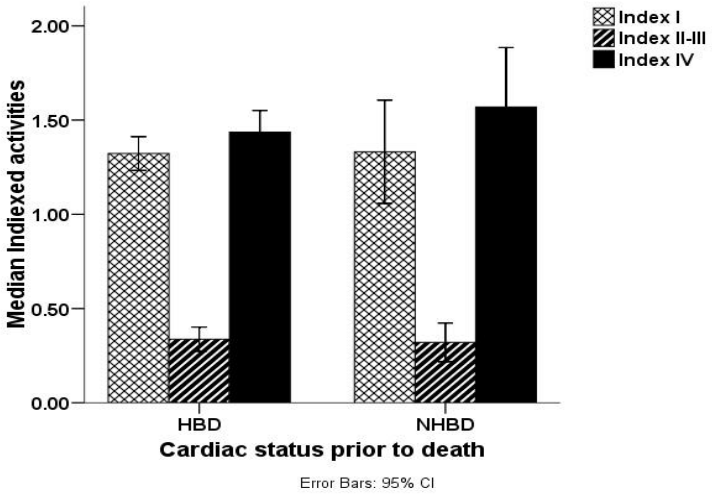


Figure 2.6: Note comparable indexed activities of mitochondrial Complexes I-IV for the two sub-groups classed according to cardiac status prior to procurement (p=ns).

Cold Ischaemia Time (CIT)

Donor kidneys were preserved for varying lengths of time before transplantation. The mean [normally distributed] CIT in hours was 17.81 ± 0.71, with a range from 8 hours to 32 hours. Cold ischaemia time was not associated with any of the Indices of Complex activities **(Figure 2.7)**.

Figure 2.7 Indices and CIT

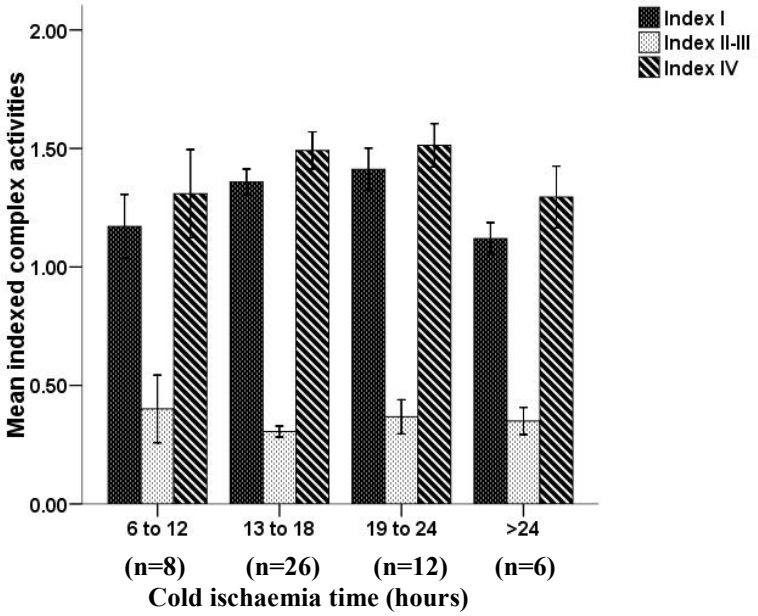


Figure 2.7: Note comparable indexed activities of mitochondrial Complexes I-IV for the increasing intervals of CIT (p=ns).

2.3.4 Recipient Demographic Details

Recipient details were obtained prior to transplantation. Two patients required a transplant nephrectomy (n=2) during the same admission for surgical/technical problems and were withdrawn from the study. Thirty (58%) recipients were males. The median age of the cohort was 43 [16-73]. Thirty-four (65%) were caucasians, 15 (29%) were indo-asians and three (6%) were black. All but eight recipients were already on dialysis prior to transplantation. Of those on dialysis, 66% were on haemodialysis and remaining patients were receiving peritoneal dialysis. Standard quadruple immunosuppression was used,

which included basiliximab as an induction agent, tacrolimus or cyclosporine, azathioprine and prednisolone.

Table 2.4 Demographic Characteristics

	Total n (%)	Male Recipients n (%)	Female Recipients n (%)
Numbers (%)	52	30 (58)	22 (42)
Mean Age, y (±SD)	43.6 (±13.8)	43.8 (±14.9)	43.2 (±12.4)
Race of recipients[+]			
Caucasian	34 (65)	20 (67)	14 (64)
Indo-asian	15 (29)	8 (27)	7 (32)
Afro-caribbean	3 (6)	2 (7)	1 (5)
PTD[+]			
Nil	4 (8)	2 (7)	2 (9)
Haemodialysis	32 (61)	20 (67)	12 (55)
Peritoneal dialysis	16 (31)	8 (27)	8 (37)
PTD+SOT[+]			
Present	50 (96)	29 (97)	21 (95)
Absent	2 (4)	1 (3)	1 (5)

Table 2.4: Recipients' demographic and peri-transplant characteristics; [+] no significant difference between sexes. PTD=pre-transplant dialysis; SOT=single organ transplant

2.3.5 Functional Outcomes

Functional outcomes were evaluated by the following means:

- primary graft function/ delayed graft function
- time taken to halve pre-operative sr. creatinine [$Crt_{1/2}$]
- transplant function at 6 months and at 12 months post-transplantation

2.3.5.1 Delayed Graft Function

Delayed graft function occurred in 37% (19/52) of recipients.

Table 2.5: Donor Characteristics and Graft Function

Variable	**DGF** **N = 19**	**IGF** **N = 33**	**P**
Donor age [median]	47.5[18-66]	46[13-67]	ns
Donor Creatinine [median]	74.5[52-107]	75[46-200]	ns
Donor sex [M:F]	11:8	21:12	ns
DCD [n]	6	2	<0.05
Anoxic death [n]	1	4	ns
Hypertensive donor [n]	2	7	ns
CIT (median hrs)	20[12-32]	16[8-22]	<0.05
Number of HLA antigens mismatched [median]	3.5[2-6]	3[0-6]	ns
DR mismatch [median]	1[0-2]	1[0-2]	ns

Table 2.5: Effect of donor characteristics on occurrence of DGF; note that DCD status of the donor and CIT are associated with DGF, donors with known history of hypertension and/ or on anti-hypertensive medications were considered as hypertensive donors.

Effects of donor age on prevalence of delayed graft function

Donor age did not have an influence on the prevalence of DGF. The median donor age of the group suffering DGF was 47.5 versus 46 in the group with IGF.

Effects of race of recipient on prevalence of delayed graft function

Recipients were classified by race into Caucasian, Indo-Asian and Black groups. There was no statistically significant difference in racial distribution between genders and no statistically significant difference in the frequency of DGF between races (χ^2=0.08, p=0.96). The odds ratio for DGF between Caucasians and Indo-Asians was 0.86 (95% Confidence Interval [CI] =0.24-3.00) and between non-Black and Black was 1.2 (CI=0.10-14.2).

Effect of Cold Ischaemia Time on Frequency of Delayed Graft Function

When graft recipients were divided into quartiles of increasing CIT, there was an increase in frequency of delayed graft function with each quartile (χ^2 for trend=6.7, p=0.01; Fig. 2.8). There was a statistical difference in median CIT between those graft recipients who did not develop DGF and those who did (No DGF median=16hrs vs DGF median=20h; U=180, p=0.02).

Figure 2.8 CIT and DGF

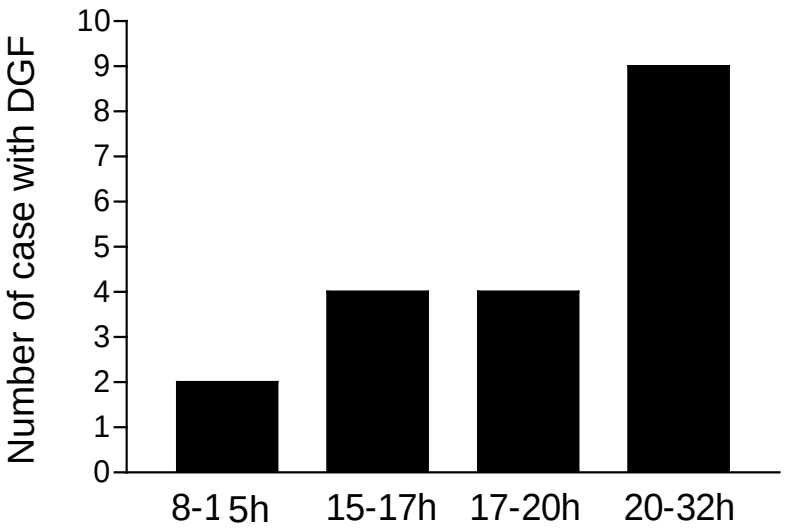


Figure 2.8: Frequency (number of cases) of delayed graft function with increasing intervals of cold ischaemia time (χ^2 for trend=6.7; P = 0.01

Effect of Mitochondrial Complex Activities on the Incidence of DGF

Mitochondrial Complex activities indexed for citrate synthase were comparable between the groups with and without DGF **(Figure 2.9)**.

Figure 2.9 Indices and Graft Function

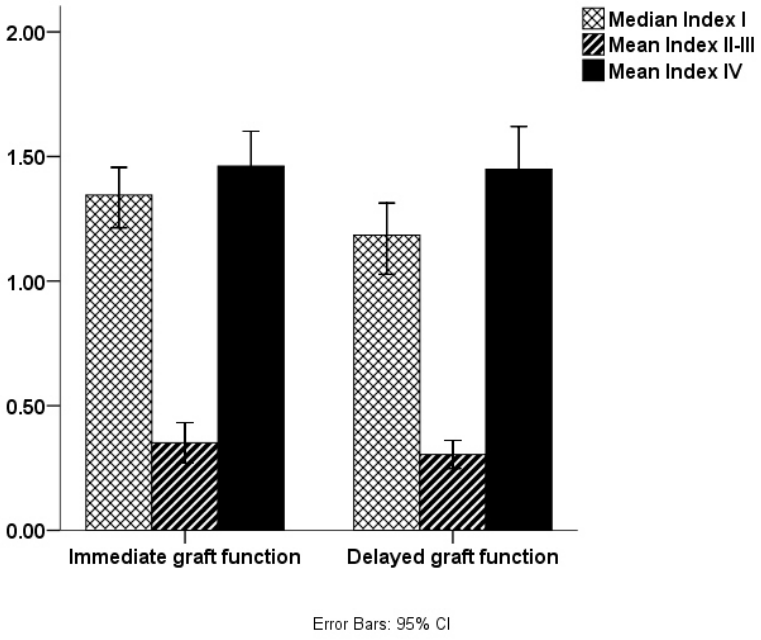


Figure 2.9: Indexed mitochondrial Complex activities are comparable between the groups with and without DGF (non-parametric tests; p = ns)

Effect of Cardiac Status of Donor on Prevalence of Delayed Graft Function

DGF occurred more frequently in recipients of grafts from DCD (6/8) than in recipients of grafts from DBD (13/44). This difference was statistically significant (Fisher's exact test: p=0.04; OR=7.15, CI=1.27-40.2) but the differences in CIT between the two groups did not reach statistical significance (mean [±SD] for DBD=17.1±4.9h, mean for DCD=20.5±4.5h; p=0.09).

2.3.5.2 Speed of Recovery: Time to Halve Preoperative Sr. Creatinine

Speed of recovery was measured by estimating the time period taken to halve pre-operative recipient Sr. creatinine.

Table 2.6 Correlations between Indices and Speed of Recovery

		Days to halve Creatinine
Index I	r	-.060
	p	.691
	n	47
Index II-III	r	-.105
	p	.487
	n	46
Index IV	r	.100
	p	.504
	n	47

Table 2.6: Note no significant correlations between speed of graft function recovery and any of the indexed mitochondrial Complex activities. (r: correlation coefficient, p: significance, n: number of cases)

Effect of Mitochondrial Complex Activities on Speed of Graft Recovery

None of the indexed mitochondrial Complex activities had an association with speed of graft recovery **(Table 2.6).**

Effect of Donor Cardiac Status Prior to Procurement on Speed of Graft Recovery

The speed of recovery of renal function (assessed by $Cr_{t½}$) was slower in recipients of grafts from DCD (median for DBD=2.86 days, median for DCD=15.39 days, U=55, p=0.01, **Figure 2.10)**. This was not related to any statistically significant difference in MCA_i.

Figure 2.10 Slow Recoveries in DCD Kidneys

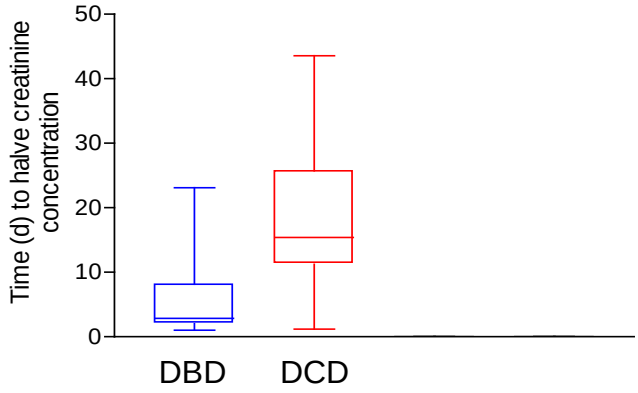


Figure 2.10: Graft recovery time (median days to halve pre transplant serum creatinine) by cardiac status of the donor: slower recovery in DCD transplants. (DBD: donation after brain death, DCD: donation after cardiac death)

Effect of CIT on Speed of Graft Recovery

There was a direct correlation between the time taken to halve preoperative serum creatinine concentration (Crt½) and CIT (r=0.43, p=0.003) **(Figure 2.11).**

Figure 2.11 A Correlation between CIT and Speed of Recovery

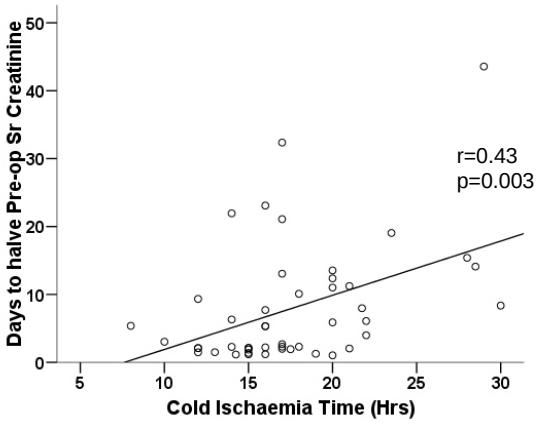


Figure 2.11: Relationship between cold ischaemia time and time taken to halve creatinine concentration. (r: correlation coefficient, p: significance)

2.3.5.3 Mid-term Graft Function

Mid-term graft function was assessed by serum creatinine at 6 and 12 months. The cohort was split in two groups: one with indexed Complex II/III activity lower than median and another with activity higher than the median value. Patients with the kidneys that had a higher indexed Complex II/III activity had significantly lower serum creatinine than those who had lower indexed Complex II/III activity **(Figure 2.12).** Mid-term graft function was not influenced by any of the other indexed activities. There was no correlation between Citrate Synthase activity and mid-term graft function.

Figure 2.12 Mid-term Graft Function

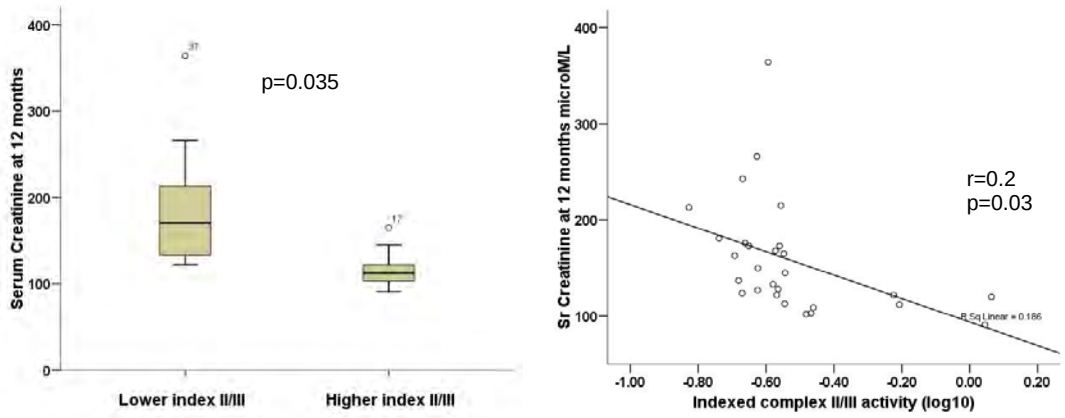


Figure 2.12: mid-term graft function by Index II/III: note better graft function in the group with higher preimplantation Index II/III activity. (r: correlation coefficient, p: significance, n: number of cases)

2.4 CONCLUSION

In summary, mitochondrial function in human kidneys can be measured by established methods. Measured in this way, mitochondrial function seems to remain stable throughout the period of cold ischaemia in the setting of donor organ preservation. Similarly it is not adversely affected by warm ischaemia inflicted by DCD donor status. Impaired mitochondrial function, defined by these activities, did not correlate with adverse early outcomes. However, higher indexed Complex II/III activity may predict superior mid-term graft function.

2.5 DISCUSSION

2.5.1 Mitochondrial Complex Activities and Immediate Graft Function

The specific aim of the study reported in this chapter was to explore whether baseline mitochondrial function can be used as a predictor of early and mid-term graft function in human kidney transplantation. Although the study was not designed to address the effect of warm ischaemia and subsequent reperfusion injury on mitochondrial function, these may have altered mitochondrial function. This is a significant shortfall of the study. Serial biopsies including pre and post implantation periods would have provided further information. Post reperfusion biopsies were indeed obtained at the beginning of the study and there were significant bleeding complications. For the safety of patients the design of the study was modified and only pre-implantation biopsies were obtained. I anticipated that mitochondrial function would reflect the overall health of the graft and expected to see varying mitochondrial function with respect to age of the donor, type of the donor (DCD vs. Brain dead donor), cold ischaemia etc. Variations in each of the Complex activities and also in citrate synthase levels in different biopsies indeed proved that mitochondrial function varies in donated kidneys. Another significant shortfall of the study is that the effect of warm ischaemia time was not accounted for.

Regardless, this is an important study and first to evaluate mitochondrial function in human kidneys (Kubal, Harris et al. 2009). Again, the aim of this study was to explore whether

mitochondrial activity can predict clinical outcomes more precisely than the predictive models used in the clinical setting. And if a correlation was to be found between mitochondrial function and graft function, perhaps interventions such as bioflavanoids to preserve mitochondrial function during cold storage might improve clinical outcomes (Ahlenstiel, Burkhardt et al. 2003).

There is sufficient published data mainly based on animal studies that led us to believe that Experimentally, post-ischaemic changes in mitochondrial morphology associated with falling ATP levels in rat proximal tubular cells have suggested that impairment of oxidative phosphorylation could affect cellular viability as could associated release of calcium causing secondary membrane changes (Trump BF 1982). Indirect assessment of mitochondrial complex I activity of rat surface tubules has shown that higher activity was associated with better subsequent renal function and preservation of cellular morphology (Coremans, Van Aken et al. 2000), wherein the authors used NADH fluorimetry to monitor kidney cortex metabolism noninvasively. Trump *et al* found that mitochondrial respiratory activity is impaired by ischaemic injury. In another animal study Ahlenstiel *et al* found that cold storage of a porcine proximal tubular cell line in culture for 20 hours has been associated with a gradual loss of mitochondrial complex I activity assessed indirectly (Ahlenstiel, Burkhardt et al. 2003). The results of this study suggest that, mitochondrial function assessed at the end of cold storage does not correlate with immediate graft function as well as the speed of graft function recovery. It was also interesting to note that the mitochondrial complex activities were not adversely affected by the warm ischaemic

injury caused in the DCD donors. In this study, delayed graft function was noted in 36% (19/52) of recipients, a frequency that is slightly higher than that reported in other studies (Irish, McCollum et al. 2003). This raises a possibility that other contributing factors such as warm ischaemia time may have contributed. Alternative explanation may be that the mitochondrial complex proteins are robust enough to resist electro-physiologic damage inflicted by cold ischaemia in the given preservation technique.

2.5.2 Effect of Mitochondrial Function on Mid-term Graft Function

This study has demonstrated for the first time that mitochondrial function has an impact on mid-term graft function after cadaveric renal transplantation. The results obtained in this study clearly show a direct and positive correlation in the whole population between mitochondrial Complex II/III activities controlled for citrate synthase and mid-term graft function. Interestingly, the correlation becomes weaker for Complex II/III activity on its own (without controlling for citrate synthase). Citrate synthase is a reliable marker of mitochondrial volume, and therefore the ratio of complex activity over citrate synthase demonstrates activity of each mitochondrion. This suggests that mid-term graft function depends on specific enrichment of the Complexes themselves within each mitochondrion rather than on mitochondrial volume within the kidney biopsy as whole. Despite the relatively large degree of scatter found in the mitochondrial enzyme-specific activities, I have found a linear relationship between these specific activities and mid-term graft function.

Previously, a correlation between all four Complexes' activities and sperm motility had been demonstrated (Ruiz-Pesini, Diez et al. 1998). In this study, the authors demonstrated that Complex II activity had the strongest correlation with sperm motility and that this was slightly weakened when correlations were performed for Complex II+III activity together. Taking this into consideration, perhaps future studies on renal mitochondrial function should assess these two complexes separately.

2.5.3 Factors Affecting Speed of Recovery of Graft Function

Using $Cr_{t½}$ as an index of speed of recovery of renal function has been directly related to CIT. However, in this study the variation in the latter only accounts for 18% of the variation in speed of recovery of renal function. Once again, the present study results suggest that, either directly or indirectly, mitochondrial function is not a factor contributing to variation in speed of recovery of graft function as no correlation was found between the activity of any of the respiratory complexes and $Cr_{t½}$.

2.5.4 Cardiac Death in Donors

The effect of the cardiac status of donors may provide insights into the mechanism of delayed graft function and speed of recovery of graft function. In this study, the frequency of DGF was increased in recipients of grafts from DCD, confirming the observation of Irish et al (Irish et al 2003; Kokkinos 2007). A meta-analysis by Kokkinos et al (2007) has reported that the incidence of delayed graft function is 3.6 times ($P<0.001$) greater in DCD

transplants (Kokkinos, Antcliffe et al. 2007). However, the increased DGF in recipients of grafts from DCD seen in the present study was not associated with increased CIT. Again this effect was not supported by adversely affected mitochondrial function as this was comparable between DBD and DCD donors.

2.6 FUTURE WORK

The caveats of this study include: one, this study did not evaluate the effects of warm ischaemia and reperfusion injury on mitochondrial complex activities. To study these effects I suggest evaluating baseline mitochondrial function prior to cold storage and then subsequent changes after reperfusion injury. Additional information can be gained by comparing mitochondrial function between deceased donor kidneys against living donor kidneys. Serial biopsies at different time points during cold storage, warm ischaemia and reperfusion injury can be measured in donor kidneys unsuitable for transplantation but consented for research (organs from deceased donors with malignancy or viral disease).

CHAPTER 3

CHRONIC KIDNEY DISEASE AFTER NON-RENAL TRANSPLANTATION: HISTOPATHOLOGY AND IN SITU DETERMINANTS OF OUTCOME

3.1 INTRODUCTION

In Chapter 2, I presented the results of a study that assessed the utility of mitochondrial Complex activity pre-transplantation in the donor kidney as a determinant of outcomes in the first year post-transplantation. The results of this analysis showed that there was an association between mitochondrial Complex activity and transplant function 12 months post-transplantation. This indicated that there are quantifiable functional parameters in the pre-transplant kidney that may be associated with longer-term outcomes. However, there is also considerable uncertainty about the relative role of specific factors in the progressive deterioration of function over time that affects most kidney transplants.

One model that may have utility in dissecting out the pathogenesis of progressive renal injury here is that of chronic kidney disease (CKD) following non-renal solid organ transplantation (NRSOT). Notably, this model may allow an assessment of Calcineurin inhibitor (CNI) toxicity in non-allogeneic settings.

As a result of the overall progress made in solid-organ transplantation, the clinical outcomes for transplant recipients have progressively improved (Jain A 2000); consequently, the long-term complication of end-stage renal failure (ESRF) is of great relevance. (Fisher 1998; van Gelder, Balk et al. 1998; Magee and Pascual 2003; Ojo, Held et al. 2003; Randhawa and Shapiro 2005; Chandrakantan, de Mattos et al. 2006; Kim, Lim

et al. 2006; O'Riordan, Wong et al. 2006). CKD itself contributes significantly to morbidity and mortality (Brown RS 1996; Moreno, Cuervas-Mons et al. 2003).

The reported cumulative incidence of CKD in non-renal solid organ transplantation varies widely. Specifically for liver transplantation, reports vary from 4% (Fisher 1998) to 83% (McCauley J 1990) (Platz KP 1994). In the largest and most cited analysis presented in this context Ojo *et al* reported the renal failure rate to be 18.1% at 5 years from liver transplantation (Ojo, Held et al. 2003). In this study, CKD (chronic renal failure) was defined as a glomerular filtration rate of 29 ml per minute per 1.73 m^2 of body-surface area or less, or as the development of ESRD. (Ojo, Held et al. 2003). This wide variation of prevalence rates in these studies may be due to variation in the design of studies, variable periods of follow-up and the lack of a standard definition of CKD. Various potential risk factors exist for an increased rate of CKD in NRSOT, including pre-transplant renal impairment, hepatitis-C, hypertension, diabetes mellitus (diabetic nephropathy), other glomerular diseases such as IgA nephropathy, and alcohol abuse (Amore A 1994; Weir MR 1999; Gayowski T 2000; Sabry, Sobh et al. 2002; Moreno, Cuervas-Mons et al. 2003; GC 1987;). However, most believe that the use of CNI therapy is the major contributor to the development of CKD in this setting (Maetz 1956; Myers, Ross et al. 1984; Bennett WM 1996; WM. 1996; Burdmann, Andoh et al. 2003).

The majority of the adverse effects of CNIs are related to afferent arteriolar vasoconstriction and interstitial ischaemia [Fig. 3.1]. This leads to interstitial scarring. The

only definitive histological indicator of CNI nephrotoxicity is the presence of focal arteriolar hyalinosis and striped interstitial fibrosis in situ. Although there is some evidence to suggest that CNI nephrotoxicity leads to CKD and CNI withdrawal improves renal function in these patients (Stewart, Hudson et al. 2001), very few studies have evaluated CNI nephrotoxicity through detailed histological analysis in non-renal solid-organ transplantation. (Pillebout, Nochy et al. 2005). Indeed, there is a risk of over-estimating CNI toxicity as a cause of CKD in non-renal transplant recipients based on serum creatinine levels or other measurements of renal function. Moreover, in the absence of a histological diagnosis there is also a risk of failing to correctly identify the underlying primary renal pathology.

