in salt-depletion and modification of effect by an antifibrotic agent

Nicholas Roger Brook BSc MSc BM MRCSEd

The Royal College of Surgeons of Edinburgh
Robertson Trust Research Fellow 2002 - 2003

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The Division of Transplant Surgery
Department of Cardiovascular Sciences, University of Leice

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The calcineurin inhibitors, cyclosporine and tacrolimus, are pivotal immunosuppressants in renal transplantation, but produce a cascade of acute (functional) and chronic (fibrotic) injurious events that contribute to chronic allograft nephropathy (CAN). Most, if not all, transplant patients treated with calcineurin inhibitors will express some degree of renal injury. The use of adjuvant agents (e.g. sirolimus) allows calcineurin-inhibitor dose-reduction and lessening of nephrotoxic exposure. There is a relative paucity of work examining the role of antifibrotic agents such as pirfenidone for halting or reversing the fibrosis of CAN.

Using the rat salt depletion model of calcineurin inhibitor toxicity, this study aimed to examine the effects of clinically relevant combinations of cyclosporine, tacrolimus and sirolimus on renal functional, structural and molecular markers of injury. Further, the effect of pirfenidone when added to these drug combinations was examined.

There were differences in the effects of cyclosporine and tacrolimus on functional and molecular variables, with tacrolimus displaying more favourable results. As sole therapy, sirolimus had no effect on renal function or messenger RNA expression. Deterioration in renal function and a deleterious effect on molecular markers of fibrosis were seen when cyclosporine and sirolimus were combined at high doses; at lower doses, favourable outcomes for these end-points were elicited. When sirolimus and tacrolimus were combined, renal function worsened and the beneficial molecular effects of tacrolimus were reversed.

Pirfenidone's actions were non-dose dependent, and beneficial effects for renal function and molecular markers were demonstrated. The effect of pirfenidone on renal function has not previously been described.

There were no differences in urinary protein or interstitial fibrosis measurements across the groups. Without fibrosis, it is impossible to say whether pirfenidone acted in a truly antifibrotic manner. However, the molecular changes possibly represent interim markers of fibrosis, suggesting such an effect may have been developing.

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STATEMENT OF ORIGINALITY

The work on which this thesis is based is my own independent work except where acknowledged.

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Presentations

The anti-fibrotic agent pirfenidone reduces cyclosporine and tacrolimus induced nephrotoxicity in a salt-depleted model of renal fibrosis. **NR Brook**, JR Waller, B Yang, SJ Marjolin, PN Furness, GR Bicknell, ML Nicholson

- Oral presentation at the British Transplantation Society Annual Congress, London. April 2003.
- Poster presentation at The Association of Surgeons of Great Britain and Ireland, Manchester. May 2003.
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- Oral presentation at the 11th ESOT Congress, Venice. September 2003.
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- Poster presentation at the American Transplant Congress, Boston. May 2004.
- Poster presentation at the 3rd International Congress on Immunosuppression. San Diego, December 2004

Cyclosporine and rapamycin act in a synergistic and dose-dependent manner in a model of immunosuppressant-induced kidney damage. **NR Brook**, JR Waller, B Yang, PN Furness, GR Bicknell, ML Nicholson

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- Poster presentation at the American Transplant Congress Meeting, Washington DC. May 30th-June 4th 2003.
- Poster presentation at the World Congress of Nephrology, Berlin. June 2003.
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- Poster presentation at the American Transplant Congress, Boston. May 2004.

Tacrolimus decreases, cyclosporine increases and rapamycin has no effect on profibrotic gene mRNA expression in renal tissue. **NR Brook**, JR Waller, GR Bicknell, ML Nicholson

- Poster presentation at the 6th International Congress on New Trends in Clinical and Experimental Immunosuppression, Salzburg. February 5th – 8th 2004.
- Poster presentation at the American Transplant Congress, Boston. May 2004.
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- Poster presentation at the 3rd International Congress on Immunosuppression. San Diego, December 2004

Prograf produces a molecular environment favouring antifibrosis, an effect reversed by the addition of rapamune. **NR Brook**, JR Waller, GR Bicknell, ML Nicholson

- Poster presentation at the 6th International Congress on New Trends in Clinical and Experimental Immunosuppression, Salzburg. February 5th – 8th 2004.
- Poster presentation at the 3rd International Congress on Immunosuppression. San Diego, December 2004
- Poster presentation at The BTS/Renal Association Joint Congress. Belfast, April 2005

Rapamycin acts in a dose-dependent manner to reduce cyclosporine-induced kidney damage. **NR Brook**, JR Waller, GR Bicknell, ML Nicholson.

- Poster presentation at the 3rd International Congress on Immunosuppression. San Diego, December 2004

LIST OF ABBREVIATIONS

ACE(I)	angiotensin converting enzyme (inhibitor)
Ach	acetylcholine
AR	acute rejection
AT	angiotensin
ATG	antithymocyte globulin
ATN	acute tubular necrosis
BCM	B cell crossmatch
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
CAN	chronic allograft nephropathy
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CR	chronic rejection
CsA	cyclosporine
CYP	cytochrome P
DGF	delayed graft function
ECM	extracellular matrix
ESRF	end-stage renal failure
FK506	tacrolimus
FKBP	FK binding protein
FSGS	focal segmental glomerulosclerosis
FSP	fibroblast specific protein
GFR	glomerular filtration rate
GVD	graft vascular disease
HBD	heart beating donor
HLA	human leukocyte antigen
HMG CoA	5-hydroxy-3-methylglutaryl Coenzyme A
IGF	insulin-like growth factor
IL	interleukin
IPF	idiopathic pulmonary fibrosis
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAd	noradrenaline
NF-AT	nuclear factor of activated T cells
NHBD	non-heart beating donor
NO	nitric oxide
OKT3	Orthoclone OKT3® (muromonab-CD3)
PAI	platelet activator inhibitor
PDGF	platelet derived growth factor
PNF	primary non-function
PRA	plasma renin activity

PTDM	post-transplant diabetes mellitus
RAS	renin angiotensin system
RT-PCR	reverse transcriptase polymaerase chain reaction
SRL	sirolimus
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor

CHAPTER 1 – LITERATURE REVIEW

I - INTRODUCTION

This chapter begins with an introduction to the topic of chronic allograft nephropathy (CAN), the primary cause of kidney allograft loss after the first post-transplant year. Evidence for disturbed extracellular matrix turnover will be considered, and the central role of pathological TGF-β expression discussed. The end-point of these changes is structural (fibrotic) change in the histological components of the kidney. A range of immunological and non-immunological agents and processes contribute to this nephropathy. These will be outlined, with an emphasis on the role of the immunosuppressive calcineurin inhibitors in the genesis of CAN. Their acute and chronic toxicity produces functional and structural changes, respectively, in the kidney.

Strategies for calcineurin inhibitor dose reduction, withdrawal and avoidance will be discussed, including the use of sirolimus. These approaches, and the use of antifibrotic agents such as pirfenidone, will be explored from the literature.
At the end of this chapter, a summary will be given indicating areas of potential further study, providing the rationale for the investigations reported in this thesis.

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II.7 TGF-β: A central controlling factor in CAN

II.1 Introduction to CAN

II.1.1 The challenge of chronic allograft nephropathy

The short-term results of kidney transplantation have improved steadily over the past two decades, mainly due to advances in immunosuppressive therapy[1] heralded by the introduction of calcineurin inhibitors into clinical practice[2]. This class of drugs has produced a revolution in the incidence of acute rejection and associated graft loss. Before the introduction of cyclosporine, one-year renal allograft survival was of the order of 50%[3], due to a high rate of graft loss secondary to uncontrolled acute rejection. The current one-year survival figure for cadaveric allografts in the United Kingdom is around 90 per cent, but thereafter there is an ongoing attrition of grafts, so that 5- and 10-year survival are 60 and 50 per cent respectively. These data are consistent with worldwide figures[4-6]. It is clear that the annual rate of graft loss after the first post-transplant year has remained unchanged in the calcineurin-inhibitor era[4-7]. With more effective immunosuppression, the process of chronic allograft nephropathy has replaced acute rejection as the most important challenge facing kidney transplant programmes. Presently, the two principal causes of graft loss after the first post-transplant year are death with a functioning graft[8] (the main cause of mortality is cardiovascular disease) and graft failure due to chronic allograft nephropathy[8]. Biopsy proven CAN is reported to be present in 40% to 50% of renal allografts by one year[9,10]. Indeed, the possibility exists that the efficacy of calcineurin inhibitors in reducing the incidence of acute rejection has been counterbalanced to some extent by their nephrotoxicity; perhaps their toxic effects explain the relatively constant long-term graft loss despite decreases in acute rejection-associated graft loss[11].

The causes and processes underlying CAN are both multiple and complex. During and after transplantation, the kidney sustains a barrage of insults. These include physiologic and metabolic changes associated with brain-death[12], warm and cold ischaemia[13], delayed graft function[11], acute rejection[13], and exposure to nephrotoxic drugs[14]. Recipient abnormalities comprising hypertension, dyslipidaemias, diabetes mellitus, and cytomegalovirus infection[15] place a further burden on the allograft. Although the magnitude and length of exposure to these stimuli varies, renal cells display a typical response in an attempt to repair damage. The stereotypic response to injury consists of an influx of leucocytes and monocytes that secrete proinflammatory cytokines, enzymes and growth factors involved in tissue repair such as TGF-β, PDGF, bFGF, IL-1β, TNF-α and angiotensin II. Similar inflammatory mediators are produced by activated graft parenchymal tissue and interstitial cells. These cytokines and growth factors mediate cellular processes that result in tissue repair and scar tissue formation. CAN is a process of excessive scar formation, producing disruption of normal tissue architecture and function[16]. Repetitive tissue injury results in excessive production of fibrogenic cytokines, with or without decreased breakdown of deposited extracellular matrix, a well defined phenomenon in interstitial fibrosis[17]. When these stimuli persist, the cells exhibit chronic dysfunction, functional tissue mass is decreased, and fibrosis occurs. CAN represents a final common pathway in response to injurious stimuli, and is manifest in the allograft recipient by a progressive and irreversible decline in renal function accompanied by hypertension and proteinuria, beginning as early as three months post-transplantation. This has traditionally been described as 'chronic rejection', and was defined by Paul[18] as follows:

- Patients must be at least 3 months post-transplant
- Regression of 1/creatinine must be significantly different than zero
- Characteristic graft histology needs to be demonstrated
- Other causes of allograft failure (vascular/urological), complications of transplant and recurrent renal disease must be excluded

Chronic rejection is now considered a misnomer, as rejection in the immunological sense is only a part of the process. Thus, once other causes of renal dysfunction have been excluded, and the diagnosis has been confirmed by biopsy, the process should be referred to as chronic allograft nephropathy.

II.1.2 Fibrosis – disturbed extracellular matrix metabolism

Renal tubulointerstitial fibrosis, characterised by accumulation of ECM (leading to tissue destruction and impairment of renal function), is a morphological hallmark of chronic progressive renal disease. Fibrosis is the final common pathway of many renal disease processes[19].

The three dimensional extracellular matrix structures determine and maintain the organisation of normal tissues. The normal interstitium consists of a loose matrix of collagens, proteoglycans, matrix producing resident fibroblasts, macrophages, dendritic and endothelial cells. This structure may become damaged during various forms of organ damage; blood vessels can regenerate after extensive damage, but some tissue components are dependant on an intact matrix framework when undergoing repair[16]. Renal tubular epithelial cells must find an intact tubular basement membrane upon which to attach their integrins, to proliferate and to organise their polarity to the tubular lumen. If they do not, they undergo a form of apoptosis signalled through integrin adhesion molecules[20]. Thus, the disruption of the extracellular matrix framework may result in an inability to restore or maintain the

graft parenchymal architecture[16]. Multiple and maintained insults to graft cells leads to their senescence and consequent failure to control fibrosis[21].

II.2 The composition of extracellular matrix in the normal kidney

The extracellular matrix is a complex of collagenous and non-collagenous compounds.

II.2.1 The collagens

The collagens are the most abundant proteins in matrix. There are three principal types in renal ECM, the fibrillar collagens I and III, and the non-fibrillar basement membrane collagen IV[22]. The tubulointerstitium contains all three of these collagens, whilst the glomeruli express only types III and IV. The interstitial deposition of collagen I is a late feature of fibrosis and only occurs to a small extent in the kidney[23].

II.2.2 The non-collagens

The non-collagenous matrix proteins include glycoproteins (laminin, fibronectin and tenascin), proteoglycans (decorin and biglycan) and glycosaminoglycans. Recent studies have shown an association between the glycoproteins and the over-expression of numerous cytokines, e.g. increased transcription of tenascin and laminin correlates with increased TGF-β transcription in a rat model of diabetes[24].

II.3 Control of matrix composition in the normal kidney

The net complement of ECM in a given tissue is a dynamic and tightly controlled process, balanced by a flux of forces of synthesis and degradation. A change in the demands on a tissue or chronic stimulation can tip the balance in favour

of either increased ECM (increased synthesis and/or decreased degradation) or decreased ECM (increased degradation and/or decreased synthesis). Key to the degradation process are four groups of proteolytic enzymes: the matrix metalloproteinases (MMPs), and serine, aspartic and cysteine proteases.

II.3.1 The matrix metalloproteinases

The MMPs are important enzymes controlling matrix turnover in chronic allograft nephropathy, and have been extensively investigated. Over 15 MMPs have been identified to date[25]. The most recently discovered are the membrane-type MMPs (MT-MMPs) that are insoluble and secreted in their active form. MMPs are a family of zinc-containing endopeptidases, capable of both matrix degradation and activation of other MMPs[26]. Secretion of MMPs results in decreased matrix accumulation due to increased matrix breakdown, with consequent reduction in fibrosis. Conversely, extracellular matrix deposition follows upregulation of the tissue inhibitors of MMPs (TIMPs)[27]. Aside from the fact they are all Zn^{2+}-dependent, three other features define the MMPs: 1) they are secreted in zymogen form and require extracellular activation (with the exception of MT-MMPs), 2) they share common amino acid sequences, 3) they are inhibited by naturally occurring tissue inhibitors (TIMPs)[23]. The activity of the metalloproteinases is tightly controlled at three levels: gene expression, proteinase activation and inhibition.

Gene expression

With the exception of MMP-9, the MMPs are produced constitutively, but gene expression is also inducible by cytokines and growth factors such as IL-2, TGF-β and PDGF. A combination of TGF-β and IL-2 upregulates all members of the MMP family[28].

Proteinase activation

Activation of the MMPs requires proteolytic cleavage by plasma proteinases and disruption of the Cys-Zn^{2+} switch. The urokinase-type plasminogen activator (uPA)-plasmin system appears to be important in this process[29]. Once activated, MMPs are capable of activating other MMPs[30;31].

Inhibition

TIMPs are the major inhibitors of MMP activity; they form a 1:1 stoichiometric complex with MMPs and inhibit both the latent and activated enzyme[32]. TIMP-1 and TIMP-2 bind to MMP-9 and MMP-2, respectively to form inactive complexes (see below).

II.3.2 Tissue inhibitors of matrix metalloproteinases

TIMP-1 is undetectable in the normal kidney, but can be detected in animal models of renal fibrosis [33-35]. Most models of renal fibrosis have demonstrated an increase in TIMP-1 activity[34;36;37]. Recent work from the Leicester unit demonstrated a direct correlation between TIMP-1 mRNA, quantified by RT-PCR, and collagen III protein expression in protocol renal transplant biopsies[38]. However, Mo *et al.*[39] discovered a reduction in TIMP-1 (and -2) in the model of bromoethylamine-induced papillary necrosis, but reasoned that this decrease may have represented an attempt by tissue to increase collagenolytic activity in response to fibrosis. The Leicester unit have also demonstrated that both tacrolimus[40] and pirfenidone[41] separately inhibit TIMP-1 mRNA expression in a mechanically-injured rat carotid artery model, with resulting inhibition of proliferation of smooth muscle cells. In the setting of renal transplant biopsies, TIMP-1 levels correlate with graft fibrosis (measured by collagen III immunostaining)[38].

TIMP-2 and TIMP-3 mRNA are detectable in normal tissue, and appear to play a less important role in renal fibrosis. The role of TIMP-4 in the kidney remains to be evaluated.

II.4 Renal fibrosis

Renal fibrosis is characterised by infiltration of mononuclear cells and expansion of the interstitial space due to accumulation of abnormal quantities and types of proteins; the tubules and peritubular capillaries ultimately disappear as fibroblasts transform and increase in number, and mononuclear cells interpolate. Fibronectin, collagens I, III, and IV (the latter is normally restricted to the tubular basement membrane[42]) all invade normal tissue. Structural derangement occurs and function is disrupted; the rate of decline of GFR in patients with chronic renal disease is closely correlated with the degree of interstitial fibrosis[43].

Renal fibrogenesis can be divided into three phases: induction, inflammatory matrix synthesis, and post-inflammatory matrix synthesis[42].

II.4.1 Induction phase

The initiation of the process occurs with the release of chemokines by damaged tubular epithelial cells, and infiltration of mononuclear cells. Consequent release of profibrogenic cytokines occurs, with activation and proliferation of resident fibroblasts, and their transformation into myofibroblasts[44].

II.4.2 Inflammatory matrix synthesis and increased matrix deposition

The extracellular matrix in fibrosis consists of an abnormal quantity of both normal extracellular matrix proteins and those that are usually limited to the basement

membrane (collagen IV and laminin). Fibronectin usually appears first; this is an adhesive glycoprotein forming a scaffold for the deposition of other proteins, and functions as a fibroblast chemoattractant. Continued release of profibrogenic cytokines by infiltrating cells maintains the drive towards matrix deposition.

II.4.3 Post-inflammatory matrix synthesis

Eventually, the primary inflammatory stimulus diminishes or stops, but there is continued secretion of profibrogenic cytokines by tubular epithelial cells. Activated (myo)fibroblasts proliferate through autocrine stimulation. It is possible that tubular cells undergo epithelial-mesenchymal transformation[42], and in this state, further tissue injury may not be required to sustain TGF-β over-expression, and the fibrotic process becomes self-perpetuating.

II.4.4 Effector cells

II.4.4.1 Fibroblasts

During fibrosis, interstitial fibroblasts proliferate and are primarily responsible for the production of interstitial proteins. A number of cell types synthesise these proteins. *In situ* hybridisation studies highlight the importance of interstitial cells[45;46], and fibroblasts probably have a dominant role in protein synthesis. Tubular cells are also a source of metalloproteinases, both apically and basolaterally[17]. Several fibroblast mitogens have been identified *in vitro*. These include interleukin-1, TNF-α and β, TGF-β, PDGF, bFGF, TGF-α, interferon-α, plasminogen activator, insulin-like growth factor, fibrinogen, and endothelin-1[17]. Very little is known about the mitogenic stimuli for renal fibroblasts *in vivo*, and the stimuli seem to vary depending on whether the fibroblasts are in a normal or fibrotic milieu. Of note, fibroblasts from

damaged kidneys produce more collagen and fibronectin than those derived from normal kidneys[44], and show an increased rate of spontaneous proliferation[17]. The ability of interstitial fibroblasts to assume an activated myofibroblast phenotype has been reported in humans with progressive renal disease[47;48].

In experimental and clinical renal scarring, myofibroblasts are detected surrounding arterioles, tubules and glomeruli[49]. It may be that vascular injury contributes to tubulointerstitial fibrosis by the migration of myofibroblasts. These cells could be activated and stimulated to proliferate and migrate by cytokines, growth factors and components of the extracellular matrix[49]. TGF-β and PDGF-B can transform fibroblasts to myofibroblasts[17], and once activated they express α-smooth muscle actin. Myofibroblasts may originate in the interstitium but there is some evidence that they are perivascular in origin, and undergo migration to the renal interstitial space[17]. Electron microscope studies have suggested that they originate from cells in the tubulointerstitium itself[50], and differential and subtractive hybridisation of transcripts from renal tubular epithelium has characterized a fibroblast specific protein (FSP-1)[51] which has a role in the motility of fibroblasts. FSP-1 activity is barely detectable in normal renal interstitial tissue, but in a mouse model of renal fibrosis a marked increase in FSP-1 activity has been demonstrated, correlating with collagen deposition[52].

II.4.4.2 Interstitial cells

As well as fibroblasts and macrophages, studies have indicated that renal tubular epithelial cells play a role in the process. These cells are particularly sensitive to the range of injurious factors affecting allografts. They are prone to hypoxia and are exposed to damaging chemicals passing through the tubules themselves (for example

proteins, glucose and cytokines). These agents stimulate epithelial cells to produce chemokines that promote interstitial infiltration of mononuclear cells[53]. These, in turn, release mediators that stimulate fibroblast proliferation with consequent production of interstitial proteins. A further action of these mononuclear-derived mediators is to stimulate epithelial cell differentiation, with a phenotypic switch to fibroblast-like cells[53]. TGF-β is released from activated mononuclear cells, and this cytokine can induce transdifferentiation of cultured rat tubular epithelial cells. Cultured human proximal tubular cells can stimulate fibroblast proliferation and collagen synthesis via production of TGF-β[54] . TGF-β antibody can prevent the process.

In a summary of the possible role of tubules, Fine *et al.*[55] stated:

> "If injured tubules produce cytokines (and they do), paracrine effects of these molecules on the interstitial fibroblasts and vascular cells should occur, and if the appropriate receptors reside on the tubular cells autocrine regulation of tubular cell growth should also ensue".

Thus, there is an element of cell-cell cross-talk, and positive feedback cycles may be initiated.

II.4.4.3 Macrophages

Macrophages also contribute to the pool of interstitial matrix, and enter the interstitium by infiltration. They synthesise both collagen and fibronectin[56]. Interstitial macrophages may also proliferate *in situ*.

II.5 Chronic allograft nephropathy

II.5.1 Definition

CAN is characterised clinically by a gradual deterioration in renal functional parameters (principally a progressive rise in serum creatinine and fall in glomerular filtration rate) along with increasing proteinuria and hypertension. It is understood to have a multifactorial aetiology, involving both alloimmune-dependent and independent factors[57;58], with a final common pathway of a stereotyped response to injury. Histopathological confirmation of the clinical picture is normally required, but since the biopsy changes are often non-specific, definite substantiation through biopsy is often difficult as a number of other conditions can produce similar histological findings.

II.5.2 Histopathology

The four principal histological features of CAN are vascular intimal hyperplasia, tubular atrophy, interstitial fibrosis, and chronic transplant glomerulopathy (primary glomerular sclerosis and basement membrane splitting)[59]. With the exception of tubular atrophy, these are characterised by an accumulation of excess extracellular matrix. Interstitial fibrosis and tubular atrophy are common responses to many forms of kidney damage, so are necessary but not specific for the diagnosis of CAN. The glomerulopathy may be a more idiosyncratic feature of CAN, and occurs in only 15% of patients[58]. All of these changes can be observed in patients with normal renal function, and consideration must always be given to the possibility that they were present in the donor kidney[59]. This point emphasises the importance of pre-transplant baseline biopsies.

Associated with the cardinal histological features of CAN is a mononuclear cellular infiltrate, mentioned above, which consists predominantly of macrophages and T-lymphocytes[23].

II.5.3 Causation of CAN

Whilst CAN has traditionally been viewed as the result of repeated low-grade immune responses directed against allogeneic tissue[60], it is now clear that a large number of both immunologic and non-immunologic factors lead to CAN. The relative contribution of each factor has yet to be defined. Further, the complex interplay between various factors precludes clear definition of the role of each. However, the following headings can be considered:

II.5.3.1 Alloantigen-dependent immune risk factors for CAN[61]

Histocompatibility factors

Even a low-level of HLA mismatch can lead to a persistent, low-level alloimmune response. Mismatching at minor histocompatability antigens may produce a similar low-level response, but this is occult. In general, the incidence of CAN increases with increasing mismatch[8]. It seems that the most important HLA locus is DR; the degree of HLA-DR mismatch is one of only two independent risk factors for graft survival (the other is acute rejection episodes) in second kidney transplants[62].

Pre-transplant anti-donor antibodies

Traditionally, it has been believed that the presence of B-cell crossmatch (BCM) positivity was associated with poorer graft survival. Recent data[63] suggests that in the majority of patients, BCM positivity is not related to the presence of anti-HLA antibodies, and graft survival is similar to that of BCM negative controls. In a

minority of patients, anti-HLA class II antibodies are responsible for the positive BCM, and their presence is associated with lower early (but not late) graft survival.

Acute rejection episodes

Acute rejection episodes are held to be particularly important in the development of CAN, but the exact role of these episodes in the genesis of allograft nephropathy remains unclear. Whilst it is accepted that the presence and extent of acute rejection is a major predictor of CAN in histological studies[64;65], the influence of timing, number of episodes and severity of acute rejection is less clear. Certainly, it appears that early vascular rejection is a strong predictor of graft loss[65]. In both heart[66] and kidney[67] allograft recipients, the number of acute rejection episodes is an independent variable correlating with the development of chronic allograft dysfunction. An isolated episode of acute rejection in the early post-operative period, with no decrement in renal function, and that is successfully treated, has little impact on the later development of CAN or late graft loss[68;69]. Conversely, multiple rejection episodes and/or long-lasting rejection, vascular rejection, and rejection episodes occurring later in the post-transplant course (after 3 months)[70] are greater risk factors for the development of CAN[71]. A paper recently published from Leicester evaluated the effect of acute renal allograft rejection upon the expression of various fibrosis-associated genes within isolated renal transplant glomeruli[72], as these are thought to be a predictor of subsequent CAN. The results demonstrated no difference in the mRNA expression of fibrosis associated genes in glomeruli taken from biopsies of patients with or without acute rejection at one week, three months and six months after renal transplantation. In a study of 1587 kidney allograft recipients, the very low incidence of graft loss caused by 'chronic rejection' in patients with no previous episodes of acute rejection (2%) stood out against an incidence of 24% in patients with previous

episodes of AR[73]. Whilst not allowing precise separation of the immunologic from non-immunologic influences, these results indicate that in the absence of acute rejection, graft failure due to CAN is uncommon.

II.5.3.2 Alloantigen independent risk factors for CAN

Alloantigen-independent immune factors have, in theory, taken on an increasingly important role, as there have been improvements in immune suppression (with a reduction in alloantigen-dependent injury), and an increased use of marginal donors[8]. Alloantigen-independent factors contribute to the progression of CAN by reducing the functional nephron mass[74], but may also trigger an autoimmune-like response by up-regulating expression of MHC genes, or by exposing cryptic antigens, thus allowing an immune response[75].

Recipient age, and Donor age and gender

Cadaveric kidneys from older (>50 years) and younger (<10 years) donors are associated with decreased graft survival in some studies. In older donors, this probably relates to aging-induced reduction of nephron mass and the presence of graft vascular disease. For younger donors, a small functioning mass is fed with an unaccustomed high blood flow and heavy metabolic demand. Other studies support the finding of a higher serum creatinine in older donors, but with no difference in graft survival[76]. The somewhat poorer graft survival of female donor kidneys in male recipients has also been ascribed to a mismatch between donor nephron supply and recipient functional demand[76]. Multivariate analysis of multicentre registry data highlights recipient age as an important independent risk factor for graft loss, but when corrected for death with a functioning graft, graft survival is independent of recipient age[77].

Delayed graft function, cold and warm ischaemia

Delayed graft function, which affects 23-34% of cadaveric renal transplants, and the subsequent requirement for dialysis in the immediate post-operative period strongly correlate with recipient outcome and development of CAN[11;78-81]. In a study of 126 cadaveric renal transplant recipients, patients with immediate function or up to 8 days of DGF experienced 5-year graft survival of 89% and 85% respectively, whilst those with DGF lasting more than 8 days had a figure of 50%[82].

With rare exceptions, post-transplant DGF is due to acute tubular necrosis caused by donor factors such as brain-death phenomena, and ischaemia/reperfusion injury sustained during organ retrieval and the recipient surgical procedure. ATN is detrimental to cadaveric grafts because it reduces working nephron mass and results in glomerular hyperfiltration, which may lead to glomerular sclerosis, proteinuria and hypertension[83]. There is also evidence that DGF exposes the patient to an increased risk of acute rejection[84] but this may be due to missed AR during the episode of DGF.

It has been postulated that neoantigens expressed by renal epithelium following cold and warm ischaemic injury facilitate immunologically mediated injury. In mice, class I and II MHC antigen expression is increased in renal tubular epithelial cells 24 hours after acute renal ischaemia (60 minutes of warm ischaemia induced by clamping the renal pedicle)[85]. This is particularly interesting since class II antigens are not constitutively expressed on tubular epithelial cells.

Injury related to brain stem death

Rowinski *et al.*[86] suggested that events around the time of brain-stem death (profound metabolic, haemodynamic and hormonal changes) play a more important role in the pathogenesis of renal damage than [warm] ischaemia. In the context of non-heart-beating donor kidneys, Alvarez *et al.*[87] showed that kidneys from ITU-

based donors (generally donors who have suffered sudden brain-stem death) had poorer short and long term function, and were associated with a greater rate of primary non-function (PNF) than donor kidneys procured from the emergency department (sudden cardiac death which precludes development of brain-death phenomena). This was strongly associated with periods of hypotension before retrieval. Moreover, the Leicester group describe a series of transplants, all with DGF, in which graft survival at 6 years was significantly better for NHBD than HBD (84% vs. 73%)[88]. One explanation is that NHBD kidneys were not subjected to the harmful events associated with brain-stem death. A pathophysiological explanation for this phenomenon was proposed by Takada *et al.*[12] whereby up-regulation of genes encoding proinflammatory mediators was demonstrated in an animal model of brain-stem death. If these findings are relevant to humans, organs from HBDs will be more prone to early host inflammatory and immune responses, in contrast to NHBDs who suffer sudden circulatory arrest, allowing no time for gene transcription.