Figure 3.1: Afferent Arteriolar Vaso-constriction

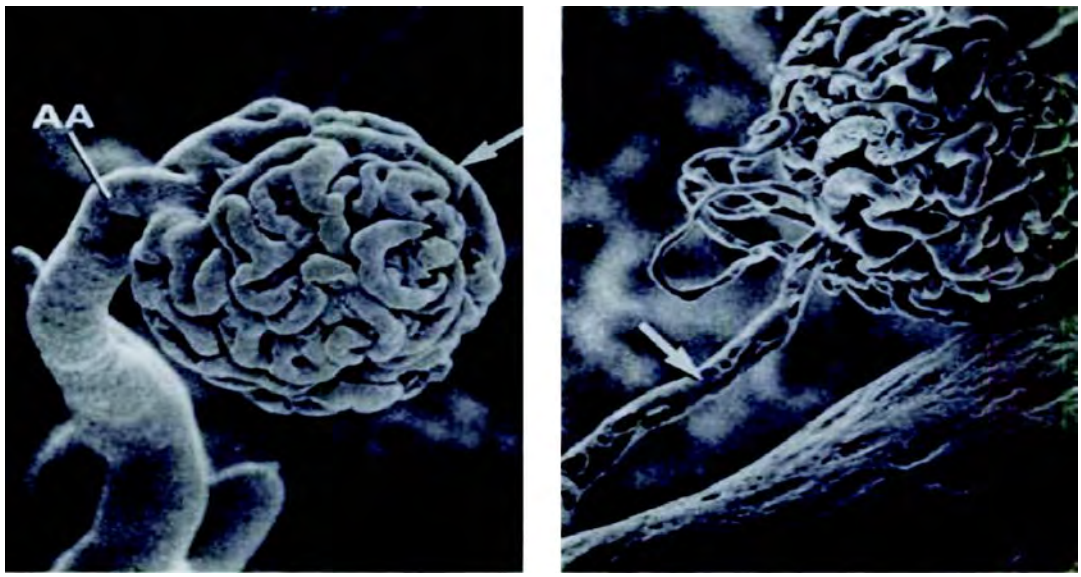


Figure 3.1: Scanning electron micrograph of an afferent arteriole (AA) and glomerular tuft from a control animal (left); and from a similar animal after 14 d of cyclosporine treatment (left). Source: English et al *Transplantation* 44(1): 135–141, 1987

In this chapter, I present the evaluation of histological diagnoses of renal biopsies from a cohort of liver, heart, and heart/lung transplant recipients with renal impairment. I have used an adaptation of the chronic allograft damage index (CADI) to evaluate renal histological injury markers in this setting for the first time. CADI has been used and validated for assessing the progressive renal (allograft) damage in renal transplantation (Isoniemi, Taskinen et al. 1994) by grading the severity of damage and to provide a semi-quantitative assessment of parameters that have been validated as clinically relevant predictors of renal allograft outcome.

A central hypothesis I address in this thesis is that the processes involved in progressive renal injury are similar, irrespective of the clinical setting. To test this hypothesis I have studied inflammation, ischaemia and scarring in situ in the NRSOT cohort that is analyzed in this chapter. Renal biopsies have been categorized based on histological patterns of injury, including evidence of CNI toxicity. To evaluate these major injury processes I assessed interstitial macrophage density (IMD) as a marker of chronic tubulointerstitial inflammation, interstitial peri-tubular capillary endothelial density (PTECD) as a marker of interstitial circulation/ ischaemia, and interstitial scarring by the validated index of chronic damage.

3.2 METHODS

3.2.1 Patients

(a) *Liver Transplant Recipients:*

Between 1986 and 2004, at University Hospital Birmingham, 1750 adults received one or more cadaveric liver transplants and 1390 recipients survived beyond one year. Of those who survived the first year post transplantation 92 (6.6%) required long-term renal replacement therapy. Thirty-one patients undergoing investigations for CKD at least three months after liver transplantation and who underwent renal biopsy amongst those investigations were included in this study.

(b) *Heart and/or Lung Transplant Recipients:*

Between 1988 and 2003, 317 adults received one or more heart, lung or heart & lung transplants. Of those who survived the first year post transplantation 43 required long-term renal replacement therapy. Thirty-one patients undergoing investigations for deteriorating renal function after at least three months post transplantation and who underwent renal biopsy amongst those investigations were included in this study.

3.2.2 Demographic and Clinical Details

Demographic and clinical details were obtained from the local transplant databases. Renal endpoint was defined by a requirement for renal replacement therapy or a fall in MDRD

eGFR by 50%. The immunosuppressive regimen used immediately post-transplant included cyclosporine (Neoral®, Novartis Pharma) in 34 (54.8%) patients, tacrolimus (FK-506, Prograf®, Astellas) in 21 (33.9%), Azathioprine (Imurel® GlaxoSmithKline) in 26 (41.9%) and Prednisolone in 57 (91.9%) patients. On deterioration of renal function, immunosuppressive regimens were changed in some patients. Calcineurin inhibitors were withdrawn in 26 (41.9%); mycophenolate mofetil (Cellcept Roche®) was introduced in 20 (32.3%) patients and sirolimus (Rapamune® Wyeth) was introduced in 7 (11.3%) patients.

3.2.3 Histopathological Evaluation

To assess the contribution of CNI nephrotoxicity to chronic renal failure in this cohort the biopsies were evaluated by an experienced transplant pathologist in a blinded fashion. The biopsies were classified into three sub-groups: [1] Chronic CNI nephrotoxicity [CNI-NT] with or without evidence of hypertensive nephropathy; [2] Hypertensive/ ischaemic nephropathy [HT/ Isch N] without chronic CNI nephrotoxicity; and [3] Primary renal disease (e.g. glomerulonephritis). Evidence of chronic CNI nephrotoxicity was defined as at least moderate arteriolar hyalinosis with or without striped fibrosis (Mihatsch, Thiel et al. 1988). Evidence for hypertensive nephropathy was defined as at least moderate intimal elastosis and/or the presence of globally sclerosed glomeruli with a shrunken tuft surrounded by fibrous tissue or shrunken glomeruli with wrinkled basement membranes. For all biopsies, sections were cut and immunohistochemical staining was performed with

anti SV40 antibody (Lee Biomolecular Research Labs. 1:10,000) to identify BK (polyoma) virus nephropathy.

3.2.4 Chronic Allograft Damage Index [CADI] and Chronic Damage Index [CDI; modified CADI]

To evaluate the severity of renal damage the biopsies were reviewed for known injury markers used in the CADI scoring system in a blinded fashion. Biopsies were considered adequate for assessment when they contained ≥7 glomeruli and at least one artery. Biopsy sections were stained with haematoxylin/eosin, periodic acid-Schiff (PAS), PAMS [periodic acid methenamine silver] and elastic haematoxylin Van Gieson (EHVG). The chronic allograft damage index system was used to assess the biopsies. The CADI parameters analysed comprised the following: diffuse or focal inflammation (‘‘i’’), interstitial fibrosis (‘‘ci’’) [Fig. 3.2 (a)], mesangial matrix increase (‘‘mm’’), sclerosis in glomeruli (‘‘g’’) [Fig. 3.2 (b)], intimal proliferation of vessels (‘‘cv’’) [Fig. 3.2 (c)], and tubular atrophy (‘‘ct’’) [Fig. 3.2 (d)]. This assessment was performed by two investigators (myself and Dr Desley Neil) blinded to patient identifiers. Each parameter of the scoring system was graded from 0 to 3, with 0 being absence and 3 being most severe. Arteriolar hyalinosis was scored (Banff 97 classification) and was added to the sum of CADI parameters to derive a chronic damage index (CDI) score.

Figure 3.2 Histopathology of Chronic Allograft Damage

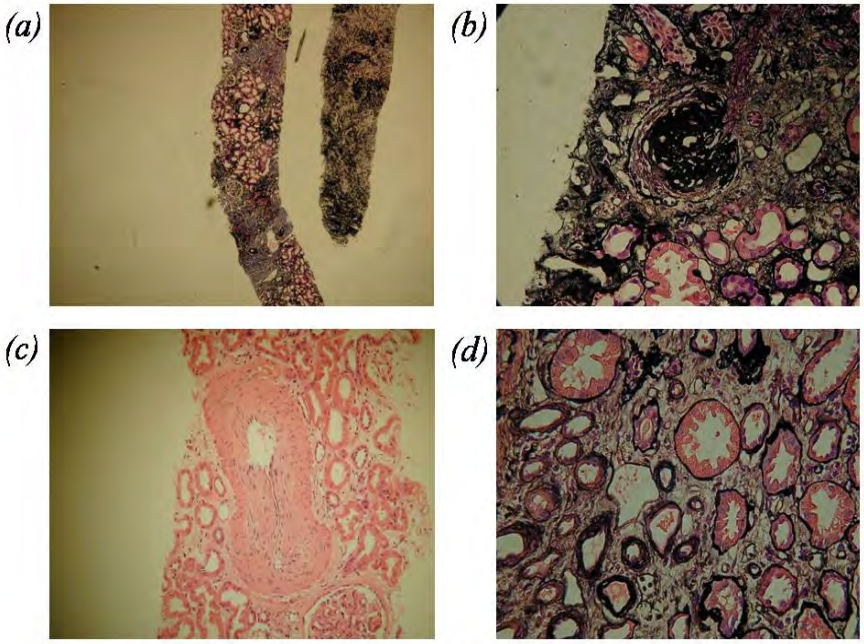


Figure 3.2: CADI scoring; (a) interstitial scarring [ci] stained with PAMS, (b) glomerulosclerosis [g], (c) intimal thickening [note >50% vascular narrowing] [cv] and (d) tubular atrophy [ct]

Figure 3.3 Arterialor Hyalinosis

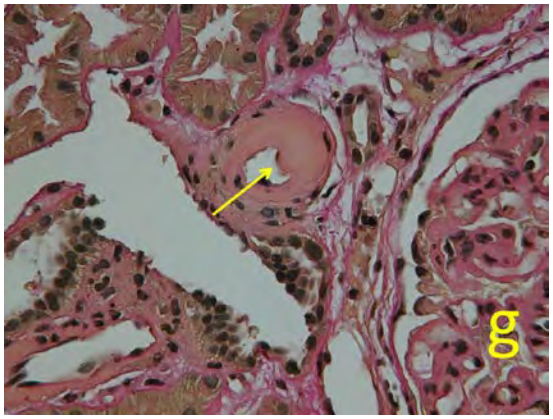


Figure 3.3: arteriolar hyalinosis; note concentric hyalinosis [arrow] in an arteriole right next to glomerulus [g] in a renal biopsy section stained with EHVG x400. Nodular hyalinosis is a classic feature of CNI nephrotoxicity

3.2.5 Morphometric Analysis of Interstitial Scarring, Ischaemia and Inflammation

3.2.5.1 Interstitial Scarring (utilising the index of chronic damage)

The index of chronic damage has previously been demonstrated to be a rigorous predictor of renal outcome (Howie, Ferreira et al. 2001). Formalin fixed paraffin embedded renal biopsy sections were stained by periodic acid-methenamine silver. Chronically scarred tissue was identified as glomeruli showing global sclerosis but not segmental sclerosis, areas of interstitial fibrosis which appear more solid and deeply stained than normal or oedematous interstitial tissues, and atrophic tubules defined as tubules smaller than normal with thickened basement membranes, or tubules larger than normal, with thin epithelium, including those large enough to be considered cysts. Arteries and arterioles were not judged to have chronic damage unless they were completely occluded. Using an Aequitas IDA image database and image archive management system (Dynamic Data Links, Cambridge, UK) images at magnification x10 were captured digitally. For analysis, each image was converted from Aequitas IDA to Aequitas IA image analysis software (Dynamic Data Links). The extent of scarring observed within the cortex of the kidney biopsy section was quantified and expressed as a percentage of total area occupied by the section analysed **(Figure 3.4)**.

Figure 3.4 Index of Chronic Damage

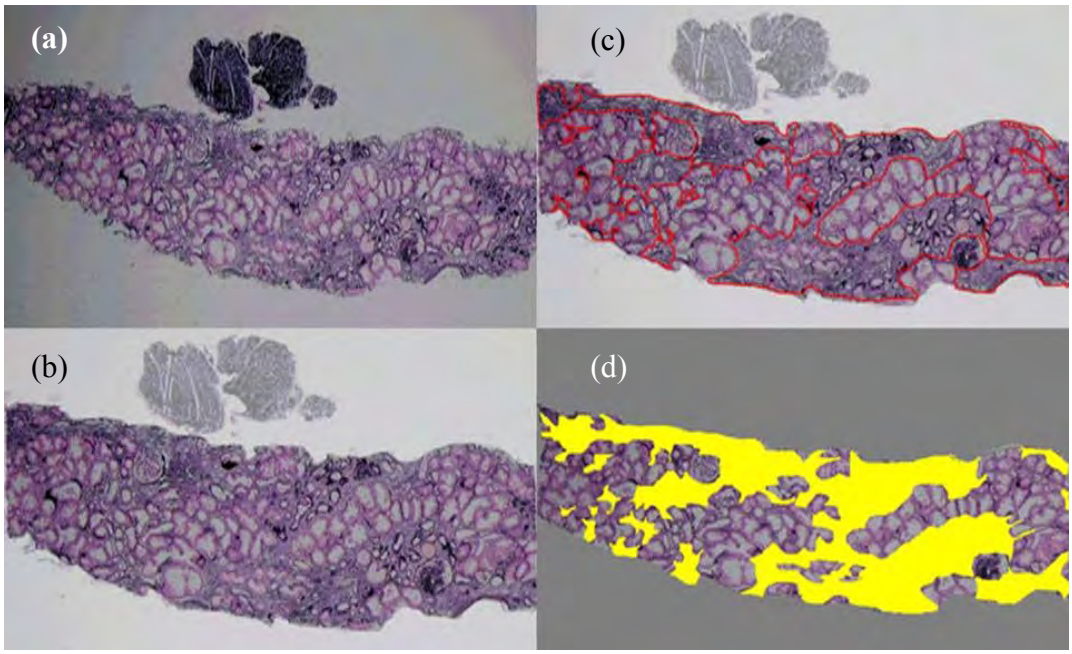


Figure 3.4: a) Section of renal biopsy from a cardiac transplant recipient stained with PAMS; b) area of interest (the biopsy) is selected and pixels for this area are counted c) areas of scarring are outlined freehand; d) the pixels present within the selected surface areas are measured, the ratio of pixels in (d) over (a) is calculated as index of scarring

To assess the validity of the method used in the quantitative microscopy studies intra- and inter-observer variability was tested. For inter-observer variation, two observers measured 20 specimens independently; one observer was an independent research fellow and the other was myself. The 20 specimens were also measured twice at intervals by myself to test for intra-observer variability. Agreement was assessed according to the method of Bland and Altman after log transformation (necessary because the differences between measurements were proportional to the mean). This method gives the bias, or mean difference, between measurements and limits of agreement, or 2 SDs either side of the

mean, with 95% confidence intervals (CIs) for the bias and limits of agreement, all expressed as ratios when back-transformed (Bland 1986).

3.2.5.2 Peri-tubular Capillary Endothelial Cell Detection by Immunohistochemistry

For the detection of endothelial cells, IHC was performed on paraffin-embedded formalin fixed tissue sectioned to 2µm. The sections were heated in an oven at 56^0C for 30 minutes and then placed in pre-heated one-step dewaxing and epitope retrieval solution W-Cap TEC Buffer pH 8.0AP (Surgipath Europe Ltd, Peterborough, UK) at 96^0C for 30 minutes. The sections were then allowed to cool in W-Cap for 20 minutes. The sections were then treated with 0.3% solution of hydrogen peroxide in methanol for 10 minutes. Slides were then washed with phosphate buffer saline (PBS). Endogenous biotin was blocked using sequential 10 min treatment with 0.1% avidin and 0.01% biotin (Dako UK Ltd, Cambridgeshire, UK). Slides were then washed with PBS and were treated with mouse anti-human monoclonal antibody directed against the endothelial cell marker CD34 (1µg/ml; clone QBEnd 10; Dako Ltd) diluted in Dako REAL™ Antibody Diluent (S2022; Dako UK Ltd, Cambridgeshire, UK) for 60 minutes at room temperature. For controls, the primary antibody was substituted with mouse IgG1 (Dako UK Ltd, Cambridgeshire, UK) isotype control at equivalent concentrations in parallel using adjacent sections on the same slide. ChemMate™ DAKO EnVision™ Detection kit (K5007; Dako UK Ltd, Cambridgeshire, UK) was used for detection of positive staining. In this two-step technique, sections were incubated with the ChemMate™ DAKO EnVision™/ HRP, rabbit/ mouse reagent for 30 minutes. Following this sections were washed with PBS and

then incubated with 1:40 dilution of ChemMate™ DAB + Chromogen in ChemMate™ Substrate Buffer for 10 minutes.(Fig. 3.4(a)) Sections for quantitative analysis were not counterstained to avoid interference in the digital quantification used. Sections were then mounted in an aqueous medium (Immu Mount).

Figure 3.5 Ischaemia and Inflammation

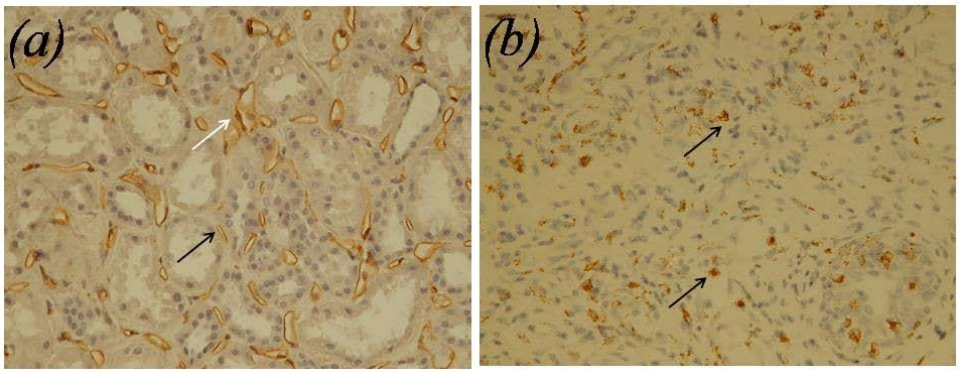
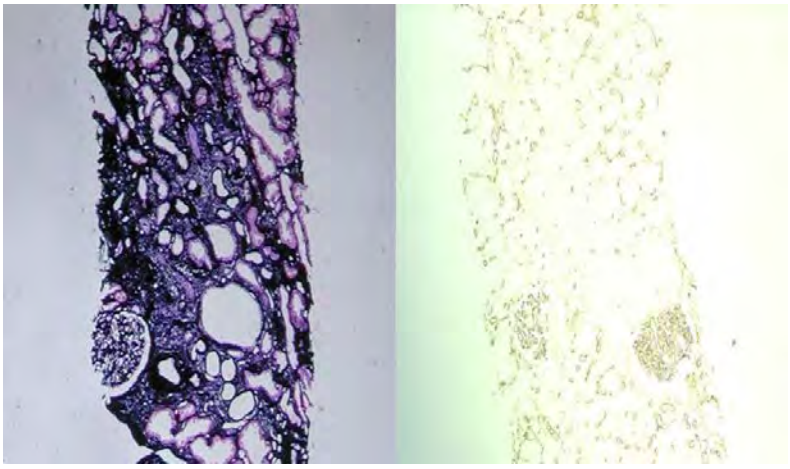


Figure 3.5: CD34 and CD68 immunostaining: (a) peri-tubular endothelial cells stained with anti-CD34 antibody using DAB, note healthy capillaries (white arrow) and atrophic/ collapsed capillaries [black arrow]; (b) interstitial\ macrophages [arrows] stained with anti-CD68 antibody using DAB. The two images represent different areas of the kidney.

Figure 3.6 Scarring and Ischaemia



(a) **(b)**

Figure 3.6: Ischaemia and scarring: In serial sections of a renal biopsy from a patient with CAD, note area of scarring stained with PAMS on panel (a); and corresponding area of scarcity of peri-tubular capillaries (CD34 staining) in panel (b).

3.2.5.3 Quantification of Peri-tubular (interstitial) Capillary Endothelial Cell Density [PTECD]

An interactive image analysis system was used for blinded assessment of PTECD. This technique has previously been found to be a reliable method for the analysis of human and animal renal biopsies (Eardley, Kubal et al. 2008).

Coded sections stained for CD34 were visualised at x200 and captured with an Aequitas IDA image database and image archive management system (Dynamic Data Links, Cambridge, UK). For analysis, each image was converted from Aequitas IDA to Aequitas IA image analysis (Dynamic Data Links), where the digitalised image is converted to a two-

colour scale image. By altering the threshold the image was processed so that positive staining was represented by black pixels measured as a percentage of the area of total image analysed. Sections where background staining made it impossible to digitally differentiate specific staining were excluded from analysis. For each patient the mean measurement of 5 randomly selected non-confluent microscopic fields was determined. Glomerular staining was excluded from the analysis by the computer software. Intra and inter-observer variability was tested and the method was validated as described earlier (see 3.2.5.1.)

Figure 3.7 Quantification of Peri-tubular Capillary Endothelial Cell Density

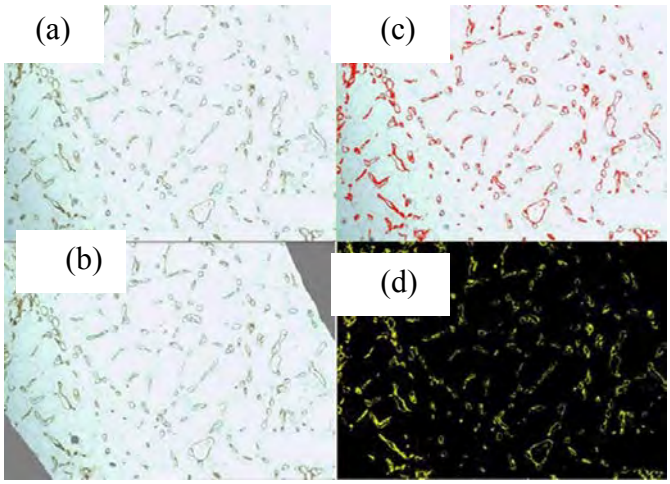


Figure 3.7: a) Section of renal biopsy from cardiac transplant recipient stained with CD34; b) area of interest is selected, the rest is masked and pixels of total area are measured digitally; c) areas of positive staining [endothelial cells] are selected digitally; d) the selected areas are converted into pixels and are measured.

3.2.5.4 Assessment of Interstitial macrophage density (IMD) by immunohistochemistry

For the detection of tissue MØ infiltration, IHC was performed on paraffin-embedded formalin fixed tissue sectioned to 2μm. The sections were heated in an oven at 56^0C for 30 minutes and then placed in pre-heated one-step dewaxing and epitope retrieval solution W-Cap TEC Buffer pH 8.0AP (Surgipath Europe Ltd, Peterborough, UK) at 96^0C for 30 minutes. The sections were then allowed to cool in W-Cap for 20 minutes. The sections were then treated with 0.3% solution of hydrogen peroxide in methanol for 10 minutes. Slides were then washed PBS. Endogenous biotin was blocked using sequential 10 min treatment with 0.1% avidin and 0.01% biotin (Dako UK Ltd, Cambridgeshire, UK). Slides were then washed with PBS and then were treated with mouse anti-human monoclonal antibody directed against the endothelial cell marker CD68 (5μg/ml; clone PG-M1; Dako Ltd) diluted in Dako REAL™ Antibody Diluent (S2022; Dako UK Ltd, Cambridgeshire, UK) for 60 minutes at room temperature. Controls used included omission of the primary antibody and substitution with mouse IgG1 (Dako UK Ltd, Cambridgeshire, UK) isotype control at equivalent concentrations in parallel using adjacent sections on the same slide. ChemMate™ DAKO EnVision™ Detection kit (K5007; Dako UK Ltd, Cambridgeshire, UK) was used for detection of positive staining. In this two-step technique, sections were incubated with the ChemMate™ DAKO EnVision™/ HRP, rabbit/ mouse reagent for 30 minutes, washed with PBS and then incubated with 1:40 dilution of ChemMate™ DAB + Chromogen in ChemMate™ Substrate Buffer for 10 minutes. Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich) [Fig. 3.4(b)], except those used for quantitative

analyses that were left unstained to avoid interference in digital quantification. Sections were then mounted in an aqueous medium (Immu Mount).

3.2.5.5 Quantification of Interstitial Macrophage Density (IMD)

The interactive image analysis system described in section 3.2.5.3 used for blinded assessment of PTECD was also used for blinded assessment of IMD. Coded sections stained for CD68 were visualised at x200 and captured with an Aequitas IDA image database and image archive management system (Dynamic Data Links, Cambridge, UK). For analysis, each image was converted from Aequitas IDA to Aequitas IA image analysis software (Dynamic Data Links) where the digitalised image is converted to a two-colour scale image. By altering the threshold the image was processed so that positive staining was represented by black pixels measured as a percentage of the area of total image analysed. Sections where background staining made it impossible to digitally differentiate specific staining were excluded from analysis. For each patient the mean measurement of 5 randomly selected non-confluent microscopic fields was determined. Glomerular staining was excluded from the analysis by the computer software. To assess the validity of the method used in the quantitative microscopy studies intra and inter-observer variability was tested as described earlier.

3.2.6 Renal Outcomes

Renal endpoint was determined as the requirement of renal replacement therapy or a 50% decline in MDRD eGFR. This was censored for patient death.

3.2.7 Statistics

All data were analysed using SPSS 15.0 software (SPSS, Chicago, USA), with results expressed as mean ± standard error of mean, and periods in median [range]. Categorical variables were compared by chi square tests. Other variables were compared by ANOVA, non parametric tests and paired t test; with values of $p \leq 0.05$ regarded as significant.

3.3 RESULTS

3.3.1 Baseline Patient Characteristics, Renal Function and Risk Factors

Sixty-two patients were included in the study. The median age was 54 [19-71] years, 47 patients were males. Thirty one patients had received one or more cadaveric liver transplants; 25 had received heart, five lung and one patient a combined heart and lung transplant. Calcineurin inhibitors were used in all patients post-transplantation with 39 (63%) receiving primary cyclosporine (Neoral®, Novartis Pharma) and the remaining primary tacrolimus (FK-506, Prograf®, Astellas Pharma). These were generally used as part of a triple therapy regimen with prednisolone and azathioprine. At the time of biopsy

53.2% were maintained on cyclosporine and 33.9% on tacrolimus based immunosuppression.

Renal biopsies were performed at a median duration of 4.04 years [0.34 to 15.92] after transplantation. At the time of biopsy the mean serum creatinine was 312.26 ± 18.26 μmol/L and MDRD eGFR 22.9 ± 1.48 ml/min/1.73 m^2) indicating advanced renal disease. After the biopsy immunosuppressive regimens were changed in some of the patients. Calcineurin inhibitors were withdrawn in 26 (41.9%) patients; mycophenolate mofetil (Cellcept Roche®) was introduced in 20 (32.3%) patients and sirolimus (Rapamune® Wyeth) in 7 (11.3%) patients. Six patients (9.67%) were dialysis dependent at the time of biopsy.

3.3.2 Histopathological Diagnoses: Contribution of CNI Nephrotoxicity (Table 3.1)

Twenty two (35.5%) biopsies showed features diagnostic of chronic CNI nephrotoxicity. Fourteen of these biopsies also had features of hypertensive nephropathy and three patients had a history of diabetes mellitus which may have contributed to the arteriolar hyalinosis. Of the remaining 40 biopsies, 27 demonstrated features of hypertensive/ischaemic nephropathy without fulfilling the criteria for chronic CNI toxicity, 12 showed another primary renal pathology [acute tubular necrosis (5/12), membranoproliferative glomerulonephritis (2/12), mesngioproliferative glomerulonephritis (2/12), diabetic nephropathy (1/12), post-infectious glomerulonephritis (1/12) and membranous

nephropathy (1/12)]. In the remaining one biopsy no significant pathology was found **(Table 3.1).**

Table 3.1 Histopathological Diagnoses

Histopathological diagnosis	**n**	**%**
Calcineurin inhibitor toxicity	22	35.5
Hypertensive nephropathy	27	43.5
Acute tubular damage	5	8.1
Mesangioproliferative GN	2	3.2
Membranoproliferative nephropathy	2	3.2
Membranous nephropathy	1	1.6
Post-infectious GN	1	1.6
Diabetic nephropathy	1	1.6
Inconclusive	1	1.6

Table 3.1: Histopathological diagnoses; note only 33% biopsies demonstrated evidence of CNI nephrotoxicity

3.3.3 CADI and CDI Scores

Forty-one biopsies displayed adequacy criteria and were included in this analysis. Median semi-quantitative grades [0-3] of inflammation, tubular atrophy, interstitial fibrosis, percent glomerulosclerosis, arteriolar hyalinosis and vascular intimal thickening were similar across

the groups. The mean CADI score was 6.8 ± 0.3 and mean CDI score 7.9 ± 0.3. Interstitial scarring and tubular atrophy scores were 1.97 ± 0.11 & 1.65 ± 0.11 respectively. There was moderate glomerulosclerosis, arteriolar hyalinosis, arterial intimal elastosis and inflammation (1.12 ± 0.13, 1.08 ± 0.15, 0.97 ± 0.12 & 0.97±0.08 respectively). Mesangial matrix expansion was mainly observed in a few biopsies in the primary renal disease group with a mean score of 0.17 ± 0.06. The distribution of individual scores (0-3) is shown in table 3.2.