Inadequate nephron dosing

Calcineurin inhibitors, acute rejection and recipient-evoked damage might each reduce nephron mass following transplantation, but the initial number of transplanted nephrons, or a surrogate marker (the mass of the graft) also determines long-term function[11;89]. In essence, this view holds that a mismatch between the metabolic demands of the host and renal excretory function would (if demand exceeded supply) lead to deleterious adaptations in the host kidney (see above for age and gender mismatching). In animal models of renal mass reduction, e.g. the remnant kidney model, pathological alterations occur in residual glomeruli during adaptation[90], principally glomerulosclerosis due to hyperperfusion and capillary hypertension[83]. Adaptations in tubular function also occur; sodium transport for residual nephrons

increases in the remnant kidney model[91], accentuating oxygen demand and consumption, and resulting in increased production of oxygen free radicals[92]. Indeed, supplementation of anti-oxidant compounds diminishes injury in this model[93]. Similar mechanisms may occur in the setting of reduced nephron numbers in human transplantation, and nephron numbers progressively reduce as CAN progresses[90]. The non-immunological factors centre on the concepts of nephron mass and sources of renal injury. Specifically, donor variables and pre-transplantation graft quality influence the initial number of nephrons in the donor kidney[94], whereas delayed graft function and recipient variables determine nephron mass after transplantation[90]. Furthermore, differences in the metabolic demands of the recipient versus the physiological capabilities of the donor kidney further complicate the processes that contribute to clinical outcome[90].

Cardiovascular disease risk factors

Recipient diabetes, hypertension and hyperlipidaemia are additional sources of injury that may reduce nephron mass and are associated with decreased long-term survival[15;89;95]. Experimental studies in rats show that kidneys with CAN exhibit glomerular hypertension[96]. Treatment with antihypertensive agents in these models decreases systemic and glomerular capillary pressures and is associated with improved graft survival[97]. There is substantial evidence that hypertension is a powerful risk factor for CAN in both paediatric[98] and adult[99;100] renal allografts.

Because the pathophysiology of CAN shares many of the features of atherosclerosis, it seems fair to assume that lipoprotein abnormalities may contribute to allograft vasculopathy[97]. An increased risk of chronic allograft nephropathy with hyperlipidaemia has been demonstrated for both heart transplant and renal allograft recipients[101]. Eddy[102] demonstrated alterations in ECM in rats with fibrosis induced by

unilateral nephrectomy and hypercholesterolaemia, noting an increase in TIMP-1 levels. In a prospective clinical study of heart transplant recipients, simvastatin treatment was correlated with improved left ventricular function, presumably due to a decreased incidence of chronic allograft vasculopathy[103].

Variations in exposure to immunosuppressive drugs

Low (insufficient) exposure to cyclosporine is a risk factor for the development of CAN[104;105], even in the absence of proven acute rejection episodes. It is possible, however, that ongoing sub-clinical acute rejection[106;107] could impart a greater propensity to chronic damage. Kahan *et al.*[108] retrospectively examined serial pharmacological profiling of 240 patients treated with cyclosporine for up to 5 years. Patients with the most variation in cyclosporine exposure displayed the highest serum creatinine and the greatest degree of organ dysfunction. It has been proposed that the importance of maintaining adequate levels is so great that the influence of acute rejection, HLA DR mismatch, positive B-cell crossmatch and HLA sensitisation can all be manipulated by maintaining the appropriate dose of cyclosporine[109].

CMV infection

Cytomegalovirus-positive donors, lack of CMV-prophylaxis, and the development of CMV disease are risk factors for chronic allograft nephropathy[103]. CMV has a spectrum of interesting actions; it encodes a protein that has sequence homology and immunologic cross-reactivity with the HLA-DR β-chain, as well as a glycoprotein homologous to the heavy chain of MHC class I antigen[110]. Further, it can induce expression of MHC class II antigens. The virus also up-regulates adhesion molecules on vascular endothelium, triggering a cytokine cascade that probably contributes towards graft deterioration[103].

Polyomavirus associated allograft dysfunction

Polyomavirus-associated nephropathy is an increasingly prevalent cause of allograft dysfunction[111]. It infects between 10 and 45% of kidney transplant recipients and results in nephropathy in approximately 6%[112].

II.6 Molecular mechanism of fibrosis in chronic allograft nephropathy

Fibrotic renal disease involves changes, both qualitatively and quantitatively, in extracellular matrix (ECM)[113]. Altered matrix metabolism in both tubular epithelial cells and interstitial fibroblasts contributes to the accumulation of matrix [113;114]. The molecular mechanisms underlying chronic allograft nephropathy-induced alterations in extracellular homeostasis are multifarious. Glomerulosclerosis and tubulointerstitial fibrosis result from the deposition of abnormal quantities of extracellular matrix[115], leading to progressive dysfunction. The accumulation of matrix proteins is determined by the rates of both their synthesis and degradation[115]. Degradation is under the control of MMPs, which are in turn regulated by their tissue inhibitors (TIMPs). Both the synthesis and secretion of MMPs and TIMPs are regulated by a number of cytokines, including IL-1, PDGF, TNF-α and TGF-β[116] that also enhance the synthesis of numerous matrix components[117;118]. Thus, the MMPs and their inhibitors may represent proteins through which biological modifiers such as cytokines and growth factors can control and influence organisation of the tubulointerstitium and glomeruli[116].

In the renal interstitium, effector cells include interstitial myofibroblasts, and in the glomerulus, mesangial cells. Various adhesion molecules, eicosanoids, oxidatively modified low-density lipoprotein cholesterol, free-radicals and vasoactive peptides may also play a role[119].

The molecular mechanisms in the renal vasculature are well-described[119]. With respect to intimal hyperplasia, endothelial cells play a pivotal role[120;121]; following injury, the cells become activated, express adhesion molecules[122] and together with infiltrating immune cells, secrete growth factors and cytokines that influence the activity and proliferation of cells that produce extracellular matrix[123]. Smooth muscle cells in the media proliferate and migrate through the internal elastic lamina to the intima, where they proliferate further[124]. There is a synchronous deposition of extracellular matrix in the intima, which contributes to the luminal obliteration. It is not clear whether a separate but similar process occurs in the interstitium leading to fibrosis, or whether it is the vascular changes that stimulate the interstitium to begin a parallel cascade of additional inflammation and injury[119].

II.7 TGF-β: A central controlling factor in CAN

TGF-β is a multifunctional peptide produced by many parenchymal cell types, by leucocytes that infiltrate injured tissues, and by platelets[19]. In the kidney, TGF-β is localised in glomeruli, renal tubules and the interstitium[125]. Its biological effects are produced by binding to a specific receptor, with consequent target-cell gene transcription and stimulation of production of interstitial proteins. It inhibits proliferation of most cell types, including epithelial, endothelial and haematopoietic cells[126]. In the kidney, inhibition of tubular cell proliferation may promote the hypertrophy that is characteristic of tubulointerstitial fibrosis[19].

The molecule is normally released transiently in response to a single isolated injury where it aids in normal tissue repair by stimulating target cells to synthesise interstitial proteins required for tissue healing[127]. In certain circumstances of excessive or sustained injurious stimulation, excess and inappropriate TGF-β secretion occurs.

In this setting, the significance of TGF-β as a key fibrogenic cytokine in many fibrotic processes is now recognised. Initially, the importance of TGF-β in renal fibrogenesis was suggested by knowledge of its role in promotion of ECM accumulation[128], and its inhibitory action on proteases. TGF-β is known to inhibit proliferation of mesangial cells, whilst stimulating them to produce matrix proteins, thus contributing to renal fibrosis. Similarly, TGF-β stimulates fibroblasts to produce matrix proteins[54;129], and increases the expression of endothelin-1, which aside from its vasoconstrictor actions, directly stimulates ECM production[130]. TGF-β also blocks the degradation of ECM by decreasing the synthesis of proteases and stimulating protease inhibitors (e.g. tissue inhibitor of matrix metalloproteinases, TIMP and plasminogen activator inhibitor, PAI-1)[131]. Thereby, excess stimulation by TGF-β can lead to excess accumulation of interstitial proteins, a characteristic of fibrosis.

TGF-β is also a chemoattractant, and can recruit inflammatory cells (monocytes, macrophages and lymphocytes) to promote interstitial injury[126]. TGF-β exerts positive feedback on its own synthesis[132] and persistent expression following prolonged insults can lead to a cycle of continued TGF-β production[133]. Furthermore, TGF-β directly stimulates the synthesis of all three of the major groups of extracellular matrix proteins[128], the collagens, fibronectins and proteoglycans. TGF-β - stimulated fibroblasts are able to produce all three[134]. Another, interesting point is that TGF-β itself promotes immune suppression[135]; transgenic mice deficient in TGF-β die from an autoimmune-like illness[136].

The exact stimuli for the synthesis and release of TGF-β and other cytokines involved in CAN are unclear. Immune activation or recruitment of inflammatory cells, as well as local vascular trauma (with platelet and monocyte release) may be stimuli[90]. Furthermore, local activation of the renin-angiotensin system with production of

angiotensin II is a catalyst for release and activation of TGF-β and other cytokines. Angiotensin II increases TGF-β expression in both tubular epithelial cells and interstitial fibroblasts[137;138], and TGF-β antibody prevents angiotensin II-induced fibronectin synthesis in fibroblasts. Antagonists of the renin-angiotensin system reduce TGF-β expression in various animal models of renal disease[139-141].

Summary table of the fibrotic actions of TGF-β (Adapted from O'Donnell[19]):

- Stimulation of fibroblast interstitial protein production
- Promotes transdifferentiation of renal tubular epithelial cells into fibroblast-like cells
- Reduced expression of MMPs
- Increased expression of TIMPs
- Promotes infiltration of inflammatory cells
- Implicated as a mediator of angiotensin II-induced fibrosis

Clinical studies in CAN have shown elevated expression of TGF-β mRNA and protein[142] in areas of the renal interstitium showing inflammation and fibrosis. Further, Sharma *et al.* demonstrated that TGF-β mRNA expression correlates with the extent of renal allograft fibrosis and CAN[143]. Nicholson *et al.*[144] found a similar correlation between glomerular expression of TGF-β1 mRNA expression and interstitial fibrosis in terms of collagen III immunohistochemical staining. Those patients with CAN expressing low levels of TGF-β had a mean decline in glomerular filtration rate of 6 ml/min per year, compared to the more rapid rate of 19ml/min per year in those expressing higher levels[145].

Ex vivo and *in vivo* models have been used to demonstrate the fibrogenic consequences of prolonged TGF-β stimulation, whereby TGF-β promotes fibrosis by increasing synthesis of individual components of the extracellular matrix whilst simultaneously blocking matrix degradation. Intravenous administration of recombinant TGF-β produces rapid glomerulosclerosis in rats and rabbits[146].

Paul *et al.*[147] have demonstrated the production of antibodies against biglycan and decorin in rats with CAN. These compounds bind and inactivate TGF-β and stimulate the release of matrix metalloproteinases by fibroblasts. The production of such antibodies could be conceived to reduce TGF-β binding and interfere with the function of these molecules in the regulation of proteolytic enzyme activity.

There is a substantial body of evidence implicating TGF-β in the development of calcineurin-inhibitor toxicity. Cyclosporine directly stimulates the expression of TGF-β1 in a number of cell culture systems[148-151]. Cuchaci *et al.*[145] reported that intrarenal allograft TGF-β levels were elevated in the majority of cyclosporine-treated renal allograft recipients. Furthermore, increasing TGF-β levels were correlated with decreasing renal function. Plasma TGF-β1 levels are also found to be higher in patients with CAN than in controls[152]. What exactly links cyclosporine and the increase in TGF-β is unclear. Since cyclosporine directly stimulates TGF-β *in vitro*, it may be that blocking of IL-2 gene transcription and renal fibrosis due to cyclosporine are both mediated by TGF-β. It is also possible that renal ischaemia produced by cyclosporine vasoconstriction can elevate downstream TGF-β expression[153]. Further, cyclosporine-induced RAS stimulation can promote TGF-β expression. Angiotensin II induces cellular proliferation, hypertrophy and the expression of immediate early genes (e.g. c-fos), TGF-β and PDGF in vascular smooth muscle cells, glomerular mesangial cells and renal proximal tubular cells[154]. *In vitro* studies have demonstrated that angiotensin II is capable of affecting the production/degradation of ECM. This may in fact be an indirect action, mediated by angiotensin II increasing active TGF-β[154]. Kagami *et al.*[155] have shown that angiotensin II promotes the conversion of latent TGF-β to the biologically active form, and Wolf *et al.*[137] showed that Ang II induces

cellular hypertrophy of cultured murine proximal tubular cells, a process mediated by the synthesis and activation of TGF-β.

By utilising gelatin zymography, human renal cortical cells have been shown to secrete matrix metalloproteinase-2 and -9[156]. These have a pivotal role in the degradation of extracellular matrix, and it is believed they modulate tubulointerstitial cellular growth and function through matrix remodelling, alteration of matrix-integrin receptor dynamics and direct growth factor-like actions[156-158]. Incubation of cortical fibroblasts with cyclosporine results in marked inhibition of MMP activity (favouring matrix accumulation), an effect that appears to be directly mediated since addition of antibodies to TGF-β and IGF-1 does not block the inhibition[157].

Duymelinck *et al.*[34] reported increased expression of TIMP in areas of focal interstitial fibrosis in a rat model. Thus, it would seem that cyclosporine-induced interstitial fibrosis is linked to suppression of MMP activity, decreasing matrix degradation and instead favouring accumulation.

CHAPTER 1 – LITERATURE REVIEW

III - THE CALCINEURIN INHIBITORS

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III.5 Tacrolimus acute and chronic nephrotoxicity

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III.7 Reducing exposure to calcineurin inhibitors

III.1 Introduction

Cyclosporine and tacrolimus are the foundation of current immunosuppressive therapy for the prevention of acute rejection in solid organ allografts. Their introduction transformed immunosuppression for transplantation by markedly reducing the rate of acute rejection, and graft loss due to AR. The drawback is the nephrotoxicity of these agents, and their tendency to potentiate chronic allograft nephropathy. A number of short- and long-term animal and human studies have confirmed the nephrotoxicity of cyclosporine[159], and virtually all CsA-treated patients develop a degree of renal dysfunction and fibrosis[160;161]. Evidence for the nephrotoxic capacity of tacrolimus has emerged more recently from short- and long-term animal and human studies of organ allografting[162-166]. By inhibiting the action of the intracellular enzyme calcineurin, these agents suppress the production of interleukin-2 and various other cytokines associated with acute graft rejection[167]. They also alter the expression of other genes (for profibrotic cytokines and growth factors) that have a role in matrix turnover[168-171]. CNIs also upregulate the transcription of TGF-β[168] and its receptor[172], and increase TGF-β secretion[172]. This upregulation may contribute to their immunosuppressive properties, since TGF-β is an inhibitor of T-lymphocyte growth and activation (in part mediated by cell cycle arrest in G_1 phase by up-regulating p21 activity), but TGF-β also plays a role in the adverse fibrotic effects associated with CNIs[173].

The histopathological changes in renal allograft biopsies, consisting of tubular vacuolisation, striped interstitial fibrosis and arteriolar hyalinosis, are associated with long-term cyclosporine and tacrolimus therapy[174]. Despite these drawbacks, the CNIs are so effective in their restraint of the immune system that they remain central agents following transplantation.

III.2 Cyclosporine and tacrolimus

Cyclosporine has remained pivotal in the prophylaxis of acute allograft rejection since its introduction into clinical practice. Graft survival in the first 12 months after transplantation improved dramatically from a mean 1-year survival of approximately 50% to greater than 90% before and after its introduction respectively[3], by a reduction in the incidence of graft loss due to treatment-resistant acute rejection.

Tacrolimus is a macrocyclic lactone antibiotic, originally isolated from *Steptomyces tsukubaenis* in 1984. It was first used for salvage therapy in liver allograft patients with cyclosporine-refractory rejection, in 1989[175]. Subsequently, Starzl *et al.*[176] reported its use in 36 renal transplant patients. The first report of a pilot study in kidney transplant patients was in 1995[177]. A number of notable studies have defined a central role for tacrolimus as a primary agent for acute rejection prophylaxis[178-181]. Four large multicentre studies[182-185] and a recent meta-analysis of randomised controlled trials[186] of tacrolimus versus cyclosporine primary therapy in renal transplantation have shown lower rates of acute rejection with tacrolimus. Moreover, long term studies demonstrate that tacrolimus is associated with improved 5-year graft survival[187] and lower rates of CAN[188] than cyclosporine.

III.2.1 Mechanism of action of calcineurin inhibitors

After entry into the cell, cyclosporine binds to the cytosolic immunophilin, cyclophillin. The cyclosporine-immunophilin complex binds to and inhibits the activity of calcineurin, a calcium/calmodulin-dependent protein phosphatase that is expressed in all mammalian tissues[189]. The enzyme contains an autoinhibitory serine-threonine phosphatase domain that is activated on presentation of an immunoreactive peptide. Once antigen is complexed to a T-lymphocyte receptor, calcium channels are

opened by phospholipase C. and the conformation of calcineurin is altered such that the phosphatase domain is exposed. After dephosphorylation by calcineurin, cytoplasmic transcriptional regulatory proteins enter the nucleus and induce the expression of cytokine genes involved in the immune response. For T-lymphocyte activation to progress, the functional domain of calcineurin must remain exposed such that transcriptional regulatory proteins can be repeatedly dephosphorylated and the expression of immunoresponsive cytokine genes induced[94]. Once bound, a pentameric unit consisting of calcineurin A, calcineurin B, calcium, calmodulin and calcineurin-inhibitor is formed and this conformational change renders the phosphatase complex inaccessible[190]. Subsequently, the translocation of various nuclear factors involved in the transcription of cytokine genes is inhibited (an example is NF-AT[190]). NF-AT is the first regulatory protein critical in promotion of DNA transcription of mRNAs that encode pro-inflammatory cytokines. The result is an interruption of the early calcium-dependent signal transduction pathway in T-cells[191:192]. Cyclic adenosine monophosphate (cAMP) -directed transcriptional events are also inhibited[193]. Thus, the transcription of early T-lymphocyte activation genes is suppressed, affecting production of cytokines such as interleukin 2 (IL2), IL3, IL-4, IL-5, interferon gamma and tumour necrosis factor-α.

In addition to its effects on T-cells, cyclosporine retards B cell activation in both a direct and indirect manner. The direct effect arises by inhibition of calcium flux in response to immunoglobulin ligation, and the indirect effect by impairment of T-cell help[194]. Polymorph degranulation is inhibited[195], and mesangial cell production of prostaglandins and nitric oxide is reduced, which may account for the ability of cyclosporine to induce renal vasoconstriction[196].

After incorporation into cells, tacrolimus binds to the intracellular lymphocyte proteins FKBP-12 and FKBP-52. The FKBP-12 combination complexes with calcium, calmodulin and calcineurin, resulting in inhibition of the phosphatase activity of calcineurin. NF-AT is inhibited, with attenuation of cytokine gene transcription. Tacrolimus is between 10 and 100 times more potent than cyclosporine in the inhibition of IL-2 synthesis in *in vitro* testing[197]. Differences in the potency of cyclosporine and tacrolimus may be because tacrolimus-induced immunosuppression is not exclusively mediated through interruption of the NF-AT pathway; other pathways are blocked by tacrolimus, including cytokine receptor expression and cytokine effects on cells[198].

III.2.2 Pharmacokinetics, dynamics and metabolism of calcineurin inhibitors

Absorption of cyclosporine is slow, variable, and incomplete. It displays approximately 30% bioavailability, mainly due to metabolism by small bowel P_{450} cytochromes. Absorption depends on the quantity of bile salts present, and blood levels peak 2-6 hours after dosing[189]. The microemulsion formulation of cyclosporine is absorbed more predictably than the older formulation[199]. It is highly fat-soluble and is therefore widely distributed throughout tissues. The drug is extensively metabolised by the hepatic cytochrome P_{450} microsomal enzyme system (principally CYP3A4)[200], and metabolism is sensitive to inducers and suppressors of P_{450}. Less than 1% of the parent drug is excreted in urine, but most metabolites (some of which are active) undergo renal excretion, and dose reduction is therefore necessary for patients with impaired renal function[189]. Frequent monitoring is required as cyclosporine activity and toxicity are related to blood levels. Unlike cyclosporine, the enteric absorption of tacrolimus is not dependent on the solubilisation of bile salts, but bioavailability is still

varied by food intake, and averages around 20%. Blood levels peak from 0.5 to 8 hours after ingestion. As with cyclosporine, the drug is primarily metabolised by cytochrome P-450 3A4, and it acts on the p-glycoprotein transporter[189].

III.2.3 Calcineurin inhibitor side effects

The calcineurin-inhibitors possess a number of side effects that contribute to graft and patient morbidity, another reason why considerable effort is directed towards reduction of exposure to these drugs. Calcineurin and NFAT tissue distribution is widespread, and this explains the range of side effects seen with these agents. Death with a functioning graft is the second major cause of graft loss after the first transplant year in living donor transplant recipients, and the leading cause in cadaveric graft recipients[201]. The majority of these deaths are related to cardiovascular disease. Although the recipients will have pre-existing disease and compounded risk factors attributable in part to their renal failure, the side effects of CNIs, principally their hypertensive, diabetogenic and hyperlipidaemic effects amplify these pre-existing risks. In rats, cyclosporine concentrations per weight of tissue protein are highest in kidney and liver and lowest in brain and testis after oral dosing, with intermediate levels in spleen, heart, and whole blood. Thus, each cyclosporine dose produces rapid and wide-spread inhibition of calcineurin in tissues, with differences in total susceptibility of each tissue[202]. Given the relationship between calcineurin inhibition and side effects, it is unsurprising that the effects associated with tacrolimus and cyclosporine are similar in terms of underlying mechanisms and associated histopathology[60].

Promotion of Atherogenesis

As well as promoting hyperlipidaemia[203], cyclosporine promotes the oxidation of low-density lipoproteins and increases their atherogenicity[204]. Platelet activation and aggregation is also enhanced[205]. It seems that cyclosporine is more potent in its promotion of hyperlipidaemia than tacrolimus[206]; in heart-transplant patients randomised to either cyclosporine- (n=46) or tacrolimus- (n=39) based immunosuppression, hyperlipidaemia and hypertension occurred less frequently and with less severity in tacrolimus treated patients. There were no differences between the groups in terms of hyperglycaemia[207].

Hypertension

Hypertension may occur with cyclosporine, even when blood levels are kept within therapeutic range[208]. The mechanism involves a shift in the balance of vasoconstriction/dilatation at the glomerular arterioles and an enhancement of sympathetic responsiveness. Platelet activation at the endothelial surface further increases renal and peripheral vascular resistance[205;208]. Post-transplant hypertension is associated with accelerated renal allograft dysfunction[209], although it is often difficult to determine if hypertension contributes to graft failure or whether it is a sign of chronic allograft nephropathy[210]. A number of studies have demonstrated that hypertension is less marked with tacrolimus than cyclosporine therapy[211-215].

Post-transplant diabetes

It has been suggested that post-transplant diabetes mellitus (PTDM) occurs far more frequently with tacrolimus rather than cyclosporine therapy[189]. A recent study comparing the effects of cyclosporine and tacrolimus on cardiovascular risk profiles in 191 patients without pre-existing diabetes demonstrated equivalent rates of post-transplant diabetes with both drugs[206]. Risk factors for the development of PTDM

include pre-transplant impaired glucose tolerance, non-white race, steroid dose and high cyclosporine[216] or tacrolimus trough levels[99].

Cosmetic changes: hypertrichosis and gingival hyperplasia

Hypertrichosis may be problematic in female and paediatric patients. Gingival hyperplasia is frequent (10-50%), and may be accentuated in patients receiving calcium channel blockers. The incidence of these two side effects appears to be less for tacrolimus than with cyclosporine[189], and conversion to tacrolimus from cyclosporine results in improvements in hypertrichosis and gingival hyperplasia[217].

III.2.4 Clinical use of calcineurin-inhibitors

In the context of renal transplantation, cyclosporine is used as mono, dual, or triple therapy for the prophylaxis of rejection. It has been the mainstay of transplant immunosuppression for nearly 20 years. Many centres still employ a cyclosporine-based triple therapy regimen with prednisolone and either azathioprine, or one of the newer agents such as mycophenolate mofetil. Tacrolimus is a more recent introduction, and has a number of different applications in transplantation:

Rescue therapy

Initial experience of tacrolimus in kidney transplantation was as rescue therapy for grafts with ongoing rejection failing to respond to cyclosporine. One of the first studies used 20 patients with recurrent rejection who were converted from cyclosporine to tacrolimus, with resolution and return of serum creatinine to pre-rejection levels in 19/20[218]. A subsequent multicentre trial of tacrolimus rescue therapy for refractory rejection involving 73 patients showed improvements in renal function in 78%, stabilisation in 11% and progressive deterioration in the remainder. There was a 7% rate of recurrent rejection and a low rate (8%) of adverse events[219].

Thus, tacrolimus can provide effective arrest of refractory acute rejection, with a low incidence of recalcitrant rejection and good tolerability.

Primary Therapy

Phase II[161] and phase III[220] clinical trials of tacrolimus- versus cyclosporine (standard formulation) - based primary immunosuppression in renal transplantation have shown parity in terms of 1-year graft and patient survival, with significantly lower rates of acute rejection in the tacrolimus groups. A newer microemulsion formula of cyclosporine is the only form now available. It displays more predictable absorption and greater bioavailability than the standard formulation[221]. Margreiter *et al.*[222] reported a multicentre phase III study of tacrolimus vs. microemulsion cyclosporine (both supplemented with azathioprine and steroids), and found a significantly lower rate of biopsy-proven acute rejection with tacrolimus (20% vs. 37%), along with lower rates of corticosteroid-resistant (9% vs. 21%) and recurrent (1% vs. 7%) acute rejection. Again, there were no differences in overall graft survival or renal function (up to six months), but the cardiovascular risk profile was more favourable in the tacrolimus group. This study's end-points were short term, but the importance of acute rejection in the later development of chronic allograft nephropathy has been discussed. Two-year patient follow-up shows that the cardiovascular advantages of tacrolimus are maintained, and that renal function is significantly improved. Further, five year follow-up[160] of the US multicentre FK506 study[161] shows significantly better graft survival (64% vs. 54%), improved renal function (1.4 vs. 1.7 mg/dl creatinine), and ameliorated cardiovascular risk profile for tacrolimus treatment compared to cyclosporine. Addressing the issues of graft half-life and 'chronic rejection', the four year follow-up [223] of the European Tacrolimus Multicentre Renal Study[182] demonstrated significantly lower rates of chronic allograft

dysfunction (7% vs. 15%) and improved estimated graft half-lives (15.8 vs. 10.8 years) for tacrolimus vs. cyclosporine.

Interesting figures come from 10-year follow-up of tacrolimus vs. cyclosporine in liver allograft recipients, where renal impairment was significantly lower with tacrolimus therapy (38% of patients on TAC vs. 65% on CsA)[213]. This result is at odds with that from the TMC trial of 606 liver transplant patients that showed renal dysfunction was equivalent in tacrolimus and cyclosporine groups at one year[224].

Switching to tacrolimus to avoid the side-effects of cyclosporine

Margreiter *et al.*[217] demonstrated that the adverse side effects of cyclosporine are reversible to some extent with a switch to tacrolimus-based therapy in renal transplantation, with improved quality of life and improved cardiovascular risk profile. These factors were hypertension, gingival hyperplasia, hypertrichosis and hyperlipidaemia. In adult liver transplant patients, switching from cyclosporine to tacrolimus benefits both hepatic and renal function, and reduces side effects[225].

III.3 Acute cyclosporine nephrotoxicity

Some degree of reduced renal blood flow occurs in all patients treated with cyclosporine. This is mediated by an increase in afferent arteriolar vasoconstriction proximal to the glomerulus[226], and is (at least partially) reversible on stopping cyclosporine[205]. The profound impairment of renal haemodynamics of even a single dose of cyclosporine in chronically treated renal transplant patients has been documented by Percio *et al.*[227]. When this cyclosporine-mediated haemodynamic phenomenon causes an increase in serum creatinine, the clinical diagnosis of acute (functional) cyclosporine nephrotoxicity is made[228] if creatinine falls after dose-reduction (more than one trough level must be high and other causes of acute renal

dysfunction must be ruled out). In stable cyclosporine-treated patients who have the drug discontinued, renal blood flow increases by 30%, with reciprocal decreases in renovascular resistance and blood pressure[205].

Acute nephrotoxicity is characterised by vasoconstriction of preglomerular arterioles and arteries, leading to reduced renal blood flow and reduced glomerular filtration rate, and increased renal vascular resistance[229]. The functional vascular effects of cyclosporine result from an imbalance between renal vasoconstrictors (angiotensin II, thromboxane, endothelin, platelet-activating factor, catecholamines) and vasodilators (prostaglandins, nitric oxide)[230]; a shift in balance that favours vasoconstriction contributes to acute downstream renal ischaemia. Cyclosporine may directly stimulate vascular smooth muscle or mesangial cell contraction processes dependent on influx of calcium. The drug increases intracellular calcium in these cells[231] and causes vasoconstriction directly in isolated arterial rings[232].

In 1994, Ryffel *et al.* stated:

"*The renal substrate of calcineurin which mediates vasoconstriction, is yet to be identified*" [233].

Initially this search seemed to be for a single effector agent. It is becoming apparent that a variety of agents act in concert, and in different settings the relative contribution of each can vary. Regardless of the exact contribution of the mediators of acute toxicity, the effects are reversible with cyclosporine dose reduction, or interruption of the effector pathways[227;234-236].