The cumulative death censored renal survival rate at 5 years after renal biopsy with a total CADI score <7 was 83%. With a CADI score 7-9 it was 33% and with a score >9 all patients met a renal endpoint (log rank $p<0.001$) [ATNs were excluded from this analysis] **(Figure 3.8).**

Table 3.2 CADI Scores

N=41	Score 0 n (%)	Score 1 n (%)	Score 2 n (%)	Score 3 n (%)	Mean ± SEM
Inflammation	06(14.6%)	30(73.2%)	05(12.2%)	00(0%)	0.97± 0.08
Interstitial fibrosis	00(0%)	11(26.8%)	20(48.8%)	10(24.4%)	1.97 ± 0.11
Mesangial expansion	35(85.4%)	05(12.2%)	01(2.4%)	00(0%)	0.17 ± 0.7
Glomerulo-sclerosis	10(24.4%)	18(43.9%)	11(26.8%)	02(4.9%)	1.12 ± 0.13
Vascular thickening	12(29.3%)	19(46.3%)	09(22.0%)	01(2.4%)	0.97 ± 0.12
Tubular atrophy	00(0%)	19(46.3%)	17(41.5%)	05(12.2%)	1.65 ± 0.1
Arteriolar hyalinosis	15(36.6%)	06(14.6%)	18(43.9%)	02(4.9%)	1.08 ± 0.15

Table 3.2: CADI scores [0 to 3]; note remarkable interstitial fibrosis and tubular atrophy in the entire cohort

Scores for individual CADI and CDI variables such as inflammation and tubular atrophy negatively correlated with renal survival; however, the correlation between total CDI score and renal survival was the strongest (-0.542; p<0.001) **(Table 3.2, Figure 3.9a)**. The cumulative death censored renal survival rate at 5 years after renal biopsy, with a total CDI score <7 was 75%; with a total score of 7-9 it was 48% and with total score >9 it was 25% (log rank <0.05) **(Fig.3.8).** Upon multivariate regression analysis a negative correlation was observed between total score and renal survival (β: -0.5, p=0.001).

Figure 3.8 Renal Survival by CDI Scores

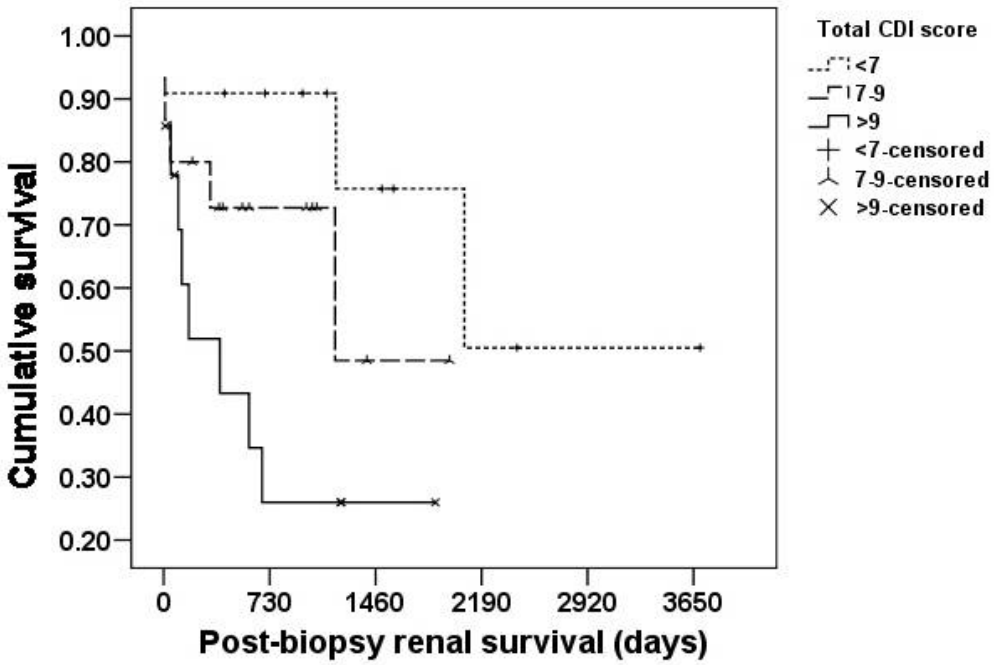


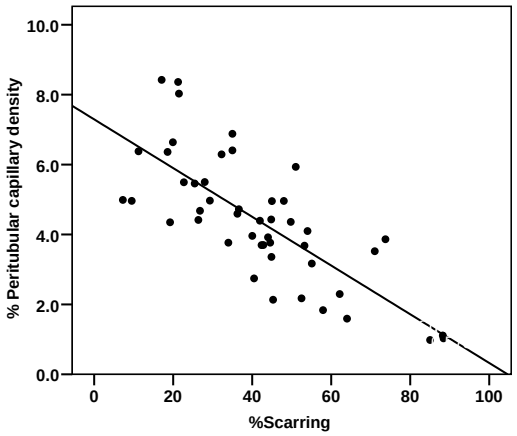
Figure 3.8: Kaplan-Meier analysis of interstitial CDI score on renal outcome. CDI scores categorized into tertiles (Log Rank Statistic: *P*<0.05).

3.3.4 Quantitative read outs for scarring, ischaemia and inflammation and their inter-relationships

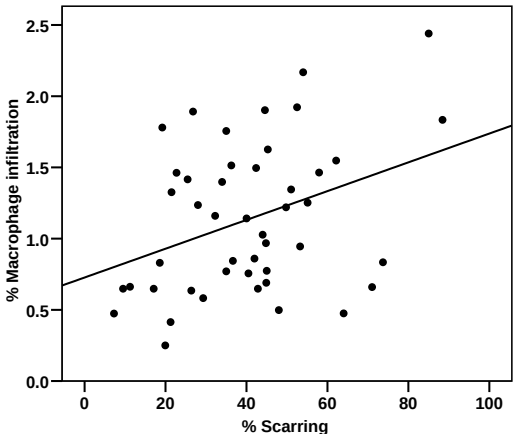
Biopsies from patients requiring dialysis at the time of biopsy and those with acute tubular necrosis were excluded from this analysis (n=8). Mean % area occupied by scarred tissue was 40.8 ± 2.7. PTECD measured as % area of the biopsy occupied by peri-tubular endothelial cells was 4.41 ± 0.26. Chronic inflammation measured by % area of the biopsy occupied by macrophages was 1.13 ± 0.07. There was a strong negative correlation between scarring and PTECD (r: -0.759, P<0.001) **(Figure 3.9a).** There was a weak positive correlation between scarring and IMD (r: 0.361, p<0.05) **(Figure 3.9b).** IMD and PTECD negatively correlated **(Figure 3.9c)** (r=-0.38; p=0.009).

Figure 3.9: Interrelationships between Injury Processes

a)



b)



c)

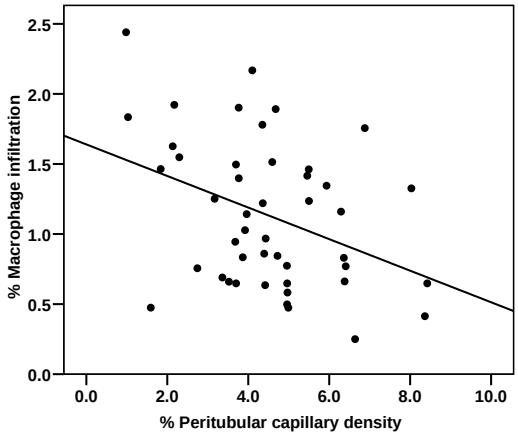


Figure 3.9: a) a strong negative correlation between PTECD and interstitial scarring (r= -0.759, P<0.001), b) a positive correlation between scarring and IMD (r=0.361, p<0.05) & c) a negative correlation between IMD and PTECD (r=-0.38; p=0.009)

3.3.5 Impact of Scarring, Ischaemia and Inflammation on Renal Survival

Cox regression analysis was performed in order to assess associations with renal survival. Since scarred interstitium is effectively avascular, ratios of unscarred interstitium over PTECD were calculated, in order to test the hypothesis that capillaries density in unscarred interstitium [viable] have an impact on renal survival. The unscarred interstitium/PTECD ratio significantly correlated with renal survival with a hazard ratio of 1.100 [CI: 1.102 – 1.228] ($p<0.05$). Higher ratios of unscarred interstitium over PTECD [representing ischaemic interstitium] were associated with worse cumulative renal survival (log rank <0.05) **(Figure 3.10).** Ratios between interstitial scarring and IMD were not significantly associated with renal survival.

Figure 3.10 Impact of Ischaemia on Renal Survival

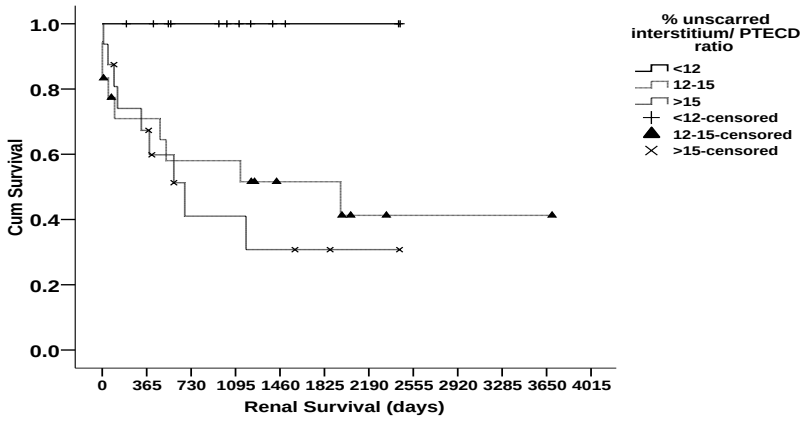


Figure 3.10: Kaplan Meier survival plots: note higher ratios of unscarred interstitium: endothelial cells [representing ischaemic interstitium] were associated with worse cumulative renal survival (log rank <0.05)

3.3.6 Features Specific to CNI Nephrotoxicity/ Ischaemic-Hypertensive Nephropathy: a sub-group analysis

The cohort was divided into three categories as described above. [1] Chronic CNI nephrotoxicity [CNI-NT] with or without evidence of hypertensive nephropathy; [2] hypertensive/ischaemic [HT/Isch] nephropathy without evidence of chronic CNI nephrotoxicity; and [3] Primary renal disease. Patient characteristics, indication for transplantation, immunosuppressive regimen used and time to renal biopsy were comparable between the groups **(Table 3.3).** The duration to renal biopsy following transplantation and renal function at the time of biopsy were comparable between the groups [(4.8[1.0-11.5] vs. 2.6 [0.6-10.0] vs. 1.9[0.8-15.9]) and (MDRD eGFR: 24.28[7.2-49.1] vs. 22.72[7.1-48.1] vs. 19.9[8.9-49.7]) respectively]. Known risk factors for the development of end-stage renal failure such as peri-transplantation acute renal failure, hepatitis-C, presence of diabetes mellitus and hypertension were also comparable.

The median score for arteriolar hyalinosis was significantly higher in biopsies with predominant CNI nephrotoxicity when compared with that in the biopsies without CNI nephrotoxicity and primary renal disease (2[2-3] vs. 1[0-1] and 0[0-1] respectively; ($p<0.05$) **(Table 3.4).** Median scores for the remaining variables (inflammation, tubular atrophy, glomerulosclerosis, mesangial matrix expansion and vascular intimal thickening) were comparable across the groups (p=ns). Median CADI scores were also comparable across the groups (7[4-10] vs. 6.5[2-9] vs. 6.5[3-11]). In the group with CNI nephrotoxicity, the presence of additional hypertensive features (n=11) was not associated

with any increase of the CADI/CDI variables. In cases where the renal endpoint was met, CADI and CDI scores demonstrated a negative correlation with renal survival (-0.625; $p<0.05$ and -0.635; $p<0.05$ respectively). Amongst CADI variables, interstitial scarring and glomerulosclerosis individually demonstrated negative correlations with renal survival (-0.518; $p<0.05$ and -0.562; $p<0.05$ respectively).

Table 3.3 Demographic Details

	Patients with predominant histological evidence of CNI nephrotoxicity (n=22)	Patients *without predominant* histological evidence of CNI nephrotoxicity *(n=39)*		*P*
		Patients with hypertensive/ ischaemic nephropathy (n=27)	Patients with other primary renal disease (n=12)	
Median age at biopsy (years)	50 (19 to 69)	57 (27-71)	51(37-68)	ns
Sex ratio (M:F)	16:6	18:9	12:0	ns
Time of renal biopsy after transplantation (years)	4.8[1.0-11.5]	2.6 [0.6-10.0]	1.9[0.8-15.9]	ns
Mean Sr. Creatinine. µmol/L	319 ± 32	303 ± 27	332 ± 43	ns
Mean MDRD eGFR	22.92 ± 2.64	22.3 ± 2.09	25.35 ± 3.7	ns
Dialysis dependent	2	1	3	ns
Change in immunosupppression				
CNI withdrawn	12(54.5%)	06 (22.2%)	11(64.7%)	<.05
MMF introduced	10(45.5%)	02 (14.8%)	06(35.3%)	<.05
Sirolimus introduced	01(4.5%)	03 (11.1%)	01(5.9%)	ns
Risk factors				
History of diabetes mellitus	03 (13.6 %)	02 (07.4%)	08 (66.7%)	<.005
Post-transplantation acute renal failure	05 (22.7%)	06 (22.2%)	05 (41.7%)	ns
Hepatitis C recurrence	02 (09.1%)	02 (07.4%)	04 (33.3%)	ns
Median post biopsy renal survival (years)	0.88 (0 – 6.67)	1.43 (0 – 10.12)	1.73 (0 – 4.34)	ns
Median post-transplant renal survival (years)	6.67 (1.78 – 14.5)	7.01 (0.92 – 18.19)	5.25 (0.34 – 19.17)	ns

3.3.5.1 Injury processes specific to each of the subgroups:

The median % area of scarring, PTECD and IMD were comparable across the three subgroups. The negative correlation between PTECD and scarring observed in the whole cohort **(Figure 3.9a)** was maintained for the CNI-NT and HTN/Isch subgroups ($r=-0.808$; $p<0.001$) and ($r=-0.783$; $p<0.001$) respectively **(Table 3.4a, 3.4b).** This correlation was weaker and not statistically significant in the primary renal disease group. A statistically significant positive correlation between IMD and scarring was observed in the CNI-NT group [$r=0.496$; $p<0.05$]; but not in HTN/Isch group. There was a negative correlation between MØ and PTECD in the HT/Isch group [$r=-0.523$; $p<0.05$] and a non-signficant ($p = 0.096$) correlation in the CNI-NT group.

Table 3.4 Injury Processes by Subgroups

a) CNI- Nephrotoxicity

		PTECD	IMD
Scarring	**r**	**-0.808**	**0.496**
	p	**0.000**	**0.036**
	n	**18**	**18**
PTECD	**r**	1	-0.404
	p		.096
	n		18

b) Hypertensive/ ischaemic nephropathy

		PTECD	IMD
Scarring	**r**	**-0.783**	0.250
	p	**0.000**	0.275
	n	**22**	21
PTECD	**r**	1	**-0.523**
	p		**0.015**
	n		**21**

c) Other primary renal diseases

		PTECD	MØ
Scarring	**r**	-0.424	0.085
	p	0.343	0.856
	n	7	7
PTECD	**r**	1	0.427
	p		0.339
	n		7

Table 3.4: Correlations between scarring, PTECD and IMD; a) CNI nephrotoxicity group: note scarring correlates with PTECD and IMD b) hypertensive/ ischaemic nephropathy: note correlations between scarring and PTECD and that between PTECD and IMD c) other primary renal diseases: no significant correlations are observed.

To assess the impact of injury processes (ischaemia, inflammation and scarring) on survival in each subgroup, Cox regression analyses were repeated. None of the injury processes had a specific impact on the diagnostic sub-class.

3.3.7 CDI Scores (Table 3.5)

Semi-quantitative grades [0-3] of inflammation, tubular atrophy, interstitial fibrosis, glomerulosclerosis, arteriolar hyalinosis and vascular intimal thickening were compared across the three groups as previously outlined. As expected, the median score for arteriolar hyalinosis was significantly higher in biopsies with predominant CNI nephrotoxicity when compared to Isch/HT biopsies without CNI nephrotoxicity and primary renal disease (2[2-3] vs. 1[0-2] and 0[0-1] respectively; ($p<0.001$)). Median scores for the remaining variables (inflammation, tubular atrophy, glomerulosclerosis, mesangial matrix expansion and vascular intimal thickening) were comparable across the groups ($p>0.05$). Median total CDI scores were also comparable across the groups (9[4-13] vs. 9[4-11] vs. 6.5[3-13]). As some (11/22) biopsies with CNI-nephrotoxicity also demonstrated mild features of hypertension, the group with CNI-nephrotoxicity was further divided according to the presence of hypertensive features (n=11 each); the CDI variables were found to be comparable between these two groups.

Table 3.5 CADI scores

	CNI nephrotoxicity	Hypertensive/ ischaemic nephropathy	Primary Renal Diseases	*p*
Inflammation	1(0-2)	1(0-2)	1(0-2)	ns
Tubular atrophy	1(1-3)	1.5(1-3)	2(1-3)	ns
Interstitial fibrosis	2(1-3)	2(1-3)	2(1-3)	ns
Glomerulosclerosis	1(0-3)	1(0-3)	1(0-2)	ns
Vascular thickening	1(0-3)	1(0-2)	0.5(0-2)	ns
Arteriolar hyalinosis	2(2-3)	1 (0-1)	0(0-1)	<0.05
Mesangial expansion	0(0-1)	0(0-1)	0(0-2)	<0.05
CADI	7(4-10)	6.5(2-9)	6.5(3-11)	ns
CDI	9(6-12)	7.5(3-9)	6.5(3-11)	<0.05

Table 3.5: Most variables are comparable between the sub-groups; arteriolar hyalinosis by definition is worse in the CNI nephrotoxicity group, also note highest CDI score in this group.

3.3.8 Follow-up and Disease Progression

The cohort was followed up at the host centre for a median 3.32 [0.01 to 10.12] years. Twenty-eight (45.2%) patients died at a median duration of 1.64 (0.1 to 6.69) years after renal biopsy. Twenty-four (38.7%) recipients required long-term dialysis; two of these patients subsequently received a renal transplant. There was no impact from the type of

non-renal solid organ transplanted on renal survival **(Figure 3.11b).** Although the median renal survival in patients with CNI nephrotoxicity was relatively shorter (0.88 [0 to 6.67] vs. 1.91 [0 to 10.12] years; p=ns) **(Figure 3.11b)** when compared with patients without CNI nephrotoxicity, this was not statistically significant. Likewise, the percentage of deaths and percentage of patients developing ESRF were comparable between the two groups. Similarly, patients in whom CNIs were withdrawn had comparable renal survival to the CNI continuation group **(Fig.3.11c)**.

Figure 3.11 a Type of Transplant and Renal Survival

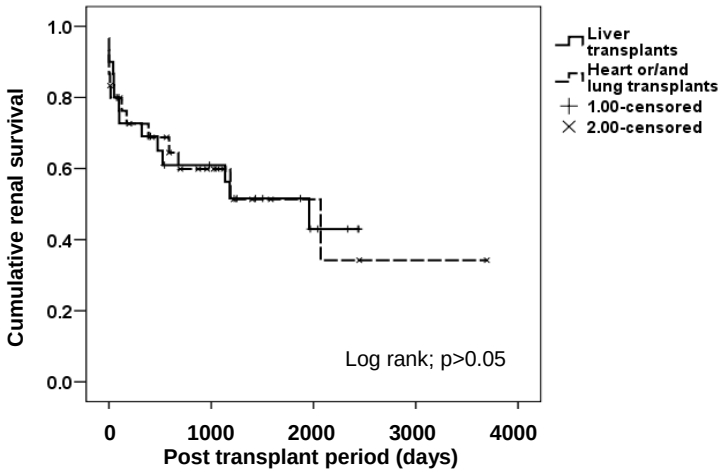


Figure 3.11a: Comparable renal survivals following liver and thoracic transplantation; note 50% cumulative renal losses occur within 5 years post transplantation.

Figure 3.11b CNI Nephrotoxicity and Renal Survival

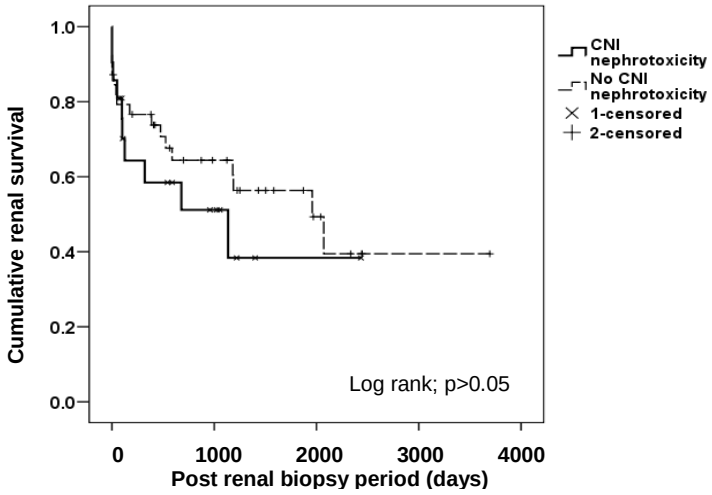


Figure 3.11b: Comparable cumulative renal survival amongst patients with and without demonstrable CNI nephrotoxicity on renal biopsy.

Figure 3.11c CNI withdrawal and Renal Survival

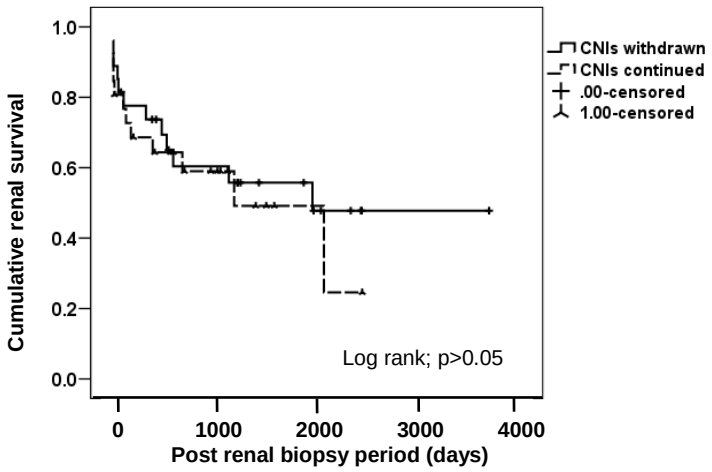


Figure 3.11c: comparable cumulative renal survival amongst patients in whom CNIs were continued and were withdrawn following on renal biopsy

3.4 DISCUSSION

Chronic kidney disease is an important complication in NRSOT (Ojo 2007). Knowledge of the pathophysiology and natural history of chronic renal failure in this setting gives an insight into mechanisms of renal injury that may be transferable to failing renal transplants. The absence of allogeneic processes in this allows an analysis of the relative contribution of CNI nephrotoxicity to renal injury. I further exploit this principle in the next chapter where I directly compare injury processes in non-renal solid organ transplantation to those in failing renal transplants.

The published data on chronic kidney disease in non-renal transplantation is mainly from clinical studies and only a few of these have performed histological analysis (Pillebout, Nochy et al. 2005; Kim, Akalin et al. 2010; Schwarz, Haller et al. 2010). Therefore, the analysis presented in this chapter is a significant contribution to our knowledge base in this area (Kubal, Cockwell et al. 2012). Although CKD is a well-known complication following heart, lung, small intestine and liver transplantation, the evidence base for the pathogenesis of CKD in these settings is limited. One of the challenges in this area is represented by inconsistancy in the definition of CKD. For example, in liver transplantation, various studies have used different thresholds to define CKD, (Fisher 1998; Ojo, Held et al. 2003; Velidedeoglu E 2004) which is associated with widely reported differences in prevalence. In this biopsy study, 6.6% of the recipients who survived at least one year after transplantation developed ESRF.

The majority of the data reported in this study was generated by using computer analysis of digital images of the kidney biopsies, including interstitial fibrosis, macrophage infiltration and peri-tubular capillary endothelial density. Computer analysis of digital images to assess interstitial fibrosis has been used by the host group (Eardley, Zehnder et al. 2006). Several other studies have shown that interstitial fibrosis quantification is predictive of renal allograft outcome and may be considered a surrogate marker (Grimm, Nickerson et al. 1999; Choi, Shin et al. 2005) . Although these methods are validated, this way of analyzing the injury processes has two issues: i) the biopsy area may not be representative of the whole organ as the disease process may can be heterogeneous as seen in Fig. 3.6 are patchy and ii) computer analysis of digital images may add an user error to the quantification. A recent study comparing four morphometric techniques to assess interstitial fibrosis in kidney biopsies on duplicate sections with each method on separate days, concluded that morphometric techniques had good to excellent inter-assay reproducibility and inter-observer reproducibility (Farris, Adams et al. 2011). To assess the validity the morphometric analysis reported in the study, intra- and inter-observer variability was tested. As regards to the computer analysis of PTECD and interstitial macrophage density, again computer analysis of digital images was used. This method has been widely used by the host group (Eardley, Zehnder et al. 2006; Eardley, Kubal et al. 2008), as well as by others (Nakayama, Sato et al. 2009). Again, similar to quantification of interstitial sclerosis, this method has shortfalls: i) the biopsy area may not be representative of the whole organ as the disease process may can be heterogeneous ii) computer analysis of digital images may add an user error to the quantification and additionally, iii) the assessment is two

dimensional as opposed to three dimensional structure of capillaries and there may be a lack of specificity of the labeling technique itself. To overcome the deficiencies related to the two dimensional assessment, recently three dimensional assessment of renal microcirculation has been used (Remuzzi, Gagliardini et al. 2006; Advani, Connelly et al. 2011).

The patients in this study, had advanced kidney disease as measured by a mean MDRD eGFR of 22.91 ± 1.4 ml/min/1.73 m^2 (mean serum creatinine of 312 ± 17 μmol/L) at the time of biopsy. This was confirmed by an index of chronic damage in excess of 20% in the majority of cases. An evaluation of a larger number of biopsies with more preserved renal function would have been interesting; however, the study did identify in situ criteria for progression to a renal end-point and allowed the description of processes that may be central for the progression of established renal disease.

3.4.1 Histological Evidence of CNI Injury

The sensitivity of arteriolar hyalinosis as the single measurable in situ determinant of CNI nephrotoxicity is of great interest. In my analysis, 35.5% of the biopsies showed criteria for CNI toxicity. Patients with biopsies with this feature did not sustain an accelerated deterioration in renal disease compared to biopsies without histological evidence of CNI use. This does not mean that CNIs do not have an important role in the pathogenesis of kidney disease; indeed, the strong inverse correlation between capillary density and

scarring across disease groups indicates that microvascular disease, possibly as a consequence of upstream arteriolar vasoconstriction to which CNIs may contribute.

In a similar study (n=26) in this area (Pillebout, Nochy et al. 2005), authors reported chronic CNI toxicity in 46% patients, at a mean follow up of 5 years post liver transplantation. As not all non-renal transplant recipients undergo renal biopsy for deteriorating renal function, blind change in immunosuppression may not only be unnecessary but potentially harmful.

3.4.2 Hypertensive Renal Disease

Histological changes secondary to hypertension were seen in 22 (32.83%) biopsies. In addition 16/23 (69.56%) biopsies with predominant CNI nephrotoxicity demonstrated hypertensive changes. It was not surprising to see these two pathophysiologic process co-exist, as more than half of the patients on cyclosporine are reported to develop new onset hypertension after OLT in long term (O'Grady JG 1988). Patients in this study with features of hypertensive damage irrespective of co-existing CNI-nephrotoxicity had a mean blood pressure of 151/86 mmHg at the time of biopsy.

3.4.3 Primary Renal Disease

The factors responsible for the appearance or worsening of CKD following non-renal transplantation are poorly understood. Any interpretation must be performed in the context of the known prevalence of CKD. A recent report indicates 16.8% of the U.S. population

aged ≥20 years have CKD (S Saydah 2007) and it is likely that some transplant recipients have pre-existing, yet undiagnosed CKD, that manifests after transplantation. Additionally, the significant loss of muscle mass, particularly in liver disease, may underestimate renal insufficiency pre-transplantation. Renal impairment may then become apparent with improved nutritional status after transplantation, in addition to the known factors associated with renal injury.

In cardiac transplantation, the majority of patients have pre-existing heart failure and thereby have various compensatory mechanisms to sustain end-organ perfusion. In the kidneys there is an overall decrease in renal plasma flow due to efferent arteriolar vasoconstriction mediated by elevated Angiotensin II and elevated vasopressin, catecholamines and endothelins. Some of these hormones have also been shown to accelerate glomerular and interstitial scarring (Weber 1997).

In this study, biopsies from 62.7% patients had no definitive evidence of CNI toxicity. This indicates that without knowledge of renal histology, there is a significant chance of making treatment changes, by altering immunosuppression based on assumptions that would not be confirmed by histology. Further, there is a significant risk of missing the correct diagnosis, where in some cases knowledge of histology may direct disease specific management. In the published literature there are other causes of renal disease reported that were not encountered in the series I report. For example, it is now known that BK nephropathy can

be seen in the setting of non-renal solid-organ transplantation (Limaye, Smith et al. 2005; Schmid, Burg et al. 2005).

3.4.4 Scarring, Ischaemia and Inflammation in this Setting

Ischaemia**,** inflammation and scarring are known injury processes involved in renal disease (Howie, Ferreira et al. 2001; Sean Eardley and Cockwell 2005; Eardley, Zehnder et al. 2006; Eardley, Kubal et al. 2008). The analysis that I have reported here indicates that these injury processes are also associated with accelerated renal damage in NRSOT. As seen in Figures 3.11 a, b and c. these processes are inter-related. The strongest correlation was seen between PTECD [ischaemia] and scarring. This may suggest that ischaemia is the dominant process of injury causing scarring. An explanation for a relatively weaker correlation between macrophage infiltration and scarring may be the use of immunosuppressive drugs in these patients, which in the case of CNIs may also contribute to the ischaemia seen in these biopsies. As seen in table 4 this correlation between ischaemia and scarring is maintained in sub-group analyses for CNI nephrotoxicity and HT/Isch nephropathy groups. This maintained level of microvascular disease in biopsies that do not have arteriolar hyalinosis can be interpreted in two ways: first, that CNI mediated injury to the microvascular bed is present irrespective of the presence or absence of arteriolar hyalinosis; second, that CNIs do not have a significant role in the pathogenesis of renal injury in solid organ transplantation. Clearly, the balance of evidence does indicate that CNIs are important, but I believe a significant reappraisal of the classification of CNI nephrotoxicity with a particular reference to arteriolar hyalinosis is required.

3.4.5 Role of CDI Scoring in Management of CKD in Non-renal Solid Organ Transplantation

Chronic kidney disease is as a major and common complication after solid organ transplantation, and renal function measurements lack accuracy in this setting (Borrows and Cockwell 2007). The work in this chapter also indicated that in patients who have renal biopsies, the CADI scoring system, which has been validated in renal transplant settings (Isoniemi, Taskinen et al. 1994), can be modified to provide a useful tool to assess renal prognosis. As seen in this study, arteriolar hyalinosis is an important histopathological variable that contributes to the overall renal injury and therefore its incorporation in the scoring models is useful. CDI scoring may be of utility in both for providing prognostic indicators for renal failure management, as well as for clinical trials where renal biopsies are part of the protocol (Kubal, Cockwell et al. 2012).

3.5 CAVEATS

This study is based on morphometric analyses using digital images of the biopsies, which may have inherent limitations to be representative of the injury pattern in the whole organ. A careful morphometric analysis of interstitial fibrosis requires sensitive parameters through which the severity can be measured. Secondly, this study was not designed to establish a dose response relationship between CNI use and renal survival or to assess the impact of the original liver, heart, heart/lung disease recurrence and hepatitis C on renal

survival. Whilst this is a large biopsy series in this setting that has been reported, the methods used to investigate in situ changes provide a level of detail that has not previously been obtained. The numbers are relatively small and the series may be underpowered to distinguish less strong, but still important, associations. Furthermore, whilst outcome data was integrated into this study it has been related to changes that may reflect the end point of the pathogenic pathway, rather than triggers and modifiers of renal injury.

CHAPTER 4

A COMPARATIVE ANALYSIS OF IN SITU DETERMINANTS IN TRANSPLANT AND NON-TRANSPLANT CHRONIC KIDNEY DISEASE

4.1 INTRODUCTION

Our understanding of the dominant processes of injury in progressive renal disease is derived from studies in non-transplant chronic kidney disease. As outlined in Chapter 3, these processes are inflammation, ischaemia and scarring. A core hypothesis of the work in this thesis is that the final common pathway of renal damage is similar in failing renal transplants and chronic kidney disease. To test this hypothesis I have used an experimental model utilising a comparable analysis between three disease groups: 1] renal transplants with CAD; 2] non-renal solid organ transplant recipients with chronic kidney damage; and 3] native non-transplant chronic kidney disease. Work in the group has recently focused on recruiting a large cohort of patients. In this, accurate characterisation of in situ changes has identified a role for albuminuria and MCP-1 directed macrophage infiltration in early chronic kidney disease, and an important role of ischaemia in more advanced CKD.

4.1.1 Tubulointerstitial Inflammation

There is a close association between the degree of renal impairment and the extent of tubulointerstitial disease in CKD, and a close relationship between tubulointerstitial disease and progression to end stage renal failure (Bohle, Strutz et al. 1994; Nath 1998). Interstitial inflammation, in particular the presence of macrophages, has a central role in this process (Nikolic-Paterson and Atkins 2001; Rodriguez-Iturbe, Pons et al. 2001).

In kidney transplantation, it is well established that persistent graft inflammation leads to chronic tubulointerstitial damage (Rush, Jeffery et al. 1995; Rush, Karpinski et al. 1999). The large majority of these patients do not have acute or chronic rejection identifiable by Banff criteria.

The role of macrophages in tubulointerstitial injury and their impact on allograft function and survival has now been established (Srinivas, Kubilis et al. 2004). Although widely studied in CKD, how macrophages cause renal injury in a transplant setting is not as well defined. Most available data is from animal studies (Wang, Cai et al. 2008) and preliminary reports suggest that the activation state of the macrophages, rather than macrophage load ,is important in mediating renal injury (Wang, Cai et al. 2008). In this chapter, I evaluate the impact of interstitial macrophage density on disease progression and also the interrelationship between macrophage density, peri-tubular capillary loss and scarring.

4.1.1.1 Role of Monocytes/ Macrophages

Tissue effector cells of the monocyte lineage have been detected increasingly as a significant subset of recruited inflammatory cells in inflammatory diseases of the kidney. Macrophage accumulation occurs following ischaemia-reperfusion injury and is seen in renal allografts. Recipient-derived macrophages have been shown to infiltrate the graft within 24 hours of transplantation (Penfield, Wang et al. 1999). With resolution of this ischaemic insult and in the absence of rejection, the number of macrophages present within

the organ decreases, but macrophages may still be present in low numbers in these allografts (Grimm, McKenna et al. 1999). In CAD, macrophage accumulation both predates and accompanies this chronic pathological process leading to end-stage organ failure (Pilmore, Painter et al. 2000). Activated macrophages may secrete a large number of proinflammatory cytokines including IL-1, IL-12, IL-18, TNF- α, IFN-γ, growth factors such as basic fibroblast growth factor (bFGF) and TGF-β (Nadeau, Azuma et al. 1995). These cells also increase expression of matrix metalloproteinases (MMP) (Palomar, Ruiz et al. 2004) which induce glomerulosclerosis, arteriosclerosis, and interstitial fibrosis. The presence of macrophages in protocol biopsy specimens is considered an important cofactor in the development of CAD (Croker, Clapp et al. 1996). Macrophages contribute to both the innate and acquired arms of the alloimmune response and thus may be involved in all aspects of acute and chronic allograft rejection. Activated macrophages may be cytotoxic to various renal parenchymal cells and have been implicated in vascular endothelial cell injury, suggesting them to be good candidates for effectors of disease progression in chronic allograft failure. Persistence of the macrophage infiltrate following resolution of acute rejection was found to be predictive of CAD. More specifically, administration of a macrophage inhibitor (Azuma, Nadeau et al. 1995) or adenoviral-mediated transfer of anti-macrophage agents (viral IL-10 and soluble antagonists of TNF-[alpha] and IL-12) abrogated the development of clinical and histological parameters of CAD in the F344-Lewis rat model (Yang, Reutzel-Selke et al. 2003).

4.1.2 Tubulointerstitial Ischaemia

In native kidney disease progressive tubulointerstitial disease is associated with the loss of interstitial capillaries and a reduced blood flow within those that remain (Seron, Alexopoulos et al. 1990; Bohle, Mackensen-Haen et al. 1996; Futrakul, Yenrudi et al. 1999; Choi, Chakraborty et al. 2000). The degree of ischaemia is a rigorous predictor of the rate of development of interstitial scarring and progression to renal failure end-points (Eardley, Kubal et al. 2008). This may be of particular importance in chronic allograft damage following renal transplantation as vascular damage is a prominent feature. Recent studies have demonstrated vascular rarefaction in failing renal transplants (Adair, Mitchell et al. 2007). In both CKD and renal transplant settings, it has been shown that the progressive tubulointerstitial disease is associated with the loss of cortical peri-tubular capillaries (Seron, Alexopoulos et al. 1990; Bohle, Mackensen-Haen et al. 1996; Choi, Chakraborty et al. 2000; Ishii, Sawada et al. 2005). In these studies peri-tubular capillary density has been used as a surrogate marker of renal circulation. Immunohistochemical techniques have been used to identify capillary endothelial cells. In the majority of studies, three-stage indirect immunohistochemical staining of the antigen CD34 was performed. Several methods of quantification were used including counting the mean number of peri-tubular capillary lumina surrounded by CD34-positive cells per interstitial field (Ishii, Sawada et al. 2005), expressing the mean number of CD34-positive cells per interstitial field (Choi, Chakraborty et al. 2000), and expressing the mean size of the CD34-positive cells visualised (Choi, Chakraborty et al. 2000). One may assume that the loss of peri-

tubular capillaries observed with disease progression is associated with increasing tissue hypoxia if there is no compensatory increase in blood flow in those capillaries that remain.

4.1.2.1 Role of Endothelial Cells

The vascular endothelium plays an important role in vascular function, through the regulation of vascular tone and integrity. Endothelial cells play a vital role in the production of cytokines and chemokines essential for chemo-attraction of "repair cells". They also play an active role in cell adhesion through increased expression of both VCAM-1 and ICAM-1 (Heemann, Tullius et al. 1994). In CAD the endothelium within the transplanted organ may play an independent role in the development of injury. Inflammation and endothelial disruption leads to increased donor antigen presentation to the host and infiltration of host immune cells (B- cells, and T- cells).

In the presence of antigen presenting cells, stimulated B- cells produce anti-donor antibodies, which are central in the development of the lesions seen in chronic rejection. Inflammation results in macrophage/monocyte infiltration and up regulation of cytokine production follows. Platelet aggregation occurs resulting in release of eicosanoids and PDGF. More specifically, the local inflammatory milieu attracts smooth muscle cells, which initiate the process of vascular re-modelling. Vascular endothelial growth factor (VEGF) is produced in increasing quantities by the damaged endothelium which results in increasing arterial hyalinization and vessel re-modelling. Vascular smooth muscle cells migrate to the site of endothelial injury, and undergo replication and differentiation

(Hayry, Alatalo et al. 1995). They may also undergo phenotypic differentiation to myofibroblasts characterised by α-smooth muscle actin production and loss of their secretory role. Although these enhance the vascular repair, it could also lead to intimal fibrosis and gradual vessel occlusion.

4.1.3 Interstitial Scarring

In CKD, interstitial scarring is the most reliable prognostic factor of long-term outcome, irrespective of the cause (Bohle, Wehrmann et al. 1992). It is accompanied by a progressive deterioration in renal function (Racusen, Solez et al. 1999). The adverse long-term consequences of interstitial scarring following renal transplantation include shortened graft survival (Isoniemi, Taskinen et al. 1994). Interstitial scarring has been documented as early as three months following transplantation (Nankivell, Fenton-Lee et al. 2001) and is thought to follow a sequence of phases similar to wound healing: initial tubular injury is followed by an early inflammatory phase with fibrogenic signaling, cellular activation and interstitial matrix generation, culminating in a relatively inactive phase of obliterative healing and tubular destruction. Interstitial fibrosis represents a common end-pathway associated with nephron destruction. In the studies reported in this chapter, I explore the relationship between macrophages, interstitial capillary density and interstitial scarring across disease groups.

4.2 METHODS

4.2.1 Patients

Three groups of patients were included in the analysis:

(a) Chronic allograft damage (n=49)

This cohort was identified from the renal transplant biopsy database in the Department of Pathology, University Hospital Birmingham. The database was scanned for 'chronic allograft nephropathy/damage' and retrieved reports were further studied. I excluded patients with previous episodes of acute rejection. All patients included in the study had a negative complement dependent cytotoxicity (CDC) crossmatch at the time of transplant. Patients developing *de novo* donor specific antibodies were not included in the study. In addition, patients with biopsy evidence of de-novo or recurrent renal disease were excluded. Patients requiring dialysis at the time of biopsy were excluded to avoid confounding factors associated with acute pathology in addition to CAD.

(b) Non-renal solid organ transplant recipients with hypertensive/ ischaemic nephropathy or CNI nephrotoxicity (n=44)

These patients were studied as a part of the patient cohort studied in Chapter 3. However, biopsies that demonstrated primary renal pathology (n=8) were not included. Biopsies from patients dependent on dialysis at the time of biopsy were also excluded.

(c) Ischaemic/ hypertensive chronic kidney disease (n=39)

A cohort of 215 patients has been extensively studied at the host centre. From this cohort biopsies that demonstrated ischaemic/hypertensive damage (n=39) were selected for the comparative analysis in this chapter.

The groups were matched for renal function measured by MDRD eGFR at the time of renal biopsy.

4.2.2 Quantification of Interstitial Scarring, Chronic Damage Index (CDI)

An interstitial scarring index [chronic damage index (CDI)] was already calculated for biopsies from non-renal transplant group (group b). The same methodology as described in 3.2.5.1 was applied to obtain quantitative readouts for CDI on renal biopsies from group (a) & (c). Briefly, sections were stained with PAMS and the index of chronic damage index (c) was calculated as % of entire biopsy area occupied by scarred tissue. The methodology for this assessment was described in Chapter 3 (3.2.5).

4.2.3 Quantification of Peri-tubular Capillary Density (PTECD) and Interstitial Macrophage Infiltration (IMD)

Peri-tubular capillary endothelial cell density (PTECD) and interstitial macrophage density (IMD) were already calculated for biopsies from the non-renal transplant group (group b).

The same methodology was applied to obtain quantitative read outs for PTECD and IMD on renal biopsies from group (a) & (c).

Briefly, peri-tubular capillaries were stained with anti-CD34 antibody and the quantitative read outs for peri-tubular capillary density [PTECD] were obtained using Aequitas IA image analysis software (Dynamic Data Links) as described in sections 3.2.5.2 and 3.2.5.3.

Interstitial macrophages were stained with anti-CD68 antibody and the quantitative read outs for IMD were obtained using Aequitas IA image analysis software (Dynamic Data Links) as described in sections 3.2.5.4 and 3.2.5.5.

4.2.4 Endothelial Cell- Macrophage Co-localization

4.2.4.1 Dual immunohistochemistry with anti CD-34 and anti CD-68 antibodies

For dual immunohistochemistry I used anti CD34 and anti CD68 antibodies on the same section utilising an established double stain system [Dako Cytomation EnVision Doublestain System (Cat. No. K1395; Dako Ltd, UK)]. This staining system is designed for the simultaneous detection of two different antigens (CD34 and CD68 in this instance) within one tissue section. A combination of horseradish peroxidase labelled polymer (HRP) and alkaline phosphatase (AP) staining systems were utilised.

Many of the previously described double staining protocols suffer from limitations such as complicated multi-step processes, lack of versatility, lack of sensitivity and cross-reactivity.

One such method utilizes indirect methods involving enzyme-labelled antibodies, avidin-biotin complexes and peroxidase anti-peroxidase combined with alkalike phosphatase-anti-alkaline phosphatase complex. The combination methods are cumbersome, long and in many cases insensitive. The system used here overcomes these limitations by utilizing an enhanced labelled-polymer technology. This system is based on HRP and AP labelled polymers which are conjugated with secondary antibodies against mouse and rabbit immunoglobulins.

The sections were heated in an oven at 56^0C for 30 minutes and then placed in a pre-heated one-step dewaxing and epitope retrieval solution W-Cap TEC Buffer pH 8.0AP (Surgipath Europe Ltd, Peterborough, UK) at 96^0C for 30 minutes. They were then allowed to cool in W-Cap for 20 minutes. The sections were then treated with 0.3% solution of hydrogen peroxide in methanol for 10 minutes. Slides were then washed with phosphate buffer solution (PBS). Endogenous biotin was blocked using a sequential 10 min treatment with 0.1% avidin and 0.01% biotin (Dako UK Ltd, Cambridgeshire, UK). Tissue specimens were initially treated with peroxidase Block to quench endogenous peroxidase activity. The sections were then incubated with mouse anti-human anti-CD68 (5μg/ml; clone PG-M1; Dako Ltd) diluted (dilution; 1: 200) in Dako REAL™ Antibody Diluent (S2022; Dako UK Ltd, Cambridgeshire, UK) for 60 minutes at room temperature. This was followed by incubation with the Labelled Polymer, HRP for 30 minutes at room temperature.

Following incubation with HRP, the initial reaction was completed with a 10-minute incubation of the Liquid DAB+ substrate-chromogen which resulted in a brown-colored precipitate at the first antigen site. Upon completion of the first reaction, the sections were treated with the Doublestain Block. The Doublestain Block served to remove any potential cross-reactivity between the reactions and to block any endogenous alkaline phosphatase that may be present. Following the Doublestain Block, specimens were incubated with the second primary antibody, anti- CD34 monoclonal antibody (1µg/ml; clone QBEnd 10; Dako Ltd) diluted (dilution; 1:100) in Dako REAL™ Antibody Diluent (S2022; Dako UK Ltd, Cambridgeshire, UK) for 60 minutes at room temperature. This was followed by incubation of the Labelled Polymer, Alkaline Phosphatase for 30 minutes. The second antigen staining was then completed with a Vector Blue Alkaline Phosphatase Substrate Kit III (Cat. No. SK-5300) for 20 minutes. Counterstaining was not required as endothelial cells and macrophages were easily identifiable **(Figure 4.1).** However, negative controls were used where only one of the two primary antibodies was used and the other was replaced with an isotype IgG. For complete negative controls, both primary antibodies were replaced with an isotype IgG.

Figure 4.2 Co-localization by Dual Immunohistochemistry

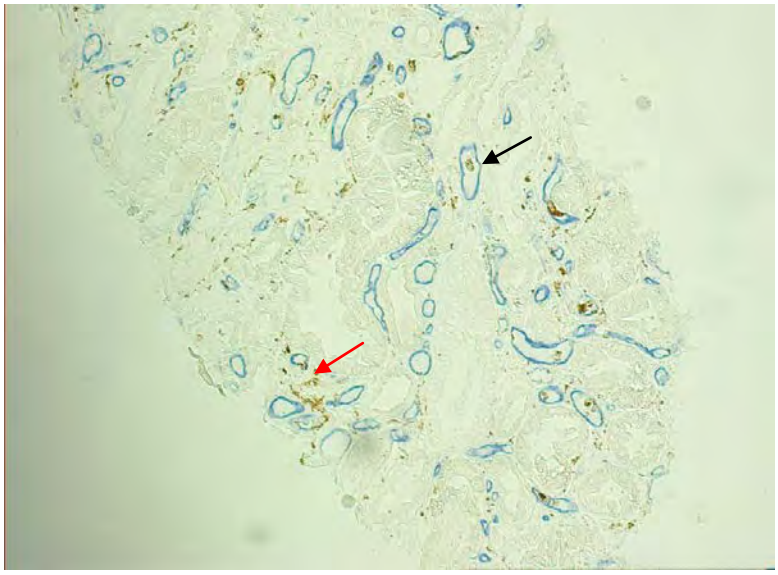


Figure 4.1: Endothelial cell-macrophage dual IHC: note peri-tubular capillary endothelial cells in blue (black arrow) and macrophages in brown (red arrow) in a renal biopsy from failing renal transplant.

4.2.4.2 Counts of macrophages attached to and within the vicinity of endothelial cells

To assess the relationship between macrophages and endothelium biopsies from the non-transplant group (n=6) and CAD group (n=6) were studied for macrophages and endothelial cells. The two groups were matched for scarring and PTECD. To evaluate the co-localization of macrophages and peri-tubular capillaries, the number of macrophages attached to endothelial cells was counted per high power field [x200]. Five HPFs were randomly selected by a blinded person and the images were coded and then archived as .zvi images using image processing software [AxioVision, Germany]. The images were then reopened by a separate investigator (R.H.) for analysis using the same software and the appropriate calibration was used for x200 magnification. The distance between endothelial

cell and macrophages was noted for each endothelial cell-macrophage association. Macrophages >5 µM apart from endothelial cells were not counted **(Figure 4.2)**. The number of macrophages attached to the endothelial cells was noted separately per HPF.

Figure 4.2 Macrophage-Endothelial Cell Association

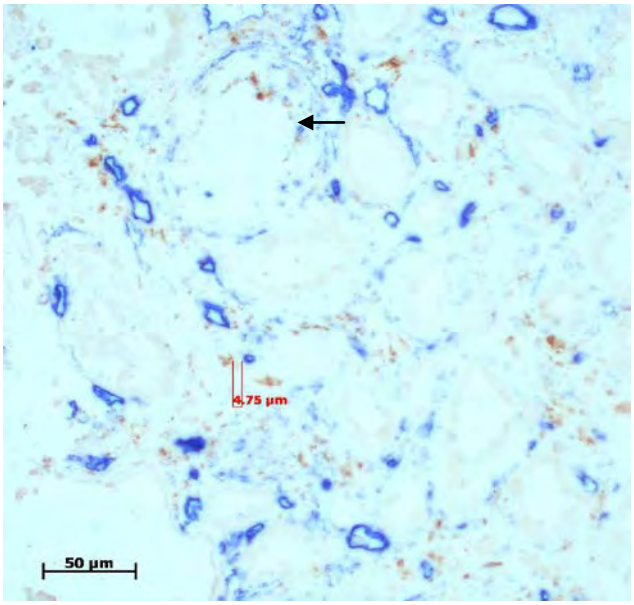


Figure 4.2: note endothelial cells in blue and macrophages in brown colour. Macrophages attached to the endothelial cells (black arrow) and those within 5µM perimeter of endothelial cells were counted separately.

4.2.5 Quantification of proteinuria

Proteinuria (albuminuria) was quantified by albumin-creatinine ration (ACR). A normal ACR is <2.5 mg/mmol for men, and <3.5 mg/mmol for women. An ACR of 100 mg/mmol approximates to a 24-h urinary albumin excretion of 1 g, whereas an ACR of 300 mg/mmol

or more approximates to nephrotic range albuminuria. Multiplying by 8.8 converts the ACR from mg/mmol creatinine to mg/mg creatinine.

4.3 RESULTS

4.3.1 Baseline Patient Characteristics, Renal Function and Proteinuria

The baseline demographic characteristics of patients in the three study groups are detailed in table 4.1. The patient population in the CAD group was relatively younger than the other two groups (median age in years: 43.5[20-69] vs. 54[19-72] & 61.5[28-83] $p<0.05$). There were more men in all the groups (75%, 77% and 72%). The median time for biopsy after renal transplantation in the CAD group was 3.8 [0.3-19.1] years, and in the NRSOT group after non-renal transplantation was 3.4 [0.6- 15.9] years. Median renal function measured by MDRD eGFR was comparable amongst the groups 22.8 [7.3-50.05] vs. 21.9 [7.14-48.13] vs. 26.15[4.96-61.14] mL/min/1.73 m^2 respectively ($p=0.24$). The ACR was significantly higher in the NRSOT group and lowest in the CKD group. There was a significant positive correlation between ACR at the time of biopsy and PTECD in the CAD group ($R=0.47$, $p=0.03$) but there was no significant correlation between these parameters in the two other groups; conversely there was a significant positive correlation between ACR at the time of biopsy and IMD in the CKD group ($R=0.51$, $p=0.007$), but no significant correlation between these parameters in the NRSOT and CAD group.

Table 4.1 Demographic Characteristics of Patient Groups

	CAD (N= 49)	**NRSOT Hypertensive/ Ischaemic, CNINT (N = 44)**	**CKD Hypertensive/ Ischaemic (N = 39)**	**p**
Age	43.5[20-69]	54[19-72]	61.5[28-83]	<0.05
Sex M:F	36:13	34:10	28:11	ns
Weight	70[45-117]	77[44-106]	78[47-135]	ns
ACR at biopsy	86 ± 26	179 ± 52	55 ± 19	<0.05
MDRD eGFR	22.8 [7.3-50]	21.9 [7.14-48]	26.15[4.96-61.1]	ns
Time since transplant	3.8[0.3-19]	3.4[0.6-16]	NA	ns

Table 4.1: Baseline patient characteristics: three study groups being compared, note comparable baseline renal function

4.3.2.1 Interstitial Inflammation

Interstitial inflammation was measured by IMD. Marked inflammation was seen in the CKD group [median IMD: 1.87 (0.94 – 4.53)]. Inflammation was relatively less in CAD and NRSOT [median IMD: 0.8 (0.32 – 3.94) &1.19(0.25 – 3.93) respectively]. The difference in IMD across the three groups was significant ($p < 0.001$) **(Figure 4.3a)**. Previously observed association between proteinuria and IMD by the host group (Eardley,

Zehnder et al. 2006) was maintained (r=0.514, p=0.007) in the CKD group, but was absent in the other two (CAD and NRSOT) groups.

Figure 4.3a Interstitial Macrophage Density (IMD)

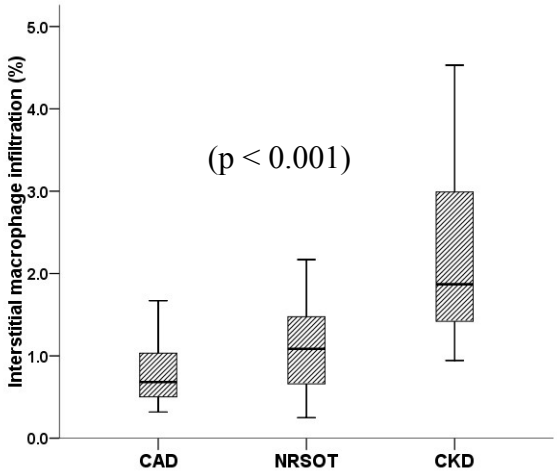


Figure 4.3a: note markedly elevated interstitial IMD in CKD group in comparison with that in the other two groups (p<0.001).