III.3.1 Renin-angiotensin system

Cyclosporine up-regulates angiotensin II receptors in a rat model, and increases plasma renin activity, with associated increases in serum creatinine[60]. ACE inhibitor therapy (and angiotensin receptor blockade) can reverse the deleterious effects of cyclosporine on renal functional parameters in a rabbit model[237], in spontaneously-hypertensive rats[238], and on renal functional and structural abnormalities in salt-depleted rats[239]. This protection against acute cyclosporine-induced nephrotoxicity is independent of the effect of ACE inhibition or angiotensin receptor blockade on renal haemodynamics; neither calcium-channel blockers[240] nor antagonists of endothelin receptors[241] are capable of similar protection against interstitial fibrosis. This implies that an important process in the development of functional and structural cyclosporine nephrotoxicity may relate to activation of the RAS, with increased angiotensin II expression. Cellular responses to angiotensin II are mediated via the membrane receptor subtypes ATI and ATII. The former are expressed on vascular smooth muscle cells ubiquitously, and are responsible for the central and peripheral effects of angiotensin II on blood pressure, osmoregulation and cell growth. The main effect on the glomerulus is pronounced vasoconstriction, affecting the efferent more than the afferent arteriole, thereby maintaining glomerular hydrostatic pressure in the face of renal hypoperfusion. ATII receptors are also present on vascular endothelium, and are thought to be responsible (in part) for signalling apoptosis, and inhibition of cell proliferation[242]. Chronic infusion of angiotensin II, and of cyclosporine induces sustained renal vasoconstriction, which, at least in a rat model, produces interstitial fibrosis[243]. Cyclosporine increases the recruitment of renin-containing cells along the afferent arteriole. Hyperplasia of the juxtaglomerular apparatus increases angiotensin II levels, which in turn stimulates TGF-β secretion[244]. Similarly, an increase in cortical

renin mRNA expression, associated with both plasma renin activity and functional disruption has been demonstrated in hypertensive rats treated with cyclosporine[245]. A further indication of the role of the RAS comes from the salt-depletion model in rats (see chapter 5). Salt-depletion is a stimulus for the renin-angiotensin system, and it enhances the functional nephrotoxic effects of CNIs in rats and humans[246].

III.3.2 Cyclooxygenase metabolites

The cyclooxygenase metabolites (thromboxane A2, prostacyclin and prostaglandin E2) contribute to cyclosporine-induced renal vasoconstriction. Cyclosporine-initiated vasoconstriction in rats is related to an increase in thromboxane levels in renal and peritoneal macrophages, and a decrease in vasodilator metabolites[247]. Cyclosporine causes increased urinary excretion of thromboxane B2, with a positive correlation between urinary excretion and decrease in renal function, an effect that is reversed by the administration of a thromboxane-synthase inhibitor[248]. In contrast to these animal data, human liver transplant patients treated with cyclosporine appear to suffer suppression of renal prostacyclin excretion, rather than stimulation of thromboxane production[249].

III.3.3 Endothelin-1

This peptide is produced by vascular endothelial and smooth muscle cells, glomerular endothelial and epithelial cells, tubular and cortical epithelial cells, renal mesangial cells, and monocytes and macrophages[130]. It mediates renal vessel constriction, contraction of mesangial cells and induction of glomerular cell proliferation[60]. Cyclosporine is known to disrupt endothelial integrity, with

consequent endothelin release, and endothelin itself may have a pathological role in acute renal vasoconstriction.

Both cyclosporine and tacrolimus have been shown to increase endothelin-1 gene expression and endothelin-1 release from endothelial and mesangial cells[250;251]. Furthermore, endothelin-1 receptor antagonists or antibodies against endothelin-1 can reduce cyclosporine-induced vasoconstriction in rats[252;253] and partially reverse cyclosporine-induced reduction in renal functional parameters in rabbits[237].

III.3.4 Nitric oxide

Nitric oxide synthase is a substrate of calcineurin; its inhibition may in part help to explain the renal dysfunction induced by cyclosporine and tacrolimus[254]. Certainly, chronic cyclosporine administration impairs NO production in rat renal arteries[236]. A calcium/calmodulin-dependent form of NO synthase is blocked by the binding of cyclosporine to calmodulin[255]. Also, structural cyclosporine toxicity can be enhanced by NO blockade, and improved by the addition of a nitric oxide donor[256;257]. Thus, CNI-induced glomerular dysfunction may be mediated, in part, through inhibition of NO synthesis. The fact that NO-donors can attenuate, and NO-inhibitors exacerbate structural and molecular markers of chronic cyclosporine toxicity suggests a link between vasoconstriction and downstream structural changes. Shihab *et al.*[258] demonstrated that the addition of L-arginine limited tubulointerstitial fibrosis and tubular atrophy, as well as acute toxicity. In other experiments, rats with unilateral ureteric obstruction treated with L-arginine showed a reduced expression of collagen IV and TIMP-1 mRNA[259]. However, these animal findings may not resemble the situation in humans; in renal allograft recipients receiving CsA, infusion of L-arginine

did not result in improvements in GFR or renal vascular resistance[260]. There is no data on potential long-term (structural) benefits of such an approach.

III.3.5 Enhancement of sympathetic activity

Cyclosporine enhances sympathetic vascular tone by inducing release of noradrenaline, and by potentiation of post-synaptic receptor binding[261]. Lessening sympathetic activity to the experimental rat kidney by α-blockade or denervation reduces this effect[262:263]. Churchill *et al.*[264] evaluated the effects of cyclosporine on renal function in the unilateral nephrectomised rat model and produced contrary findings. When treated with cyclosporine for 4 weeks, the native (innervated) and transplanted (denervated) kidneys showed no difference in GFR or renal plasma flow, suggesting that denervation does not protect the kidney from the adverse haemodynamic effects of cyclosporine. Another study has shown that denervation does not affect the positive correlation between renal vein cyclosporine concentration and renal perfusion pressure[265].

III.4 Chronic cyclosporine nephrotoxicity

Chronic (structural) CNI nephrotoxicity is a complex entity, best described as a clinicopathologic phenomenon produced by exposure of the patient to cyclosporine and characterised by tubulointersitial fibrosis in a striped pattern[159]. The afferent glomerular arterioles also undergo degenerative hyaline change[266]. Usually, the pathological changes are associated with renal dysfunction, but this may not always be the case. Chronic toxicity occurs in most, if not all patients treated with cyclosporine and can be detected in 52% of patients after 24 months of therapy[267]. Unlike the acute form, blood drug concentrations bear little correlation to the development of chronic

toxicity[268]. Four proposed pathways may be responsible for chronic CNI toxicity: chronic ischaemia, direct stimulation of profibrotic mediators, direct stimulation of apoptotic genes, and inhibition of p-glycoprotein.

Chronic ischaemia

Chronic vasoconstriction of glomerular afferent arterioles with long-standing downstream ischaemia could produce chronic nephropathy. Arteriolopathy may lead to eventual vascular occlusion, with resultant (striped) fibrosis in the area supplied by the affected vessels[266;269]. The subsequent nephron dropout and tubular atrophy would eventually produce functional compromise. Cyclosporine is thought to act directly on vascular endothelial cells causing the release of vasoactive compounds that initiate various processes leading to an obliterative arteriolopathy. This causes chronic renal ischaemia that in turn causes the release of factors such as cytokines and growth factors that contribute to renal parenchymal damage.

Animal models of chronic cyclosporine nephrotoxicity have been difficult to develop and have generally failed to reproduce the clinicopathologic findings in humans[159]. High dose treatment for long periods causes alterations in renal haemodynamics that do not lead to major structural abnormalities of the kidney[270], and tubular function is preserved[226]. The exception is the salt-depleted model, discussed later. Thus, connections between afferent vasoconstriction and structural damage have been difficult to draw. Indeed clinical experience suggests that there is often no clear correlation between renal fibrosis and functional parameters[271].

Direct stimulation of fibrogenic mediators

Wolf *et al.* demonstrated that very small doses of cyclosporine (less than would be expected to produce clinical nephrotoxicity) stimulate collagen mRNA synthesis in mouse kidney[272], suggesting a direct fibrogenic effect of cyclosporine.

Cyclosporine may also directly stimulate expression of TGF-β and angiotensin II messenger RNA[273]. Aside from pre-clinical data[273], there is indirect human evidence; reductions in cyclosporine levels (by using concomitant mycophenolate immunosuppression) produce correlated decreases in plasma TGF-β levels[274]. A full fibrotic response to cyclosporine administration in animal models was not demonstrated until Rosen *et al.*[275] developed the salt-depleted model of cyclosporine nephrotoxicity. One week of salt depletion, followed by cyclosporine treatment, produces renal haemodynamic changes and structural lesions that resemble human cyclosporine nephrotoxicity. Once drug treatment is stopped, GFR returns to baseline levels, but the histological changes persist, thus dissociating structure from function[276]. The up-regulation of components of the renin-angiotensin system (RAS), seen with salt-depletion, sets this model apart from others and suggests a role for the RAS in structural changes. Angiotensin II plays an important role in the progression of glomerular and tubulointerstitial disease. It stimulates ECM synthesis, opposing the effect of NO[258], an action at least partly mediated through TGF-β. The expression of TGF-β and ECM synthesis is attenuated by ACE inhibition or ATII receptor blockade[277]. The importance of the RAS is supported by the experiments of Johnson *et al.*[243] where angiotensin II infusion produced similar renal lesions to those seen in cyclosporine toxicity. Moreover, the ATI receptor is located in the outer medulla and medullary stripe where damage is first seen in the chronic cyclosporine model[278]. Angiotensin II receptor blockade (ATII RB) in this model reduces both arteriolopathy and tubulointerstitial fibrosis without correcting the GFR[239;270].

This evidence for the role of angiotensin does not particularly help to clarify the mechanisms involved in chronic toxicity, because its actions are multifaceted. Losartan (an ATII receptor blocker) reduces apoptosis in tubular and interstitial cells

in the salt-depleted model of cyclosporine toxicity[279], and previous studies have demonstrated that both ACE inhibitors[239;270] and angiotensin II receptor antagonists[239;280] block interstitial fibrosis in the same model. A mechanism for this protective effect may be prevention of the afferent arteriolar lesions, thereby reducing downstream interstitial ischaemia. Further, angiotensin II mediates vasoconstriction of the vasa recta[281] and the peritubular capillaries[282]. However, angiotensin II may have non-haemodynamic effects that promote the development of interstitial injury. Angiotensin II induces cellular proliferation and hypertrophy (via ATII receptors), expression of immediate early genes (e.g. c-*fos*), and both TGF-β and PDGF in vascular smooth muscle cells, glomerular mesangial cells and renal proximal tubular cells[154]. *In vitro* studies have demonstrated that angiotensin II alters the turnover of ECM (*vide supra*). This may in fact be an indirect action[279], mediated by angiotensin II increasing active TGF-β[154]. Kagami *et al.*[155] have shown that angiotensin II promotes the conversion of latent TGF-β to the biologically active form, and Wolf *et al.*[137] showed that angiotensin II induces cellular hypertrophy of cultured murine proximal tubular cells, and that this process is mediated by the synthesis and activation of TGF-β.

Stimulation of apoptotic genes

Cyclosporine activates certain apoptotic genes, and increases apoptosis in rat tubular and interstitial cells, with a positive correlation between cyclosporine-induced apoptosis and interstitial fibrosis[279;283]. The 'death' genes p53, BAX and Fas-L are upregulated by cyclosporine in the salt-depleted model, and the expression of the 'survival' gene Bcl-2 is decreased. Caspase-III expression is also increased in this model. Increased apoptosis may explain the tubular dropout and loss of cellularity

with fibrosis, which could then impair the ability of the tubulointerstitium to remodel[283].

As with the functional (acute) form, nitric oxide may play a role in chronic toxicity; agents that attenuate the cyclosporine-induced increase in NO-synthase activity also reduce cyclosporine-induced apoptosis[284].

Inhibition of p-glycoprotein

The plasma membrane-bound transporter p-glycoprotein participates in removal of drugs from cells. Inhibition of the transporter by cyclosporine will allow the drug to accumulate intracellularly, with toxic effects[285]. Presumably, this would increase the exposure of calcineurin to this inhibitor. Tacrolimus (and sirolimus) also inhibit p-glycoprotein[286].

III.5 Tacrolimus acute and chronic nephrotoxicity

Tacrolimus has been introduced into clinical practice more recently than cyclosporine, so data on its functional and structural nephrotoxic potential is less abundant. Information now consists principally of some animal data and molecular biological/histopathological findings in human renal allografts. De Lima *et al.*[287] produced an interesting study demonstrating that tacrolimus treatment of both rat and human resistance arteries for 24 hours increases the responsiveness to noradrenaline and decreases responsiveness to acetylcholine. This suggests that tacrolimus is toxic to vasculature, affects smooth muscle relaxation and alters vascular haemodynamics.

William Bennett's laboratory applied tacrolimus to the salt-depleted model, demonstrating proximal tubular vacuolisation, striped tubulointerstitial fibrosis and arteriolopathy similar to that found in humans treated with tacrolimus[37;288;289], at similar whole blood trough levels. These structural changes are linked to worsening

renal function, decreased concentrating ability and enzymuria. Peripheral and renal renin concentrations are elevated in tacrolimus experimental toxicity[37], as is expression of TGF-β1 and the common matrix proteins.

It would appear that the mechanisms underlying the detrimental vascular effects of both of the calcineurin-inhibitors are the same. Certainly the renal lesions in kidney allograft recipients are similar, if not identical, for cyclosporine and tacrolimus treated patients[59;290]. However, whilst tacrolimus may be associated with renal structural injury and a rise in intragraft TGF-β and renin mRNA expression, it may be less toxic than cyclosporine[291]. Studies of isolated human glomeruli from renal transplant biopsies[169] show that tacrolimus is less stimulatory to profibrotic gene expression than cyclosporine. Indeed, in a rat model of renal ischaemia/reperfusion injury[292], tacrolimus *inhibited* profibrotic gene expression, favouring extracellular matrix degradation.

In the clinical setting, four large multicentre studies[182;293-295] and a recent meta-analysis[296] of tacrolimus vs. cyclosporine primary therapy in renal transplantation have shown lower rates of acute rejection with tacrolimus. Moreover, long term studies demonstrate tacrolimus is associated with improved 5-year graft survival[297] and lower rates of 'chronic rejection'[298] than cyclosporine. It is, however, a vexed issue whether tacrolimus is actually less nephrotoxic than cyclosporine. A large study of 370 liver transplant patients demonstrated 134 cases (36%) of renal dysfunction during the first month of tacrolimus therapy, and 115 cases (31%) in patients on cyclosporine therapy[299]. The incidence of human renal allograft fibrosis was examined by Solez et al.[300]; 72% of cyclosporine-treated and 62% of tacrolimus-treated patients displayed fibrosis from a total of 144 protocol biopsies taken two years post-renal transplant. Concordantly, Kyo et al.[301] reported a 65% incidence of

drug-induced nephropathy in rejection-free renal allograft biopsies, with no significant qualitative or quantitative differences between tacrolimus and cyclosporine-induced nephropathy. Murphy et al.[302] demonstrated greater degrees of allograft fibrosis in cyclosporine compared to tacrolimus treated patients in one-year protocol biopsies.

Cyclosporine and tacrolimus have differential effects on the humoral immune response, which may have clinical implications. Production of antibodies against graft tissue may affect long-term outcome and antibody formation against HLA and non-HLA antigens has been linked to the development of chronic allograft vasculopathy[303;304]. Convincing evidence for the role of antibodies in the process of 'chronic rejection' comes from comparisons of the intimal lesions in antibody-deficient and normal mouse heart models of chronic graft dysfunction; although antibody-deficient mice suffer intimitis, it is very different from that seen in wild-type mice in that it lacks a fibrotic or collagen component[305]. This suggests that the chronic production of antibodies stimulates the production of fibrogenic growth factors and extracellular matrix. Both cyclosporine and tacrolimus inhibit T-cell dependent alloantibody responses[306] and there may be differences in the effects of cyclosporine and tacrolimus on B-cell differentiation. With tacrolimus, antibody levels are lower, and this may contribute to the reduction in the incidence of CAN which has been observed with tacrolimus in a number of clinical studies[182;307].

The calcineurin-inhibitors also differ in their effects on apoptosis of donor antigen-stimulated T-cells. T-lymphocytes raised against donor cell antigens cause graft damage that may be part of the overall picture of CAN. Tacrolimus, but not cyclosporine, augments anti-CD3-induced peripheral T-cell apoptosis and potentiates steroid-induced apoptosis[308].

III.6 The role of calcineurin-inhibitors in chronic allograft nephropathy

Some of the initial evidence for the nephrotoxicity of cyclosporine came from heart transplant patients treated with cyclosporine, where there was a dose-dependent reduction in GFR and an increase in serum creatinine over time. Most histopathologic changes in renal biopsies from these patients show progressive arteriolopathy and glomerulosclerosis, but unlike the functional changes, were not dose-related[309]. In one ten-year study, 10% of heart transplant patients treated with cyclosporine developed end-stage renal failure (ESRF). There were no differences in cardiac function in the ESRF and non-ESRF groups that could otherwise explain the renal dysfunction[309 310]. Cyclosporine nephrotoxicity is also a major cause of progressive renal injury in lung[311] and liver[310] transplantation. Other data comes from non-transplant patients receiving long-term cyclosporine therapy for autoimmune disease; these patients display evidence of renal functional impairment, and structural damage on biopsy samples. Of 17 cyclosporine-treated patients with autoimmune uveitis (with normal renal function before commencing cyclosporine therapy), all demonstrated progressive tubulointerstitial and arteriolar changes, and 14/17 demonstrated marked reduction in renal function[312]. Psoriatic patients treated with cyclosporine and subjected to serial renal biopsies at one-year intervals demonstrated increases in renal fibrosis, inversely correlated with creatinine clearance[313]. In cyclosporine-treated rheumatoid patients both renal structural (interstitial fibrosis, tubular atrophy and arteriolar hyalinosis) and functional changes have been reported[314]. Similar data exists for patients with myasthenia gravis and other autoimmune diseases studied in blinded clinical trials[315-317]. Interestingly, these studies demonstrated no relationship between pathological changes and dose, blood levels or duration of treatment.

In renal transplantation, an understanding of the effect of cyclosporine on the function, structure, and survival of the transplanted kidney took longer to develop, due to in part to the complex differential diagnosis of renal dysfunction in this cohort[266]. As mentioned previously, a spectrum of injurious factors impinges on the kidney and contributes to chronic allograft nephropathy. Isolating the role of CNIs in an allograft under immunological attack[159] and exposed to many non-immunological insults is difficult. Results from 2-year protocol biopsies from the US FK506 Kidney Transplant Study show that CAN was present in 72% of biopsies from patients treated with cyclosporine and 62% of those treated with tacrolimus[300]. Further, the incidence of CAN was significantly higher in patients who had experienced episodes of CNI nephrotoxicity.

Ruiz *et al.*[318] studied 59 cyclosporine-treated kidney transplant patients, and compared them to 46 recipients who did not receive cyclosporine. Interstitial fibrosis was greater in both groups compared to pre-transplant biopsies, but was more extensive (and the serum creatinine was significantly higher) in the cyclosporine-treated group after 6 months. Comparing function and histology in renal transplant patients treated with either cyclosporine or azathioprine, Klintmalm *et al.*[319] found higher serum creatinine and more severe interstitial fibrosis and tubular atrophy in the cyclosporine group. The biopsy findings correlated with high trough cyclosporine levels and cumulative cyclosporine dose in the first six months of therapy. The stability of cyclosporine levels may influence the incidence of CAN. Kahan *et al.*[105] have recently reported that the incidence of CR at 5 years is 24% in patients with a stable pharmacokinetic cyclosporine profile, but 40% in those who are unstable.

Bennett *et al.*[159] have summarised the findings from studies so far on the long term effects of cyclosporine on renal allograft structure and function, and it is clear

that despite a number of years experience of cyclosporine in renal transplantation, there is still no clear consensus on the magnitude of its effect.

III.6.1 Histopathology of calcineurin inhibitor toxicity: differentiation from CAN

The histological attributes of calcineurin-inhibitor toxicity resemble those of CAN, thus differentiation is often difficult[59], further confounded because the two frequently occur together. The tubulointerstitial changes ("banded fibrosis") that can occur in CNI nephrotoxicity are non-specific, but the appearance of the peritubular capillary basement membranes may help with the diagnosis. In CNI toxicity, the membranes are thickened, or may even appear normal, whereas they are split or multilayered in CAN[320]. These abnormalities seem highly specific for CAN and are observed in approximately 60% of chronically failing grafts[94], but electron microscopy of the biopsy sample is required for detection[321]. Some other differences are identifiable; in toxicity, specific features include proximal tubular vacuolisation with giant mitochondria, afferent arteriolar hyalinosis and striped interstitial fibrosis[322]. Kidneys from patients treated with CNIs sometimes produce characteristic changes in the glomerular afferent arterioles[323]. In drug toxicity, afferent arteriolar lesions with nodular focal or circular protein deposits in the tunica media are characteristic, whilst the hallmarks of CAN are intravascular fibrinoid necrosis, inflammatory cell infiltration, cellular proliferation, and sclerosis. These differences may be obvious in the early course of either process, but after months or years of renal impairment, the lesions become progressively more difficult to differentiate. Abrass *et al.*[324] suggest that the content of the extracellular matrix may vary depending on whether the damage is primarily caused by immunological insults or CNIs, specifically stating that in the former there are collagens I and III in the interstitium,

and in the latter collagen IV and laminin-beta2. This is the only study reporting this finding, biopsy numbers were small, and it is unlikely that grafts will show a pure response. The result is more likely to be a predominance of one type over the other[59]. Definitive pathological diagnosis of CAN remains difficult and is complicated by biopsy sampling errors, pre-existing histological damage to the donor kidney and an overall lack of reproducibility in biopsy interpretation[94].

III.6.2 Molecular mechanisms of calcineurin inhibitor toxicity

The multiple molecular mechanisms underlying chronic CNI-induced alterations in extracellular homeostasis are complex and closely inter-related; glomerulosclerosis and tubulointerstitial fibrosis result from the deposition of abnormal quantities of extracellular matrix, producing structural changes and leading to progressive dysfunction. Profibrotic cytokines participate in the synthesis of matrix, and TGF-β is thought to be particularly important. Various animal and human studies of chronic cyclosporine toxicity and CAN have demonstrated an increase in the expression of profibrotic cytokines and growth factors, and a decrease in the level of MMPs[168-171].

A precise understanding of the mechanisms of chronic calcineurin-inhibitor toxicity has not yet been achieved; they probably act in both a direct and an indirect manner. The indirect effect occurs via long-term afferent arteriolar vasoconstriction, whilst there is a direct effect on tubulointerstitial cells. At the molecular level, this is due to a combination of suppressed MMP activity and augmented fibroblast collagen synthesis. The latter effect is mediated by cyclosporine's ability to stimulate autocrine secretion of insulin-like growth factor-1 by fibroblasts, and paracrine secretion of TGF-β1 and platelet-derived growth factor by proximal tubular cells. The renin-

angiotensin system plays an intermediary role; some of these fibrogenic effects can be completely reversed by administration of angiotensin-converting-enzyme-inhibitors[157;235;270;277].

III.7 Reducing exposure to calcineurin inhibitors

Approaches for reduction, elimination, or avoidance of calcineurin inhibitors have been investigated in an attempt to reduce the exposure of renal allografts to the damaging effects of these drugs. In a study of renal transplant patients maintained on cyclosporine and prednisolone, patients with clinically and histologically diagnosed chronic cyclosporine nephropathy were dose-reduced by 50-70% over 1 month, with re-introduction of azathioprine. A significant increase in GFR and decrease in mean arterial pressure and serum creatinine were noted up to five years after reduction[325], with no increase in acute rejection in the cohort.

The use of adjuvant mycophenolate in cyclosporine-based immunosuppressive regimens allows reduction of cyclosporine dose, resulting in decreased serum creatinine, increased GFR[326;327], reduced blood pressure and plasma TGF-β levels[326] with no increase in the incidence of acute rejection. MMF also inhibits smooth muscle cell proliferation[328], which may benefit vasculopathy. In a rat model of chronic renal allograft rejection, MMF prevented functional and morphological deterioration at 16-52 weeks when administered at the time of engraftment as well as when first administered 8 weeks after transplantation[329].

Sirolimus is a relatively new antiproliferative immunosuppressant. Its use has extended the possibilities for curtailment of CNI exposure. This agent is discussed in the following section.

CHAPTER 1 – LITERATURE REVIEW

IV – SIROLIMUS

IV.1 Introduction to sirolimus

- IV.1.1 Mechanism of action
- IV.1.2 Pharmacokinetics and interaction with the calcineurin inhibitors
- IV.1.3 Side effects

IV.2 Clinical uses of sirolimus in transplantation

- IV.2.1 Sirolimus without calcineurin inhibitors (primary therapy)
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- IV.2.4 Sirolimus (± CNI) with anti-IL2 antibody induction
- IV.2.5 Sirolimus for treatment of refractory rejection
- IV.2.6 Sirolimus with tacrolimus

IV.3 Is sirolimus non-nephrotoxic?

IV.4 The antiproliferative action of sirolimus

IV.5 Evidence for sirolimus antiproliferation in the setting of fibrosis

IV.1 Introduction to sirolimus

A search for newer immunosuppressive agents has been driven by the high incidence of chronic allograft nephropathy in CNI-treated patients, and the side effects associated with these agents. Sirolimus, a macrocyclic lactone isolated from *Streptomyces hygroscopicus*, promotes neither post-transplant diabetes nor hypertension in phase I and II studies[330;331], and has only marginal effects on glomerular dynamics in animals[332]. When used as base therapy without CNIs, sirolimus supports long-term stable graft function with good graft survival, and is not associated with the nephrotoxicity of the calcineurin inhibitors.

IV.1.2 Mechanism of action

Sirolimus inhibits calcium-dependent and -independent proliferation of T- and B-lymphocytes, and inhibits proliferation of some non-lymphoid cells. It decreases antibody production by interfering with transduction of IL-2, IL-4 and IL-15 signals[333-335]. To a lesser extent, sirolimus blocks signals delivered by non-lymphoid cytokines such as fibroblast growth factor, colony stimulating factor, platelet-derived growth factor and insulin-like growth factor[335]. It achieves these effects by binding to and forming active complexes with FK-binding protein 12 and subsequently inhibiting a multifunctional phosphatidyl-inositol kinase, named mammalian target of rapamycin (mTOR). This kinase is required for cell-cycle progression from G_1 to S phase, in response to interleukin-2 stimulation[336].

IV.1.3 Pharmacokinetics and interaction with the calcineurin inhibitors

Sirolimus is metabolised by the cytochrome P_{450} 3A4 and 3A9 pathways, thereby reducing metabolism of cyclosporine[200]. Both of these agents compete for the

p-glycoprotein transporter[337]. Thus, both drugs in the sirolimus/cyclosporine combination potentiate the other by an increase in drug level. One early study demonstrated the facultative effect of sirolimus on cyclosporine-induced renal damage in rats[338]. This augmented acute nephrotoxicity with the sirolimus/cyclosporine combination was later shown to be due to a pharmacokinetic interaction of the two agents, whilst the augmented toxicity in terms of myelosuppression and hyperlipidaemia is due to pharmacodynamic effects[339]. A study of cyclosporine and sirolimus tissue distribution after concomitant oral administration in rats revealed that sirolimus produces a two-fold increase in the tissue concentration of cyclosporine with no effect on the equilibrium of cyclosporine distribution, and that cyclosporine disturbs the equilibrium of sirolimus between blood and tissue compartments[340].

Sirolimus, cyclosporine and tacrolimus are metabolised by the liver microsomal cytochrome P_{450} system, thus there is an element of metabolic competition between the agents. Yoshimura *et al.*[341] demonstrated histochemical evidence of P_{450} in the proximal tubules of rats treated with cyclosporine, but not those treated with tacrolimus or sirolimus, suggesting that the latter two drugs have no effect on the induction of *renal* P_{450}.

Sirolimus and tacrolimus have similar structures and compete for FKBP-12. Despite this, they have distinct biological effects; tacrolimus inhibits IL-2 production whilst sirolimus inhibits IL-2-induced cell proliferation. Molar excess of one can displace the other from the binding site *in vitro*[342], suggesting antagonism. However, *in vivo* data has suggested a synergistic effect[343], likely to be due to abundance of FKBP in most cells; only 5 to 10% site occupancy is required for an immunosuppressive effect[344]. Half-maximal suppression of mouse and human T-cells requires only 3 to 5% occupancy of intracellular FKBP[345].

Interestingly, the initial indication that tacrolimus and sirolimus should be taken four hours apart has been shown to be unnecessary; the pharmacokinetic profiles of the drugs are not altered by simultaneous administration and there is no enhancement of myelosuppression or nephrotoxcity[346]. There is some evidence that over time, the dose of sirolimus may have to be increased slightly to maintain constant exposure[347] when administered alongside tacrolimus.

IV.1.4 Side effects

The most common side effects of sirolimus therapy are thrombocytopaenia, leucopaenia, and hyperlipidaemia. The thrombocytopaenia may be either a direct effect of sirolimus on megakaryopoiesis, or by platelet destruction due to clumping and aggregation[335]. Myelosuppression tends to occur within the first four weeks of therapy, and is generally concentration dependent, but seems to resolve spontaneously[335]. Hyperlipidaemia is a marked effect of sirolimus treatment, and combination with cyclosporine may exacerbate cyclosporine-induced hypercholesterolaemia, and steroid-induced hypertriglyceridaemia. The mechanism involves inhibition of lipoprotein lipase and a disruption of signal transduction by insulin-like growth factors, thereby attenuating the uptake of fatty acids into cells. A moderate rise in serum lipids is seen in about 40% of patients, with the peak effect observed at 2 months[335]. This hyperlipidaemic effect was described by Branström *et al.*[348] who noted reversal with dose-reduction. In Groth's study[349], hyperlipidaemia responded more favourably to fibrates than HMG-CoA reductase inhibitors. Increases in serum cholesterol and triglycerides are transient, tend to resolve after 12 months and mostly do not require statin therapy[344]. Post-transplant hypertension occurs less frequently with sirolimus than cyclosporine therapy, and there is no difference in the

rate of PTDM[350]. Mouth ulcers occur frequently with sirolimus[351] but the mechanism of action is unclear. It may be due to the long dwell time of the oral suspension (tablets are now available), or to a decreased facility for the regularly damaged oral mucosa to repair because of systemic inhibition of proliferative processes. Sirolimus is associated with high rates of wound-related complications and lymphocele[352], and this may be due to similar inhibition.