4.3.2.2 Ischaemia

Interstitial ischaemia was assessed by inverse of PTECD (an inverse measure of interstitial ischaemia). Peri-tubular capillary endothelial cells were better preserved in the CKD group (% PTECD: 6.47 ± 2.3). In comparison there was a lower capillary density in the NRSOT and CAD groups (%PTECD: 3.01 ± 0.01). The differences were significant (ANOVA; p<0.001) **(Figure. 4.3b).**

Fig. 4.3b Peri-tubular Capillary Endothelial Cell Density (PTECD)

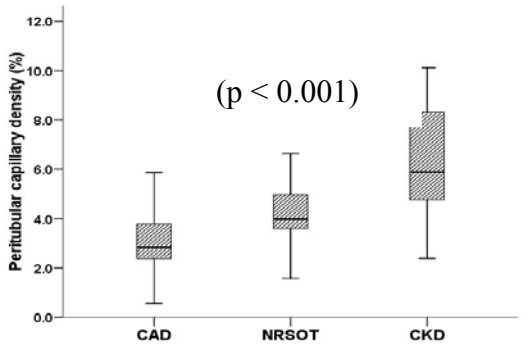


Figure 4.3b: PTECD across the three groups: note marked ischaemia in CAD group and relatively preserved interstitial vascularity in CKD group (ANOVA; p<0.001).

4.3.2.3 Interstitial Scarring

Interstitial scarring was comparable across the groups [mean interstitial scarring: 47.37± 3.3 vs. 37.66 ± 2.9 vs. 43.89 ± 4.24 in CAD, NRSOT and CKD respectively (p = ns)] **(Figure 4.3c)**.

Figure 4.3c Chronic Damage Index

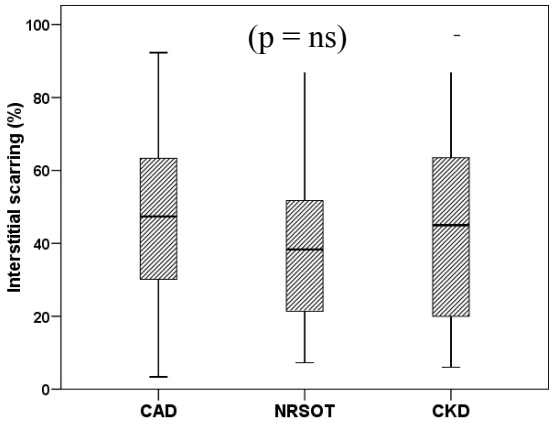


Figure 4.3c: Interstitial scarring (% area) across the three study groups; p = ns.

4.3.2.4 Inflammation and Ischaemia controlled for Scarring

To evaluate the differential relationships between ischaemia and inflammation, comparative analyses were carried out for these processes after controlling for scarring. The ratios of scarring/PTECD and scarring/IMD were compared across the three groups **(Figure 4.4).** The interstitial scarring/ PTECD ratio was significantly higher in the CAD group in comparison with NRSOT and CKD [16.54(3.01 – 65.79) vs. 8.8(1.46 – 40.25) & 8.19(0.59 – 38.08)] [Kruskal Wallis; $p<0.001$]. Also, the interstitial scarring/IMD ratio was significantly higher in CAD group when compared with NRSOT and CKD [74.62(15.28 – 184.96) vs. 27.56(6.11 – 133.33) & 20.86(4.32 – 32.68)] [Kruskal Wallis; $p = 0.001$].

Figure 4.4a Inflammation Controlled for Scarring

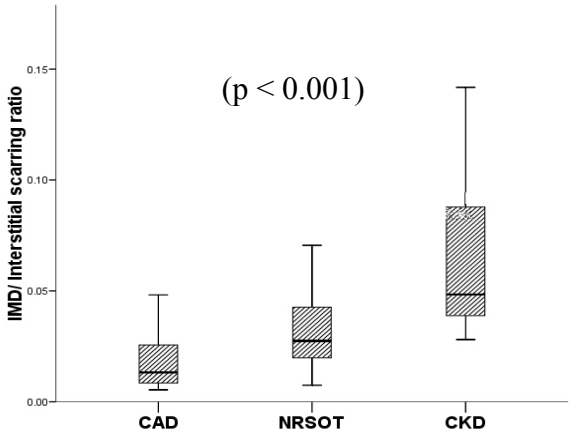


Figure 4.4a: Significantly lower interstitial IMD/ scarring ratio in CAD ($p < 0.001$), suggesting predominant inflammation in NRSOT and CKD groups.

Figure 4.4b Ischaemia Controlled for Scarring

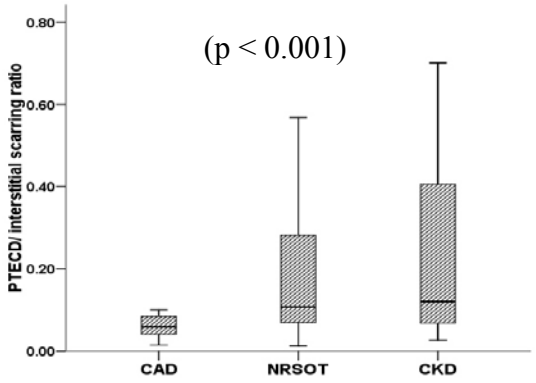


Figure 4.4b: Significantly lower PTECD/ interstitial scarring ratio in CAD (p = 0.001), suggesting predominant ischaemia in this group.

4.3.3 Inter-relationships between Inflammation, Ischaemia & Scarring (Figure 4.6 & Table 4.2)

To assess the inter-relationship between each of the injury processes, subgroup analyses were carried out. There was a strong negative association observed between PTECD and interstitial scarring in NRSOT and CKD groups [(r = -0.728 and -0.837 respectively (p < 0.001 in both instances)] [Fig 4.6 & table 4.2]. This association was not observed in CAD (r = -0.089, p = ns). There was a strong positive association between IMD and interstitial scarring (r = 0.811; p<0.001) in CKD. This association was not observed in either of the transplant settings (CAD & NRSOT). There was a negative association between IMD and PTECD observed in all three groups, this was strongest in the CKD group (r = - 0.704; p<0.001) compared to CAD and NRSOT [(r = - 0.392 & - 0.367 respectively (p<0.05)].

Table 4.2 Inflammation, Ischaemia & Scarring: Sub-group Analysis

			% Interstitial scarring	PTECD	IMD(log10)
CAD	**% Interstitial scarring**	r p n	1	-0.089 ns 26	0.208 ns 26
	PTECD	r p n		1	**-0.329** **0.036** **29**
	IMD(log10)	r p n			1
NRSOT	**% Interstitial scarring**	r p n	1	**-0.728** **0.000** **39**	0.235 ns
	PTECD	r p n		1	**-0.367** **0.023** **38**
	IMD	R p n			1
CKD	**% Interstitial scarring**	r p n	1	**-0.837** **0.000** **32**	**0.811** **0.000** **33**
	PTECD	r p n		1	**-0.704** **0.000** **31**
	IMD	r p n			1

Table 4.2: Univariate analysis of correlation between interstitial scarring, peri-tubular capillary density and macrophage infiltration by disease groups (r: correlation coefficient, p: significance, n: number of cases).

Figure 4.6 Inter-relationships between Inflammation, Ischaemia & Scarring

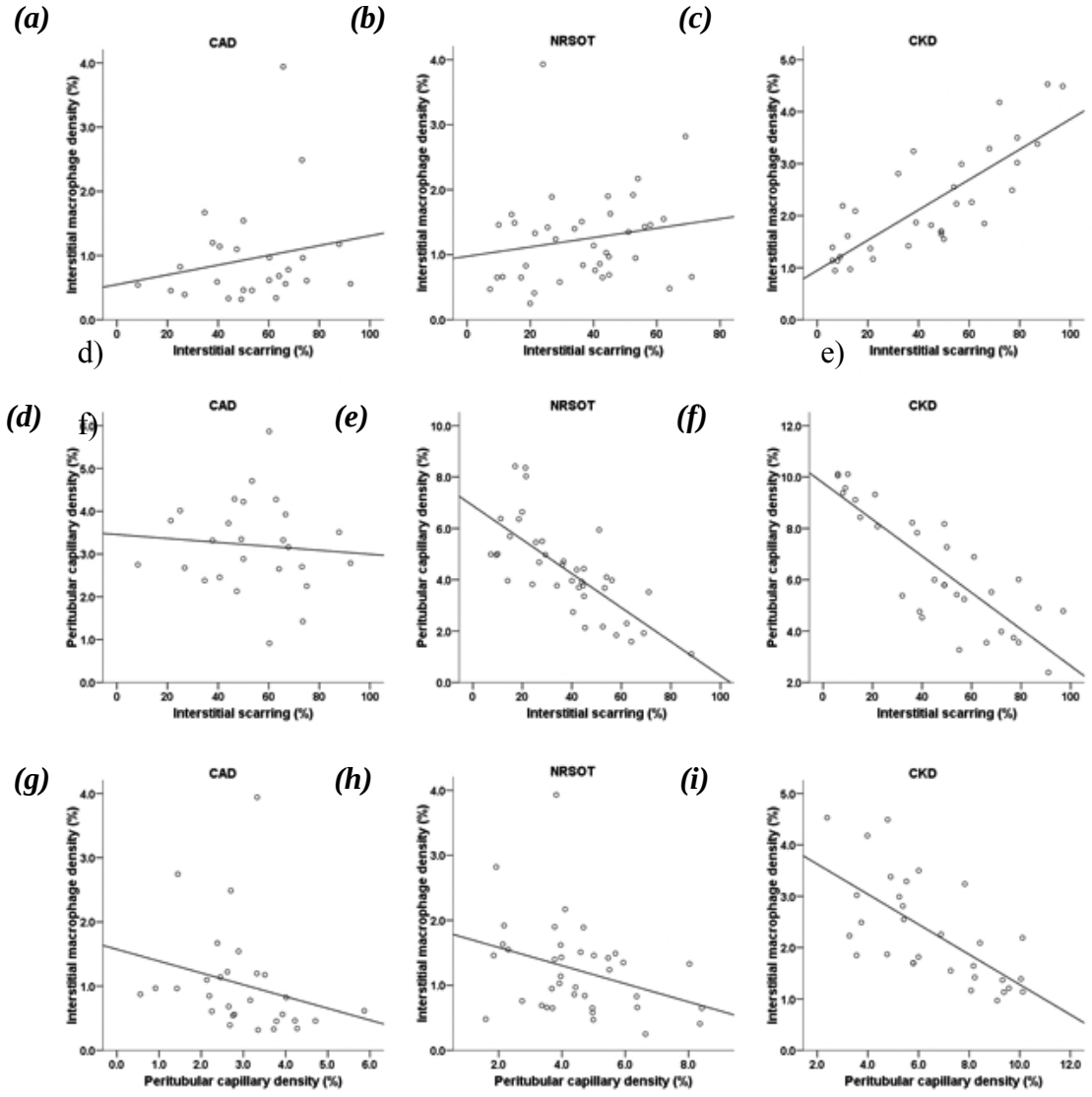


Figure 4.6: Inter-relationships between injury processes in three study groups: [top row] <u>interstitial inflammation and scarring (a-c)</u>: a strong positive correlation in CKD group [r = -0.811; p<0.001], [middle row] <u>PTECD and scarring (d-f)</u>: a strong negative correlations in the NRSOT and CKD group [r = -0.728, -0.837 respectively (p<0.001)] [bottom row] <u>PTECD and inflammation (g-i)</u>: positive correlations between PTECD and inflammation in all study groups, which is strongest in CKD (r = 0.811; p<0.001).

By multivariate linear regression analysis with interstitial scarring as the dependent variable, there was a highly significant association with PTECD in NRSOT and CKD groups and a significant association with IMD in the CKD group **(Table 4.3).**

Table 4.3 Inflammation, Ischaemia & Scarring: Multivariate Analysis

	PTECD	**IMD**
CAD	B: -0.783 p = ns	B: 4.63 p = ns
NRSOT	B: -7.46 p <0.001	B: -1.455 p = ns
CKD	B: -7.64 p <0.001	B: 10.578 p = 0.001

Table 4.3: Multivariate regression analysis with interstitial scarring as a dependent: note significant association with PTECD in NRSOT and CKD and with IMD in CKD; B: regression coefficient.

4.3.4 Impact of Injury Processes on Renal Outcomes

Cumulative renal survival was assessed for each of the study groups by Kaplan Meier plots **(Figure 4.7)**. Cumulative renal survival was 55%, 62% and 71% at 2 years and 40%, 38% and 47% at five years from biopsy in CAD, NRSOT and CKD respectively. The median

renal survival was 3.79, 5.2 and 3.8 years for CAD, NRSOT and CKD respectively (log rank; p = ns).

To assess the impact of injury processes on renal survival, a Cox regression analysis was performed using interstitial scarring, PTECD and IMD as co-variates. None of the injury processes were found to have any significant impact on renal survival in each of the study groups tested separately **(Table 4.4).**

However upon considering a single injury process in tertiles, interstitial scarring and PTECD were the determinants of renal survival in the CAD and CKD group respectively [log rank; $p<0.05$] **(Figures 4.8a and 4.8b).**

Figure 4.7 Renal Survival

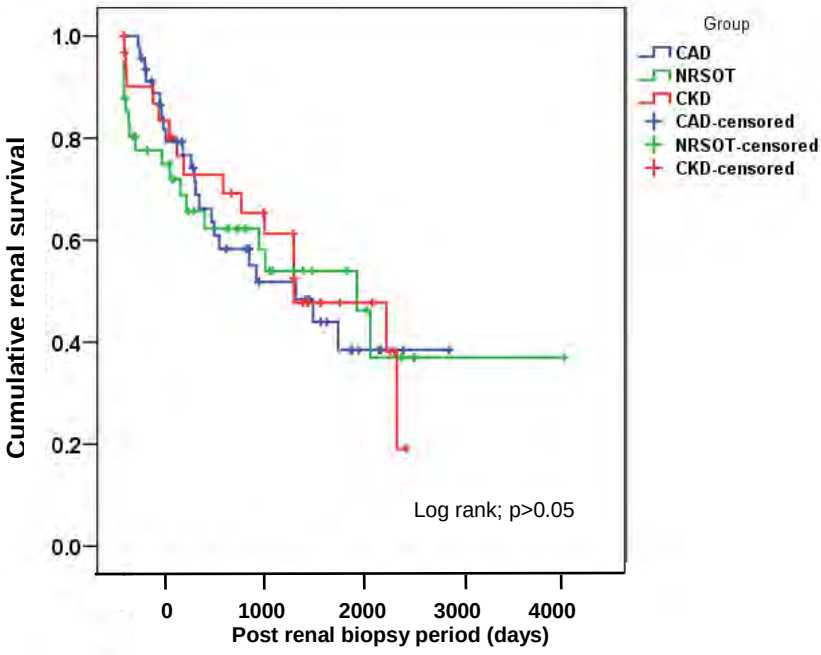


Figure 4.7: Comparable cumulative renal survival in the three groups (log rank >0.05)

Table 4.4 Impact of Injury Processes on Renal Survival

	Interstitial scarring	**Peri-tubular capillary density**	**Interstitial macrophage infiltration**
CAD	Exp (B): 1.013 p = 0.32	Exp (B): 0.785 p = 0.48	Exp (B): 0.953 p = 0.92
NRSOT	Exp (B): 0.990 p = 0.65	Exp (B): 0.68 p = 0.16	Exp (B): 2.01 p = 0.063
CKD	Exp (B): 1.029 p = 0.31	Exp (B): 1.64 p = 0.076	Exp (B): 2.703 p = 0.076

Table 4.4: Disease specific impact of individual injury processes on renal survival: Multivariate analysis reveals no significant impact of individual injury processes on renal survival, B: regression coefficient.

Figure 4.8a Scarring and Renal Survival

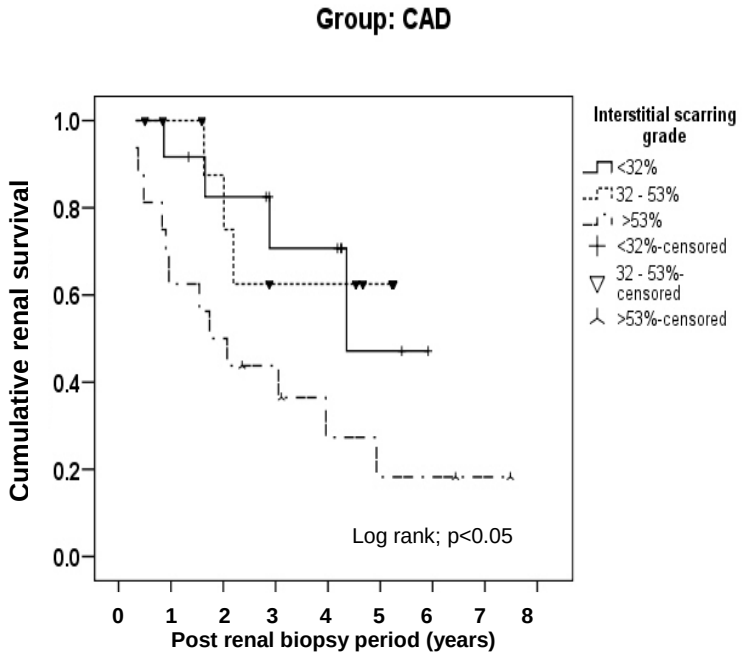


Figure 4.8a: The impact of interstitial scarring on renal survival in CAD: note better cumulative renal survival in less scarred kidneys (log rank < 0.05)

Figure 4.8b Ischaemia and Renal Survival

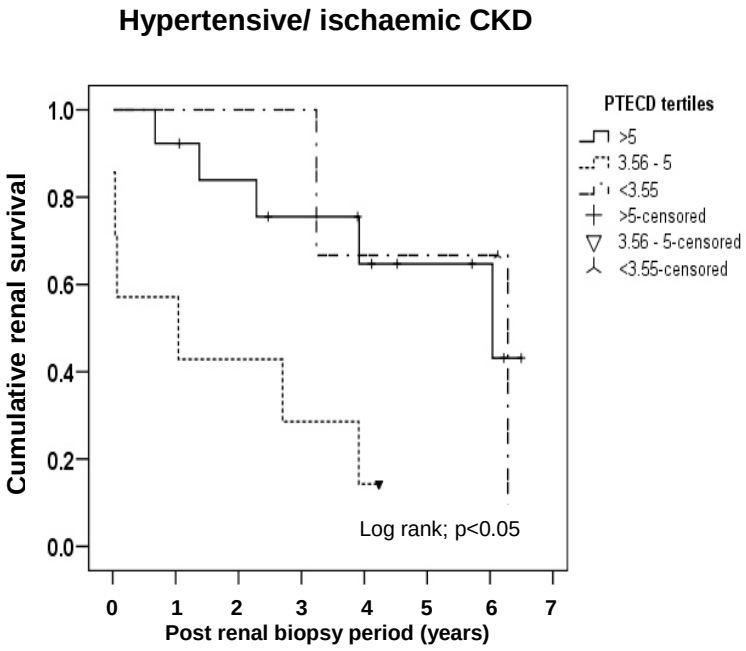


Figure 4.8b: Impact of PTECD on renal survival in hypertensive/ ischaemic CKD: note better cumulative renal survival in kidneys with less ischaemia (log rank < 0.05)

4.3.5 Macrophages and Endothelial Cell Co-localization

The counts of macrophages attached to and within 5 μM of endothelial cells (EC) for each group were normally distributed. There were significant inter-group differences noted.

The mean count of macrophages in contact with endothelial cells was significantly higher in biopsies from the CAD group than that in NRSOT group (n = 6 each) (13.8 ± 1.97 vs. 6.93 ± 1.31; $p<0.05$). The mean count of macrophages within 5μM from endothelial cells

(EC) were also significantly higher in biopsies from the CAD group when compared against the NRSOT group (14.67 ± 1.84 vs. 6.87 ± 0.32; p = 0.002).

To assess the effect of macrophages attached and within 5 microns of endothelial cells, these counts were controlled for ischaemia and inflammation present in the sections. This was done by assessing the ratios of cell counts to PTECD and IMD. The median ratio of PTECD/ macrophages within 5μM of EC was significantly higher in CAD than in NRSOT [0.22(0.05 – 0.53) vs. 0.41(0.25 – 0.72); Mann Whitney: $p<0.05$] **(Figure 4.9a).** However, median ratio of PTECD/ macrophages attached to EC in the CAD group was comparable with that in NRSOT [0.21(0.07 – 0.44) vs. 0.39(0.22 – 1.04); Mann Whitney: p = 0.06]. But when counts were controlled for inflammation, both ratios were significantly higher in CAD than in NRSOT. The median ratio of IMD/ macrophages attached to ECs in CAD and NRSOT were [0.067(0.02 – 0.14) vs. 0.15(0.07 – 0.52) respectively; Mann Whitney: $p<0.05$]. The median ratio of IMD/ macrophages within 5μM of ECs were: [0.064(0.02 – 0.22) vs. 0.17(0.08 – 0.45); Mann Whitney: $p <0.05$] **(Figure 4.9b).**

Figure 4.9a Association of ECs and Macrophages, controlled for Ischaemia

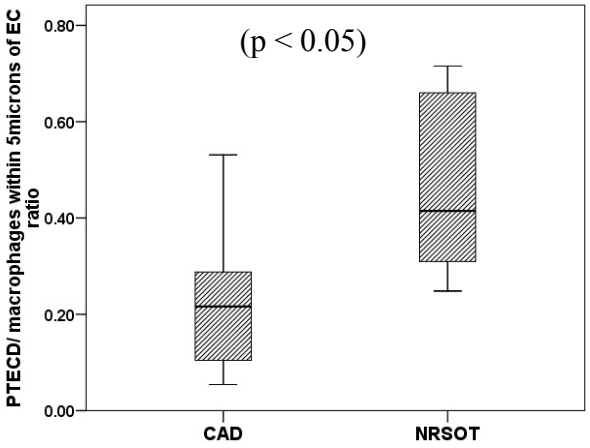


Figure 4.9a: Ratio of PTECD/ macrophages within 5 μM from EC in two study groups: note the significantly lower ratio in CAD to suggest higher number of macrophages around capillaries for given amount of ischaemia.

Figure 4.9b Association of ECs and Macrophages, controlled for Inflammation

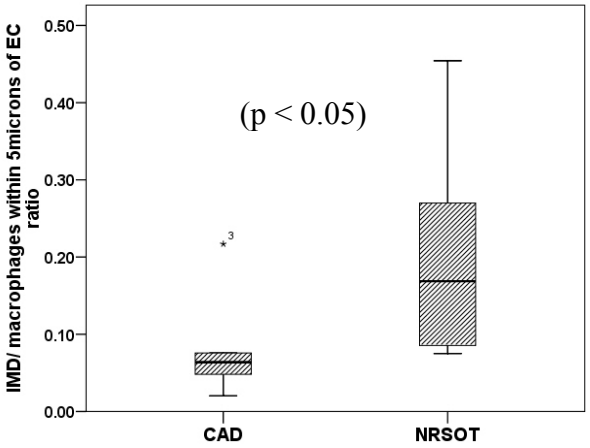


Figure 4.9b: Ratio of IMD/ macrophages within 5 μM from EC in two study groups: note the significantly lower ratio in CAD which indicated a higher number of macrophages around capillaries for a given amount of inflammation.

To evaluate the impact of these counts when controlled for ischaemia/inflammation on injury processes (interstitial scarring, ischaemia and inflammation) both groups were combined and a univariate analysis and linear regression analyses were carried out.

Both ratios of PTECD/ macrophages attached to EC and PTECD/macrophages within 5μM of EC **(Figure 4.9c)** correlated inversely with interstitial scarring (r = -0.601; p = 0.038 and r = -0.723; p = 0.008 respectively). However, ratio of PTECD/macrophages within 5μM of EC correlated positively with PTECD (inverse of ischaemia) (r = -0.682; p = 0.01) **(Figure 4.9d).**

Figure 4.9c Macrophages within 5 μM from EC and Scarring, controlled for Ischaemia

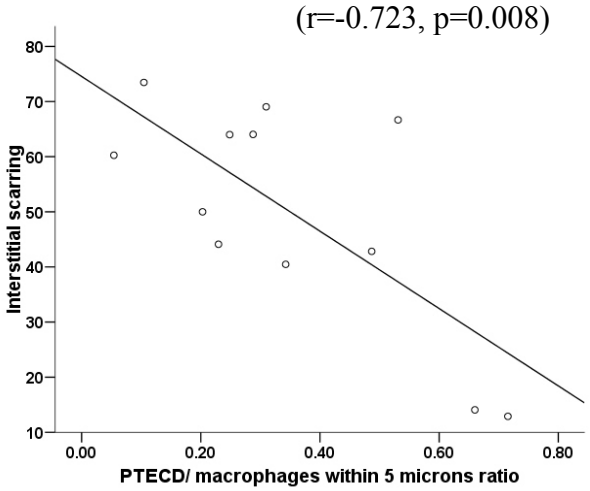


Figure 4.9c: Inverse correlation between interstitial scarring and PTECD/ macrophages within 5 μM from EC [r = -0.723; p = 0.008]

Figure 4.9d Macrophages within 5 µM from EC and Ischaemia, controlled for Ischaemia

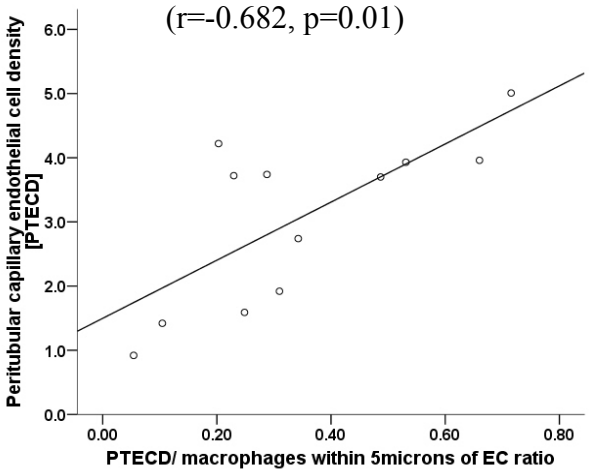


Figure 4.9d: Positive correlation between PTECD and PTECD/macrophages within 5 µM from EC [r=-0.682; p=0.01]

4.4 CONCLUSIONS

For matched renal function, a similar degree of interstitial scarring was observed across the groups irrespective of the clinical setting. However, ischaemia is a predominant injury process in failing renal transplants, as demonstrated by lower PTECD in these biopsies compared with other groups **(Figure 4.3b).** Conversely, inflammation is a predominant injury process in CKD **(Figure 4.3a).** When controlled for interstitial scarring, ischaemia

remains the predominant association in failing renal transplants **(Figure 4.4b)** and inflammation the predominant injury process in NRSOT and CKD groups **(Figure 4.4a)**. However, the relationship between macrophages and endothelial cells **(Table 4.2)** indicates that despite a lower macrophage load, there may be an allogeneic impact of macrophages on endothelial cells.

4.5 DISCUSSION

In the previous chapter, I demonstrated that the injury processes accelerating renal damage in CKD are also present in NRSOT. In this chapter, I hypothesised that these injury processes are involved in deterioration of renal transplants. Although efforts have been made to identify mechanisms of allograft attrition after renal transplantation over the last two decades, this is the first attempt to evaluate injury processes by a direct comparison across disease groups. In this study, I attempted to exclude allo-specific injury processes per se, to delineate mechanisms of renal injury that are common to the three study groups. To do so in the CAD group I excluded patients with history of acute cellular rejection and have had donor specific antibodies. It is now understood that anti- HLA antibodies are produced even late after transplantation are detrimental to allograft outcome (Lachmann, Terasaki et al. 2009). Admittedly in this study, there was no protocol in place to test DSAs periodically and not all patients had DSAs tested at the time of renal biopsy. This is a significant shortfall of the analysis presented in this chapter. In the study patients, DSAs

were mainly checked when clinically indicated to evaluate allograft dysfunction. The role played by DSA in progression of allograft dysfunction has been recognized, in a recent study almost 75% of the biopsies with transplant glomerulopathy (Zollinger, Moppert et al. 1973), an important phenotype of late kidney deterioration demonstrated evidence of antibody-mediated injury, with C4d deposition and/or circulating anti-HLA antibodies anti-HLA alloantibodies (Sis, Campbell et al. 2007). DSA were proven to have a strong adverse impact on graft survival.

The three study groups were matched for renal function at the time of allograft biopsy. Quantitative analysis clearly demonstrated that scarring, loss of interstitial capillaries and interstitial inflammation were present in all three study groups. As observed in our earlier work in CKD (Eardley, Kubal et al. 2008), there was a significant association in the subgroups. This association was also observed in the NRSOT setting. Importantly, this association was lost in the CAD group. Proteinuria was measured by ACR. There was significant proteinuria in noticed in all study groups; this was highest in the NRSOT group. There was a positive correlation between proteinuria and PTECD in the CAD group, suggesting proteinuria decreased with worsening capillary rarefaction. This may indicate that proteinuria is a dominant process early on in the disease course in this group. However the numbers are small and further studies are required to draw conclusions in this area. A positive correlation between proteinuria and macrophage density was noted and is in keeping with previous work by the host group (Eardley, Zehnder et al. 2006; Eardley, Kubal et al. 2008).

Similarly, an association between scarring and inflammatory load was observed in CKD (Eardley, Kubal et al. 2008). This association was not observed in NRSOT and CAD settings, and the possible explanation may be the use of immunosuppressants in these groups. The association between inflammation and ischaemia however was observed in all study groups and was strongest in the CKD group.

Although interstitial scarring was comparable between these function matched study groups, variations in the other two processes were prominent. For a given amount of scarring, failing renal transplant biopsies demonstrated significant peri-tubular capillary loss. This accelerated ischaemia in the renal transplant setting is felt to be associated with CNI nephrotoxicity and chronic vascular rejection. However there is little evidence available from histological analyses to support this observation (Ishii, Sawada et al. 2005; Adair, Mitchell et al. 2007). The quantitative data in this chapter clearly demonstrates increased peri-tubular capillary loss in NRSOT and CAD in comparison with CKD, and this may be at least in part a result of CNI use.

Understanding the role of an ongoing chronic inflammatory process in the absence of proven rejection is vital. In this chapter, I have attempted to address this issue by selecting renal transplant recipients who have not had acute rejection episodes and have no donor specific antibodies.

Macrophage infiltration has long been recognized as a feature of allograft rejection, yet the role of macrophages in the development of CAD remains underappreciated. Macrophages contribute to both the innate and acquired arms of the allo-immune response and thus may be involved in all aspects of acute and chronic allograft injury/ rejection. Role of macrophages in the development of ischaemia in transplanted kidneys has been suggested by previous work (Pilmore, Eris et al. 1999). Expression of iNOS and VEGF by infiltrating macrophages has been demonstrated (Adair, Mitchell et al. 2007) and this may suggest macrophages induce both vascular endothelial cell injury and the generation of new lymphatic vessels in end-stage transplant nephrectomies.

My findings in the co-localization studies show an increased number of macrophages within 5 microns from endothelial cells in failing renal transplants when compared to chronic kidney disease. Furthermore, this study also demonstrated significant correlations between the number of macrophages within the close vicinity of endothelial cells and ischaemia and interstitial scarring. These findings may provide an insight into a novel mechanism of injury in this setting. This association between macrophages and endothelial cells can be further explored and new therapeutic targets can be developed.

The data that is reported here is observational and associative, but does indicate that ischaemia is a predominant injury process in failing renal transplants and this may be driven by chronic inflammation. This macrophage driven obliteration of the interstitial capillary bed may represent an important pathway in understanding renal allograft attrition.

4.6 CAVEATS

The most important criticism of this study is that I have compared injury processes in heterogeneous study groups. Patients in the CAD group have one functional/failing kidney and patients in the NRSOT/CKD group have two functional/failing kidneys. Although the groups were matched for renal function, the relative contribution of the kidney undergoing biopsy in NRSOT/CKD group to the overall renal function could not be estimated. The CAD group had a comparatively smaller renal mass and therefore a smaller number of total nephrons. Secondly, allo-specific injury was not completely ruled out in the patients with CAD. With the lack of prospective DSA monitoring and C4d staining allo-antibody mediated injury was not ruled out in this group of biopsies.

CHAPTER 5

ADAPTIVE CHANGES IN RENAL MICROENVIRONMENT IN CHRONIC KIDNEY DISEASE

5.1 INTRODUCTION

Recent studies from the host group have shown that interstitial inflammation, peri-tubular ischaemia and interstitial scarring are closely associated with the progression of human CKD. (Eardley, Zehnder et al. 2006; Eardley, Kubal et al. 2008) The data presented in Chapter 3 showed that in CKD following NRSOT, these processes have differential relationships based on histological subgroups. In Chapter 4, I then showed that there are major differences in peri-tubular capillary loss and macrophage load between CAD, CKD in NRSOT and native CKD.

In native CKD, there was a heavier macrophage load and less capillary loss, when corrected for scarring, than in the transplant groups. In this chapter I have utilised cDNA reverse transcribed from mRNA derived from renal biopsies from a cohort of patients with CKD to analyse the patterns of mRNA expression in respect to ultra structural parameters. In particular, I have focused on those relationships that may represent molecular responses associated with macrophage load. Furthermore, I have assessed the expression of the ischaemia marker carbonic anhydrase IX (CA-IX) and chemokine receptors important for the trafficking and retention of macrophages. In this chapter, I also evaluate the pro-inflammatory phenotype of macrophages and its relationship with ischaemia. To assess this I quantified mRNA expression of arginase and iNOS and used arginase/ iNOS ratio as a marker of pro-inflammatory phenotype of macrophages. I also measured expression of fibroblast specific protein-1 (FSP-1) to evaluate expression of fibrosis and expression of BCL-2 to evaluate anti-apoptotic processes.

5.1.1 Putative Makers of Adaptive Changes in the Renal Microenvironment in Tubulointerstitial Inflammation

The variable role of macrophages in inflammation and tissue remodelling in the context of renal tubulointerstitial inflammation is not fully understood. Functionally distinct subpopulations of macrophages may exist in the same tissue and play critical roles in both the initiation and recovery phases of interstitial fibrosis. The origin and activation state of the macrophage and the microenvironment in which they reside are critical determinants of their response to injury. Macrophages that secrete anti-inflammatory cytokines, promote angiogenesis, and play a positive role in wound healing and tissue remodelling have been generally referred to as possessing an "alternative" phenotype. They are renowned for their heterogeneity and plasticity, which are reflected by their specialized functions in tissue inflammation and resolving injury. During enhanced recruitment in response to disease states, inflammatory monocytes are recruited in response to cytokine cues and undergo differentiation into two broad but distinct subsets of macrophages that are categorized as either classically activated (M1) or alternatively activated (M2). M2 macrophages represent various phenotypes that are further subdivided into M2a (upon exposure to IL-4 or IL-13), M2b (induced by immune complexes in combination with IL-1β or LPS), and M2c cells (following exposure to IL-10, TGF-β, or glucocorticoids).

Inflammatory cues within the regional microenvironment can prime macrophage phenotype and determine whether these cells will have a beneficial or deleterious effect during tissue repair and remodelling. Macrophage phenotype and function ultimately determine the

outcome of inflammation and the development of irreversible tissue scarring. Profiling techniques and genetic models have provided markers of macrophage polarization. Polarization associated genes such as Fizz, YM-1 have been identified. Additionally, tissue-resident macrophages can be maintained through local proliferation or differentiation in situ from circulating monocytic precursors (Varol, Yona et al. 2009). Importantly, discrete subsets of blood monocytes have been described. Murine monocytes can be classified as $Ly6C^{low}CX3CR1^{hi}$ ($CCR2^{-}CD62L^{-}$) or $Ly6C^{hi}CX3CR1^{low}$ ($CCR2^{+}CD62L^{+}$) and are shown to have distinct functions and migration patterns (Auffray, Sieweke et al. 2009).

5.1.1.1 iNOS and Arginase

The macrophage is a pluripotent cell representing a front line host defense system that can express diverse activities depending on the stimuli they encounter. Many of these activities appear mutually antagonistic in nature, such as pro-inflammatory versus anti-inflammatory, immunogenic versus tolerogenic, and tissue destructive versus tissue restorative. Cytokine expression patterns or the ratio of nitric oxide synthase (Kokkinos, Antcliffe et al.) to arginase I (Arg I) gene expression are commonly used as readouts of macrophage functional status. However, these functional phenotypes are reversible.

Recently it has been shown that macrophages sequentially change their functional phenotype in response to changes in their immediate microenvironment (Stout, Jiang et al. 2005): based on the ability of Th1 and Th2 cytokines to promote opposing activities in macrophages, it has been proposed that macrophages develop into either a pro-

inflammatory M1 or immunomodulatory M2 subsets (Gordon 2003). A useful indicator of the balance between M1 and M2 phenotypes is provided by the relative level of arginase and inducible nitric oxide synthase (Kokkinos, Antcliffe et al.). These molecules share the substrate L-arginine, so arginase down-regulates NO production by competing with iNOS for arginine. iNOS is up-regulated in response to inflammatory stimuli as macrophages shift towards the M1 phenotype and become involved in phagocytosis, bacterial killing and initiation of the immune response. M1 macrophages, but not M2 macrophages, up-regulate iNOS to generate free radicals for phagocytosis (Song, Ouyang et al. 2000). The ratio of iNOS to arginase is profoundly elevated in M1 macrophage activation and reduced in M-2 macrophage activation (Munder, Eichmann et al. 1998; Gordon 2003; Mosser 2003). However there are exceptions to this assumption, arginase is not expressed prominently in human IL-13-induced M2 cells (Scotton, Martinez et al. 2005). To test this correlation of the renal micro-environment, I evaluated expression of iNOS and arginase in renal biopsies with varying renal damage and inflammatory load.

5.1.1.2 Podoplanin

Podoplanin, originally detected on the surface of podocytes, belongs to the family of type-1 transmembrane sialomucin-like glycoproteins. Podoplanin is important in renal homeostasis and the role of podoplanin is also well described in the development of proteinuria (Breiteneder-Geleff, Matsui et al. 1997; Matsui, Breiteneder-Geleff et al. 1998). More recently, expression has been described on lymphatic endothelial cells. However, it is not expressed on blood vessel endothelium, so it has been widely used as a specific marker

for lymphatic endothelial cells and lymphangiogenesis. Neolymphangiogenesis has been described as a prominent finding in the remnant kidney model (Matsui, Nagy-Bojarsky et al. 2003) and in human renal allograft (Kerjaschki, Regele et al. 2004). Data suggests that chronic inflammation is required to initiate neolymphangiogenesis with formation of lymphatic vessels under the influence of mononuclear cell-derived growth factors, predominantly of the VEGF isoforms. In addition to expression on lymphatic vascular (LV) endothelium, podoplanin is also expressed in normal tissues such as kidney podocytes (Breiteneder-Geleff, Soleiman et al. 1999), skeletal muscle, placenta, lung, heart (Martin-Villar, Scholl et al. 2005), myofibroblasts of the breast and salivary glands, in osteoblasts and mesothelial cells (Schacht, Dadras et al. 2005).

In this chapter, I have used podoplanin mRNA expression as the evidence of renal injury. I have related podoplanin expression to inflammatory load and mRNA expression of other genes that are expressed in renal micro-environment.

5.1.1.3 Chemokines and Chemokine Receptors

The chemokine monocyte chemoattractant protein (MCP)-1/CCL2 and its main target, receptor CCR2, direct the infiltration of circulating monocytes into the kidney. There is good evidence that this chemokine/receptor pair contributes to progressive fibrosis. In an animal study, dual immunohistochemical staining revealed that most infiltrating TGF-ß1-positive cells were also positive for CCR2 (Kitagawa, Wada et al. 2004). Recent evidence from the host group has indicated that MCP-1/CCL2 is most strongly linked with

macrophage infiltration in early renal scarring. However, other chemokines and their receptors may also be important (Segerer, Nelson et al. 2000).

Another important chemokine/receptor in renal tubulointerstitial inflammation is CX3CL1/CX3CR1. CX3CL1 was initially called fractalkine, and was the first chemokine identified as having adhesion molecules as well as chemotacic and activating properties. CX3CL1 is expressed by endothelial cells and tubular epithelial cells on activation. The targeted receptor, CX3CR1, is expressed on monocyte/macrophages, NK cells and certain T-cell subsets, particularly CD8+ T cells CX3CR1 has been shown to play a role in fibrosis following induction of CX3CL1 expression in the outer medulla of the kidney. Moreover, in an animal renal injury model decreased α-smooth muscle actin (SMA) expression has been shown in CX3CR1-deficient animals (Furuichi, Gao et al. 2006). In this chapter I have assessed tissue expression of CCR2 and CX3CR1 and mRNA expression of CX3CL1, and correlated these with inflammatory load and scarring.

5.1.1.4 Carbonic anhydrase IX

Carbonic anhydrase IX (CA-IX) is highly over expressed in many cancer types including renal cancer and is present in normal tissues such as the gastrointestinal tract. Its expression is strongly induced by the hypoxic drive present in many tumours, with regulation by the HIF transcription factor. Furthermore, CA-IX expression correlated with a poor response to current chemotherapy and radiotherapy regimens. CA-IX has been shown to contribute to acidification of the tumour environment, by efficiently catalyzing the hydration of carbon

dioxide to bicarbonate and protons, leading both to the acquisition of metastasic phenotypes and to chemo resistance with weakly basic anticancer drugs. Inhibition of this enzymatic activity by specific and potent inhibitors was shown to reverse these acidification processes, establishing a clear-cut role for CA-IX in tumorigenesis (Pastorekova, Casini et al. 2004). It is now established that outside the setting of cancer, the adaptive physiological response to hypoxia also involves increased expression of CA-IX (Jubb, Pham et al. 2004). From a renal perspective, most of the work on this hypoxia marker has focused on renal cell carcinoma; however, the expression patterns of CA-IX in CKD and the relationship of this expression to the markers of disease in situ have not been described to date. In this chapter, I address this by describing CA-IX expression by immunohistochemistry on renal biopsies from patients with CKD.

5.2 METHODS

5.2.1 Patients and Biopsies

Renal biopsies of the CKD group studied in Chapter 4 were snap- frozen immediately after the biopsy and used for the analyses described in this chapter. The clinical and pathological parameters of these patients have already been characterised (Section 4.2.1 (c) & table 4.1). Histological diagnoses were made on formal saline and glutaraldehyde fixed biopsy specimens. 2 μm sections were examined in orthodox ways and immunostaining done with an immunoperoxidase method for IgG, IgA, IgM and the complement component C9. The

glutaraldehyde fixed specimen was embedded in Araldite and sectioned for electron microscopy when indicated. Some of the patients had additional biopsy material taken which was processed for molecular analyses.

5.2.2 RT-PCR for iNOS, arginase, FSP-1, BCL-2, podoplanin, MCP-1/CCL2, CX3CL1, iNOS, arginase, FSP-1 and BCL-2

5.2.2.1 mRNA extraction from biopsy samples

The TRI Reagent RNA extraction system (Sigma-Aldrich, Cat No T9424) was used for RNA extraction. Briefly, mechanically homogenised biopsy material was transferred into 1 ml tri-reagent in an eppendorf. This mix was frozen for a minimum of 12 hours and then thawed and 200µL chloroform added and mixed thoroughly. The material was then centrifuged at 12,000 rpm for 30 minutes at 4^0C and the aqueous phase was taken into a fresh tube. To this solution, 1µL glycoblue was added and then 500 µL isopropanolol per ml of tri-reagent. This mix was then centrifuged at 12,000 rpm for 30 minutes at 4^0C. Supernatant was removed and 500µL 70% ethanol was added. The mix was then centrifuged for 5 minutes 12,000 rpm for at 4^0C and the supernatant was removed. The pellet was resuspended in RNAse free water and stored at -80^0C.

5.2.2.2 Generation of cDNA using reverse transcriptase

RNA extracted from sample tissues were used to generate a stable complementary DNA (cDNA). A DNA polymerase enzyme was used to transcribe RNA into double stranded complementary DNA. Reverse transcription was performed using MultiScribe™ Reverse

Transcriptase (Applied Biosytems, California, USA). Briefly 1 µg of RNA was added to a reaction mix with a final volume of 25 µl. Reaction mix consists of 2.5 µl 10X reaction buffer, 6 µl of MgCl (25 nmol/l), 5 µl dNTPs (10 nmol/l), 1.25 µl random hexamers, 0.5 µl RNase inhibitor, 1.55 µl Multiscribe Reverse transcriptase 50 u/µl and made up to 25 µl with RNA free water. Reactions occurred as follows: 24°C for 10 min, 37°C for 60 min, 48°C for 30 min and 95°C for 5 min. cDNA was stored at -20°C.

5.2.2.3 Quantitative Real Time PCR

Quantitative real-time polymerase chain reaction (PCR) is a method that allows accurate measurement of specific DNA and RNA sequences from cell and tissue samples (Bustin 2000). The technique is based around detection of a fluorescence signal during the course of a PCR amplification of the product. This is achieved through the presence of a probe that anneals to a sequence of DNA between the forward and reverse primers. The probe is labelled with a fluorescent reporter (VIC) on the 5' end and a quencher (6-carboxy-tetramethyl-rhodamine, TAMRA) on the 3' end. With both fluorophore and quencher attached, there is no fluorescence emitted by the probe when hybridised to the DNA. As the PCR reaction progresses and *Taq* polymerase extends the primer sequences, the 5'-3' nuclease activity degrades the probe, freeing the reporter from the quencher. This results in a detectable fluorescent signal, which accumulates with each successive cycle of the PCR reaction, and when the reaction is in an exponential phase, the cycle number is recorded as the threshold cycle (Ct) number, demonstrated in figure 5.1. This value is inversely

proportional to the copy number of the target DNA template. Quantitative real-time PCR can be performed as a multiplex reaction, with a target sequence analysed in unison with an internal standard such as 18S in the same reaction. This allows quantification of the target DNA relative to the internal standard, providing a ΔCt value around which different RNA values can be normalised. Comparison of ΔCt values between samples provides a means of measuring fold changes in mRNA expression between samples.

Figure 5.1 Real-time PCR Plot

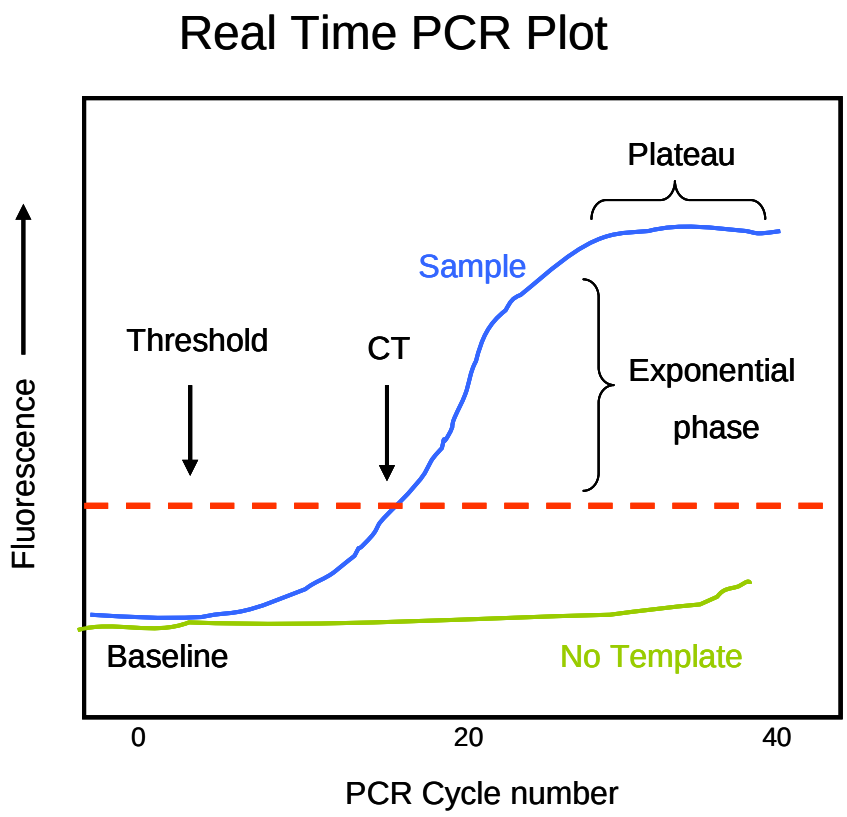


Figure 5.1: Real-time PCR plot demonstrating quantification of RNA at different points during amplification of RNA.

Real-time quantitative PCR was performed using an ABI Prism 7700 Sequence Detection System™ (PE Applied Biosystems, UK). Primers and probes for the mRNA of interest were ordered, pre-designed and optimised, from Applied Biosytems, California, USA.

5.2.2.3.1 Method

The reactions were performed in 25 µl aliquots on a 96 well optical reaction plate (Sigma). Primers for 18S were used in multiplex with target primers as an internal reference. The reactions contained TaqMan universal PCR master mix (Applied Biosytems), 900 nmol/l primers, 100-200 nmol/l TaqMan probe and 25-50 ng cDNA. Target gene probes were labelled with FAM and 18S probes were labelled with VIC. Reactions occurred as follows: 50°C for 2 min, 95°C for 10 min, 44 cycles of 95°C for 15 s and 60°C for 1 min. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines, and used to determine ΔCt values (Ct of target gene – Ct of housekeeping gene) as raw data for gene expression. Data was analysed at the ΔCt stage and mean $\pm$SD ΔCt values transformed through the equation $2^{-\Delta\Delta Ct}$ to give relative mRNA levels.

5.2.2.3.2 Validation and calibration

The amplification of the house-keeping gene 18S (supplied by the manufacturer) was examined to ensure linearity of amplification using human kidney cDNA as a template. Using 18S as housekeeping gene the rate of change in Ct with template concentration

should be similar for both target and house-keeping genes. These relationships were examined for all the genes studied and the respective house-keeping gene 18Ss with no difference in gradients (Figure 5.2: example gradients for CX3CL1). Therefore, 18S was used as the house-keeping gene in all studies.

Figure 5.2: Efficiency Curves

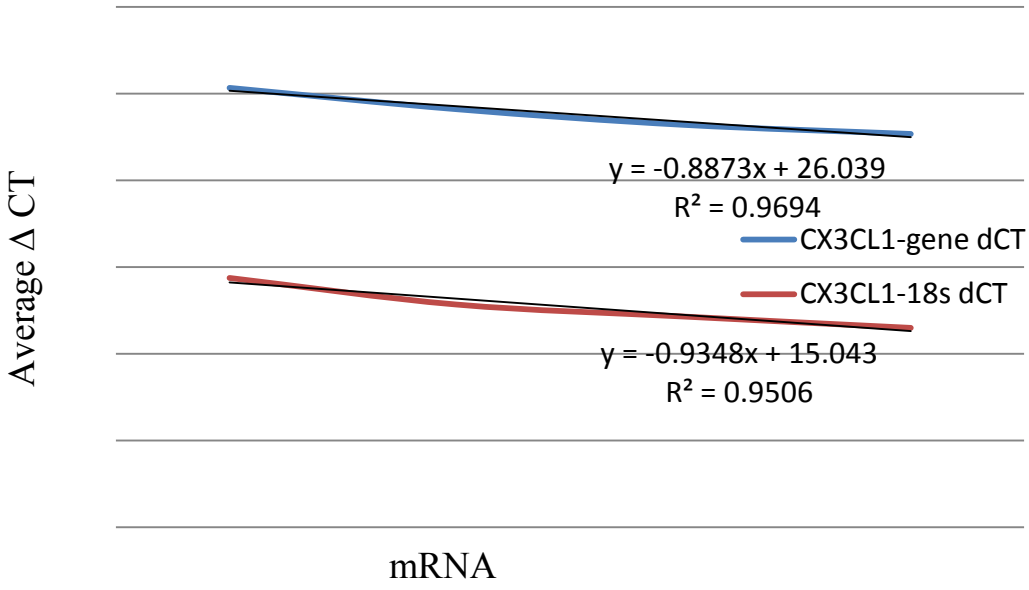


Figure 5.2: Efficiency curves examining the relationship between CX3CL1 and 18S. The difference in gradient between the two curves was very low

All TaqMan® Gene Expression Assays have been designed using a validated bioinformatics pipeline, and run with the same PCR protocol, eliminating the need for primer design or PCR optimization.

5.2.2.3.3 Probe and Primer Details

All primers utilised in Taqman® Gene Expression Assays were provided by Applied Biosytems, California, USA:

- iNOS: TaqMan gene expression assay for nitric oxide synthase 2A (inducible, hepatocytes); [Assay ID: Hs 00167257_m1]
- Arginase: TaqMan gene expression assay for arginase, type II [Assay ID: Hs 00265750_m1]
- FSP-1 TaqMan gene expression assay for S100 calcium binding protein A4; [Assay ID: Hs 00243202_m1]
- BCL-2: TaqMan gene expression assay for B-cell CLL/lymphoma 2; [Assay ID: Hs 00153350_m1]
- Podoplanin TaqMan gene expression assay for podoplanin; [Assay ID: Hs 00366766_m1]
- MCP-1/CCL-2: TaqMan gene expression assay for chemokine (C-C motif) ligand 2 [Assay ID: Hs 00234140_ml]
- CX3CL1: TaqMan gene expression assay for chemokine (C-X-X-X-C motif) ligand 1 [Assay ID: Hs 00171086_m1]

5.2.3 Immunohistochemistry for CD34, CD68 and PAMS Staining for Scarring

This was as described in section 3.2.5.

5.2.4 Immunohistochemistry for CA-IX, CCR2 and CX3CR1

For this analysis I used biopsy sections from patients with ischaemic/hypertensive nephropathy. Sections were heated in an oven at 56^0C for 30 minutes and then placed in one-step dewaxing and epitope retrieval solution W-Cap TEC Buffer pH 8.0AP (Surgipath Europe Ltd, Peterborough, UK) at 96^0C for 30 minutes. Sections were then allowed to cool in W-Cap for 20 minutes and then treated with 0.3% solution of hydrogen peroxide in methanol for 10 minutes. Endogenous biotin was blocked using sequential 10 min treatment with 0.1% avidin and 0.01% biotin (Dako UK Ltd, Cambridgeshire, UK). For CA-IX antigen retrieval, the citrate buffer epitope retrieval method was used instead of W-Cap one-step dewaxing and epitope retrieval technique.

Chemokine receptor expression was analysed utilising a CCR2 rabbit anti-human monoclonal antibody ([ab32144]; Abcam plc, Cambridge, UK) at 1:300 dilution and a CX3CR1 rabbit anti-human polyclonal antibody ([AB1891]; Chemicon Europe Ltd, Hampshire UK) at a 1:125 dilution. Carbonic anhydrase IX was studied by a rabbit anti-human polyclonal antibody ([ab15086]; Abcam plc, Cambridge, UK, dilution 1:500), diluted in Dako REAL™ Antibody Diluent (S2022; Dako UK Ltd, Cambridgeshire, UK). Samples were incubated with the primary antibodies for 60 minutes at room temperature. A

ChemMate™ DAKO EnVision™ Detection kit (K5007; Dako UK Ltd, Cambridgeshire, UK) was used for detection of positive staining. In this two-step technique, sections were incubated with the ChemMate™ DAKO EnVision™/ HRP, rabbit/mouse reagent for 30 minutes. Following which, sections were washed with PBS and then incubated with 1:40 dilution of ChemMate™ DAB + Chromogen in ChemMate™ Substrate Buffer for 10 minutes. Sections were then mounted in an aqueous medium (Immu Mount).

Controls used included omission of the primary antibody and substitution with an irrelevant rabbit IgG (Dako UK Ltd, Cambridgeshire, UK) isotype control at equivalent concentrations in parallel using adjacent sections on the same slide. Sections from renal cell carcinoma tissue were used as positive controls for Carbonic Anhydrase IX staining.

5.2.5 In situ Quantification

An interactive image analysis system was used for quantitative assessment of in situ antigens as previously described. Digital images were captured from coded sections, converted to a two-colour scale image and processed. For each patient the mean measurement of 5 randomly selected non-confluent microscopic fields of renal cortex was determined. Glomerular staining was excluded from the analysis by the computer software.