IV.2 Clinical uses of sirolimus in transplantation

IV.2.1 Sirolimus without calcineurin inhibitors (primary therapy)

Initial experience of sirolimus indicated that calcineurin inhibitors are required in the early post-operative period to minimise the risk of acute rejection. Two of these early studies employing sirolimus without CNIs demonstrated acute rejection rates of around 40%; Groth *et al.*[349] used cyclosporine or sirolimus plus azathioprine and prednisolone, whilst Kries *et al.*[353] reported a multicentre study comparing cyclosporine and sirolimus, both in combination with mycophenolate plus prednisolone. Both studies reported a similar biopsy-proven severity and temporal occurrence of acute rejection, and similar requirement for OKT-3 or ATG in the cyclosporine and sirolimus arms. Sirolimus was only as effective as cyclosporine-based therapy for prophylaxis of acute rejection. Of note however, the mean serum creatinine from sirolimus-treated grafts was significantly lower (as early as 3 months and extending to the end of the studies) than with cyclosporine. Renal function at 6 months is a powerful predictor of CAN and long-term graft survival[354], thus there are theoretical benefits of sirolimus, which could translate into the reduction of late allograft failure. Hyperuricaemia (3% vs. 18%), cytomegalovirus infection (5% vs. 21%), and tremor (5% vs. 21%) were observed significantly more often, and blood

pressure was higher, in the cyclosporine-treated group, whilst incidences of herpes simplex (24% vs. 10%) and pneumonia (17% vs. 2%) were higher with sirolimus. Laboratory abnormalities that are reported significantly more often with sirolimus than cyclosporine include hypertriglyceridemia (51% vs. 12%), hypercholesterolemia (44% vs. 14%), thrombocytopenia (37% vs. 0%), leucopaenia (39% vs. 14%), increased liver enzymes and hypokalemia.

IV.2.2 Sirolimus with calcineurin inhibitor reduction

The early trials of cyclosporine/sirolimus combination therapy demonstrated a potent and synergistic effect of the drug combination in prophylaxis of acute rejection. The first showed that increasing doses of sirolimus, with carefully controlled blood concentrations of cyclosporine, reduced acute rejection (in the first three years following renal transplantation) from 32% for cyclosporine/prednisolone combination therapy, to 7% for the cyclosporine/sirolimus/prednisolone combination[330]. A subsequent phase II trial showed that this reduction in acute rejection was accompanied by a reduction in dose of cyclosporine by approximately 40%[355]. Two phase III, multicentre trials comparing cyclosporine/azathioprine/ prednisolone with cyclosporine/sirolimus (2mg or 5mg) /prednisolone demonstrated significantly reduced acute rejection rates for the sirolimus-treated group. Mean serum creatinine was higher, and creatinine clearance was lower, in those patients treated with sirolimus. As previous animal studies had shown sirolimus to be non-nephrotoxic, it seems likely that this effect was due to a synergistic combination with cyclosporine[4]. The augmented toxicity was related to increased renal tissue concentrations of cyclosporine[339].

Sirolimus acts in a synergistic manner with both cyclosporine[356] and tacrolimus[343]. Using sub-therapeutic doses of sirolimus plus cyclosporine to treat heart- and kidney-allografted rats, survival has been prolonged compared to treatment with either agent used alone[357]. Similar synergistic effects have been observed in cardiac allograft survival in mice[358], renal allograft survival in dogs[359], and rat lung allografts[360]. The pharmacodynamic explanation for this synergy lies in the different modes of action of the two drugs; cyclosporine blocks the progression of T-lymphocytes in the G_0 to G_1 phase of the cell cycle, and sirolimus inhibits IL-2 transcription in the G_0 to G_1 phase and inhibits the cytokine transduction pathway during late G_1, preventing progression to S phase[4]. There are also two pharmacokinetic explanations for the synergy. Firstly, cyclosporine and sirolimus compete for the cytochrome P_{450} 3A4 and 3A9 pathways[200]. Second, both drugs act on the p-glycoprotein transporter, a membrane-bound ATP-binding protein which serves to reduce drug accumulation within cells[337]. Thus, each drug has a potentiating effect on the other.

Reports of the nature of the interaction between tacrolimus and sirolimus are inconsistent. *In vitro* assays have suggested an antagonistic action between the compounds[361], but *in vivo* studies in rat heart allografts have suggested a synergistic action[343].

IV.2.3 Sirolimus with calcineurin inhibitor elimination

There is some evidence that even low doses of CNIs cause interstitial fibrosis that is not detectable by a decrease in renal function[362]. (This dissociation of structural and functional effects is discussed in III.4). Therefore, lowering the dose of CNI may not be sufficient to reduce the incidence of graft fibrosis and CAN. Based on this

theory, researchers have examined methods of CNI withdrawal from therapeutic schemes. The two foundation trials were the '212' study[363] and the Rapamune® Maintenance Regimen (RMR) study[364]. In both, patients were randomised to receive either triple therapy (sirolimus/cyclosporine/prednisolone), or to have cyclosporine eliminated after 2 months (212) or 3 months (RMR). In the elimination groups, the trough levels of sirolimus were adjusted to approximately twice those of the patients randomised to remain on triple-therapy. In both studies, measures of renal function were significantly improved at 6 and 12 months in the groups with cyclosporine withdrawal, whilst graft and patient survival were comparable (97.2% at one year for the cyclosporine withdrawal group and 95.8% for the triple therapy group). Acute rejection rates were not statistically different across the groups, but there was a tendency towards higher acute rejection in the withdrawal group (13.5% vs. 20.2% at 12 months for cyclosporine-maintained and cyclosporine-withdrawn respectively). However, the rate of AR once cyclosporine was withdrawn was much lower than that quoted for lone sirolimus therapy[349;353]. Most importantly, the RMR study demonstrated improved renal function in those patients who had cyclosporine withdrawn (1.25mg/dl vs. 1.4mg/dl for withdrawal vs. continued cyclosporine, $P<0.001$).

Renal and liver transplant patients demonstrating chronic calcineurin-inhibitor toxicity have been converted from cyclosporine to sirolimus by two methods, either abrupt cessation, with same or next-day introduction of sirolimus, or gradual withdrawal then elimination and a gradual increase in sirolimus dosing. Interestingly, the most beneficial method for kidneys (gradual reduction of cyclosporine) differs from the most advantageous for livers (sudden cessation)[344]. When these respective techniques were employed, renal and liver transplant patients showed a 15% decrease

in serum creatinine, improvements in systolic and diastolic arterial pressure, and better glucose control. Both groups demonstrated a mild, transient rise in serum cholesterol and triglycerides, which resolved after 12 months and did not require statin therapy.

Interim data from the UK and Ireland Rapamune Study Group (cyclosporine withdrawal or minimisation in combination with sirolimus), suggests that continuing cyclosporine treatment beyond three months post-transplantation is detrimental to renal function, and confers no additional immunosuppressive efficacy over sirolimus and prednisolone therapy[365].

IV.2.4 Sirolimus (± CNI) with anti-IL2 antibody induction

This combination provides the basis for a new treatment paradigm in immunosuppression. Basiliximab or dacluzimab are used to block binding of IL-2, a CNI attenuates IL-2 gene transcription and inhibits T-lymphocyte maturation, and sirolimus is used to prevent signal transduction that follows IL-2 binding[4]. The calcineurin-inhibitor may be introduced later[366], often one month after transplant, because the induction agent and high-dose sirolimus provide cover during this period, and the newly transplanted kidney avoids nephrotoxic damage from calcineurin inhibitors. Further, this approach allows a reduction in CNI dose and overall exposure, whilst lowering the rate of acute rejection[4]. IL-2 receptor antibody induction followed by sirolimus-based therapy without a calcineurin inhibitor seems to provide good results; this may be related to the fact that sirolimus delays repopulation of basiliximab-depleted CD25 T cells compared to cyclosporine[367].

IV.2.5 Sirolimus for treatment of refractory rejection

Acute rejection that does not respond to intravenous methylprednisolone is treated with a 14- to 21-day course of an antilymphocyte antibody. Occasionally, the rejection remains resistant to treatment, and this has been successfully treated with conversion of patients to sirolimus. In one study, sirolimus produced 92% reversal of anti-lymphocyte antibody refractory rejection[368].

IV.2.6 Sirolimus with tacrolimus

Sirolimus has been used as rescue therapy for tacrolimus-treated transplanted children with either refractory rejection or tacrolimus toxicity, and appears safe in both of these settings, but the study numbers were small and included more than one organ type[369]. A recent paper from van Hooff *et al.*[370] evaluated the efficacy and safety of tacrolimus and sirolimus therapy (at three different doses of 0.5, 2 and 5mg/day), with steroids. Biopsy proven acute rejection decreased as sirolimus dose increased (8%, 8% and 3.8% respectively) and was much lower than with tacrolimus plus steroids alone (28.6%); graft and patient survival and infection rates were not statistically different, but hypercholesterolaemia occurred more frequently in the sirolimus groups.

IV.3 Is sirolimus non-nephrotoxic?

The importance of preserving renal function is illustrated by the finding that serum creatinine at 1 year-post transplant is the most accurate predictor of graft survival, and that a post transplant serum creatinine of >1.5 mg/dl (approx. 130 μmol/l) is associated with a reduction in long term survival[371]. In therapeutic doses,

sirolimus is thought to be non-nephrotoxic. Some studies have indicated that even high dose sirolimus (1.5mg/kg/day[372] and 10mg/kg/day[373]) is non-nephrotoxic in animals. However, in spontaneously hypertensive rats although sirolimus displays no effect on renal function at doses sufficient to prevent heart and kidney allograft rejection (0.01-0.08 mg/kg/day i.v), it does accelerate the histological changes of necrotising vasculopathy and tubular atrophy seen in this model. Higher doses (0.8mg/kg) did cause functional changes[374].

In salt-depleted rats, sirolimus has no effect on glomerular dynamics and renal function, but does demonstrate some elements of nephrotoxicity including renal magnesium wasting, tubular collapse, vacuolisation and nephrocalcinosis[254].

IV.4 The antiproliferative action of sirolimus

Vascular intimal hyperplasia and fibroblast proliferation are necessary steps in the generation of the obliterative processes seen in chronic allograft nephropathy. Besides its suppressive effect on B- and T-lymphocytes, sirolimus attenuates the proliferation of many non-immune cells[375]; this is unsurprising since the inhibitory effect of sirolimus disables virtually all responses to cytokine-driven mediators, due to the widespread involvement of mTOR in cell signalling pathways. *In vitro* studies have shown that sirolimus produces a non-cytotoxic inhibition of vascular smooth muscle cell proliferation, via inhibition of basic fibroblast growth factor (bFGF), angiotensin II and PDGF[334]. This effect was seen even when sirolimus was added after the cells were stimulated with growth factors, suggesting that it is capable of suppressing the ongoing vascular remodelling process *in vivo*.

Sirolimus has been applied to the rat carotid balloon-injury model to study its effect in the setting of a non-immune insult. Despite this model being a one-off

mechanical injury (very different from the chronic, progressive injury seen in chronic allograft dysfunction), it is useful for examining the effects and underlying molecular mechanisms of immunosuppressants. The model produces substantial intimal thickening within 2 weeks in controls, and sirolimus reduces the intimal hyperplastic response by about 50%[40;376], an effect not seen with cyclosporine or tacrolimus.

In experimental non-human primate aortic grafting, sirolimus therapy introduced in the medium term (45 days after aortic allografting) halts the *progression* of graft vascular disease, as detected by intra-vascular ultrasound evaluation of intimal thickening[377]. More recently using the same model, primary sirolimus monotherapy at clinically relevant doses *prevented* graft vascular disease, suggesting a role for controlling GVD in clinical transplantation[378]. In humans, sirolimus reduces the rate of re-stenosis of stented coronary[379] and superficial femoral[380] arteries. Examining the mechanisms underpinning the antiproliferative effect of sirolimus, Randall Morris' team concluded that the pharmacological actions of sirolimus involve both immune and non-immune cells, whereas the actions of cyclosporine and tacrolimus are primarily restricted to the suppression of T-cell function[334]. Thus, inhibition of non-immune growth factors may have an application in human CAN.

Of importance is a recent study suggesting that sirolimus, despite the belief that it displays limited or no nephrotoxicity, is a risk factor for prolonged DGF in renal transplant patients when used as primary therapy[381]. This suggests that it is either toxic to regenerating tubular epithelial cells, or inhibits regeneration through its antiproliferative actions.

IV.5 Evidence for sirolimus antiproliferation in the setting of fibrosis

The clinical evidence for a beneficial effect of sirolimus in renal transplant immunosuppressive protocols has been outlined above, and there is a good deal of pre-clinical animal data suggesting reduced or absent histological evidence of drug-induced toxicity with sirolimus. A clear example of this is a study showing significantly less noticeable renal tubular atrophy and interstitial fibrosis in heart-transplanted rabbits treated with sirolimus compared to those treated with cyclosporine[382]. In view of its antiproliferative actions, the question remains whether sirolimus can act in an antifibrotic manner. In the carbon-tetrachloride model of hepatic fibrosis, sirolimus inhibited both PDGF-induced proliferation of hepatic stellate cells and extracellular matrix deposition[383]. Furthermore, *in vitro* cultured human fibroblast proliferation in response to PDGF and bFGF is inhibited by sirolimus[384]. At the molecular level, sirolimus reduces the expression of fibrosis-associated genes in the rat renal ischaemia-reperfusion model[385].

CHAPTER 1 – LITERATURE REVIEW

V – ANTIFIBROSIS: A TREATMENT STRATEGY FOR CHRONIC ALLOGRAFT NEPHROPATHY

V.1 Introduction

V.2 Potential therapeutic interventions for fibrosis

- V.2.1 ACE inhibitors and angiotensin II receptor blockers
- V.2.2 Agents that inhibit fibroblast proliferation

V.3 Pirfenidone

- V.3.1 Pre-clinical experience of pirfenidone
- V.3.2 Pre-clinical transplant models
- V.3.3 Smooth muscle cell proliferation
- V.3.4 Effect of pirfenidone on normal matrix
- V.3.5 Clinical studies of pirfenidone

V.1 Introduction

Chronic allograft nephropathy presents a considerable challenge in renal transplantation. The lack of agents to effectively treat or reverse the problem has led to the search for novel therapeutic strategies. For new transplants, there is the emerging potential of complete avoidance of CNIs, as novel agents are surfacing from basic science research and early clinical studies. Presently, cyclosporine and tacrolimus remain the platform for immunosuppressive strategies. Also, for the large number of transplant patients treated with calcineurin inhibitors in the past (sometimes in high doses, such as heart and lung allograft recipients), renal fibrosis is an established pathology. Chronic CNI nephrotoxicity may be partially reversible, but only if the exposure is reduced as soon as renal dysfunction becomes apparent. Moreover, there is evidence that even a small number of CNI doses may cause irreversible renal structural damage[159]. For those allografts with genuine long-term injury, the possibility of arrest of further fibrosis, and the potential for reversing at least some of the established fibrosis may offer hope of extending graft half-life. This is all the more important in the face of the chronic shortage of organs[386].

Because there is so far no effective treatment for patients with established CAN, efforts have been concentrated on the control of co-morbid factors that are known to contribute to and accelerate CAN. This includes control of blood pressure, lipid abnormalities, diabetes, prevention of viral infection and careful control of CNI levels. These efforts go hand in hand with attempts to reduce exposure to CNIs; several approaches are being examined in ongoing multicentre trials, and have been discussed earlier.

The scheme proposed for the pathogenesis of CAN indicates a number of possible strategies for treatment: inhibition of the immune response and inflammation,

and suppression of cytokines, lipid mediators, and growth factors. The array of cells, chemicals, and interstitial components involved in the process offers multiple sites of possible drug intervention[19], but the complexity of the fibrotic process is such that it is unlikely that a single therapy targeting one site will surmount the problem. However, because the common pathway leading to fibrosis is the same regardless of the initial insult, it is likely that inhibition of components in this pathway will be more successful than attempts to remove the multiplicity of injurious stimuli, or attempts to tackle individual contributing factors.

V.2 Potential therapeutic interventions for fibrosis

Some of the experimental forms of therapy for CAN that have been reported are summarised below.

V.2.1 ACE inhibitors and angiotensin II receptor blockers

The role of antagonists of the renin-angiotensin system in the pathogenesis of renal fibrosis has been discussed in earlier chapters. ACE inhibitors reduce renal fibrosis in animal models of renal disease and slow the rate of decline of renal function in patients with chronic renal disease[154]. Similarly, angiotensin receptor antagonists reduce tubulointerstitial injury in experimental models of renal disease[387]. Shihab *et al.* treated salt-depleted rats with placebo, nilvadipine, hydralazine/hydrochlorothiazide, enalapril and losartan. Some antihypertensive effect and reduction in GFR was noted in all groups, but only enalapril and losartan decreased the expression of TGF-β[277].

Two clinical trials have been studied the effects of angiotensin II receptor blockers in renal transplant recipients. Calvino *et al.*[388] studied the antiproteinuric

effect of losartan on 18 stable renal transplant patients with hypertension for a mean follow-up period of 76 months. In addition to its antihypertensive properties, proteinuria was significantly reduced from 1.0 to 0.4 g/l ($P<0.03$). In the other trial, 14 patients with histological evidence of CAN were treated with losartan and followed for 8 weeks. Both blood pressure and proteinuria were significantly reduced; these effects were accompanied by a 55% reduction in TGF-β plasma levels[389].

Peters *et al.*[390] provided evidence that the doses of ACEI or angiotensin II receptor blockers used for antihypertension are insufficient to diminish renal fibrosis, and even at higher doses, pharmacological antagonism of the renin-angiotensin system is not sufficient to fully prevent the TGF-β over-expression in renal disease. To date however, there have been no randomised clinical trials of these agents in either renal fibrosis or CAN, and the issue remains unresolved.

V.2.2 Agents that inhibit fibroblast proliferation

Fibroblast proliferation is a seminal process in the development of fibrosis. Recent evidence points to the intracellular signalling protein Ki-*ras* as being necessary for fibroblast proliferation[391]. Ki-*ras* must undergo prenylation to allow it to act, and inhibitors of this process are under development[19]. The HMG-CoA reductase inhibitors interfere with the process of prenylation, and can improve experimental tubulointerstitial injury[392].

V.3 Pirfenidone

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone, Deskar®) is a synthetic, low molecular weight pyridone that possesses antifibrotic properties, as demonstrated in a number of animal models of fibrotic disease. Little is known about the exact

mechanism of action, but it inhibits the expression and/or activation of TGF-β, and another important mediator of fibrosis, tumour necrosis factor alpha (TNF-α).

V.3.1 Pre-clinical experience of pirfenidone

Various models, discussed below, suggest that pirfenidone has three modes of action in fibrosis:

- Prevention of the formation of fibrotic lesions
- Arrest of development of existing fibrotic lesions
- Partial or complete reversal of existing fibrotic lesions

Its mechanism of action relates to selective regulation of gene expression triggered by molecular signals from cytokines; pirfenidone is an inhibitor of the polypeptide TNF-α and the fibrogenic cytokines TGF-β, platelet derived growth factor, basic fibroblast growth factor and epithelial growth factor. It acts at transcriptional[393] and/or translational[394:395] levels.

In vitro culture of human cells derived from lung fibroblasts, dermal fibroblasts, prostatic stroma and renal mesenchyme demonstrates pirfenidone's ability to inhibit both cellular proliferation and excess production of extracellular matrix[396]. Furthermore, cell proliferation stimulated by the addition of cytokines to cell culture can be completely blocked by pirfenidone at one-tenth to one-twentieth of its toxic dose. Importantly, pirfenidone down regulates *in vitro* activation and proliferation of renal fibroblasts[397]. Other actions include inhibition of lipid peroxidation and reduced generation of reactive oxygen radicals[398].

In vivo studies using animal models of fibrotic disease support these *in vitro* findings. Oral pirfenidone treatment both prevents and treats bleomycin-induced pulmonary fibrosis in hamsters[399-404] and rats[396], with improvement in lung function

parameters and reduction in profibrotic cytokine mRNA expression[405;406]. Also, pirfenidone attenuates cyclophosphamide-induced pulmonary fibrosis in mice[407] and demonstrates a reduction in asbestos-induced fibrosis in hamsters[396]. It reduces dimethynitrosamine-induced liver fibrosis[408], and inhibits hepatic stellate cell proliferation and collagen production[409]. Intraperitoneal injection of pirfenidone prevents chemically-induced sclerosing peritonitis in rats[410], and has reduced post-surgical peritoneal adhesions in rabbits[396]. Growth of human keloid xenografts in nude mice[411] is inhibited, and in models of both experimental wound contraction and hypertension-induced deposition of collagen in heart tissue[412;413], pirfenidone decreased collagen accumulation and matrix deposition.

A number of animal studies of experimentally-induced kidney damage in rats (5/6 nephrectomy, unilateral ureteric obstruction, monoclonal antibody-induced nephritis, puromycin aminonucleoside-induced nephrosis, diabetic renal fibrosis) have demonstrated reduction or resolution of fibrotic renal lesions with pirfenidone treatment[414-416]. The beneficial effect in these kidney models includes reduction in markers of renal damage (serum urea and creatinine and proteinuria), and attenuation of glomerular and interstitial lesions. Additionally, immunohistochemical staining for components of inflammatory lesions is reduced.

Recently, pirfenidone has been shown to decrease the expression of p53, Fas-ligand and caspase 3 mRNA, and increase that of Bcl-xL, in rat salt-depleted cyclosporine nephrotoxicity[417]. This anti-apoptotic action may be due to a direct effect, or a secondary action through inhibition of TGF-β.

V.3.2 Pre-clinical transplant models

Little work has been reported on the effect of pirfenidone in transplant models. One study involving tracheal transplants in rodents has shown that prifenidone both delays the onset of airway dysfunction after transplant[418], and reduces the severity of bronchiolitis[419] compared to controls.

V.3.3 Smooth muscle cell proliferation

The process of intimal hyperplasia (smooth muscle cell proliferation) is closely associated with fibroblast stimulation and resultant fibrosis in transplantation[59]. Recent publications from Leicester have demonstrated that pirfenidone reduces intimal hyperplasia by inhibiting smooth muscle cell proliferation in the rat carotid balloon angioplasty model[420-422]. The underlying mechanism is a reduction in extracellular matrix accumulation and collagen deposition in carotid artery intima. Pirfenidone also decreased mRNA expression of proteases involved in breakdown of the internal elastic lamina (a necessary step in migration of proliferated smooth muscle cells), and decreased expression of collagen III mRNA, the major component of the extracellular matrix. In the setting of leiomyomas, pirfenidone reduces smooth muscle cell proliferation and collagen I and III mRNA production by these cells[423].

V.3.4 Effect of pirfenidone on normal matrix

A number of these pre-clinical studies (in rats, mice, hamsters, dogs, and monkeys) have shown that pirfenidone has no effect on the structural integrity of normal collagen-containing tissues[396]. This is consistent with what is so far reported regarding it mechanism of action, whereby it inhibits only abnormal, cytokine-stimulated fibrosis.

V.3.5 Clinical studies of pirfenidone

Presently, pirfenidone is undergoing a number of phase II clinical trials in conditions associated with fibrosis. These studies demonstrate some potential for treatment of fibrotic disease, whilst also indicating that pirfenidone is a safe compound, relatively free from major side effects.

Idiopathic focal segmental glomerulosclerosis

Fifteen patients with idiopathic FSGS were enrolled in an on-going, open label phase I/II study. Inclusion criteria were GFR slope of >0.4 ml/min/month during a baseline period lasting >6 months while receiving angiotensin antagonist therapy (AAT, unless intolerant), with blood pressure controlled to <140/80. Pirfenidone therapy was initiated at 800 mg three times daily and AAT was continued. The primary outcome was change in the GFR slope. Side effects were limited to gastrointestinal symptoms, including dyspepsia and early satiety. Pharmacokinetic studies in seven patients showed that pirfenidone clearance (319±136 ml/min, mean ± SD) was larger than inulin clearance (30±10 ml/min). Pirfenidone is primarily metabolised by the liver, with some renal excretion. Although most clearance was extra-renal, there was a positive correlation between inulin and pirfenidone clearance (R=0.61, P=0.15). A revised pirfenidone-dosing schedule was instituted and adjusted for GFR (40 mg/kg/d for GFR 50-80 ml/min, 30 mg/kg/d for GFR 30-50 ml/min, and 20 mg/kg/d for GFR <30 ml/min), which reduced gastrointestinal symptoms. In 12 patients who completed at least 4 months of therapy (mean 9 months, range 4-25 months), the GFR slope was minus 0.76±0.40 ml/min/mo during the baseline period and GFR decline slope was minus 0.40±0.63 ml/min/mo during therapy (P=0.15 by paired t-test). Pirfenidone therapy had no effect on proteinuria (4.0±4.1 g/d at

baseline, 4.2±3.8 g/d on pirfenidone). The authors concluded that pirfenidone had an acceptable safety profile in patients with renal insufficiency, but that the dose should be adjusted for GFR. While the study was not powered to demonstrate a statistically significant benefit, it suggests that pirfenidone slows renal functional decline in patients receiving AAT, with an effect size of ≈50% (comparable to that of AAT alone). The study authors summarised that pirfenidone merits further consideration as anti-fibrotic therapy for progressive renal disease[424].

Idiopathic pulmonary fibrosis (IPF)

IPF is a progressive clinical syndrome of unknown etiology and fatal outcome. Currently available therapies are ineffective and associated with significant adverse effects. Pirfenidone was evaluated for its tolerability and usefulness in terminally ill patients with advanced IPF. Consecutive patients with IPF and deterioration despite conventional therapy, or those who were unable to tolerate or unwilling to try conventional therapy were treated with oral pirfenidone. Treatment was administered on a compassionate use basis (open-label). Fifty-four patients were followed for mortality, change in lung function, and adverse effects. Mean age was 62, mean duration of symptoms was 4.6 years, and time since lung biopsy diagnosis was 3.2 yr. Conventional therapy was discontinued in 38 of 46 patients; 8 were able to decrease their prednisolone dosage and 8 had no previous conventional treatment. One- and two-year survival was 78% (95% CI, 66%-89%) and 63% (95% CI, 50%-76%), respectively. Patients whose lung function had deteriorated prior to enrollment appeared to stabilize after beginning treatment. Adverse effects were relatively minor. The results of this study are encouraging, suggesting pirfenidone is a promising, well tolerated treatment for IPF[425].

Multiple sclerosis

Current treatment of this progressive, demyelinating process is unsatisfactory in stabilizing or reversing the disabilities associated with the disease. Pirfenidone has been shown *in vitro* and *in vivo* to decrease synthesis of TNF-α and to block receptors for TNF-α. Since TNF-α seems to be a key cytokine in demyelination, a pilot study of oral pirfenidone was undertaken in an open-label, baseline vs. treatment protocol over a 2-year period in 20 patients. Fourteen patients (70%) remained in the study for 2 years. Three patients dropped out early because of gastrointestinal adverse reactions, and another three patients dropped out after 1 year for reasons unrelated to side effects. The remainder did not manifest any other drug-related adverse reactions or complications. Improvement or stabilization occurred in most patients at 3 months, and was sustained at 6, 12 and 24 months as evaluated by both primary and secondary outcome measures. Magnetic resonance imaging failed to reveal any new lesions in those treated with pirfenidone. Most patients reported subjective improvement in their neurological disability[426].

Myelofibrosis

The anti-fibrotic and cytokine modulatory properties of pirfenidone suggest potential in the treatment of myelofibrosis with myeloid metaplasia (MMM). In a prospective study, 28 patients with MMM were treated with oral pirfenidone. Twelve patients completed 1 year of therapy; 13 were withdrawn because of disease progression and 3 because of drug intolerance. Only one patient experienced a clinically relevant benefit with respect to anaemia and splenomegaly. The overall lack of clinical benefit correlated with no significant improvement in the bone marrow

morphological features of the disease. Pirfenidone seems to have no significant clinical or biological activity in MMM[427].

Hermansky-Pudlak syndrome (HPS)

HPS consists of oculocutaneous albinism, a platelet storage pool deficiency and, in patients with HPS1 gene mutations, a progressive, fatal pulmonary fibrosis. This study investigated the safety and efficacy of pirfenidone (800 mg, tds.), in 21 adult Puerto Rican HPS patients, 20 of who were homozygous for the HPS1 mutation. Patients were examined every 4 months for up to 44 months in a randomized, placebo-controlled trial, with rate of change in pulmonary function values as outcome parameters. The pirfenidone-treated group lost FVC ($P<0.022$), FEV_1 ($P<0.0007$), and TLC ($P<0.001$) at a rate approximately 8% per year slower than the placebo group. Clinical side effects and laboratory-detected abnormalities were similar in the two groups[428].

CHAPTER 1 – LITERATURE REVIEW

SECTION VI – THE SALT DEPLETED MODEL

VI.1 The salt depleted model

This study was constructed to examine the effects of immunosuppressants and an antifibrotic agent on markers and surrogate markers of acute and chronic renal injury in the rat salt-depleted model of nephrotoxicity.

Animal models of chronic allograft nephropathy and cyclosporine nephrotoxicity have been difficult to develop, and have generally failed to reproduce the clinicopathologic findings in humans[159]. High-dose cyclosporine treatment for long periods produces renal haemodynamic alterations that are not associated with major structural abnormalities of the animal kidney[270], and tubular function is preserved[226]. Structural changes may occur, but often take three months or more to develop. Even then vascular changes are not apparent after five months of high dose (40mg/kg/day) treatment[429]. However, Rosen *et al.*[430] introduced a rat model of cyclosporine-induced nephrotoxicity, whereby salt-depletion and administration of cyclosporine produces renal functional and structural changes similar to those seen in humans on long-term cyclosporine treatment[431]. Importantly, sodium-depletion accelerates cyclosporine nephropathy, so that structural changes may be seen within 3 to 4 weeks[430]. The reverse also holds true; if cyclosporine-treated animals are subsequently salt-depleted, similar pathological lesions can be seen[159].

This salt-depleted model has become a paradigm for the study of chronic calcineurin-inhibitor nephrotoxicity because it allows examination of the underlying mechanisms without the influence of multiple confounding factors that can confuse clinical studies. The model has elucidated some of the structural, functional[432:433] and

molecular mechanisms[434] of calcineurin-inhibitor nephrotoxicity, which include alterations in the levels of profibrotic cytokines and changes in extracellular matrix metabolism.