5.3 RESULTS

5.3.1 Patients

One hundred and six patients with chronic non-proliferative nephropathies were included from a large (215) patient cohort that had been accurately characterised for macrophage load, interstitial peri-tubular capillary density and index of chronic damage. This analysis had previously been carried out by Dr Kevin Eardley (Eardley, Zehnder et al. 2006; Eardley, Kubal et al. 2008). From this group (215), I excluded patients with (i) thin glomerular basement membrane disease (*n*=44); (ii) diabetic nephropathy (*n*=18), (iii) lupus nephropathy (*n*=4), (iv) extensive background staining that prevented analysis of tissue (*n*=28), and (v) no cortex in the biopsy specimen (*n*=10). Of the remaining patients, 89 had cDNA available and were included in the studies reported in this chapter. The cDNA was reverse transcribed from mRNA that had been isolated from biopsies by Dr Daniel Zehnder. This mRNA had previously been analysed for other molecular readouts in studies assessing the intra-renal Vitamin D network in CKD (Zehnder, Bland et al. 2001; Zehnder, Bland et al. 2002; Zehnder, Evans et al. 2002). Table 5.1 shows the histological diagnoses of the patients included in this present study and subset breakdown of the parameters analysed in this chapter.

5.3.2 In situ Analysis of Interstitial Macrophages and Peri-tubular Capillaries

I analysed the patterns of macrophages in the biopsies included in the analysis. In tissue with no evidence of tubulointerstitial damage, there were low numbers of interstitial

Macrophages that were evenly dispersed throughout the interstitium **(Figure 5.3b)** and there was a uniform distribution of capillaries between tubular epithelial cells **(Figure 5.3a)**. Where tubulointerstitial disease was patchy, there were increased interstitial macrophage numbers and these were associated with a reduced density of peri-tubular capillaries; these changes often co-localized to areas of scarring. In biopsies with an advanced stage of damage, macrophage infiltration was and reduced interstitial capillary density was widespread **(Figure 5.3 e, f)**.

Figure 5.3 Capillaries and Macrophages

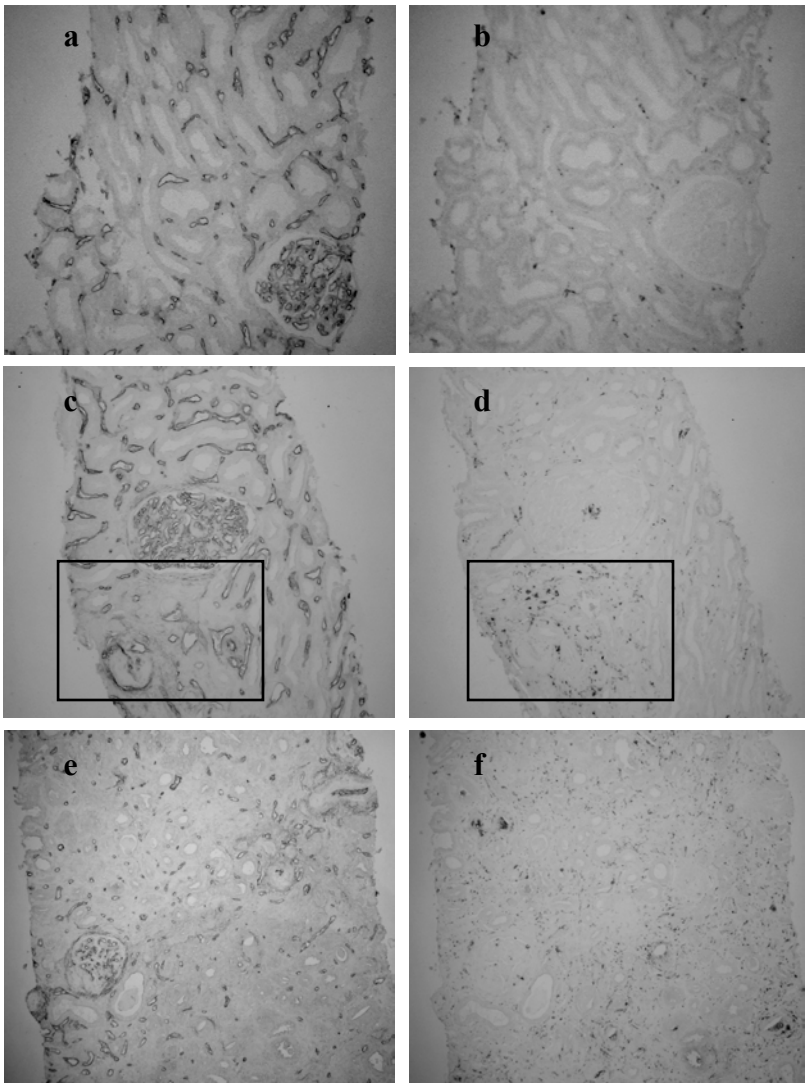


Figure 5.3 Capillaries (a, c & e) and infiltrating macrophages (b, d & e) in serial sections of renal biopsies with chronic kidney disease. No damage; uniform distribution of capillaries and macrophages (c & d). Moderate damage; patchy areas (inset box) of decreased capillary density and increased macrophages density (e & f). Advanced damage; widespread decrease in capillary density and increase in macrophage density. (I am grateful to Dr Kevin Eardley for these photomicrographs)

Table 5.1: Patient characteristics: clinical diagnoses and pathological variables

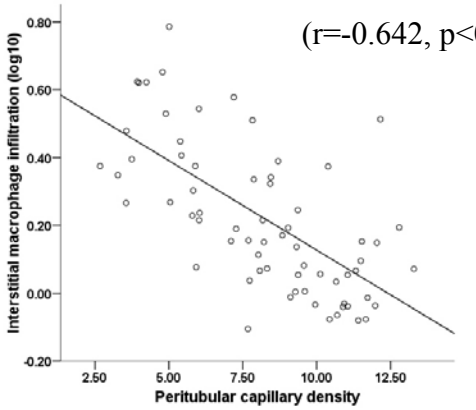
Histological diagnosis (n)	Age (yr) mean ± SEM	Creatinine (μmol/L) mean ± SEM	Index of chronic damage (%) mean ± SEM	Interstitial macrophages (%) mean ± SEM	Interstitial capillaries (%) mean ± SEM
IgA nephropathy (25)	42 ± 2.7 (25)	162 ± 37 (25)	20 ± 5.6 (25)	1.34 ± 0.16 (22)	9.44 ± 0.6 (19)
Ischaemic/hypertensive nephropathy (33)	56 ± 2.5 (33)	227 ± 19.3 (33)	41.18 ± 4.89 (33)	2.08 ± 0.2 (28)	6.79 ± 0.46 (27)
Focal segmental glomerulosclerosis (17)	58 ± 3.5 (17)	161 ± 27.67 (17)	17.71 ± 2.94 (17)	1.95 ± 0.27 (12)	8.22 ± 0.57 (12)
Membranous nephropathy (6)	60 ± 2.6 (6)	143.5 ± 34.4 (6)	30.33 ± 11.36 (6)	3.6 ± 0.8 (5)	9.7 ± 1.8 (4)
Light chain nephropathy (3)	73 ± 6.6 (3)	129.6 ± 28.9 (3)	30.33 ± 8.45 (3)	1.89 ± 0.12 (2)	7.6 ± 1.8 (2)
Minimal change nephropathy (2)	27 ± 7 (2)	81 ± 12 (2)	1 ± 1 (2)	1.23 ± 0.16 (2)	10.66 ± 1.3 (1)
Other (3)	50 ± 15 (3)	209 ± 214 (3)	31 ± 31 (3)	2.13 ± 1.04 (3)	6.93 ± 3.01 (2)
All (89)	50 ± 1.68 (89)	181.9 ± 15.11 (89)	27.37 ± 2.84 (89)	1.88 ± 0.13 (74)	8.17 ± 0.33 (67)

5.3.3 Quantitative analysis of interstitial macrophages and peri-tubular (interstitial) capillaries and correlations with chronic damage

Interstitial macrophage load was quantified in 74 patients (83.1%) and peri-tubular (interstitial) capillary density was successfully quantified in 70 patients (78.6%). The results were similar to those reported in Chapter 4, where this analysis had been restricted to biopsies from patients with ischaemic/hypertensive chronic kidney disease. Interstitial macrophage infiltration correlated (inversely) with capillary density ($r = -0.642$; $p<0.001$) **(Figure 5.4a)**. There was a positive correlation between macrophage infiltration and interstitial scarring ($r = 0.797$; $p<0.001$) **(Figure 5.4b)**. Capillary density correlated inversely with interstitial scarring ($r = -0.8$; $p< 0.001$) **(Figure 5.4bc).**

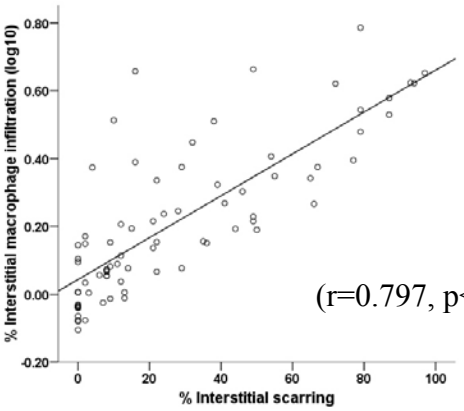
Figure 5.4 Inter-relationships between Inflammation, Ischaemia and Scarring

a)



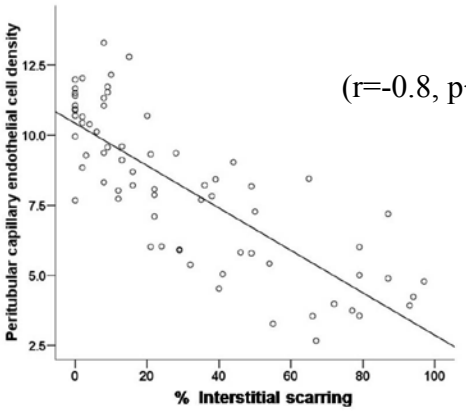
(r=-0.642, p<0.001)

b)



(r=0.797, p<0.001)

c)



(r=-0.8, p<0.001)

Figure 5.4: Correlations between: **(a)** macrophage infiltration and interstitial capillary density (r = -0.642; p<0.001) **(b)** macrophage infiltration and interstitial scarring (r = 0.797; p<0.001) and **(c)** interstitial capillary density and scarring (r = -0.8; p< 0.001)

5.3.4 Determinants of Macrophage Infiltration: The Relationship between Macrophage Load and Intra-renal mRNA Levels

5.3.4.1 MCP-1/CCL2 and CX3CL1

MCP-1/CCL2 is the major chemokine for the infiltration of macrophages to tissue sites (Kulkarni, Pawar et al. 2007). I assessed the relationship between mRNA for this chemokine and interstitial macrophage load in CKD.

There was a significant inverse correlation between interstitial macrophage load and MCP-1/CCL2 ΔCT mRNA. Where results are expressed as ΔCT, a negative correlation with a non ΔCT parameter where the linear or non linear relationship of that parameter has a positive relationship to expression represents an increase in mRNA expression with an increase in the parameter. The inverse correlation I found between macrophage load and MCP1/CCL2 ΔCT mRNA ($r = -0.452$; $p < 0.01$) **(Figure 5.5)** is an example of this, indicating that increasing macrophage load is associated with increasing MCP-1 mRNA levels.

There was no statistically significant correlation between CX3CL1 mRNA expression and macrophage load ($r = 0.163$; $p = 0.09$). There was a positive correlation between CX3CL1 and MCP-1/CCL2, however this did not reach statistical significance ($r = 0.403$; $p = 0.07$). There was a strong positive correlation between CX3CL1 mRNA expression and mRNA expression for the anti-apoptotic gene BCL-2 ($r = 0.738$; $p<0.001$) **(Figure 5.6)**.

Figure 5.5 Macrophage Load and MCP1/CCL2 ΔCT

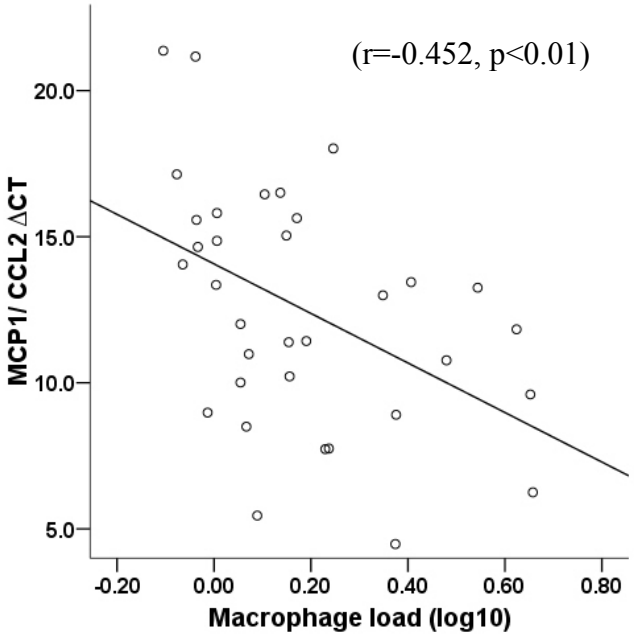


Figure 5.5: a) a negative correlation between macrophage load and MCP1/CCL2 ΔCT (r = -0.452; p < 0.01); a higher macrophage load correlates with lower MCP1/CCL2 ΔCT (i.e. higher MCP1/CCL2 mRNA expression).

Figure 5.6 CX3CL1 ΔCT & BCL-2 ΔCT

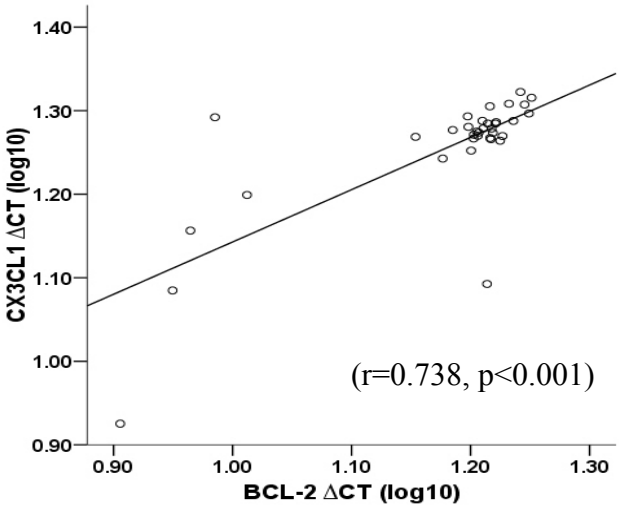


Figure 5.6 A positive correlation between CX3CL1 ΔCT & BCL-2 ΔCT (r = 0.738; p <0.001); a higher CX3CL1 mRNA expression correlates with higher BCL-2 mRNA expression.

This pattern is consistent with previous analyses reported by the group where urinary MCP-1/CCL2 correlated with in situ macrophage load (Eardley, Zehnder et al. 2006). Other studies have shown that the likely major source for MCP-1/CCL2, in non-proliferative glomerulonephritis, is from tubular epithelial cells (Vielhauer, Anders et al. 2001).

5.3.4.2 Arginase/ iNOS mRNA Levels

iNOS is up-regulated in macrophages in response to inflammatory stimuli to generate free radicals as the cells shift towards an M1 phenotype and become involved in phagocytosis, bacterial killing and initiation of the immune response. iNOS is not up regulated in M2 macrophages. Therefore, a lower ratio of arginase/iNOS mRNA is associated with an M1 macrophage phenotype, and a higher ratio represents an M2 phenotype. The ratio of iNOS ΔCT/arginase ΔCT used in these analyses represents arginase/iNOS mRNA expression (as iNOS mRNA is inversely proportional to iNOS ΔCT). Higher iNOS ΔCT/arginase ΔCT ratios were noted with increasing macrophage infiltration [$r = 0.433$; $p<0.05$] **(Figure 5.7)**. This represents an increasing type 2 intra-renal microenvironment with increasing macrophage infiltration.

Figure 5.7 Macrophage Load and arginase/ iNOS mRNA

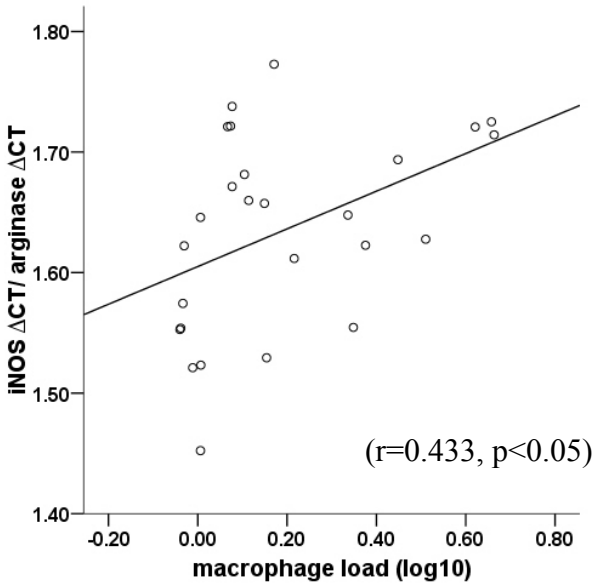


Figure 5.7: A positive correlation between macrophage load and iNOS ΔCT/ arginase ΔCT ratio (r = 0.433; p<0.05); higher macrophage load correlates with higher iNOS ΔCT/ arginase ΔCT ratio (i.e. higher arginase/ iNOS mRNA expression ratio, representing M2 macrophage phenotype).

5.3.4.2 Arginase/ iNOS mRNA and effector mechanisms

To assess the relationship between inflammatory phenotype and fibrosis I correlated macrophages and iNOS ΔCT/ arginase ΔCT with FSP-1 (for fibrosis), podoplanin (for lymphangeogenesis) and BCL-2 (anti-apototic) gene. These studies demonstrated distinct patterns of expression.

5.3.4.2a Fibroblast specific protein-1 (FSP-1)

FSP-1 mRNA expression showed a positive correlation with chronic damage, but this was statistically insignificant (r = 0.473; p = 0.077). However, it was up-regulated with an increasing iNOSΔCT/arginaseΔCT ratio **(Figure 5.8)**, indicating that with an increased M2 in situ macrophage phenotype, there was an overall increase in pro-fibrotic gene expression. However this relationship was independent of macrophage load. FSP-1 also correlated positively with expression of BCL-2 (r = 0.397; p<0.05).

Figure 5.8 Arginase/ iNOS and FSP-1

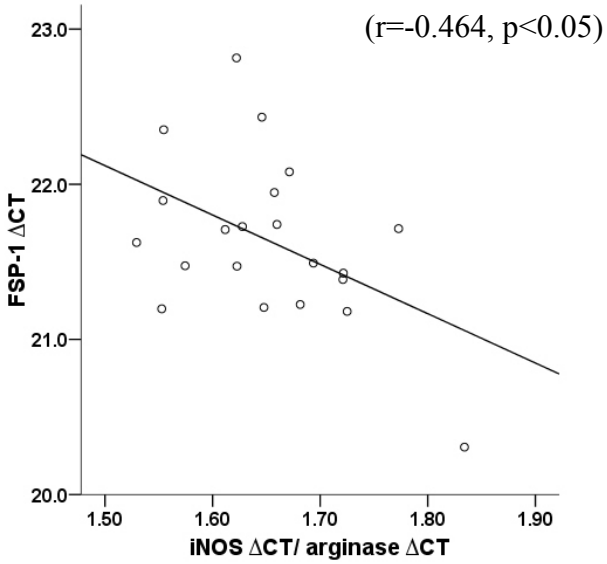


Figure 5.8: Higher ratios of iNOS ΔCT/ arginase ΔCT (M2 macrophage phenotype) correlate with lower FSP-1 ΔCT (higher mRNA expression); (r = -0.464; p<0.05).

5.3.4.2b Podoplanin

Macrophage load and iNOS ΔCT/ arginase ΔCT ratio correlated with podoplanin expression (r= 0.414, p<0.05). This indicates that a Th 2 micro-environment and increasing macrophage load are associated with increased gene expression associated with lymphangiogenesis (**Figure 5.9)**. This will be discussed later in this chapter.

Figure 5.9 Podoplanin and arginase/ iNOS and FSP-1

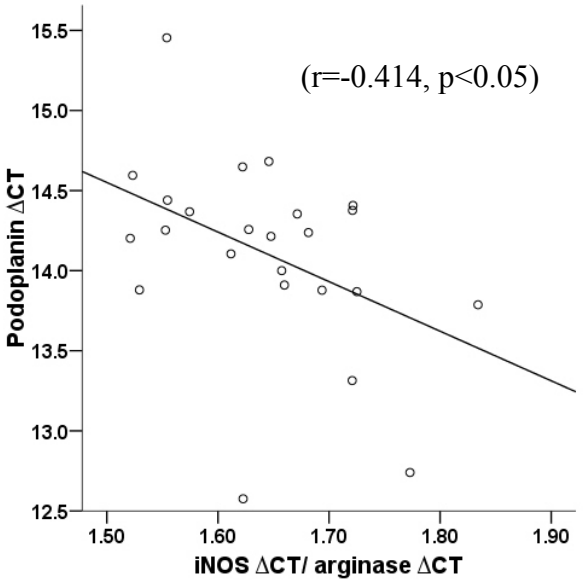


Figure 5.9: Higher ratios of iNOS ΔCT/ arginase ΔCT (M2 macrophage phenotype) correlate with lower podoplanin ΔCT (i.e. higher podoplanin mRNA); (r=-0.414, p<0.05).

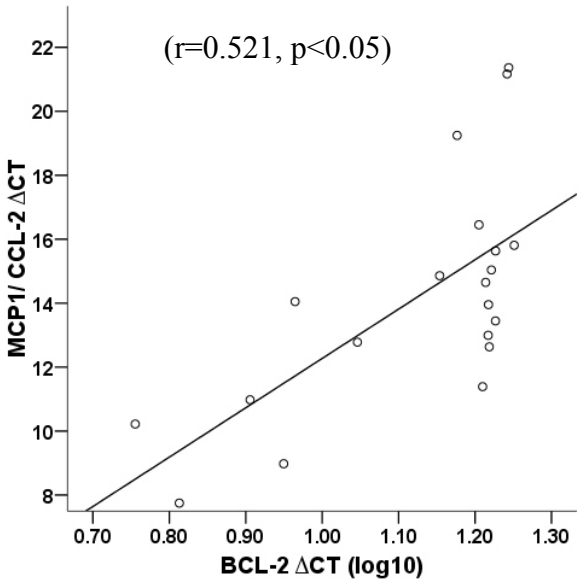
5.3.4.2c BCL-2

There was no significant correlation between BCL-2 ΔCT and iNOS ΔCT/ arginase ΔCT ratio (r=0.336, p=0.08). However, positive correlations were observed between BCL-2 ΔCT and ΔCT for MCP-1/CCL-2 (r=0.521, p<0.05) and FSP-1(r=0.383, p<0.05) **(Figure**

5. 10 a & b), and CX3CL-1 (r = 0.738; p <0.001) **(Figure 5.6)**. These relationships are discussed later in this chapter.

Figure 5.10 MCP-1/CCL-2, FSP-1 and BCL-2

a)



b)

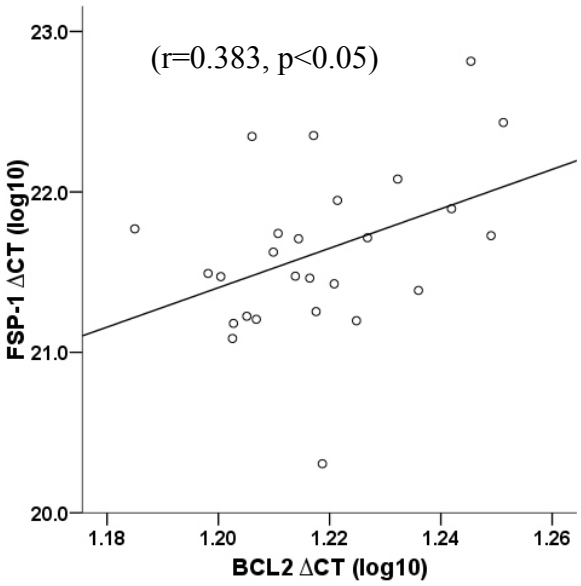


Figure 5.10: positive correlations between ΔCTs of BCL-2 and MCP-1/CCL-2 (a), and FSP-1 (b)

5.3.4.3 Macrophage load & effector mechanisms

Apart from iNOS/ arginase ΔCT ratio, macrophage load also correlated negatively with podoplanin ΔCT (r= -0.428; p<0.05) **(Figure 5.11)**. This supports the suggestion that there may be increasing neo-lymphangiogenesis with increasing macrophage load.

Figure 5.11: Inflammation and podoplanin

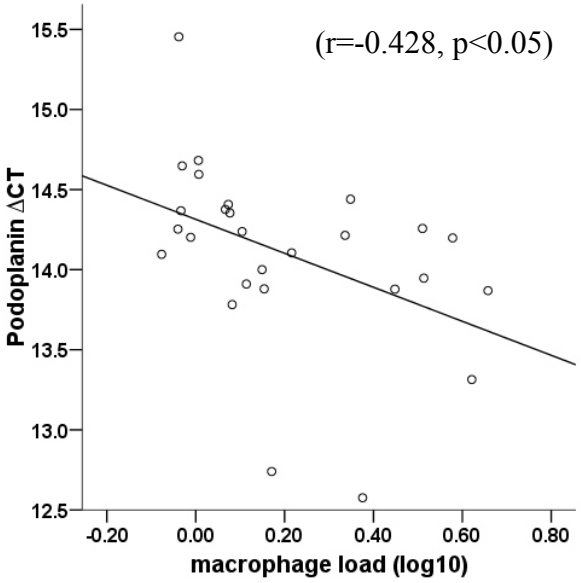


Figure 5.11: Inter-relationship between inflammatory load and podoplanin ΔCT: a negative correlation indicates increased podoplanin mRNA expression with increasing macrophage load (r= -0.428, p<0.05).

5.3.4.4 Determinants of macrophage recruitment/ retention: expression CCR2 and CX3CR1

To further explore changes in the micro-environment in CKD, I assessed the expression of two receptors (CCR2 and CX3CR1) that direct the recruitment and retention of macrophages. CCR2 **(Figure 5.12a & b)** and CX3CR1 **(Figure 5.12 c & d)** receptor

expression appeared localized to cell infiltrates. The ratio between macrophage infiltration and CCR2 expression was increased in biopsies with ≥20% interstitial scarring compared to biopsies with less than 20% scarring **(Figure 5.13)**, whilst the macrophage/CX3CR1 ratio was maintained **(Figure 5.13).** This indicates that CCR2 may have a more important role for the recruitment of macrophages in early chronic damage, such that as chronic damage progresses, this ratio increases. A role for CX3CR1 in the recruitment and retention of cells is maintained throughout the progression of damage.