Animals are fed a low salt diet for a week before treatment. This in itself does not cause renal damage, but the subsequent dosing with CNIs results in structural lesions that resemble the human renal pathology of chronic cyclosporine nephropathy[275;435]. The model affects renal haemodynamics, producing a fall in glomerular filtration that returns to normal after cessation of the drug. However, the tubulointerstitial pathological lesions persist; thus, structure and function may be dissociated[159].

The mechanism by which sodium-depletion accelerates cyclosporine-induced injury is not entirely clear. In a study of paired, cyclosporine-treated rats fed either normal or low-salt diets, tubulointerstitial fibrosis and arteriolopathy was observed, and plasma renin activity (PRA) was increased in salt-depleted rats, but not in those fed a normal sodium load[436]. Decreased creatinine clearance was seen in both groups, suggesting that the structural changes are not necessary for functional abnormality to occur. That the structural changes seen in this model depend on prior salt depletion attests to the involvement of the renin-angiotensin system in chronic calcineurin-inhibitor toxicity[437]. Indeed, both ACE and angiotensin II receptor blockade reduce the arteriolopathy and interstitial fibrosis in the model, but fail to normalise GFR[235]. The tubulointerstitial fibrosis in the model is associated with apoptosis (partially mediated by angiotensin II), and is related to renal ischaemia[279]. Further evidence for the role of the RAS comes from the fact that angiotensin I and II receptors are present in high concentration (in both humans and rats) in the inner zone of the medulla and medullary rays[278], the area that is initially damaged in the salt-depleted model.

Previous studies have demonstrated an increased tissue renin expression and plasma rennin activity in association with cyclosporine-induced injury in rats[239;280]. Feeding rats a low salt diet upregulates renal renin mRNA and renal and plasma angiotensin II and angiotensin-converting enzyme[436]. Of course, the situation in rats and humans may not be entirely analogous; whilst cyclosporine consistently upregulates various components of the RAS in rats, the findings in humans are inconsistent. Humans treated with cyclosporine may have an unchanged or even a slight decreased PRA[438], but increased plasma pro-renin and renin concentrations have been found in cyclosporine-treated heart and liver allograft recipients[439].

Whilst evidence suggests that RAS blockade reverses structural but not functional changes in the salt-depleted model, the reverse is true for endothelin A and B blockade[241]. This exciting finding might help define (in animals at least) the role of these agents in acute and chronic CNI-toxicity. Sirolimus has been applied to the salt-depleted model as sole therapy and in combination with other immunosuppressants[254;440]. Kidneys in this model demonstrate no glomerular dysfunction after sirolimus treatment but do display hypomagnesaemia and tubular injury.

The salt-depleted model has advanced understanding of the role of TGF-β in chronic cyclosporine[436] and tacrolimus[37] toxicity, and the role of the RAS in stimulating TGF-β expression[239;280;441]. Furthermore, a direct effect of TGF-β1 on matrix deposition has been observed in this model, and is implicated in both animal and human forms of chronic renal allograft nephropathy[442;442]. Elevated expression of PAI-1[436] and certain extracellular matrix components is also observed in kidneys of rats on a low-salt diet administered cyclosporine for 28 days[168]. Other effects of the model include early macrophage infiltration with up-regulation of the macrophage

chemoattractant, osteopontin[443], prior to structural changes. Such infiltrating cells may well be the source of vasoconstrictors and mediators of inflammation[444]. Thus, a proposed mechanism for salt-depletion is stimulation of angiotensin II-dependent growth factors for fibroblasts, lymphokines and cytokines, and this may be an explanation for the link between the vascular changes and fibrosis.

It can be argued that the model bears a poor resemblance to the processes in human transplant kidneys because of the requirement for salt-depletion, but sodium-depletion may have a pathophysiological role, as it is a well-established risk factor for the development of acute renal failure under various experimental and clinical conditions. It also potentiates the effects of various nephrotoxins[436;445].

CHAPTER 1 – LITERATURE REVIEW

VI - SUMMARY

This literature review has shown that chronic allograft nephropathy is the primary cause of graft failure after the first post-transplant year. In turn, calcineurin inhibitor toxicity is one of the important factors in the development of CAN. At the molecular level, modifications in effector signals (produced by acute and chronic exposure to these drugs) alter the composition and quantity of extracellular matrix in the kidney, favouring fibrosis. Approaches to tackle CAN have focused on minimising exposure to calcineurin-inhibitors, with dose-reduction, withdrawal or complete avoidance of these agents. This has been made possible by the introduction of newer drugs such as sirolimus, which may be used alongside, or in-place of, CNIs. Whilst the literature reports many examples of such attempts to reduce the future development of CAN, the reversal of established CAN by the use of antifibrotic agents has received little attention. Such an approach appears to be logical because fibrosis underpins CAN, and most transplanted patients (probably all those treated with CNIs) will already have developed CAN.

Pirfenidone is an experimental antifibrotic that has demonstrated arrest and partial reversal of fibrosis in pre-clinical models and clinical examples of fibrosis. Its mode of action may be related to the interruption of pro-fibrotic signals acting on extracellular matrix. The rat salt-depletion model of CNI-induced fibrosis provides a framework for the investigation of the effect of pirfenidone on renal functional, structural and molecular variables when clinically relevant combinations of immunosuppressants are applied to the model.

On this basis, the aims and hypotheses for this work were established.

CHAPTER 2: AIMS, STUDY STRUCTURE, OBJECTIVES AND HYPOTHESES

2.1 Study outline

2.2 Study structure with aims, hypotheses and groups tested

2.2.1 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

2.2.2 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

2.2.3 Section C: The effect of the combination of tacrolimus and sirolimus

2.2.4 Section D: Effects of pirfenidone when added to treatment with calcineurin inhibitors

2.2.5 Section E: The effect of pirfenidone when added to a combination of CNI and sirolimus

2.1 Study aims

The aim of this investigation was to examine the effect on renal function, histology and molecular biology of monotherapy and combination therapy using the common clinical agents cyclosporine, tacrolimus, and sirolimus, with or without pirfenidone. Within this overall aim, the detailed objectives of each study and the hypotheses tested are given below. The indices of renal function, structure, and molecular biology were serum creatinine, urinary protein measurement, extracellular matrix deposition (sirius red staining), and messenger ribonucleic acid expression of some of the effectors of extracellular matrix turnover (TGF-β, collagen III, MMP-2, MMP-9 and TIMP-1). Overall, 40 groups of six rats were utlilised (20 groups at seven and 28 days). These are outlined below.

The drugs and doses used were:

- Control (low salt diet alone)
- Cyclosporine (15mg and 7.5mg /kg/day)
- Tacrolimus (6mg/kg/day)
- Sirolimus (1mg, 0.5mg and 0.1 mg/kg/day)
- Pirfenidone (250mg, 500mg and 750 mg/kg/day)

2.2 Study structure with aims, hypotheses and groups tested

2.2.1 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

Cyclosporine, tacrolimus and sirolimus were initially examined as sole therapy, and comparisons were made between the drugs. Whilst basic science and clinical studies have already demonstrated the nephrotoxicity of CsA and (probably to a lesser degree) of TAC, this section served as a baseline for the subsequent sections when comparisons to sole therapy were required. Likewise, sirolimus is believed to be minimally- or non- nephrotoxic, but a direct comparison to the calcineurin-inhibitors, in the same model and setting, was required.

The hypothesis was that CsA, TAC and SRL vary in their effects on structural, functional and molecular indices of renal injury.

The groups tested were:

- Control group (low salt diet); sacrificed at 7 days (n=6) and 28 days (n=6)
- Cyclosporine 15mg/kg/day; sacrificed at 7 days and 28 days.
- Tacrolimus 6mg/kg/day; sacrificed at 7 days and 28 days.
- Sirolimus 1mg/kg/day; sacrificed at 7 days and 28 days.

2.2.2 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

Because calcineurin-inhibitor dose-reduction by addition of sirolimus is an emerging strategy in transplant immunosuppression, the combination of CsA plus SRL was examined. As well as permitting dose-reduction, utilising sirolimus may confer other benefits. It is known to act in an antiproliferative manner at molecular and morphological levels in arterial disease, although little is known about its effect on renal fibrosis.

Both additive effects (due to different modes of action on T cell inhibition) and synergistic effects (due to competition for cytochrome and p-glycoprotein) of CsA and SRL occur. This poses difficulty in correct dosing when both drugs are used, and nephrotoxicity may be enhanced. At the outset of this section of the study, only two combinations were examined (CsA 15 + SRL 1mg/kg/day, and CsA15 + SRL 0.5mg/kg/day). The former group were euthanised at day 14 because of poor condition. This prompted more thorough examination of dose combinations for the measured variables in the model.

The hypotheses were a) dose manipulation produces variable effects on the structural, functional and molecular indices of renal injury, and b) at the correct doses, the addition of SRL to CsA is beneficial compared to CsA alone.

The groups tested were:

- Cyclosporine 7.5mg/kg/day; sacrificed at 7 days and 28 days.
- Cyclosporine 15 mg + sirolimus 1 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 15 mg + sirolimus 0.5 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 15 mg + sirolimus 0.1 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 7.5 mg + sirolimus 1 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 7.5 mg + sirolimus 0.5 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 7.5 mg + sirolimus 0.1 mg; sacrificed at 7 days and 28 days.

2.2.3 Section C: The effect of the combination of tacrolimus and sirolimus

This section was similar to section B, but utilised TAC + SRL. Less information is available regarding the pharmacodynamics of the TAC + SRL combination, principally because TAC is a newer drug than CsA. The small amount of information that is available indicates that neither pharmacokinetic profiles of SRL nor TAC are altered by simultaneous administration[346]. Therefore, only one dose combination was examined.

The hypothesis tested was that the addition of SRL to TAC is favourable compared to TAC alone for the variables of renal structure and function tested.

The group tested in this section was:

- Tacrolimus 6 mg + sirolimus 1 mg; sacrificed at 7 days and 28 days.

2.2.4 Section D: Effects of pirfenidone when added to treatment with calcineurin inhibitors

This section was designed in an attempt to translate into the present model what is known about the antifibrotic effects of pirfenidone. Both CsA and TAC were tested with three doses of pirfenidone (see below) to elicit potential dose-dependency. Comparisons were made to sole drug treatment.

The hypotheses tested were that a) pirfenidone reduces markers of CNI-induced injury in the salt-depleted model, and b) that pirfenidone has a dose-dependent effect.

The groups tested were:

- Cyclosporine 15 mg + prifenidone 250 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 15 mg + prifenidone 500 mg; sacrificed at 7 days and 28 days.
- Cyclosporine 15 mg + prifenidone 750 mg; sacrificed at 7 days and 28 days.
- Tacrolimus 6mg + prifenidone 250 mg; sacrificed at 7 days and 28 days.
- Tacrolimus 6mg + prifenidone 500 mg; sacrificed at 7 days and 28 days.
- Tacrolimus 6mg + prifenidone 750 mg; sacrificed at 7 days and 28 days.

2.2.5 Section E: The effect of pirfenidone when added to a combination of CNI and sirolimus

Earlier sections of the study demonstrated beneficial effects of (separately) adding SRL or pirfenidone to the calcineurin inhibitors. This section was constructed to investigate any further effect of adding both sirolimus and pirfenidone. As the previous section demonstrated no pirfenidone dose-dependency, the middle dose of 500mg/kg/day was chosen.

The hypothesis was that pirfenidone confers further benefits on markers of renal injury when added to sirolimus plus the calcineurin inhibitors.

The groups tested were:

- Cyclosporine 7.5 mg + sirolimus 1 mg + prifenidone 500 mg; sacrificed at 7 days and 28 days.
- Tacrolimus 6 mg + sirolimus 1 mg + prifenidone 500 mg; sacrificed at 7 days and 28 days.

CHAPTER 3 - MATERIALS AND METHODS

3.0 Statistical analysis

3.1 Treatment schedule

3.2 Serum creatinine

3.3 Urinary protein quantification

3.4 Extracellular matrix evaluation

3.5 Molecular analysis

3.5.1 Introduction
3.5.2 Molecular analysis – detailed description

3.0 Statistical analysis

Statistical analysis was performed using GraphPad Instat® for Macintosh, Version 3.0b software (San Diego, USA). Animal weight, urinary protein measurements and percent area fraction staining (extracellular matrix) demonstrated Gaussian distribution and were analysed with repeated measures one-way analysis of variance.

Some, but not all groups demonstrated Gaussian distribution for serum creatinine. Likewise, some data for arbitrary units of messenger RNA expression demonstrated Gaussian distribution. However, non-parametric Kruskall-Wallis analysis was applied to all groups for creatinine and mRNA analysis. This is because the data sets were small, and there is less risk of creating false significant results if a non-parametric test is applied, even if data is Gaussian. Mann-Whitney *U* post-testing was applied for two-group analysis where appropriate.

For all tests, $P<0.05$ was considered significant. Data from some groups is presented repeatedly in later sections when comparison is necessary.

3.1 Treatment schedule

Male Sprague Dawley rats (350-500g), obtained from Harland (Cambridge, U.K.) were housed and cared for in accordance with the Animals (Scientific Procedures) Act 1986, in cages of three animals in a temperature and light controlled environment, with water *ad libitum*. The rats were acclimatized for seven days on a 12:12 hour light:dark cycle. They were fed a salt-depleted diet for 7 days (0.05% sodium, Special Diets Services, Witham, Essex, UK), before the introduction of a regimen of treatment involving mono or dual therapy with the immunosuppressive agents cyclosporine, tacrolimus and sirolimus, with or without the addition of pirfenidone (Marnac Inc, Dallas, Texas) at varying doses. The animals were randomly assigned to the groups listed in chapter 2.

Cyclosporine (Sandimmun®, Sandoz Pharmaceuticals, Camberley, Surrey, UK), tacrolimus (Prograf®, Fujisawa Ltd, Staines, Middlesex, UK) and sirolimus (Rapamycin®, Wyeth Biotechnology, Taplow, Maidenhead, UK) were administered by oral gavage on a dose/weight schedule. Pirfenidone (250mg, 500mg or 750 mg/kg/day) was mixed with the low salt-diet using a planetary mixer and stored at 4°C.

Animals were weighed daily. On a weekly basis, tail bleeds were performed for measurement of serum creatinine and animals were placed in metabolic cages (Techniplast, Kettering, Northants, UK) for 24-hour urine collection, with subsequent measurement of urinary total protein. At seven or 28 days, animals were anaesthetised with inhaled halothane, and both kidneys were harvested through a midline laparotomy incision. They were killed whilst under anaesthesia. Sections of renal cortex were either snap frozen in liquid nitrogen for later molecular biological

analysis, or placed in 10% formal saline solution for subsequent histological examination.

3.2 Serum creatinine

Rat blood samples (0.5ml) were obtained by tail-bleeding, and blood was placed in sterile lithium-heparin tubes prior to cooling to 4°C and transfer to the Clinical Biochemistry Department of Leicester General Hospital for measurement of serum creatinine using the sodium picrate reaction. Measurement of creatinine-picarate complex at a wavelength of 500nm was performed using the Abbott Diagnositc Aeroset Analyser (Abbott Laboratories, Maidenhead, UK).

3.3 Urinary protein quantification

Urinary protein concentration was measured by an automated immunoprecipitation analysis. Standards, controls and experimental samples were pipetted into the reaction cuvettes together with a polymer enhancement solution. Following an initial incubation and measurement of sample blank, neat antibody is added to the cuvette and mixed. Insoluble antigen/antibody complexes form, producing turbidity in the mixture. This increases the amount of light scattered by the solution. Following incubation, the absorbance of the solution is measured at 340nm. Assay of 5 standards (known protein concentration) generates a calibration curve , and experimental sample values are interpolated from the calibration curve.

3.4 Extracellular matrix evaluation

Tissue for evaluation of extracellular matrix staining was stored in 10% formal saline for 18 hours, after which time it was transferred to phosphate buffered saline

solution. Tissue was embedded in paraffin, cut into 4μm sections, and slide-mounted. The paraffin was removed in xylene for 10 minutes and dehydrated in serial washes of 100% alcohol for two minutes performed twice, then 95%, 80%, and 60% alcohol for 2 minutes each. Sections were held under running cold water for 10 minutes and finally rinsed briefly with distilled water. Staining was performed for 12 hours in pico-sirius red F3BA (0.1% Sirius red F3BA in saturated aqueous picric acid). Rapid dehydration was repeated with an initial wash of 0.01 M HCl for 2 minutes and serial washes with 70% alcohol for 45 seconds, followed by 80%, 95%, and 100% for 2 minutes each. Slides were cleared with two washes of xylene for 2 minutes, excess xylene was removed, and slides mounted with XAM organic mountant. Sections were viewed on a Nikon Eclipse E800 microscope, and images were transferred via a digital video system to the in-built frame-grabber board of an Apple Macintosh microcomputer. Images were imported directly to the freeware image analysis programme NIH-Image (US National Institutes of Health; www.rsb.info.nih.gov/nih-image/). Sequential greyscale images of renal cortex were captured using the X 10 objective, by moving along the central line of each specimen from one end of the available cortex to the other without overlapping. Twenty fields were counted for each section, with six sections counted per group, representing 120 total counts per drug treatment. To calculate the area fraction of extracellular matrix staining, a threshold was applied to each image at a constant level that distinguished between stained component and the unstained background. The proportion of black to white pixels in the image was calculated as a percentage, representing the percentage area fraction of the tissue which is occupied by stained element[446]. This technique has previously been validated in the laboratory at Leicester[447].

3.5 Molecular analysis

3.5.1 Introduction

The methods used to quantify levels of mRNA expression using non-competitive reverse transcriptase-polymerase chain reaction (RT-PCR) have been described previously[448;449]. Messenger RNA was extracted using oligo-dT-linked Dynabeads (Dynal, Bromborough, U.K.). Genes chosen for quantification in this study were matrix metalloproteinases (MMP)-2 and 9, tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix protein collagen III, and transforming growth factor-beta (TGF-β). All probes and primers were designed from sequences available on the EMBL database (Heidelberg, Germany) using the program GCG Prime (Genetics Computer Group, Madison, WI, USA) and synthesised by Life Technologies (Paisley, U.K.) (see table below). Quantification of RT-PCR products was performed using an enzyme-linked immunosorbent assay[169]. Differences in tissue cellularity were corrected for by expressing values of RT-PCR product as a ratio to that of the constitutively expressed housekeeping gene, β-actin. All samples were screened against genomic DNA contamination prior to use.

3.5.2 Molecular analysis – detailed description

Quantification of transcripts at specific time points provides an opportunity to assess cellular messaging dynamically. The use of animal tissue from 7 and 28 days is a useful tool for tracking changes over time, allowing description of the evolution of changes. By measuring these transcripts, it is possible to speculate as to the possible mechanisms, by which the various drugs exhibit their effects. Reverse transcriptase polymerase chain reaction (RT-PCR) is a powerful technique that amplifies gene transcripts.

House-keeping gene

Differences in tissue cellularity were corrected for by expressing values of complementary DNA product as a ratio to that of constitutively expressed housekeeping gene, β-actin.

Primers

All probes and primers (see table below) were designed from sequences available on the EMBL database using the program GCG Prime (Genetics Computer Group, Madison, Wisconson, USA) and synthesised by Life Technologies (Paisley, UK). Forward primers were synthesized with 5'-biotinylation to enable quantification of RT-PCR products by an enzyme-linked immunosorbent assay. The primer sequences were designed to bind specifically to the complementary DNA sequence of interest. They are required by DNA synthetases to begin DNA synthesis. Primer sequences generally consisted of 20 nucleotides to minimise non-specific DNA amplification. Short primers increase the frequency of non-specific binding, whilst longer sequences may lead to non-specific binding within the primer itself to form a tertiary structure, which inhibits the ability of the primer to bind to the cDNA sequence of interest. The number of PCR cycles and annealing temperatures were optimised before this study and standardised to 59°C and 40 cycles, except for β-Actin that only required 35 cycles.

Sample storage

Renal cortical samples were submersed in liquid nitrogen to 'snap-freeze' the specimens, thus preserving mRNA, and tissue was placed in a cryotube and stored in liquid nitrogen.

Extraction of mRNA from kidney tissue

Tissue was removed from storage in liquid nitrogen and a small sample was homogenised in 100µl of lysis binding buffer [450ml solution of lysis binding buffer- 45mls 1M Tris pH 8.0, 200mls DEPC water, 9mls 0.5M EDTA pH 8.0, 9.537g LiCl, 45mls 10% sodium dodecyl sulphate (SDS), 2250µl 1M dithiothreitol (DTT)] using a micro-homogeniser. Twenty-five microlitres of 1mg/ml proteinase K (5ml solution of proteinase K- 5mg proteinase K, 5mls 0.05M Tris pH 7.65) was added to the lysate and incubated for 1 hour at 50°C. The lysate was centrifuged for ~45 seconds at 10,000*g* to separate any debris. All reusable instruments were pre-treated with 3% hydrogen peroxide (H_2O_2) for at least 20 minutes and rinsed in diethylpyrocarbonate (DEPC) water in order to reduce RNAase contamination.

Preparation of Dynabeads®

Thirty microlitres of dT_{25} Dynabeads® (Dynal, Bromborough, UK) were pelleted with a Dynal magnetic particle concentrator until the supernatant was clear. The supernatant was discarded and the Dynabeads re-suspended in 10µl of lysis binding buffer. This cleaning procedure was repeated once to ensure that all traces of preservative solution were removed.

Extraction of mRNA

The lysate was added to the Dynabeads and allowed to anneal at room temperature for 10 minutes. dT_{25} Dynabeads capture RNA by annealing to the poly-A tail of mRNA. Other cellular constituents, including DNA and other RNAs are washed away in five easy steps. Dynabeads® were pelleted with the Dynal MPC until the solution was clear and the supernatant discarded and this process was repeated once. The Dynabeads were resuspended thoroughly in 50µl washing buffer (50ml solution- 0.5ml 1M Tris pH 8.0, 25ml DEPC water, 0.1ml 0.5M EDTA pH 8.0, 0.318g LiCl,

0.5ml 10% SDS, made up to 50ml with DEPC water). The Dynabeads were further pelleted and washed in 50µl washing buffer (450ml stock solution- 4.5ml 1M Tris pH 8.0, 0.9ml 0.5M EDTA pH 8.0, 2.862g LiCl, made up to 450ml with DEPC water) three times.

Reverse Transcription of Dynabead-Extracted mRNA

Dynabeads were pelleted and resuspended in 10µl DEPC-treated water. Two microlitres of Dynabeads from this solution were mixed with 6.5µl of DEPC-treated water in a second tube. This represented the "–RT" sample, used to check for genomic DNA contamination. The remaining 8µl represented the "+RT" sample. Sixteen point five microlitres of RT mastermix (5µl of proprietary Avian Myeloblastosis Viral Reverse Transcriptase (AMVRT) 5X buffer, 2.5µl 10mM DEPC-treated dNTPs (1mM), 0.6µl 40U/µl RNAsin (25U), 8.4µl DEPC-treated water) were added to each –RT sample and mixed thoroughly. Seventeen microlitres of mastermix (supplemented with 0.5µl of 10U/µl AMVRT (5U) for every +RT sample) was added to each +RT sample. These samples were then incubated in the thermal cycler at 42°C for 1 hour (PTC-225, DNA Engine Tetrad, MJ Research Inc, Watertown, MA, USA). The Dynabeads poly-T tails acted as a primer for reverse transcriptase and allowed the synthesis of cDNA directly onto the beads for all mRNA species present. Following reverse transcription, the cDNA loaded Dynabeads were stored in 25 µl TE buffer (1ml 100X TE stock, 99ml sterile water) at 4°C.

Polymerase chain reaction

All sample volumes were checked, so that each microlitre represented 1/25th of the initial volumes. A tube of Alec Jeffries (AJ) 10X buffer was thawed, vortexed and spun at 3000-4000*g* for 1 minute to pellet any BSA. Precipitated BSA was removed as its presence inhibits the polymerase chain reaction. A PCR mastermix was made using

the AJ 10X buffer as follows: for each sample, 5µl of AJ 10X buffer, 2µl of (5pmol/µl forward + 5pmol/µl reverse primer mix), 42µl sterile distilled water, and 0.4µl of 2.5U/µl JumpStart Taq (Sigma). Forty-nine microlitres of this mastermix was added to 1µl of +RT Dynabeads (or, in the case of checking for genomic DNA contamination, 4µl of –RT Dynabeads resuspended to 1µl). Each PCR reaction was then covered with a drop of sterile mineral oil to prevent evaporation during thermal cycling. Reactions were amplified using primers as detailed in the table below. For each PCR 'run', a positive (previously amplified product, cleaned and diluted) and negative (sterile distilled water) control was performed.

Analysis of PCR by agarose gel electrophoresis

Fifteen µl of each PCR reaction was mixed with 2µl 5X loading buffer (20ml-20mg bromophenol blue in 8mls sterile distilled water, 2mls 50X TAE, 10mls glycerol) and pipetted into a well of a 3% agarose gel containing 15µl of (10mg/ml) ethidium bromide. The gel was run at 150 volts for 30 minutes using a 100 base pair DNA ladder in buffer (20mg bromophenol blue, 8ml sterile distilled water, 2ml 50X TAE, 10ml glycerol) as a standard. Bands of DNA were visualised under ultra violet illumination and recorded digitally.

PCR Assay using Enzyme Linked Immunosorbent Assay

Pre-avidinylated microtitre plates (Thermo Life Sciences, Basingstoke, UK) were washed once with binding buffer (1% bovine serum albumin in phosphate buffered saline), and the PCR products from individual PCR samples were captured (2 µl in 100 µl binding buffer) in duplicate in the washed wells for 30 minutes. One hundred microlitres of 0.25 M sodium hydroxide was added to each sample for 10 minutes to denature the unbiotinylated (reverse) DNA strand of each PCR product. Samples were washed three times with a washing buffer (0.02% Tween in phosphate

buffered saline) to remove the unbiotinylated strand. Samples were then incubated with a specific, digoxigenin-labelled DNA probe (see table below; 0.2 pmol probe in Rapid Hyb buffer (Amersham Life Sciences, Amersham, UK)) for 90 minutes at 42°C. After three washes in washing buffer (to remove unbound probe), samples were incubated at room temperature for 30 minutes with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche, Lewes, UK, 1:500 in binding buffer). Samples were washed a further three times with washing buffer (to remove unbound antibody), and incubated with paranitrophenyl phosphate (1 mg/ml in 10% diethanolamine, pH 9.8) at 37°C. The resulting yellow colour was measured periodically with a colorimeter at 405 nm (with 630 nm differential) until readings were at the high end of the linearity. An average of each set of duplicates was used for statistical analysis. Correction for changes in PCR and ELISA efficiency was achieved by reference to the positive control from each PCR run.

Gene	Forward Primer	Reverse Primer	ELISA Probe
β-Actin	TCA TCA CCA TTG GCA ATG AGCG	CTA GAA GCA TTT GCGBGTGBGACG	GGA GTA CTT GCG CTC AGG AGG
Collagen III	GAA ATT CTG CCA CCC TGA AC	GGC TGG AAA GAA GTC TGA GG	CTT CTC AGC ACC AGC ATC TG
MMP-2	ATT GAT GCG GTA TAC GAG GC	GGC ACC CTT GAA GAA GTA GC	CTC CAG AAT TTG TCT CCA GC
MMP-9	GCA TTT CTT CAA GGA CGG TC	CGC CAG AGA ACT CGT TAT CC	AGC CTA GCC CCA ACT TAT CC
TIMP-1	GTT CCC CAG AAA TCA TCG AG	TGA ACA GGG AAA CAC TGT GC	GCA GTG ATG TGC AAA TTT CC
TGFβ	TAC GTC AGA CAT TCG GGA AG	GAA GCG AAA GCC CTG TAT TC	TCA AAA GAC AGC CAC TCA GG

Forward and reverse primers and ELISA probes for genes investigated.

CHAPTER 4 – RESULTS

4.1 Section A: The effect of single agent therapy:
Cyclosporine (15mg/kg/day), tacrolimus (6mg/kg/day) and sirolimus (1mg/kg/day).

- 4.1.1 Animal weight
- 4.1.2 Serum creatinine
- 4.1.3 Urinary protein
- 4.1.4 Interstitial fibrosis
- 4.1.5 RT-PCR messenger RNA expression

4.2 Section B: The effect of combination therapy - cyclosporine and sirolimus at varying doses.

- 4.2.1 Serum creatinine
- 4.2.2 Urinary Protein
- 4.2.3 Interstitial fibrosis
- 4.2.4 RT-PCR messenger RNA expression

4.3 Section C: The effect of the combination of tacrolimus and sirolimus

- 4.3.1 Serum creatinine
- 4.3.2 Urinary Protein
- 4.3.3 Interstitial fibrosis
- 4.3.4 RT-PCR messenger RNA expression

4.4 Section D: Effects of pirfenidone when added to treatment with cyclosporine or tacrolimus.

- 4.4.1 Serum creatinine
- 4.4.2 Urinary Protein
- 4.4.3 Interstitial fibrosis
- 4.4.4 RT-PCR messenger RNA expression

4.5 Section E: The effect of pirfenidone when added to a combination of calcineurin inhibitor plus sirolimus.

- 4.5.1 Serum creatinine
- 4.5.2 Urinary Protein
- 4.5.3 Interstitial fibrosis
- 4.5.4 RT-PCR messenger RNA expression

4.1 Section A: The effect of single agent therapy: Cyclosporine (15mg/kg/day), tacrolimus (6mg/kg/day) and sirolimus(1mg/kg/day).

4.1.1 Animal weight

Figure 4.1 summarises change in rat weight over time for the different treatments. Weight gain was expected as rats matured. There was a progressive increase in the mean weight of each group of rats, and statistical analysis (one way analysis of variance) revealed no significant difference in weight between the groups at any time point. Furthermore, there were no significant differences in animal weights for all other groups tested (data not presented).

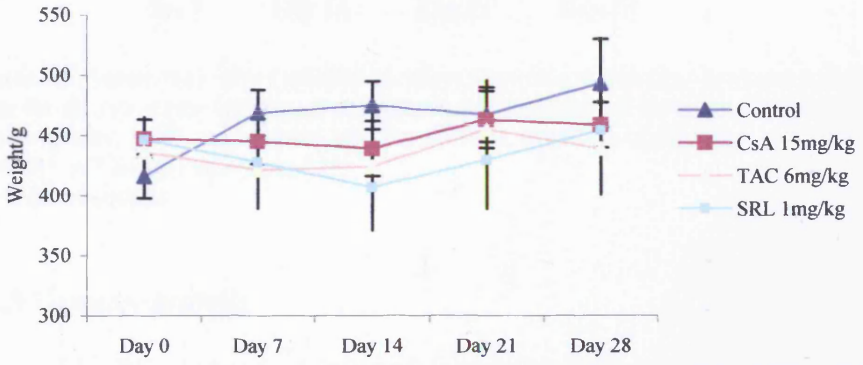


Figure 4.1. Rat weight in grams at time points during treatment schedule. Each point represents the mean weight of 6 rats, and error bars represent one standard deviation of the mean. Control = low salt diet, CsA = cyclosporine, TAC = tacrolimus, SRL = sirolimus.

4.1.2 Serum creatinine

Serum creatinine for the groups under consideration in this section is shown in Figure 4.2. Serum creatinine was elevated compared to controls by calcineurin inhibitor treatment, reaching statistical significance for cyclosporine (15mg/kg/day) at 28 days (94 ± vs. 61 ± 7 μmol/litre, P=0.0001) and for tacrolimus (6mg/kg/day) at both 21 days (75 ± 10 vs. 61 ± 7.2 μmol/l, P=0.006) and 28 days (75 ± 8 vs. 61 ± 7.2 μmol/l, P=0.008). A higher serum creatinine was produced by cyclosporine than tacrolimus treatment at 7 days (64 vs. 51 μmol/l, P=0.03), 14 days (77 vs. 60 μmol/l

P=0.01) and 28 days (94 vs. 76 μmol/l, P=0.002). Sirolimus (1mg/kg/day) treatment produced no significant difference in creatinine compared to the control group.

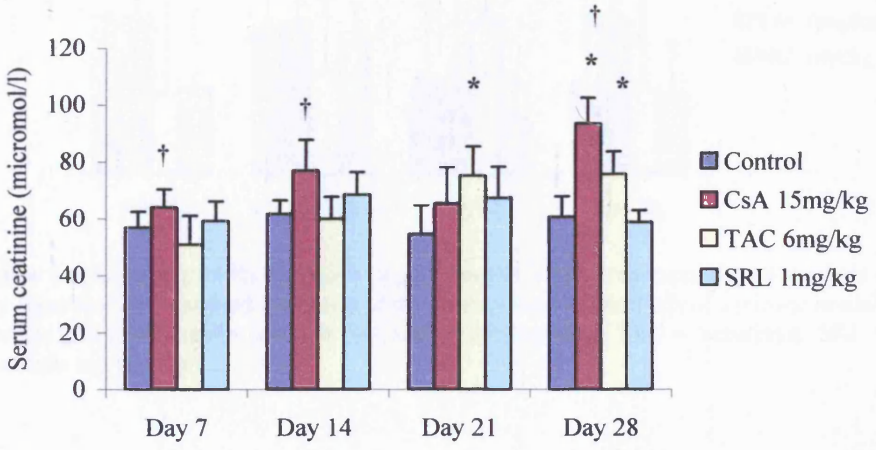


Figure 4.2. Serum creatinine (μmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, CsA = cyclosporine, TAC = tacrolimus, SRL = sirolimus. Doses are mg/kg/day.
† P<0.05 vs TAC at same time point
* P<0.01 vs control

4.1.3 Urinary protein

The figures for mean urinary protein excretion, expressed as total protein mg/24hours, representing the mean of six samples, corrected for animal weight, are presented in the Figure 4.3. One-way analysis of variance revealed no significant differences between values for different groups at any time point.

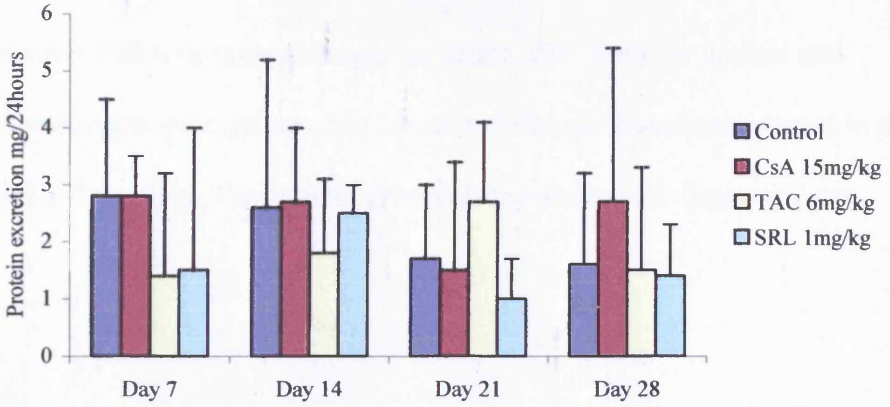


Figure 4.3. Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, CsA = cyclosporine, TAC = tacrolimus, SRL = sirolimus. Doses are mg/kg/day.

4.1.4 Interstitial fibrosis

Interstitial fibrosis, measured as percent area fraction staining by pico-sirius red, was not significantly different across groups. Data are presented in Figure 4.4.

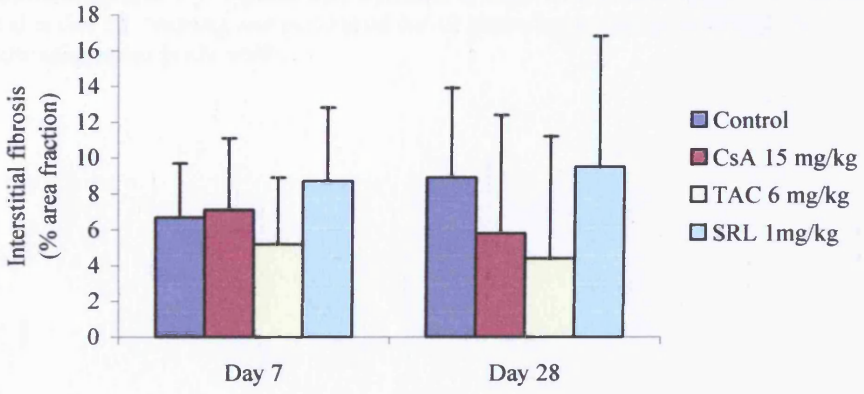
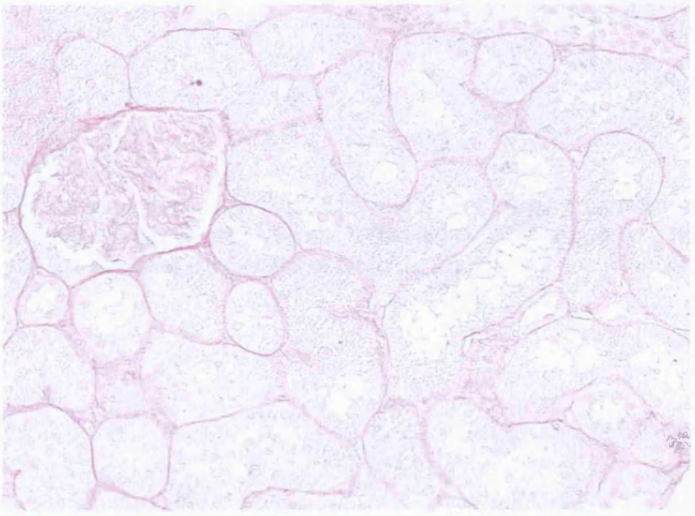
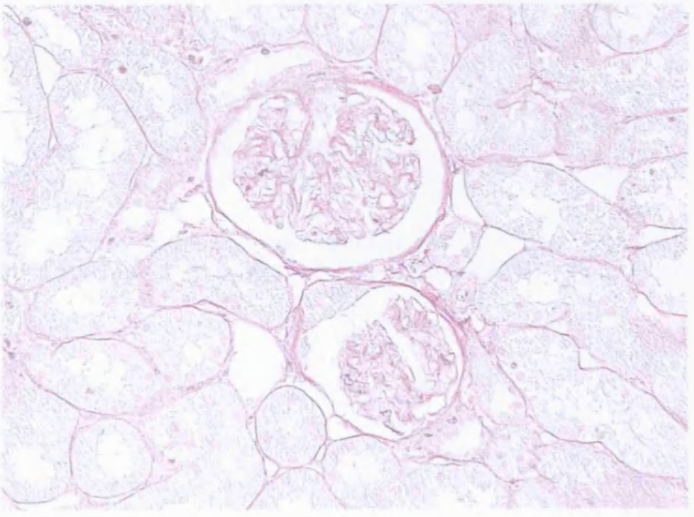


Figure 4.4. Interstitial fibrosis as measured by sirius red staining of renal cortical slices taken from rats treated with a low salt diet only, or low salt diet with cyclosporine (CsA), tacrolimus (TAC) or sirolimus (SRL) treatment. Each value represents the mean of six measurements, and error bars represent one standard deviation of the mean. Doses are mg/kg/day.

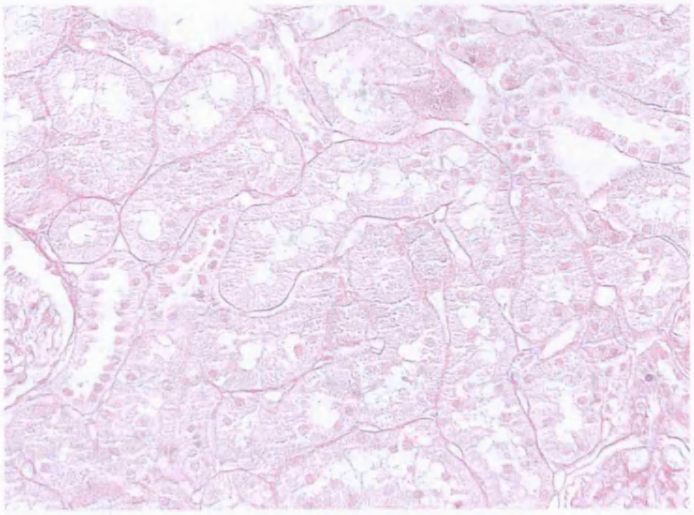
The photomicrographs below are representative sections of renal cortex, stained with sirius red F3BA in saturated aqueous picric acid, from the control and immunosuppressant groups. No interstitial fibrosis was demonstrated in these or any group in the study. These other groups are therefore not displayed later.



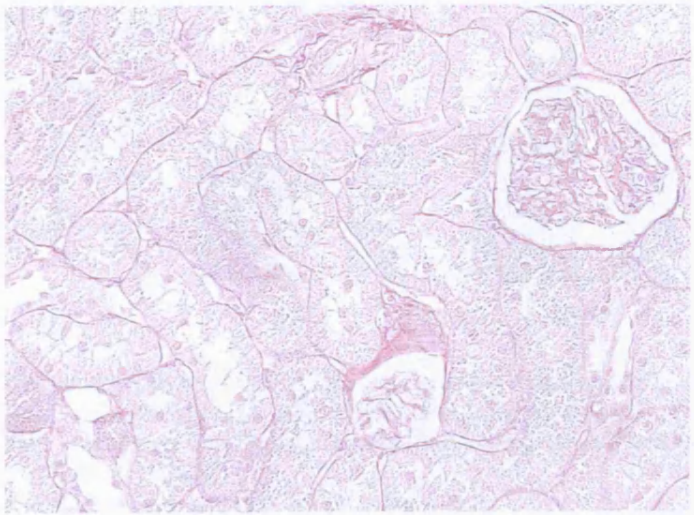
Photomicrograph 1. A representative section of renal cortex tissue from a salt-depleted rat (control) killed at day 28. Staining was performed for 12 hours in pico-sirius red F3BA (0.1% Sirius red F3BA in saturated aqueous picric acid).



Photomicrograph 2. A representative section of renal cortex tissue from a salt-depleted rat, treated with cyclosporine 15mg/kg/day and killed at day 28. There is very little extracellular matrix deposition, and the stigmata of calcineurin-inhibitor toxicity are not apparent.



Photomicrograph 3. A representative section of renal cortex tissue from a salt-depleted rat, treated with tacrolimus 6mg/kg/day and killed at day 28. There is very little extracellular matrix deposition, and the stigmata of calcineurin-inhibitor toxicity are not apparent.



Photomicrograph 4. A representative section of renal cortex tissue from a salt-depleted rat, treated with sirolimus 1mg/kg/day and killed at day 28. There is very little extracellular matrix deposition.

4.1.5 RT-PCR messenger RNA expression

Seven days after commencement of treatment, no differences in the messenger RNA expression of TGF-β were observed between treatment groups. For animals sacrificed at day 28, renal cortical TGF-β mRNA expression was greater in cyclosporine than control groups (1.28 ± 0.53 vs. 0.76 ± 0.13, P=0.046). Sirolimus treatment had no effect compared to controls (P=0.37). Tacrolimus significantly inhibited TGF-β expression compared to controls (0.32 ± 0.1 vs. 0.76 ± 0.13, P=0.004), and compared to both cyclosporine (0.32 ± 0.1 vs. 1.28 ± 0.53, P=0.007) and sirolimus (0.32 ± 0.1vs. 0.76 ± 0.13, P=0.001). See Figure 4.5

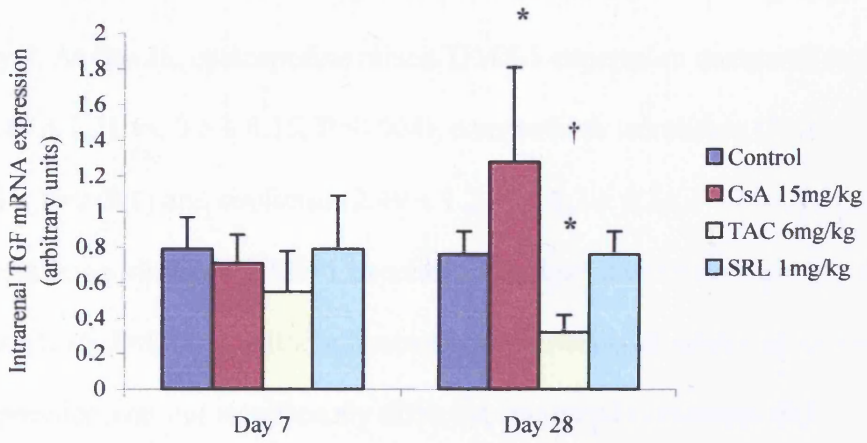


Figure 4.5 Intrarenal TGF-β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. CsA – cyclosporine, TAC – tacrolimus, SRL – sirolimus. Error bars represent one standard deviation of the mean.
* P<0.01 vs Control
† P<0.05 vs CsA and SRL at same time point

Collagen III mRNA expression was statistically similar between groups for those animals sacrificed at day 7. By day 28, collagen III expression was significantly raised by cyclosporine treatment compared to expression in control animals (1.59 ± 0.68 vs. 0.37 ± 0.19, P=0.002), in animals treated with tacrolimus (1.59 ± 0.68 vs. 0.27 ± 0.1, P=0.001) and sirolimus (1.59 ± 0.68 vs. 0.46 ± 0.22, P=0.003). See Figure 4.6.

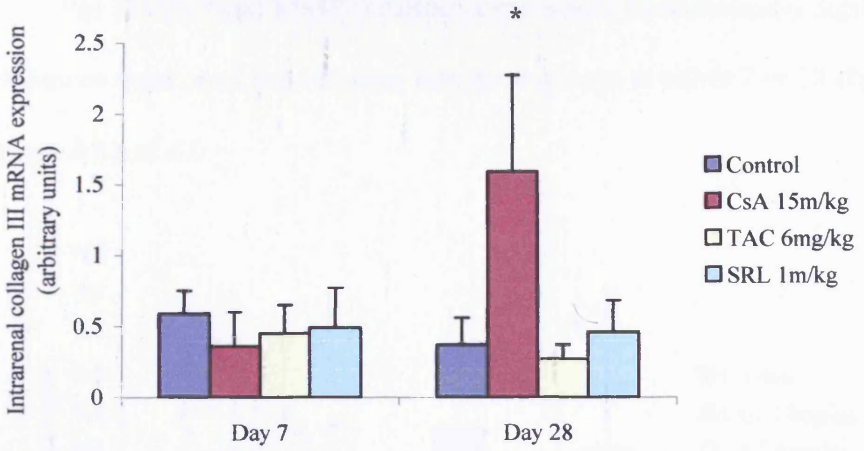


Figure 4.6 Intrarenal Collagen III mRNA expression. See Figure 4.5 legend for description.
* P<0.05 vs Control, TAC and SRL at same time point

For TIMP-1 mRNA expression the results showed no significant differences at day 7. At day 28, cyclosporine raised TIMP-1 expression compared to control animals (2.49 ± 1.21 vs. 0.6 ± 0.16, P=0.004), compared to tacrolimus (2.49 ± 1.21 vs. 0.12 ± 0.14, P=0.001) and sirolimus (2.49 ± 1.21 vs. 0.3 ± 0.34, P=0.002) treatment. Tacrolimus inhibited TIMP-1 expression compared to control animals (0.12 ± 0.14 vs. 0.6 ± 0.16, P=0.003). Although numerically lower with sirolimus treatment, TIMP-1 expression was not significantly different compared to controls (0.3 ± 0.34 vs. 0.6 ± 0.16, P=0.079). See Figure 4.7.

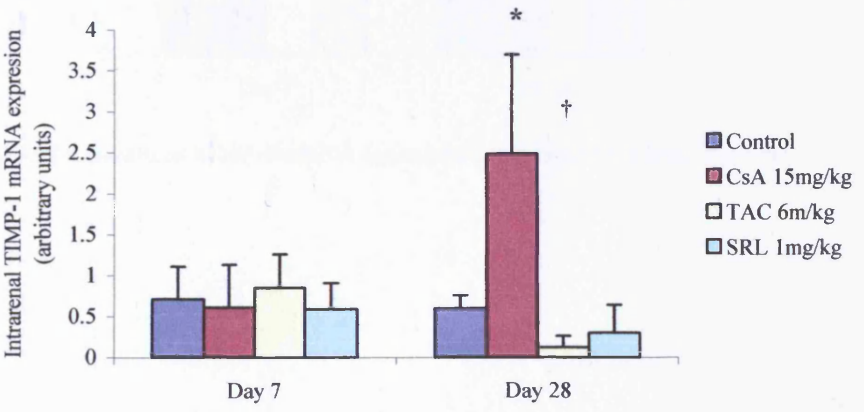


Figure 4.7 Intrarenal TIMP-1 mRNA expression. See Figure 4.5 legend for description.
* P<0.05 vs Control, TAC and SRL at same time point
† P=0.03 vs Control at same time point

For MMP-2 and MMP-9 mRNA expression, no statistically significant differences were observed between treatment groups at either 7 or 28 days. See Figures 4.8 and 4.9.

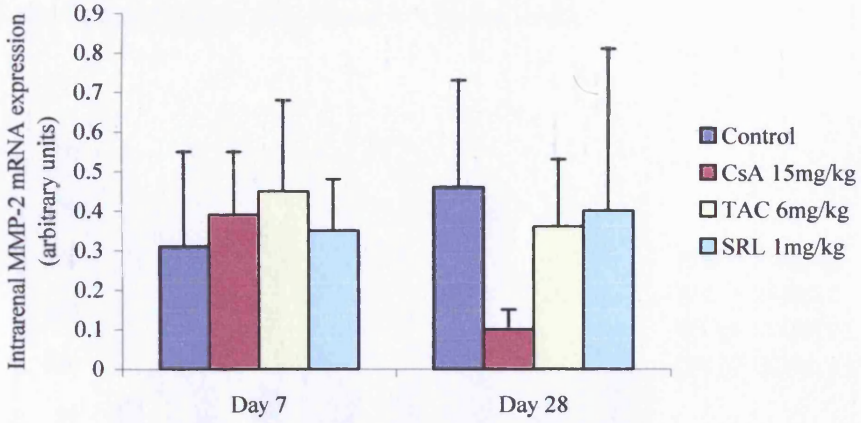


Figure 4.8 Intrarenal MMP-2 mRNA expression. See Figure 4.5 legend for description.

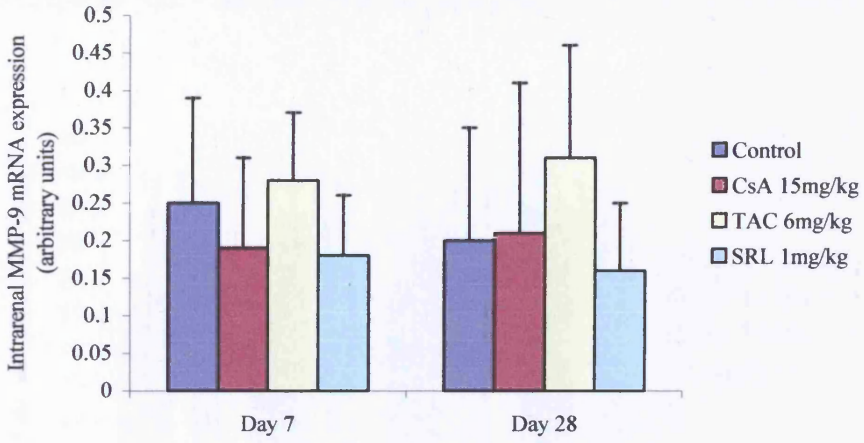


Figure 4.9 Intrarenal MMP-9 mRNA expression. See Figure 4.5 legend for description.

4.2 Section B: The effect of combination therapy - cyclosporine and sirolimus at varying doses.

4.2.1 Serum Creatinine

Cyclosporine was tested at doses of 15 and 7.5mg/kg/day and sirolimus at doses of 1, 0.5 and 0.1mg/kg/day. See Figures 4.10 and 4.11.

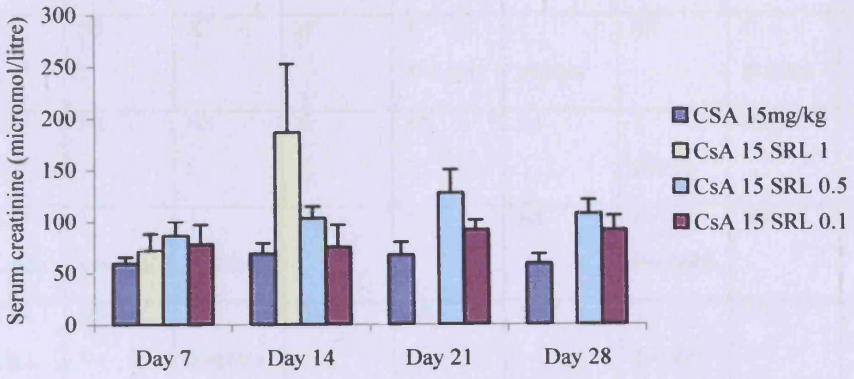


Figure 4.10 Serum creatinine (μmol/litre) for various drug treatments. Each bar represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, CsA = cyclosporine, SRL = sirolimus. Doses are mg/kg/day. See Figures 4.12-4.15 for P values.

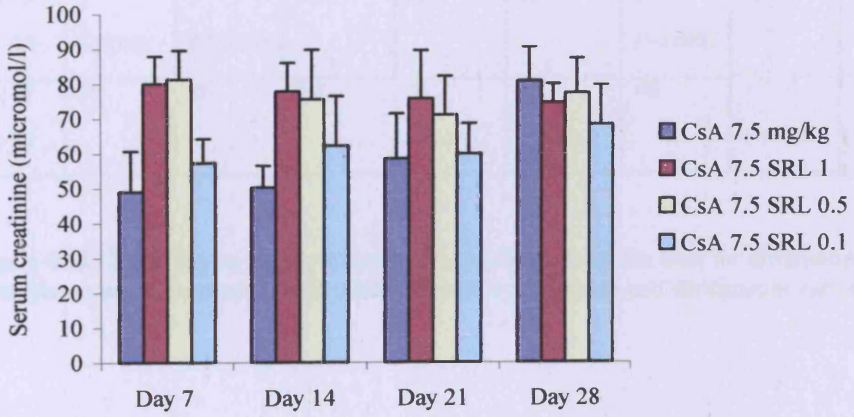


Figure 4.11 See Figure 4.10 legend for description

The tables below (Figures 4.12 to 4.15) show the significances for the groups tested against each other at seven, 14, 21 and 28 days.

	CsA 15	**SRL 1**	**CsA 15 SRL 1**	**CsA 15 SRL 0.5**	**CsA 15 SRL 0.1**	**CsA 7.5**	**CsA 7.5 SRL 1**	**CsA7.5 SRL 0.5**	**CSA 7.5 SRL 0.1**
CsA 15	**X**	NS	NS	↑ P=0.004	NS	↓ P=0.02	↑ P=0.003	↑ P=0.003	NS
SRL 1	NS	**X**	NS	↑ P=0.001	↑ P=0.004	NS	↑ P=0.001	↑ P=0.0006	NS
CsA 15 SRL 1	NS	NS	**X**	NS	NS	↑ P=0.02	NS	NS	NS
CsA 15 SRL 0.5	↓ P=0.004	↓ P=0.001	NS	**X**	NS	↓ P=0.0004	NS	NS	↓ P=0.001
CsA 15 SRL 0.1	NS	↓ P=0.004	NS	NS	**X**	↓ P=0.001	NS	NS	↓ P=0.03
CsA 7.5	↑ P=0.02	NS	↓ P=0.02	↑ P=0.0004	↑ P=0.001	**X**	↑ P=0.0002	↑ P=0.0002	NS
CsA 7.5 SRL 1	↓ P=0.003	↓ P=0.001	NS	NS	NS	↓ P=0.0002	**X**	NS	↓ P=0.0004
CsA 7.5 SRL 0.5	↑ P=0.003	↓ P=0.0006	NS	NS	NS	↓ P=0.0002	NS	**X**	↓ P=0.0003
CsA 7.5 SRL 0.1	NS	NS	NS	↑ P=0.001	↑ P=0.03	NS	↑ P=0.0004	↑ P=0.0003	**X**

Figure 4.12. Table displaying significance levels (Kruskall-Wallis test) for differences in serum creatinine at day 7 for combination treatment with cyclosporine and sirolimus at various doses.

	CsA 15	SRL 1	CsA 15 SRL 1	CsA 15 SRL 0.5	CsA 15 SRL 0.1	CsA 7.5	CsA 7.5 SRL 1.0	CsA 7.5 SRL 0.5	CsA 7.5 SRL 0.1
CsA 15	X	NS	↑ P=0.01	↑ P=0.003	NS	↓ P=0.001	NS	NS	NS
SRL 1	NS	X	↑ P=0.08	↑ P=0.0003	NS	↓ P=0.002	NS	NS	NS
CsA 15 SRL 1	↓ P=0.01	↓ P=0.008	X	↓ P=0.003	↓ P=0.008	↓ P=0.004	↓ P=0.001	↓ P=0.01	↓ P=0.007
CsA 15 SRL 0.5	↓ P=0.003	↓ P=0.0003	↑ P=0.003	X	↓ P=0.02	↓ P=0.0001	↓ P=0.002	↓ P=0.005	↓ P=0.0004
CsA 15 SRL 0.1	NS	NS	↑ P=0.008	↑ P=0.02	X	↓ P=0.04	NS	NS	NS
CsA 7.5	↑ P=0.001	↑ P=0.002	↑ P=0.004	↑ P=0.001	↑ P=0.004	X	↑ P=0.001	↑ P=0.007	NS
CsA 7.5 SRL 1.0	NS	NS	↑ P=0.01	↑ P=0.002	NS	↓ P=0.001	X	NS	↓ P=0.05
CsA 7.5 SRL 0.5	NS	NS	↑ P=0.001	↑ P=0.005	NS	↓ P=0.007	NS	X	NS
CsA 7.5 SRL 0.1	NS	NS	↑ P=0.007	↑ P=0.004	NS	NS	↑ P=0.05	NS	X

Figure 4.13. Table displaying significance levels (Kruskall-Wallis test) for differences in serum creatinine at day 14 for combination treatment with cyclosporine and sirolimus at various doses.

	CsA 15	SRL 1	CsA 15 SRL 1	CsA 15 SRL 0.5	CsA 15 SRL 0.1	CsA 7.5	CsA 7.5 SRL 1	CsA 7.5 SRL 0.5	CsA 7.5 SRL 0.1
CsA 15	X	NS	RIP	↑ P=0.0006	↑ P=0.003	NS	NS	NS	NS
SRL 1	NS	X	RIP	↑ P=0.006	↑ P=0.002	NS	NS	NS	NS
CsA 15 SRL 1	RIP	RIP	X	RIP	RIP	RIP	RIP	RIP	RIP
CsA 15 SRL 0.5	↓ P=0.0006	↓ P=0.006	RIP	X	↓ P=0.01	↓ P=0.003	↓ P=0.001	↓ P=0.009	↓ P=0.001
CsA 15 SRL 0.1	↓ P=0.003	↓ P=0.002	RIP	↑ P=0.01	X	↓ P=0.006	↓ P=0.04	↓ P=0.006	↓ P=0.002
CsA 7.5	NS	NS	RIP	↑ P=0.003	↑ P=0.006	X	↑ P=0.05	NS	NS
CsA 7.5 SRL 1	NS	NS	RIP	↑ P=0.001	↑ P=0.004	↓ P=0.05	X	NS	↓ P=0.04
CsA 7.5 SRL 0.5	NS	NS	RIP	↑ P=0.009	↑ P=0.006	NS	NS	X	NS
CsA 7.5 SRL 0.1	NS	NS	RIP	↑ P=0.001	↑ P=0.002	NS	↑ P=0.04	NS	X

Figure 4.14. Table displaying significance levels (Kruskall-Wallis test) for differences in serum creatinine at day 21 for combination treatment with cyclosporine and sirolimus at various doses. RIP- animals died before day 21

	CsA 15	SRL 1	CsA 15 SRL 1	CsA 15 SRL 0.5	CsA 15 SRL 0.1	CsA 7.5	CsA 7.5 SRL 1	CsA 7.5 SRL 0.5	CsA 7.5 SRL 0.1
CsA 15	X	↓ P=0.0001	RIP	NS	NS	↓ P=0.04	↓ P=0.002	↓ P=0.02	↓ P=0.002
SRL 1	↑ P=0.0001	X	RIP	↑ P=0.004	↑ P=0.003	↑ P=0.002	↑ P=0.0003	↑ P=0.006	NS
CsA 15 SRL 1	RIP	RIP	X	RIP	RIP	RIP	RIP	RIP	RIP
CsA 15 SRL 0.5	NS	↓ P=0.004	RIP	X	NS	↓ P=0.003	↓ P=0.001	↓ P=0.002	↓ P=0.004
CsA 15 SRL 0.1	NS	↓ P=0.003	RIP	NS	X	NS	↓ P=0.03	NS	↓ P=0.01
CsA 7.5	↑ P=0.04	↓ P=0.002	RIP	↑ P=0.003	NS	X	NS	NS	NS
CsA 7.5 SRL 1	↑ P=0.002	↓ P=0.003	RIP	↑ P=0.001	↑ P=0.03	NS	X	NS	NS
CsA 7.5 SRL 0.5	↑ P=0.02	↓ P=0.006	RIP	↑ P=0.002	NS	NS	NS	X	NS
CsA 7.5 SRL 0.1	↑ P=0.002	NS	RIP	↑ P=0.004	↑ P=0.01	NS	NS	NS	X

Figure 4.15. Table displaying significance levels (Kruskall-Wallis test) for differences in serum creatinine at day 28 for combination treatment with cyclosporine and sirolimus at various doses. RIP-animals died before day 28.