Figure 5.12 Immunostaining for CCR2 and CX3CR1

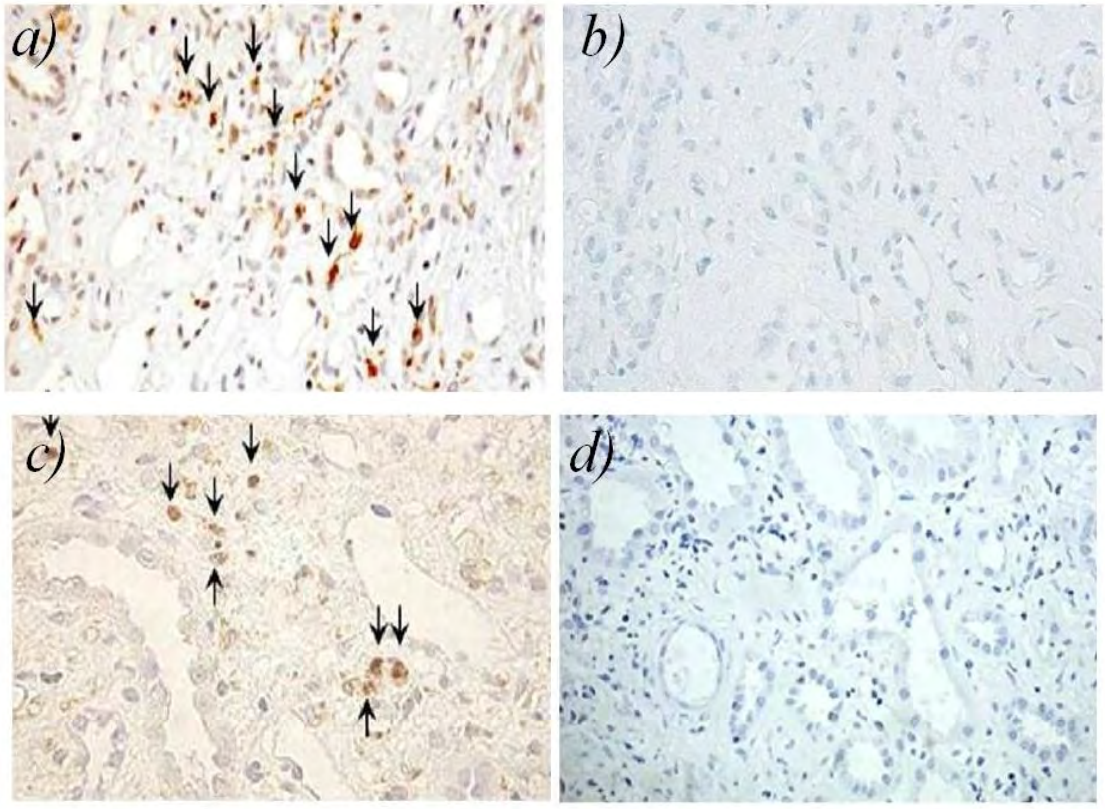


Figure 5.12: Immunostaining for CCR2 **(a)** and CX3CR1 **(c)** localized to cell infiltrates, with their respective isotype controls **(b)** and **(d)**.

Figure 5.13: Chemokine Receptor Expression in Early vs. Late Damage

CCR2 CX3R1

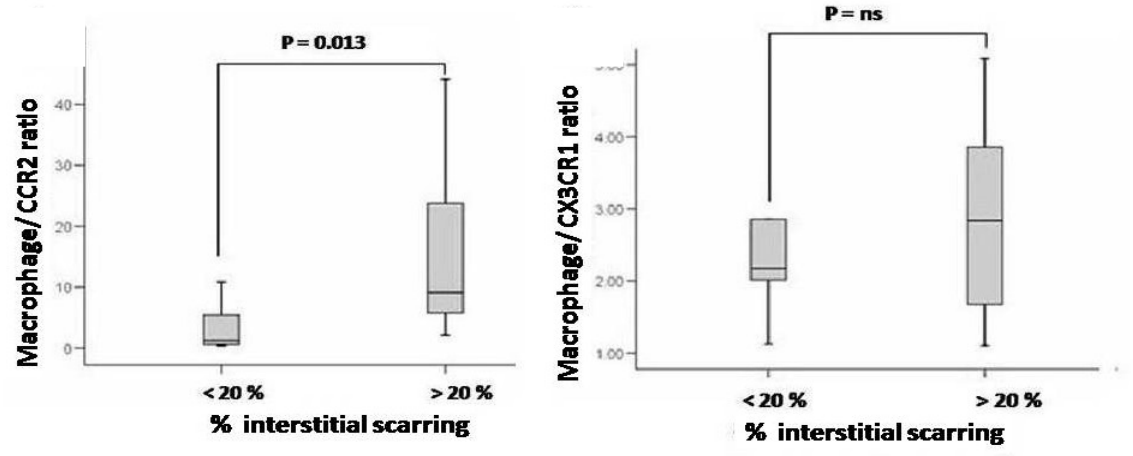


Figure 5.13: Patterns of chemokine receptor expression controlled for inflammatory load in early vs. late damage: note increased CCR2 expression in early damage **(left panel)**, CX3CR1 expression remains unchanged with increasing scarring **(right panel).**

5.3.5 Tubulointerstitial Hypoxia: Relationships with Injury Processes

In order to assess the relationship between hypoxia and in situ determinants of renal damage, I analysed tissue from patients with ischaemic/hypertensive nephropathy (n=20) for Carbonic anhydrase IX (CA IX) – a transmembrane glycoprotein which is hypoxia inducible and may therefore be an endogenous marker of hypoxia. There was a significant difference between expression of CA IX in tissue with less than 20% interstitial scarring and ≥20% interstitial scarring **(Figure 5.14c).** The large majority of expression of CA IX was restricted to tubular epithelium **(Figure 5.14 a & b)**.

Figure 5.14 Carbonic Anhydrase- IX (CA-IX)

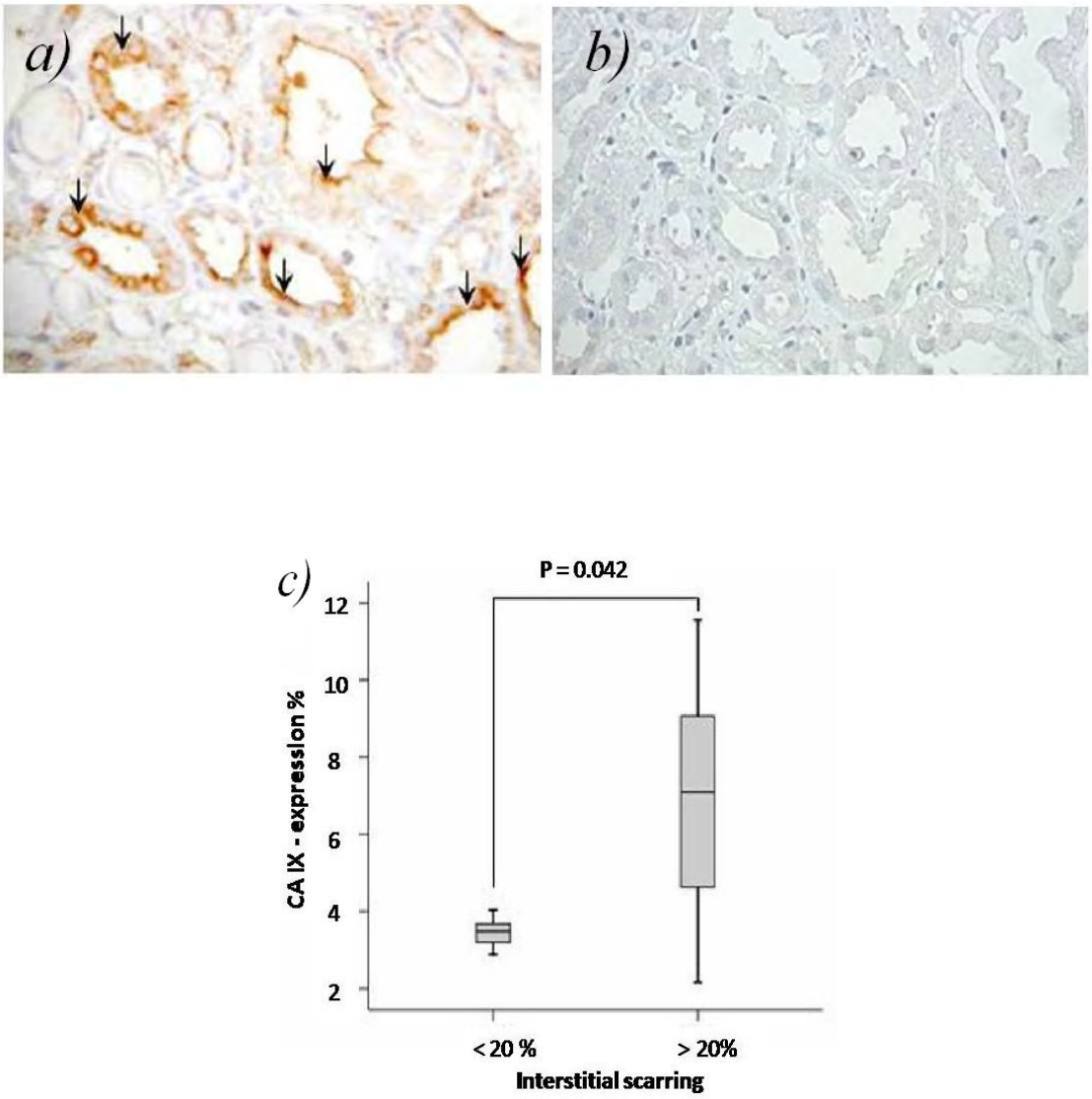


Figure 5.14 CA-IX expression: a) note positive staining of apical borders of tubular cells b) negative isotype control c) markedly stronger CA-IX expression in biopsies with > 20% interstitial scarring

5.3.6 Changes in renal micro-environment by disease state: relative role of inflammation and ischaemia

I compared iscahemic/hypertensive nephropathies with other disease groups to assess the variation in the micro-environment by disease state. This analysis was limited by the small numbers in the two groups (n=20 and n=69 respectively). Some of the correlations observed amongst mRNA expression of genes studied in the whole cohort became weaker or insignificant due to the smaller numbers. The inter-relationships maintained in the sub-group analyses were those between macrophage load, peri-tubular capillary loss and interstitial scarring. However, there were distinct patterns present for other parameters for the 2 groups **[Table 5.2]**.

Table 5.2: significant associations by disease state

Ischaemic/hypertensive	Others
Podoplanin ΔCT: FSP-1ΔCT	Macrophage load: MCP-1/CCL2 ΔCT
Podoplanin ΔCT: BCL2ΔCT	Macrophage load: iNOS ΔCT/ arginase ΔCT
FSP-1ΔCT: Interstitial scarring	MCP-1/CCL2 ΔCT: BCL2ΔCT
BCL2ΔCT: TGF-β ΔCT	Podoplanin ΔCT: BCL2ΔCT

5.4 DISCUSSION

The majority of the cohort studied comprised patients with IgA nephropathy, hypertensive/ischaemic nephropathy and FSGS. Patients had both early and advanced renal failure. The correlations between injury processes of macrophage infiltration, ischaemia and interstitial scarring observed in earlier chapters were maintained in this cohort. I hypothesised that macrophage infiltration is a primary determinant of progressive renal damage and that phenotypic variation in the micro-environment in relationship to macrophage load may be related to in situ adaptive processes.

I used RT-PCR as the main methodology to assess renal micro-environment by studying relevant gene expression. The rate of production and degradation of mRNA may differ significantly from that of its protein. Post-translational modification of the protein may also alter the association between mRNA and protein levels

5.4.1 Adaptive Responses to Increasing Inflammatory Load

Several gene mRNA levels were increased in association with an increasing macrophage load. My results showed that with increasing macrophage load there is significant loss of peri-tubular capillary loss, increased renal scaring and up-regulation of expression of a number of genes. MCP-1/CCL2 mRNA levels correlated with CX3CL1, BCL-2 and arginase mRNA expression. Furthermore, up-regulation of podoplanin mRNA expression with increasing macrophage load supports an association between macrophage load and neo-lymphangiogenesis. A change in the arginase/ iNOS mRNA ratio suggests that macrophage phenotype evolves from M1 to M2 as renal scarring progresses.

Furthermore, the arginase/iNOS ratio correlated positively with FSP-1 mRNA and podoplanin mRNA, suggesting that this evolution of phenotype may be associated with fibrosis and neo-lymphangiogenesis respectively. However, macrophage load and FSP-1 mRNA did not correlate. The reasons for this are speculative, but one potential explanation is that arginase/iNOS ratio is a more direct readout of the micro-environment than overall macrophage numbers.

5.4.2 In situ Macrophage Phenotypes and the Arginase/iNOS Ratio

The results presented in this experimental chapter demonstrate an increased M2 phenotype associated with an increasing macrophage load. The M2 phenotype was suggested by an increased arginase/iNOS mRNA ratio (iNOS ΔCT/ Arginase ΔCT ratio). The pattern demonstrated in human CKD by this study is consistent with previous work by Stout *et al* in animal models (Stout, Jiang et al. 2005). It is well known that M2 cells play a role in reparative processes involved in wound healing (Martinez, Sica et al. 2008), directing Th2 humoral responses, allergic and parasitic responses and the coordination of repair following an inflammatory reaction (Song, Ouyang et al. 2000). In this study, M2 phenotype was found to be associated with mRNA expression of FSP-1 and podoplanin. This indicates that a M2 phenotype is associated with the activation of genes for fibrosis and neo-lymphangiogenesis in this setting. It may be possible that an M2 phenotype in the renal micro-environment supports fibrosis. Targeting macrophage activation and phenotype is now leading to new therapies in treatment of many acute and chronic kidney diseases (Duffield 2010). Although polarization of macrophages to M1 or M2 phenotypes is well known, it is not fully understood how macrophages become polarized in vivo and

the degree to which they retain plasticity or remain committed to a particular activation state (Winnepenninckx, Lazar et al. 2006; Dijkman, van Doorn et al. 2007). Critically, the coordinated polarization of macrophages determines whether infection or injury is resolved successfully (Hoefnagel, Dijkman et al. 2005). The ability to redirect inappropriate macrophage activation, therefore, presents a potentially effective therapeutic approach for treating macrophage-mediated diseases (Vezzoli, Novara et al. 2011). More recently M2/ M2 classification has been modified to reflect the increasing controversy in this area and the increased awareness that other discrete macrophage phenotypes may exist. Major advances have been made in this area; Whyte *et al* showed that suppressor of cytokine signaling (SOCS)1 expression in alternatively activated M2 macrophages and its role in differential macrophage activation and function (Deonarine, Panelli et al. 2007). Additionally, emerging data demonstrates global changes in the expression of genes during monocyte to macrophage differentiation, and how the patterns of change identify the mechanism contributing to macrophage differentiation or function (Gershenwald and Bar-Eli 2004). With the advent of microarray technology, the transcriptional profile of in vitro differentiation of primary human monocytes into macrophages has been demonstrated. Distinct patterns of gene expression have been demonstrated with a phenotypic shift of macrophages from M1 to M2 (Deonarine, Panelli et al. 2007; Nahrendorf, Swirski et al. 2007). Distinct patterns of gene expressions have been identified with macrophage polarization (Martinez, Gordon et al. 2006). A hallmark of M1 polarization is the synthesis of the proinflammatory cytokines IL-6, IL-12, and IL-15 and receptors for IL-2R α-chain, IL-15R α-chain, and IL-7R (Mantovani, Sica et al. 2005), on the other hand, M2 are characterized by the overexpression of several

scavenger receptors able to bind a diverse array of endogenous and foreign molecules (Peiser and Gordon 2001). Additionally recent studies have identified Krüppel-like factor 4 (KLF4) as a critical regulator of macrophage polarization (Liao, Sharma et al. 2011). The ability for KLF4 to regulate both M1 and M2 polarization has potentially broad implications for numerous inflammatory disease states and wound healing.

5.4.3 Neo-lymphangiogenesis

Podoplanin has been used as a specific marker of lymphangiogenesis (Breiteneder-Geleff, Soleiman et al. 1999). In 2005, in a mouse corneal transplantation model, $CD11b^{+}$ macrophages were shown to transdifferentiate into lymphatic endothelial cells (Maruyama, Ii et al. 2005). It is now clear that macrophages support lymphangiogenesis in two different ways, either by transdifferentiating and directly incorporating into the endothelial layer or by stimulating division of pre-existent local lymphatic endothelial cells (Kerjaschki 2005). The results from my work suggest increasing podoplanin mRNA expression with increasing macrophage load. The results presented here support the previous evidence in this area. Correlation between Arginase/iNOS mRNA ratio and podoplanin mRNA expression suggests that M2 rather than M1 macrophages are involved in this process of neo-lymphangiogenesis as part of a reparative process.

5.4.4 Apoptosis

BCL-2 is an anti-apoptotic gene and prevents cell death by apoptosis. In the context of chronic kidney disease, over-expression of BCL-2 may mean relatively increased proportion of cell death by necrosis. In this study, BCL-2 over-expression was found to

be associated with increased mRNA expression of MCP-1/ CCL-2, FSP-1 and CX3CL1 genes. It is difficult to draw conclusions based on BCL-2 mRNA expression alone in this setting and further analysis of cell death by apoptosis/necrosis in relation with the injury processes is required.

5.4.5 Hypoxia in Renal Micro-environment

I used tissue expression of CA-IX to evaluate hypoxia in the renal micro-environment. Although CA-IX has been used in the settings of renal cell carcinoma, I have demonstrated this in benign kidney disease for the first time. CA-IX expression was particularly present on tubular epithelium and was heavily expressed in biopsies with heavy interstitial scarring. However, there was no correlation between CA-IX and peri-tubular capillary loss.

5.4.6 Renal Micro-environment by Disease State

Upon sub-group analysis for ischaemic and non-ischaemic nephropathies, different patterns of micro-environmental processes were observed. In both groups the relationships between inflammation, ischaemia and interstitial scarring were maintained, although they were stronger in the ischaemic group. Different patterns of inter-relationships amongst gene expressions specific to each sub-group may suggest predominant mechanisms of injury specific to that disease state. For example, the correlation between iNOS ΔCT/Arginase ΔCT ratio and macrophage infiltration was not observed in the ischaemic sub-group. This may suggest that phenotypic transformation of macrophages predominantly occurs in glomeronephritis/inflammatory diseases rather

than ischaemic/hypertensive disease states. Similarly, MCP-1/CCL2 mRNA expression did not correlate with either macrophage infiltration or BCL-2 mRNA expression in the ischaemic sub-group. These findings suggest that MCP-1/CCL2 is an actively involved chemokine in inflammatory renal disease and may induce BCL-2. Conversely, relationships between FSP-1 and interstitial scarring, and FSP-1 & podoplanin, were specific to the ischaemic sub-group, which may suggest FSP-1 plays an important role in fibrosis and lymphangiogenesis is this setting. The above patterns may be important in understanding the mechanisms of injury specific to each disease state, particularly because the mRNA expressions for the genes studied were comparable between the two disease sub-groups.

5.5 CAVEATS

Although this study has demonstrated novel findings in the setting of chronic kidney disease, a major limiting factor has been the small numbers of datasets in the assessment of mRNA expression. Although some associations are clear, important relationships may have been lost as a result of small numbers. Another potential criticism is the use of RT-PCR as a method to assess the renal micro-environment as mRNA levels may not accurately reflect the amount of the mature protein which it codes for. Post-translational modification of the protein may also disconnect the association between mRNA and protein levels. Further studies to relate mRNA to tissue protein expression may be required to obtain further insight into the mechanisms suggested here.

CHAPTER 6

DISCUSSION

The principal aim of the research described in the experimental chapters in this thesis was to assess the role of different pathophysiological processes in the evolution of chronic allograft damage and native chronic kidney disease (CKD). To study this, I evaluated the impact of the pre-transplant status of cadaveric renal grafts (Chapter 2), the role of calcineurin inhibitor toxicity (Chapter 3), the histological changes in the kidney in transplant and native kidneys (Chapter 4), and assessed the renal microenvironment by mRNA expression of specific genes in CKD (Chapter 5).

6.1 Summary of Main Research Findings

In Chapter 2, I demonstrated that mitochondrial function could be measured by complex I-IV activity in human kidney samples. This was the first study to make this observation. Previously, a correlation has been demonstrated between all four Complexes activities and sperm motility (Ruiz-Pesini, Diez et al. 1998). I then demonstrated that mitochondrial function in the pre-transplant kidney had an impact on clinical outcomes following cadaveric renal transplantation, with a positive correlation between mitochondrial Complex II/III activities controlled for citrate synthase and mid-term graft function. Despite the relatively large degree of scatter found in the mitochondrial enzyme-specific activities, I found a linear relationship between these specific activities and mid-term graft function. However, mitochondrial function did not have an impact on immediate post-transplant recovery of the graft function and the mitochondrial effects of subsequent warm ischaemia and reperfusion after engraftment remained to be determined. Variations in each of the Complex activities and also in citrate synthase levels in different biopsies prove that mitochondrial function varies in donated kidneys.

Extended ischaemia time may result in decreased activity of the complexes of the electron transport chain such as complex IV and complex I (Rouslin 1983). The experiments in this study indicated that mitochondrial complex activities did not correspond to the period of cold ischaemia used for organ preservation, perhaps due to: 1) mitochondrial complex proteins are robust enough to resist electrophysiologic damage inflicted by cold ischaemia in the given preservation technique; and 2) mitochondrial ROS produced during initial ischaemia induces protection from further damage from prolonged ischaemia. These concepts need further investigation.

In Chapter 3, I studied the relative contribution of CNI nephrotoxicity to kidney injury following non-renal solid organ transplants. In my analysis, 35.5% of the biopsies showed criteria for CNI toxicity. Patients with biopsies with this finding did not sustain an accelerated deterioration in renal disease compared to biopsies without histological evidence of CNI use. This does not mean that CNIs do not have an important role in the pathogenesis of kidney disease; indeed, there was a strong inverse correlation between capillary density and scarring across disease groups indicating microvascular disease, possibly as a consequence of upstream arteriolar vasoconstriction to which CNIs may contribute. In that chapter I quantified ischaemia**,** inflammation and scarring, and, as seen in figure 3.11 (a, b, and c), these processes were inter-related. The strongest correlation was between ischaemia and scarring. This may suggest that ischaemia is the dominant injury process, with a weaker correlation between macrophage infiltration and scarring, perhaps due to the use of immunosuppressive drugs in these patients. As seen in table 3.4, the correlation between ischaemia and scarring was maintained in sub-group analyses for

CNI nephrotoxicity and hypertensive/ischaemic nephropathy. However, an important point is that the biopsies in this study were performed at a stage where renal disease was advanced and therefore some of the changes may reflect the end point of the pathogenic pathway, rather than triggers and modifiers of renal injury. To further explore this model, in Chapter 4, I described a comparative analysis that I performed, assessing differential injury processes in kidney transplant biopsies and biopsies from native kidneys (non-renal solid organ transplants and native kidney disease). For matched renal function, a similar degree of interstitial scarring was observed across the three study groups. Ischaemia was predominant in failing renal transplants; conversely inflammation was predominant in native CKD**.** When controlled for interstitial scarring, ischaemia remained the predominant association in failing renal transplants. The relationship between macrophages and endothelial cells indicated that, despite a lower macrophage load, there may be an allogeneic impact of macrophages on endothelial cells. The role of macrophages in the development of ischaemia in transplanted kidneys has been suggested by previous work (Pilmore, Eris et al. 1999). Co-localization studies, showed an increased number of macrophages within 5 microns from endothelial cells in failing renal transplants when compared to chronic kidney disease. Furthermore, this study also demonstrated significant correlations between the number of macrophages within the vicinity of endothelial cells and ischaemia and interstitial scarring. Expression of iNOS and VEGF by infiltrating macrophages has been demonstrated (Adair, Mitchell et al. 2007) and this may suggest macrophages induce both vascular endothelial cell injury and the generation of new lymphatic vessels in end-stage transplant nephrectomies. To study this, in Chapter 5, I found that several gene mRNA levels (iNOS, arginase, CX3CL1,

BCL-2, MCP-1/CCL2 and FSP-1) were increased in association with an increasing macrophage load. MCP-1/CCL2 mRNA levels correlated with CX3CL1, BCL-2 and arginase mRNA expression. The most important finding was that an increased Arginase/iNOS mRNA ratio (iNOS ΔCT/Arginase ΔCT ratio) was associated with increasing macrophage load. This study was the first to demonstrate this pattern in human CKD and is consistent with previous work by Stout et al in animal models (Stout, Jiang et al. 2005). It is known that M2 phenotype macrophages play a role in reparative processes involved in wound healing (Martinez, Sica et al. 2008), directing Th2 humoral responses, allergic and parasitic responses and the coordination of repair following an inflammatory reaction (Song, Ouyang et al. 2000). I demonstrated that an M2 phenotype was associated with the activation of genes for fibrosis and neo-lymphangiogenesis in this setting. It is possible that an M2 phenotype in the renal micro-environment supports fibrosis. Upon sub-group analysis, the correlations between iNOS ΔCT/ Arginase ΔCT ratio or MCP-1/CCL2 mRNA expression and macrophage infiltration were not observed in the ischaemic sub-group. This suggests that phenotypic transformation of macrophages predominantly occurs in glomeronephritis/inflammatory diseases rather than ischaemic/hypertensive disease states. However, it must be noted that M1/ M2 phenotypic change is not straight forward. Recently identified profiling techniques and genetic approaches have shed new light on the M1/M2 paradigm. For instance, unexpectedly, arginase is not expressed prominently in human IL-13-induced M2 cells (Scotton, Martinez et al. 2005). M2 cells express high levels of the chitinase-like YM-1. Chitinases represent an antiparasite strategy conserved in evolution, and there is now evidence that acidic mammalian chitinase induced by IL-13 in macrophages is an

important mediator of type 2 inflammation (Zhu, Zheng et al. 2004). Polarization of macrophage function should be viewed as an operationally useful, simplified, conceptual framework describing a continuum of diverse functional states (Mantovani, Sica et al. 2004). The genetic approaches now available provide tools to dissect the actual in vivo function of polarized macrophages and to explore the limitations of the M1/M2 paradigm to define the complexity and plasticity of mononuclear phagocytes.

6.2 Proposed Theory of Predominant Ischaemia in Failing Renal Transplants

This work has provided an insight into the mechanisms involved in failing renal transplants. In particular, it has produced evidence of an association between macrophage infiltration and ischaemia in failing renal transplants. The co-localization studies indicate a possible role for adhesion molecules and/or chemokines and their receptors mediating endothelial cell damage and loss. Similarly, the observation of changing phenotype of macrophages associated with increasing macrophage load is interesting, even though this requires validation in the setting of kidney transplantation.

6.3 Future Studies

From a clinical perspective, mitochondrial complex assays with longer follow-up in a larger cohort of patients will produce a better understanding of mitochondrial function and its utility in renal transplantation. However, one must carefully consider immunologic causes of graft losses in longer follow-up studies. The question of whether or not mitochondrial function deteriorates during the process of organ harvesting and/or

after reperfusion injury is worth pursuing. If it is proven that mitochondrial function is affected by harvesting or reperfusion injury, there may be a role for drugs such as trimetazidine (Hauet, Goujon et al. 2000) and N acetylcystine (Kuo, Lee et al. 2009) in the preservation of mitochondrial function.

Further studies to establish the role of macrophages in mediating ischaemia may provide better insights into this novel mechanism of injury in this setting. This association between macrophages and endothelial cells can be further explored and new therapeutic targets can be developed. Adnesion molecules, chemokines and chemokine receptors may play a role in mediating this process.

Recently there has been an interest in the molecule hypoxia-inducible factor-1α (HIF-1α), a transcription factor that functions as a master regulator of oxygen homeostasis that has been implicated in fibrinogenesis. Its role has been recently demonstrated in a mouse bleomycin-induced pulmonary fibrosis model, where the expression of HIF-1α and plasminogen activator inhibitor-1 (PAI-1) was predominantly induced in alveolar macrophages (Ueno, Maeno et al. 2011). Quantification of renal tubulo-interstitial expression of HIF-1α at different stages of inflammation, ischaemia and scarring and its role in the setting of renal transplantation may be worth pursuing.

6.4 In Conclusion

Inflammation and ischaemia lead to a final common pathway of renal damage; interstitial scarring in the absence of otherwise obvious immunological or inflammatory

etiopathogenesis. Ischaemia and inflammation play varying roles dependent on the disease state; for instance ischaemia was predominant in failing renal transplants, whereas inflammation was predominant in cases of CKD. Interstitial macrophages may cause “direct injury” to the endothelial cells and this may trigger tubulointerstitial ischaemia. Particularly in the setting of renal transplants, this phenomenon may be “allogeneic”. A phenotypic transformation of macrophages is seen from from M1 to M2 as renal scarring progresses. A detailed understanding of the biology of this area may lead to the further development of therapies that will improve the outcome of renal disease. The work presented in this thesis adds to our understanding.

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