4.2.2 Urinary protein

The figures for mean urinary protein excretion, expressed as total protein mg/24hours, representing the mean of six samples, are presented in the Figures 4.16 and 4.17. One-way analysis of variance revealed no significant differences between values for groups at any time point.

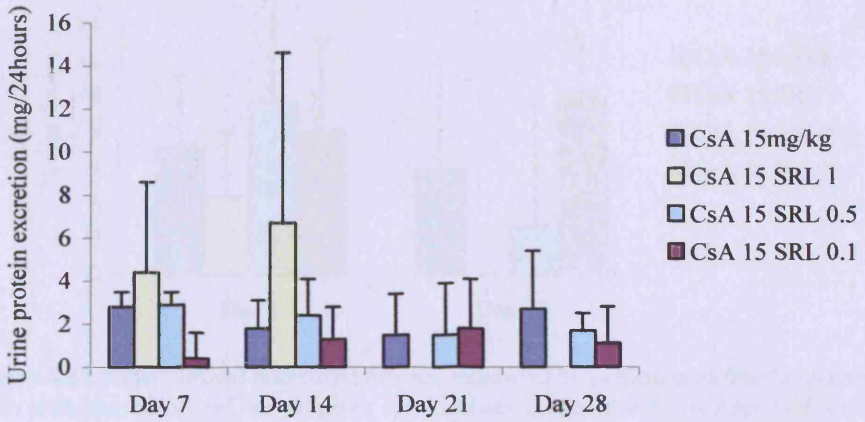


Figure 4.16 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, CsA = cyclosporine, SRL = sirolimus. Doses are mg/kg/day.

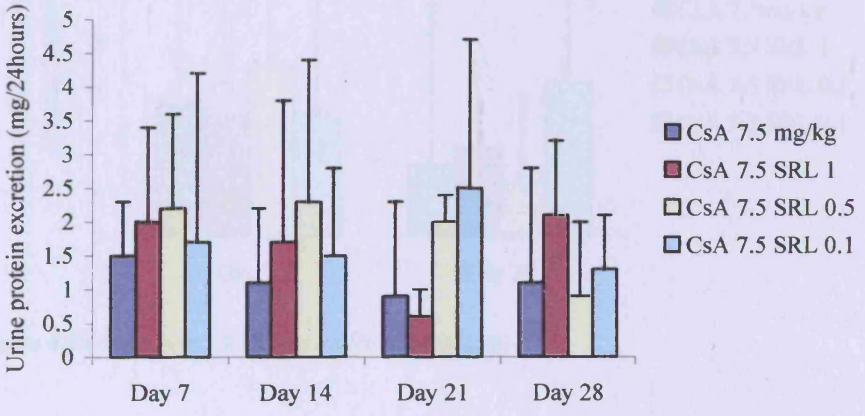


Figure 4.17 See Figure 4.16 legend for description.

4.2.3 Interstitial fibrosis

Interstitial fibrosis, measured as percentage area fraction staining by pico-sirius red, demonstrated no significant differences across groups. Data are presented in Figure 4.18 and 4.19.

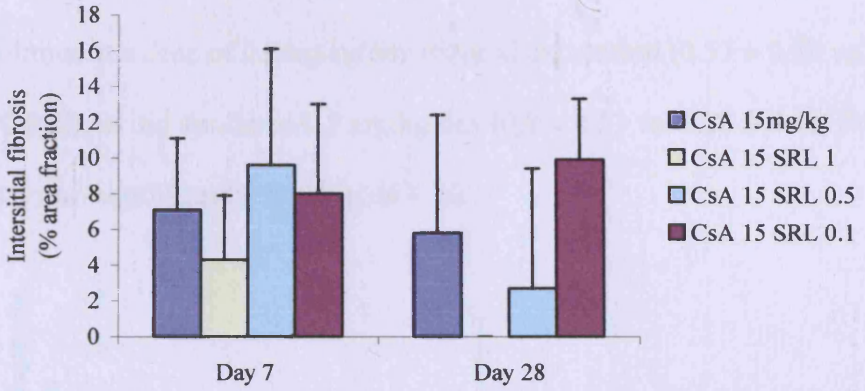


Figure 4.18 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with cyclosporine (CsA) and sirolimus (SRL) at varying doses (mg/kg/day). Error bars represent one standard deviation of the mean.

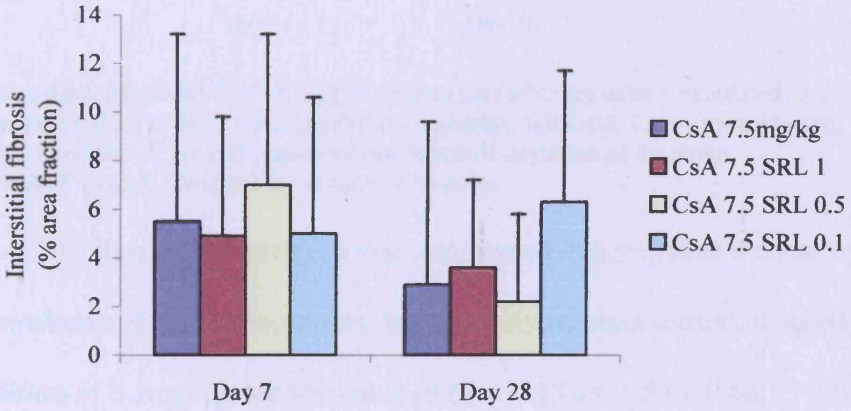


Figure 4.19 See Figure 4.18 legend for description.

4.2.4 RT-PCR messenger RNA expression

Renal cortical tissue from animals sacrificed at day 7 demonstrated no significant differences in the mRNA expression of TGF-β. At day 28, addition of sirolimus 1mg/kg day to cyclosporine (15mg/kg/day) caused a numerical but non-significant rise in TGF-β expression (1.91 ± 1.38 vs. 1.28 ± 0.53, P=0.32). Combining sirolimus at a dose of 0.5mg/kg/day reduced expression (0.57 ± 0.50 vs. 1.28 ± 0.53, P=0.018), as did sirolimus 0.1 mg/kg/day (0.8 ± 0.53 vs. 1.28 ± 0.53, P=0.148), the latter non-significantly. See Figure 4.20.

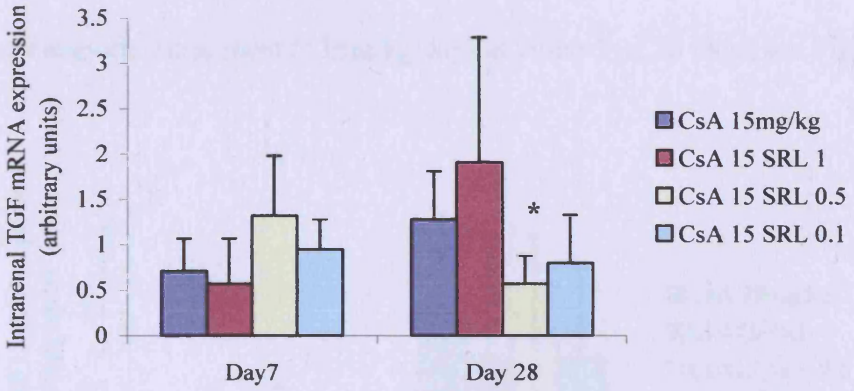


Figure 4.20. Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. CsA – cyclosporine, TAC – tacrolimus, SRL – sirolimus. Error bars represent one standard deviation of the mean.
* P=0.018 vs CsA 15mg/kg/day at same time point.

Collagen III expression was suppressed in a stepwise manner by the addition of sirolimus in decreasing doses, but this only reached statistical significance with the addition of 0.1mg/kg/day sirolimus (0.65 ± 0.53 vs. 1.59 ± 0.68, P=0.024). See Figure 4.21.

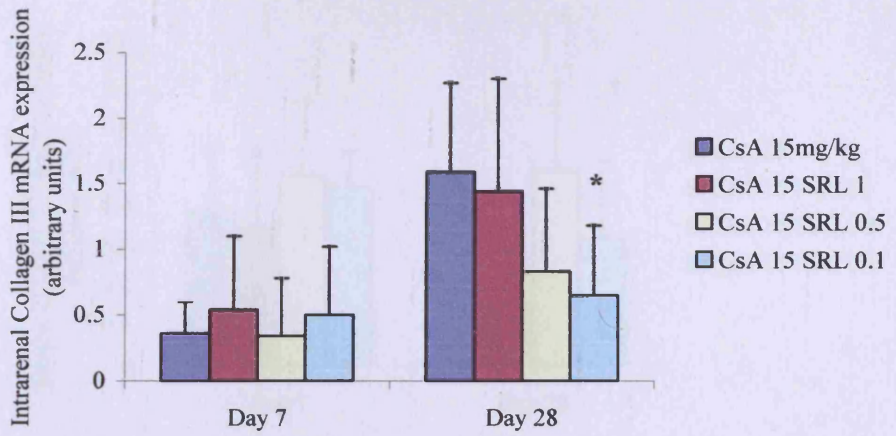


Figure 4.21 Intrarenal Collagen III mRNA expression. See Figure 4.20 for description.
*P=0.024 vs CsA 15mg/kg/day at same time point

The mRNA expression of TIMP-1 was unaltered by the addition of sirolimus to cyclosporine treatment (15mg/kg/day) at either 7 or 28 days, see Figure 4.22.

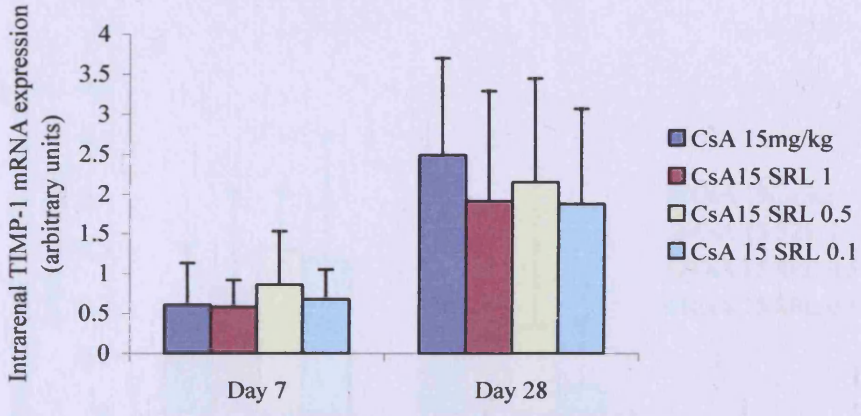


Figure 4.22 Intrarenal TIMP-1 mRNA expression. See Figure 4.21 for description.

The suppression of MMP-2 expression produced by 15mg/kg/day of cyclosporine was significantly reversed by the addition of 0.5 mg/kg/day of sirolimus (0.1 ± 0.05 vs. 0.49 ± 0.22, P=0.0017) and by 0.1 mg/kg/day sirolimus (0.1 ± 0.05 vs. 0.32 ± 0.19, P=0.021). Addition of the higher dose of sirolimus (1mg/kg/day) made no difference to MMP-2 expression compared to cyclosporine alone (0.15 ± 0.14 vs. 0.1 ± 0.05, P=0.43), see Figure 4.23

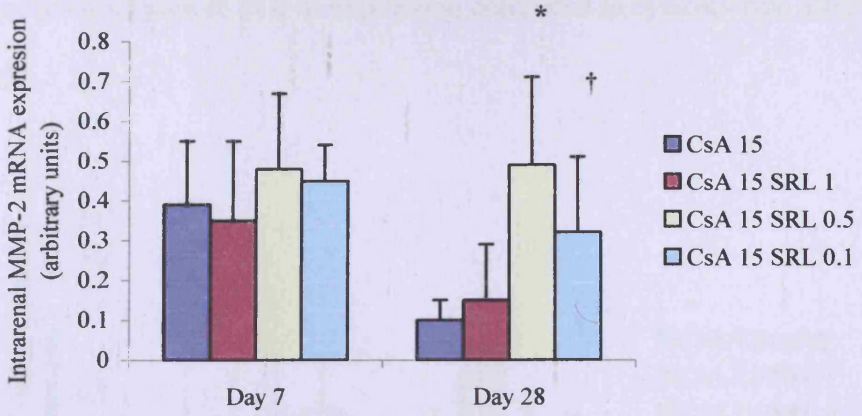


Figure 4.23 Intrarenal MMP-2 mRNA expression. See Figure 4.21 for description.
* P=0.0017 vs CsA 15mg/kg/day at same time point
† P=0.021 vs CsA 15mg/kg/day at same time point

The expression of MMP-9 was unaltered by the addition of sirolimus to cyclosporine at varying doses (Figure 4.24).

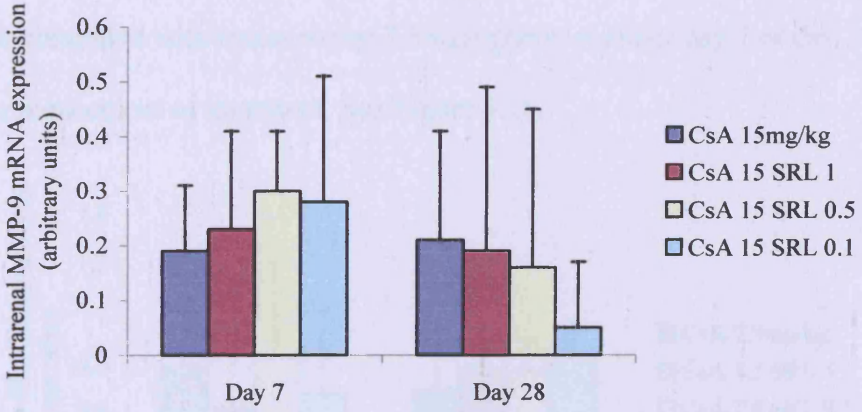


Figure 4.24 Intrarenal MMP-9 mRNA expression. See Figure 4.21 for description.

Examining the effect of the addition of varying doses of sirolimus to the lower dose of cyclosporine (7.5mg/kg), TGF-β expression seven days after starting treatment was unaltered by sirolimus. At day 28, addition of sirolimus 1mg/kg/day caused a significant rise in TGF-β expression (1.02 ± 0.38 vs. 0.51 ± 0.22, P=0.017). Neither 0.5 mg/kg/day sirolimus (P=0.488) nor 0.1 mg/kg/day (P=0.875) caused a

significant change in TGF-β expression compared to cyclosporine alone. See Figure 4.25.

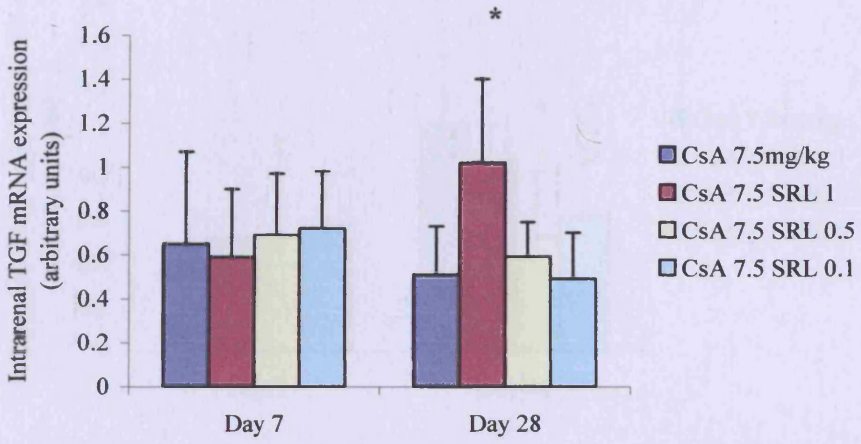


Figure 4.25 Intrarenal TGF-β mRNA expression. See Figure 4.21 for description.
* P=0.017 vs CsA 7.5mg/kg/day at same time point

There were no significant alterations in collagen III expression when sirolimus was combined with cyclosporine 7.5mg/kg/day, at either day 7 or day 28 after commencement of treatment. See Figure 4.26.

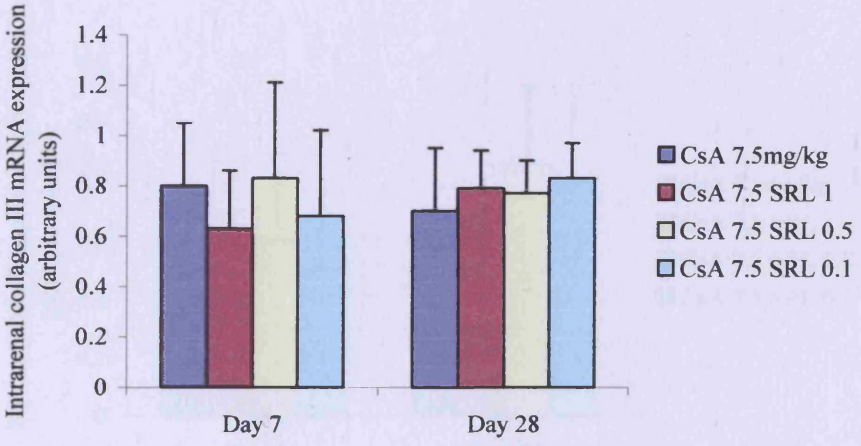


Figure 4.26 Intrarenal Collagen III mRNA expression. See Figure 4.21 for description.

At 28 days, sirolimus 0.5mg/kg/day suppressed TIMP-1 expression when combined with cyclosporine (0.51 ± 0.3 vs. 1.04 ± 0.3, P=0.012), as did sirolimus 0.1mg/kg/day (0.61 ± 0.25 vs. 1.04 ± 0.3, P=0.022) compared to cyclosporine alone.

The higher dose of sirolimus (1mg/kg/day) had no effect (0.87 ± 0.16 vs. 1.04 ± 0.3, P=0.249). See Figure 4.27.

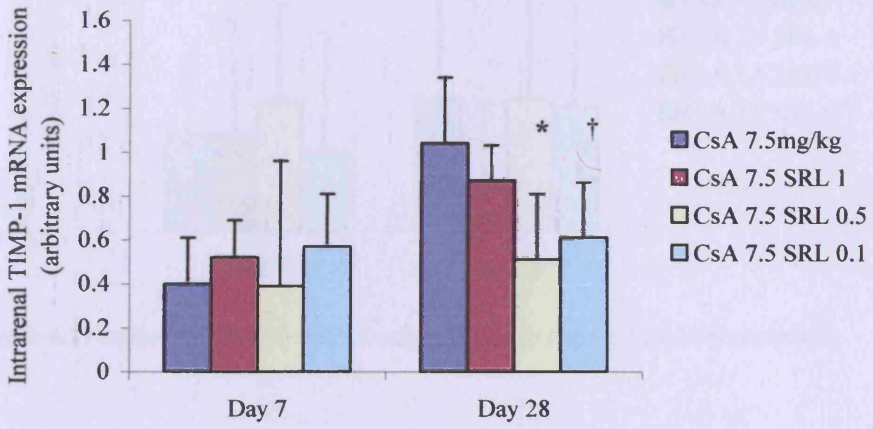


Figure 4.27 Intrarenal TIMP-1 mRNA expression. See Figure 4.21 for description.
* P=0.012 vs CsA 7.5mg/kg/day at same time point
† P=0.022 vs CsA 7.5mg/kg/day at same time point

For both forms of MMP, the addition of sirolimus had no effect on mRNA expression when added to cyclosporine 7.5mg/kg/day at either 7 or 28 days. See Figures 4.28 and 4.29.

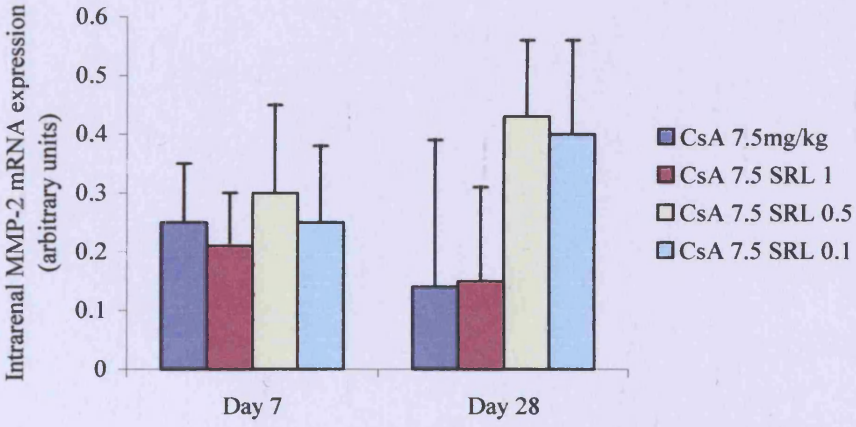


Figure 4.28 Intrarenal MMP-2 mRNA expression. See Figure 4.21 for description.

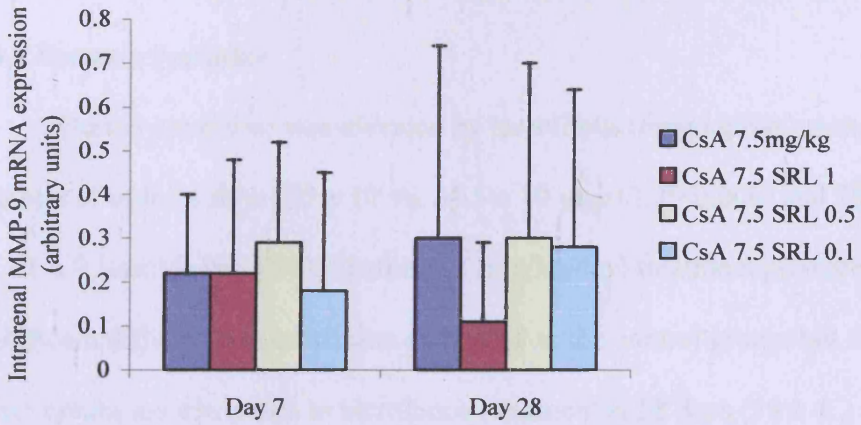


Figure 4.29 Intrarenal MMP-9 mRNA expression. See Figure 4.21 for description.

4.3 Section C: The effect of the combination of tacrolimus and sirolimus

4.3.1 Serum creatinine

Serum creatinine was elevated by tacrolimus (6mg/kg/day) compared to controls at both 21 days (75 ± 10 vs. 54.5 ± 10 μmol/l, P=0.006) and 28 days (75 ± 8 vs. 61 ± 7 μmol/l, P=0.008). Sirolimus (1mg/kg/day) treatment produced no significant difference in creatinine compared to the control group, but resulted in a lower creatinine compared to tacrolimus treatment at 28 days (59 ± 4.2 vs. 76 ± 8 μmol/l, P=0.001). Combination treatment with tacrolimus and sirolimus significantly raised serum creatinine compared to tacrolimus treatment alone at 7 days (68 ± 5.2 vs. 51 ± 10.1 μmol/l, P=0.004) and 14 days (77 ± 10.4 vs. 60 ± 7.8 μmol/l, P=0.008) but not at 21 days (P=0.86) or 28 days (P=0.07). See Figure 4.30.

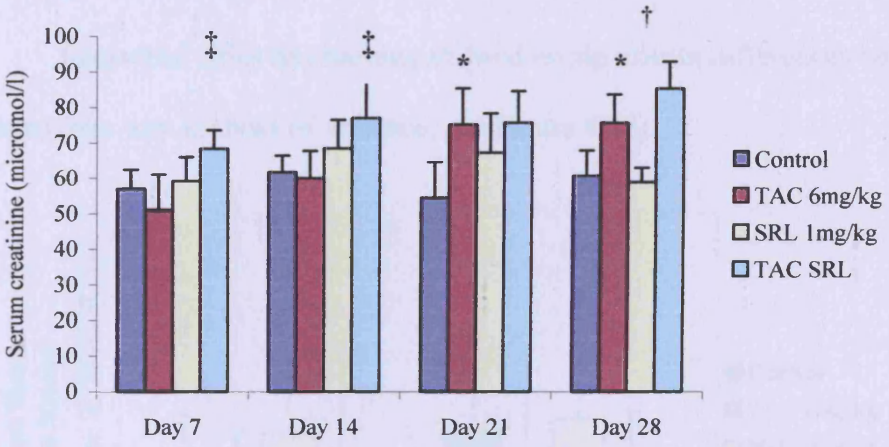


Figure 4.30 Serum creatinine (μmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, TAC = tacrolimus, SRL = sirolimus. Doses are mg/kg/day.
* P<0.05 vs controls at same time point
† P=0.001 vs TAC at same time point
‡ P<0.05 vs TAC at same point

4.3.2 Urinary protein

Urinary protein excretion showed no significant difference between groups (one way analysis of variance). See Figure 4.31, which displays mean urinary protein excretion (mg/24hr) ± standard deviation of the mean, for treatment with tacrolimus

6mg/kg/day (TAC), sirolimus 1mg/kg/day (SRL) and the combination of tacrolimus and sirolimus (TAC SRL).

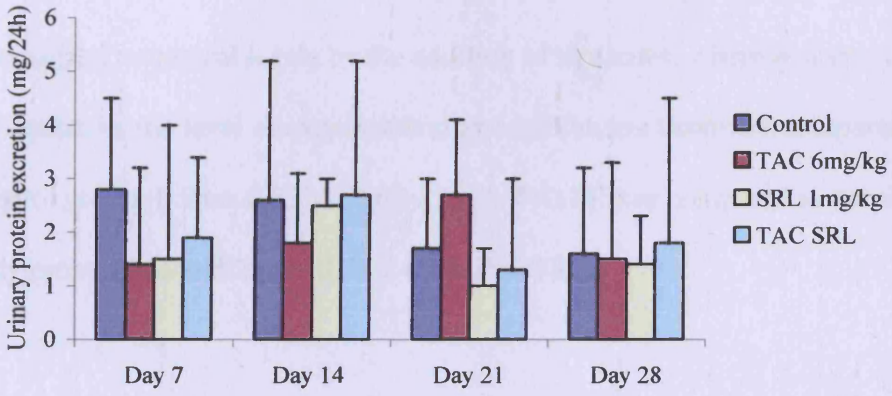


Figure 4.31 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, TAC = tacrolimus, SRL = sirolimus. Doses are mg/kg/day.

4.3.3 Interstitial fibrosis

Interstitial sirius red staining showed no significant differences between groups (one way analysis of variance, see Figure 4.32).

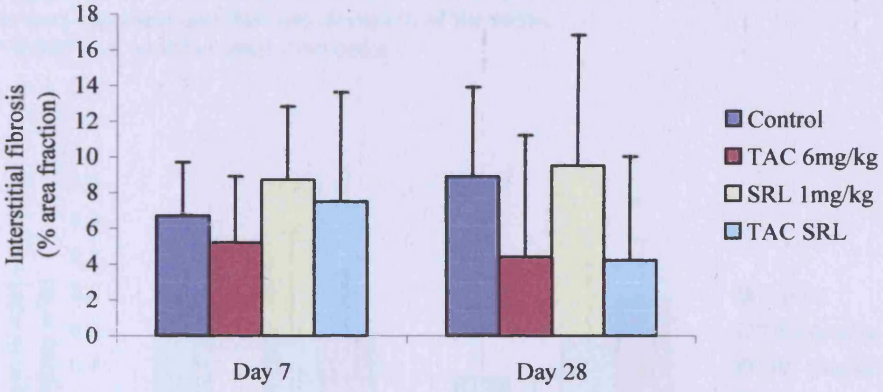


Figure 4.32 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with tacrolimus (TAC) and sirolimus (SRL). Doses are mg/kg/day. Error bars represent one standard deviation of the mean.

4.3.4 RT-PCR messenger RNA expression

The statistically significant suppression of TGF-β expression by tacrolimus compared to control animals at 28 days (0.32 ± 0.1 vs. 0.69 ± 0.13, P=0.005) was normalised to control levels by the addition of sirolimus, whereby there was no difference in the level of expression after combination treatment compared to the control group (0.88 ± 0.25 vs. 0.69 ± 0.13, P=0.143) or compared to the sirolimus-only group (0.88 ± 0.25 vs. 0.76 ± 0.13, P=0.332).

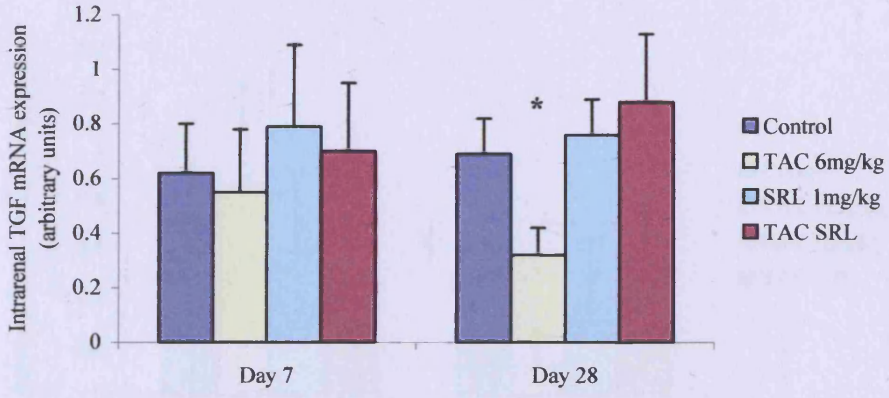


Figure 4.33 Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. TAC – tacrolimus, SRL – sirolimus. Error bars represent one standard deviation of the mean.
* P=0.005 vs Control at same time point

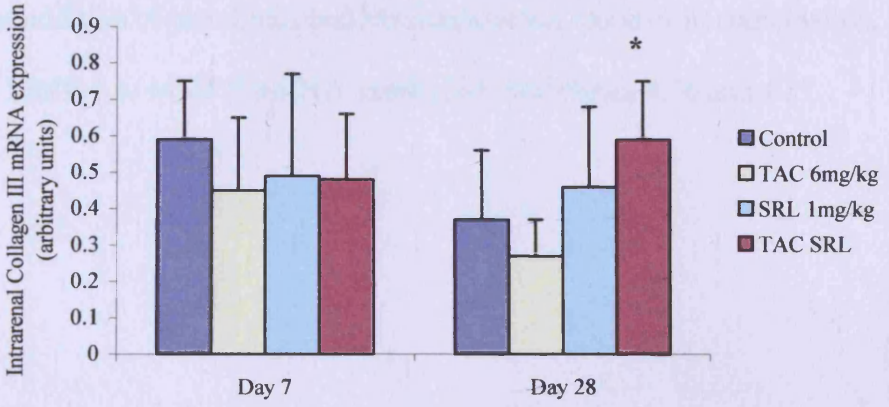


Figure 4.34 Intrarenal Collagen III mRNA expression. See Figure 4.33 legend for description
* P=0.03 vs TAC at same time point

A similar picture was observed for collagen III expression. Particularly noteworthy was that the combination of tacrolimus and sirolimus caused a statistically greater expression of collagen III than tacrolimus alone (0.59 ± 0.16 vs. 0.27 ± 0.1, P=0.003, see Figure 4.34).

TIMP-1 expression followed a similar pattern. A statistically significant depression in the expression of TIMP-1 by tacrolimus (0.12 ± 0.14 vs. 0.6 ± 0.16, P=0.004) was reversed when sirolimus is combined with tacrolimus (0.12 ± 0.14 vs. 0.56 ± 0.26, P=0.008). See Figure 4.35.

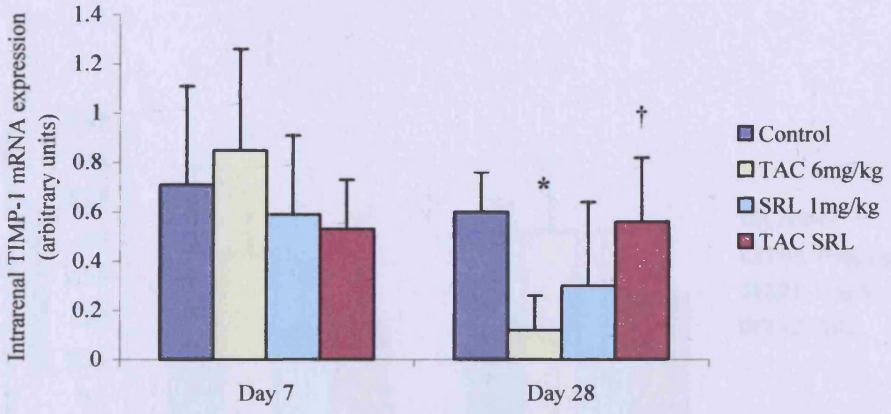


Figure 4.35 Intrarenal TIMP-1 mRNA expression. See Figure 4.33 legend for description
* P=0.004 vs Control
† P=0.008 vs TAC

The addition of tacrolimus and sirolimus, either alone or in combination, had no effect on MMP-2 or MMP-9 mRNA expression. See Figure 4.36 and 4.37.

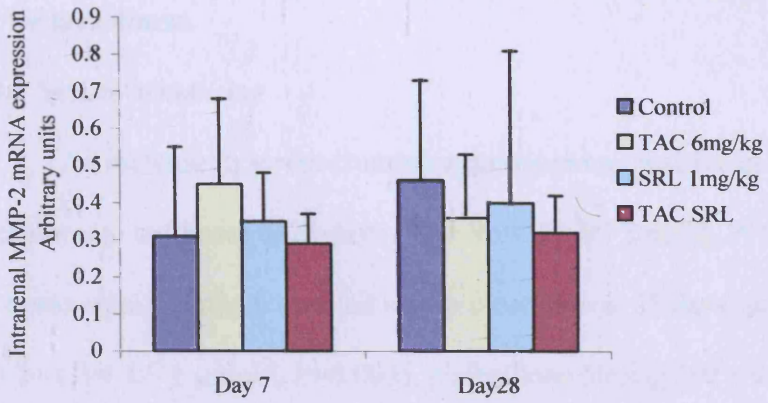


Figure 4.36 Intrarenal MMP-2 mRNA expression. See Figure 4.33 legend for description

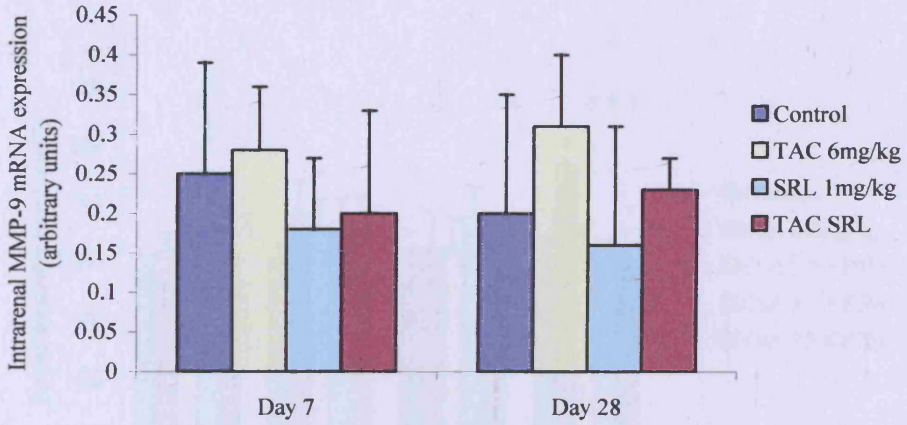


Figure 4.37 Intrarenal MMP-9 mRNA expression. See Figure 4.33 legend for description

4.4 Section D: Effects of pirfenidone when added to treatment with cyclosporine or tacrolimus.

4.4.1 Serum creatinine

An increase in serum creatinine compared to positive controls was seen for cyclosporine treatment at 28 days (94 ± 9 vs. 61 ± 7 µmol/l, P=0.0001). Pirfenidone at all doses significantly attenuated serum creatinine at 28 days; pirfenidone 250mg (64 ± 9.2 vs. 94 ± 9.1 µmol/l, P=0.003), pirfenidone 500mg (59 ± 9 vs. 94 ± 9 µmol/l, P<0.001), pirfenidone 750mg (62 ± 10 vs. 94 ± 9 µmol/l, P<0.001). There were no significant differences in serum creatinine at any time point for the different doses of pirfenidone (Figure 4.38).

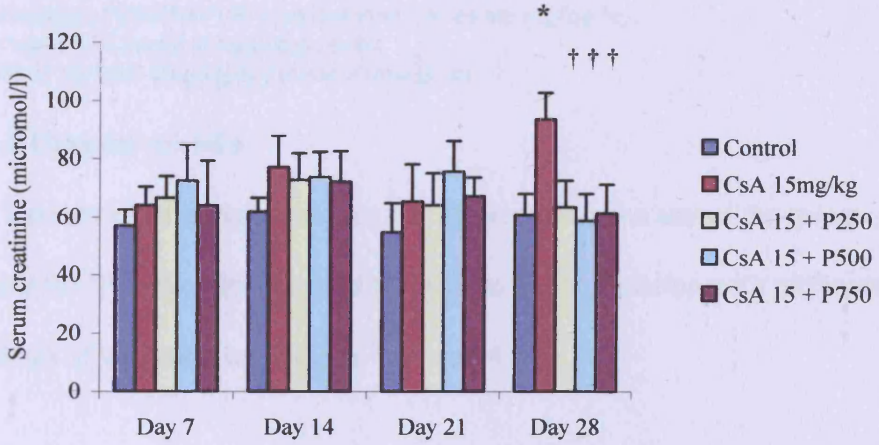


Figure 4.38 Serum creatinine (µmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, CsA = cyclosporine, P250/500/750 = pirfenidone. Doses are mg/kg/day.
* P=0.0001 vs Control at same time point
† P<0.01 vs CsA 15mg/kg/day at same time point

Tacrolimus caused a rise in serum creatinine compared to the low salt-diet treated controls at 21 days (75 ± 10.2 vs. 55 ± 10 µmol/l, P=0.001) and 28 days (75 ± 8 vs. 61 ± 7 µmol/l, P=0.01). Addition of pirfenidone (at all doses) significantly reduced serum creatinine compared to tacrolimus treatment alone (54 ± 8 vs. 76 ± 8

μmol/l, P=0.002 for pirfenidone 250mg/kg/day, 55 ± 10 vs. 76 ± 8 μmol/l, P=0.009 for pirfenidone 500mg/kg/day, and 57 ± 7 vs. 76 ± 8 μmol/l, P=0.004 for pirfenidone 750 mg/kg/day). See Figure 4.39

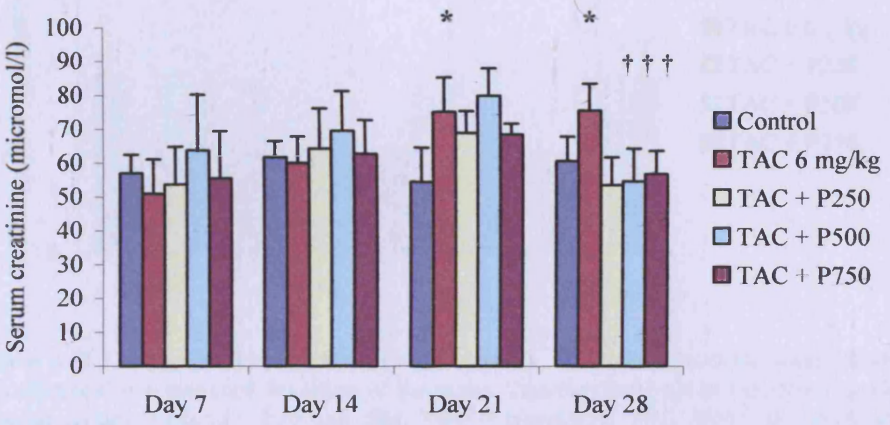


Figure 4.39 Serum creatinine (μmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, TAC = tacrolimus, P250/500/750 = pirfenidone. Doses are mg/kg/day.
* P<=0.01 vs Control at same time point
† P<0.01 vs TAC 6mg/kg/day at same time point

4.4.2 Urinary protein

Urinary protein excretion was no different between any of the groups at all time points for both cyclosporine and tacrolimus in combination with pirfenidone (one way analysis of variance, see Figures 4.40 and 4.41).

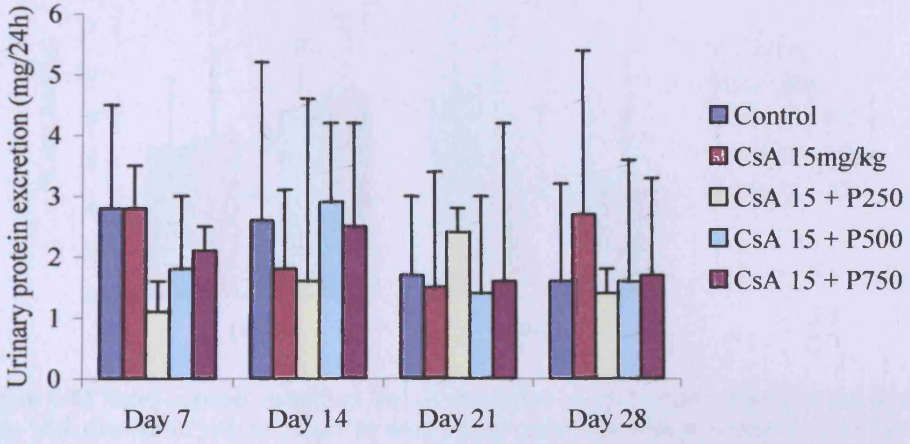


Figure 4.40 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, CsA = cyclosporine, P250/500/750 = pirfenidone. Doses are mg/kg/day.

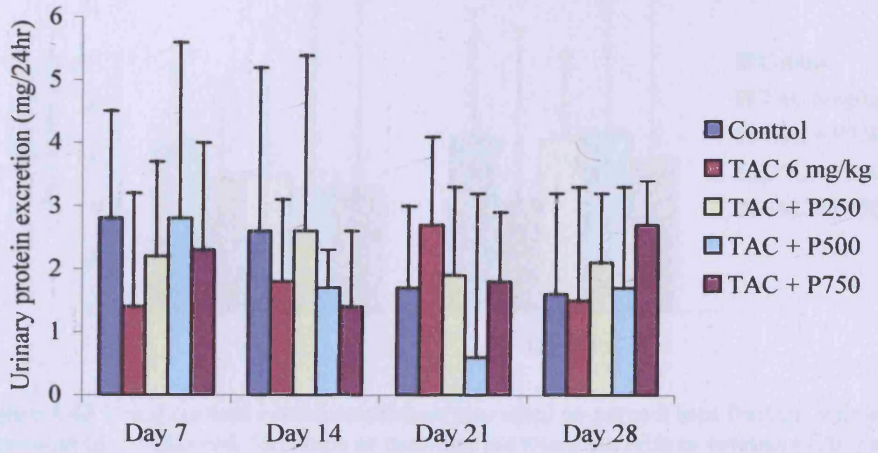


Figure 4.41 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, TAC = tacrolimus, P250/500/750 = pirfenidone. Doses are mg/kg/day.

4.4.3 Interstitial Fibrosis

Interstitial fibrosis was not significantly different between groups for both cyclosporine and tacrolimus in combination with pirfenidone at all time points (one way analysis of variance, see Figures 4.42 and 4.43).

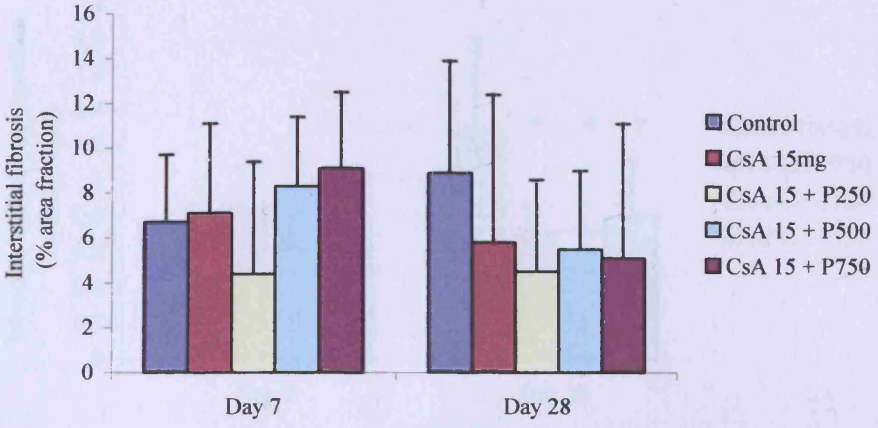


Figure 4.42 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with cyclosporine (CsA) and pirfenidone at varying doses (mg/kg/day). Error bars represent one standard deviation of the mean.

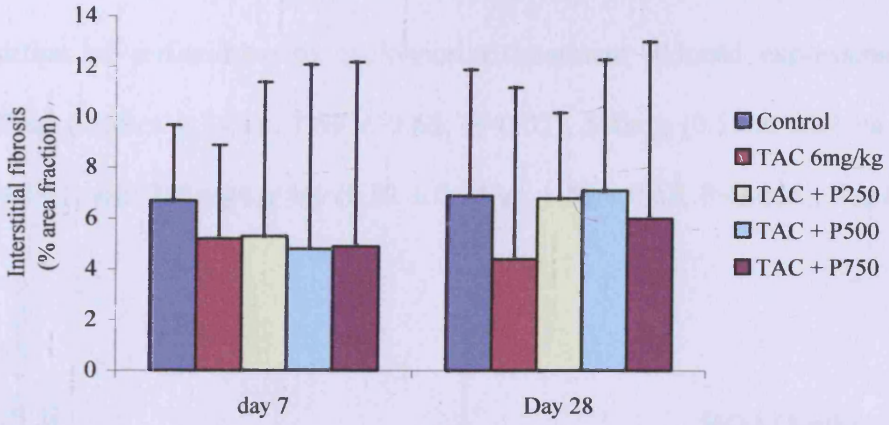


Figure 4.43 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with tacrolimus (TAC) and pirfenidone at varying doses (mg/kg/day) Error bars represent one standard deviation of the mean.

4.4.4 RT-PCR quantification of mRNA expression

Whilst there were no significant differences for TGF-β expression at day 7, addition of pirfenidone (at all doses) to cyclosporine treatment reduced the expression of TGF-β mRNA (day 28). This was significant for 500mg pirfenidone (0.71 ± 0.16 vs. 1.28 ± 0.53, P=0.05), and 250mg (0.71 ± 0.1 vs. 1.28 ± 0.53, P=0.053) but not for 750mg (0.80 ± 0.31 vs. 1.28 ± 0.53, P=0.09) pirfenidone. See Figure 4.44.

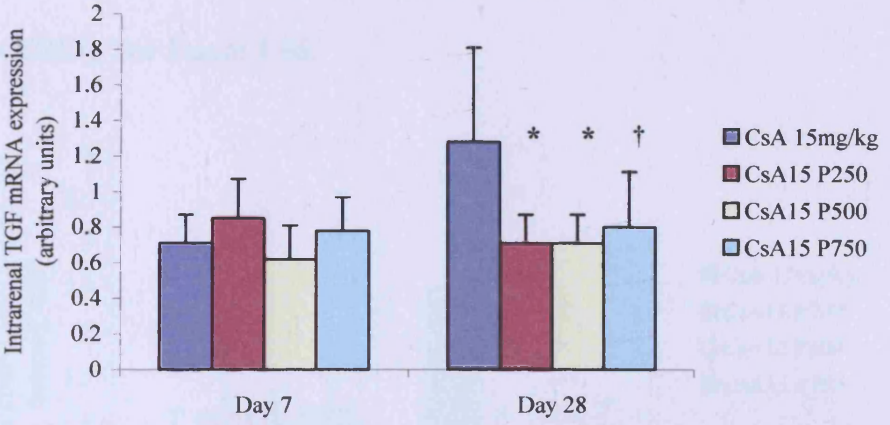


Figure 4.44 Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. CsA – cyclosporine, P250/500/750 – pirfenidone. Doses are mg/kg/day. Error bars represent one standard deviation of the mean.
* P=0.05 vs CsA 15mg/kg/day at same time point
† P=0.09 vs CsA 15mg/kg/day at same time point

A similar picture was demonstrated with collagen III mRNA expression (day 28). The addition of pirfenidone to cyclosporine treatment reduced expression at doses of 250mg (0.68 ± 0.34 vs. 1.59 ± 0.68, P=0.02), 500mg (0.51 ± 0.37 vs. 1.59 ± 0.68, P=0.011) and 750mg/kg/day (0.81 ± 0.24 vs. 1.59 ± 0.68, P=0.038). See Figure 4.45.

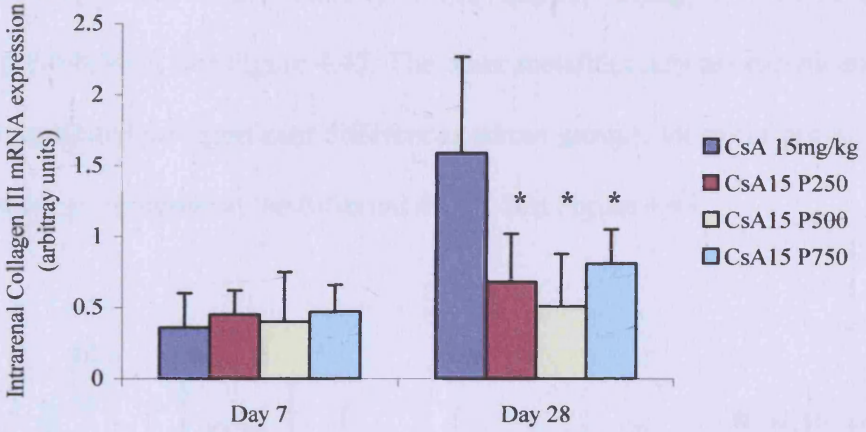


Figure 4.45 Intrarenal Collagen III mRNA expression. See Figure 4.44 legend for description
* P<0.05 vs CsA 15mg/kg/day at same time point

The pattern was the same for TIMP-1 expression at day 28; addition of pirfenidone to cyclosporine treatment reduced the expression of TIMP-1 mRNA at pirfenidone doses of 250mg (0.80 ± 0.34 vs. 2.49 ± 1.21, P=0.0216), 500mg (1.03 ± 0.43 vs. 2.49 ± 1.21, P=0.0318) and 750mg/kg/day (0.88 ± 0.37 vs. 2.49 ± 1.21, P=0.0263). See Figure 4.46.

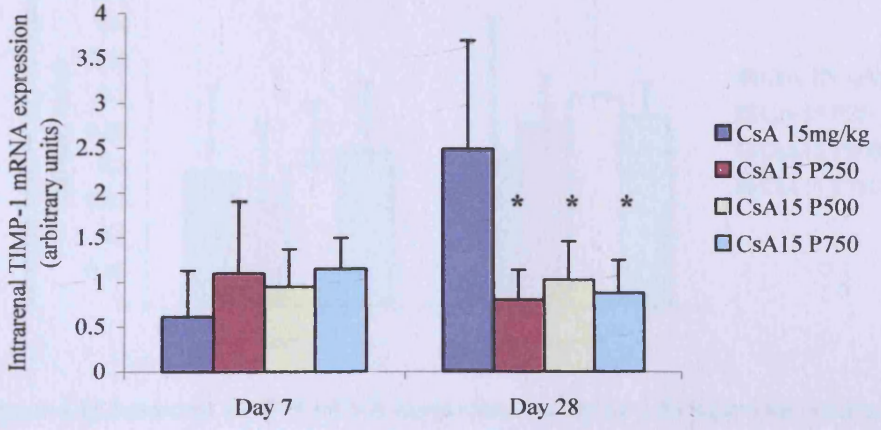


Figure 4.46 Intrarenal TIMP-1 mRNA expression. See Figure 4.44 legend for description
* P<0.05 vs CsA 15mg/kg/day at same time point

The suppressed expression of MMP-2 mRNA produced by cyclosporine was significantly reversed by pirfenidone in a non-dose dependent manner. Pirfenidone, at a dose of 250mg/kg/day increased MMP-2 expression compared to cyclosporine treatment alone (0.33 ± 0.12 vs. 0.1 ± 0.05, P=0.005). For the 500mg dose, the values were 0.42 ± 0.16 vs. 0.1 ± 0.05 (P=0.005) and for 750mg, were 0.34 ± 0.11 vs. 0.1 ± 0.05 (P=0.003). See Figure 4.47. The other metalloproteinase examined, MMP-9, demonstrated no significant differences across groups, for cyclosporine with or without pirfenidone at the different doses. See Figure 4.48.

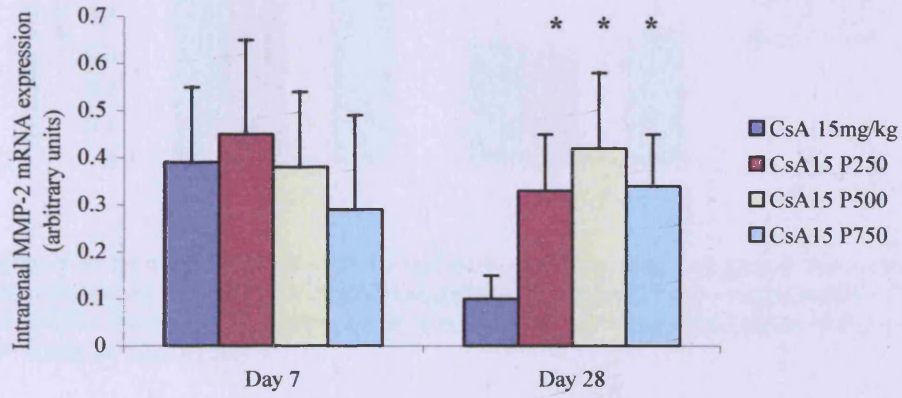


Figure 4.47 Intrarenal MMP-2 mRNA expression. See Figure 4.44 legend for description
* P<=0.05 vs CsA 15mg/kg/day at same time point

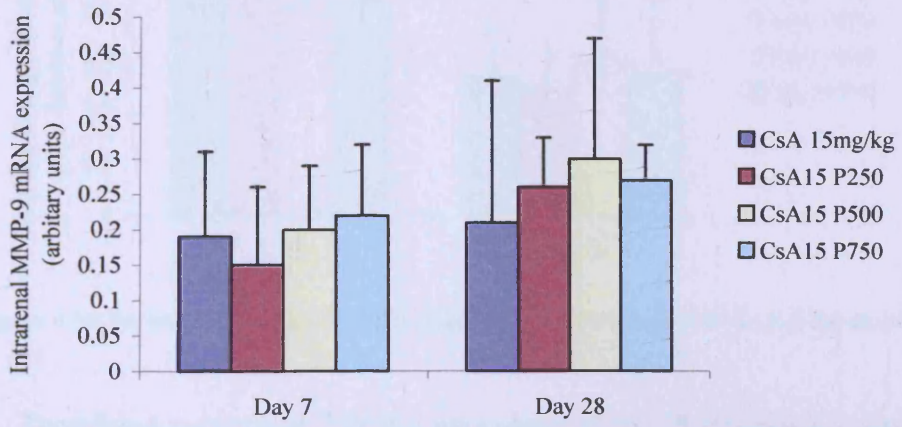


Figure 4.48 Intrarenal MMP-9 mRNA expression. See Figure 4.44 legend for description

Despite the suppressant effect (marginal significance) of tacrolimus on TGF-β expression at day 28 compared to day 7 (0.55 ± 0.23 vs. 0.32 ± 0.1, P=0.068), there were no significant differences for the pirfenidone groups compared to tacrolimus alone at the two time points (Figure 4.49). A similar pattern was demonstrated for collagen III expression (Figure 4.50).

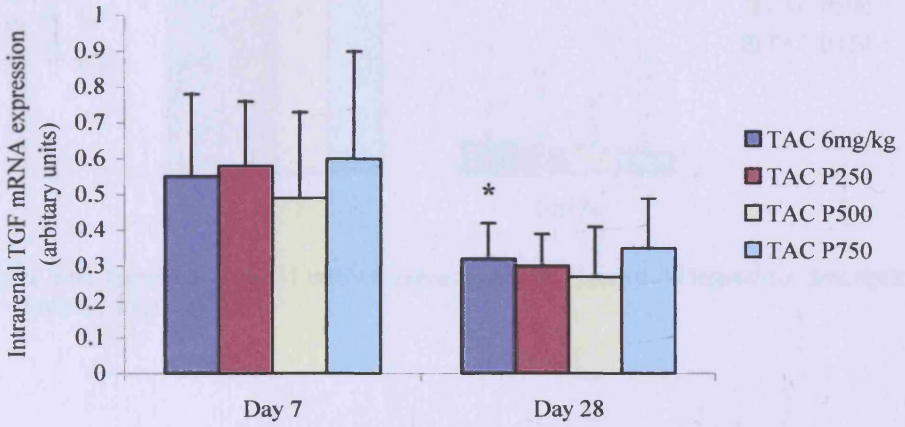


Figure 4.49 Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. TAC – tacrolimus, P250/500/750 – pirfenidone. Doses are mg/kg/day. Error bars represent one standard deviation of the mean.
* P=0.068 vs TAC at Day 7

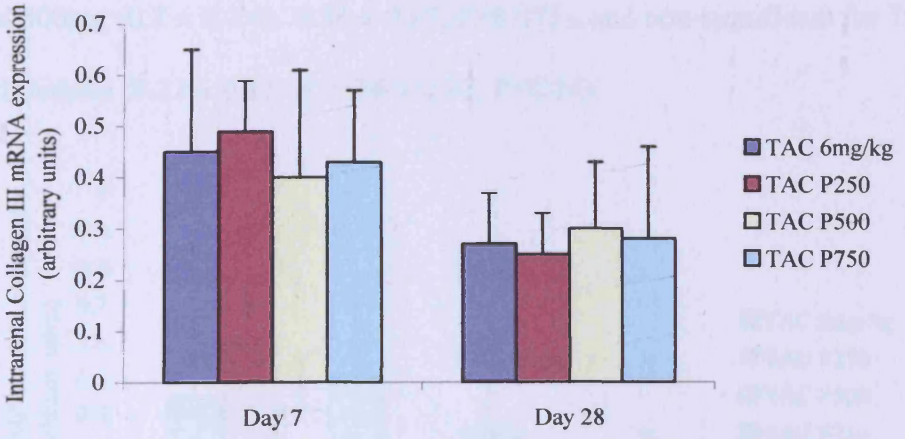


Figure 4.50 Intrarenal Collagen III mRNA expression. See Figure 4.49 legend for description.

Tacrolimus suppressed TIMP-1 expression at day 28 compared to day 7 (0.85 ± 0.41 vs. 0.12 ± 0.14 arbitrary units, P=0.0062), but there were no significant

differences for the pirfenidone groups compared to tacrolimus alone at the two time points, i.e. pirfenidone did not further suppress TIMP1 expression (see Figure 4.51).

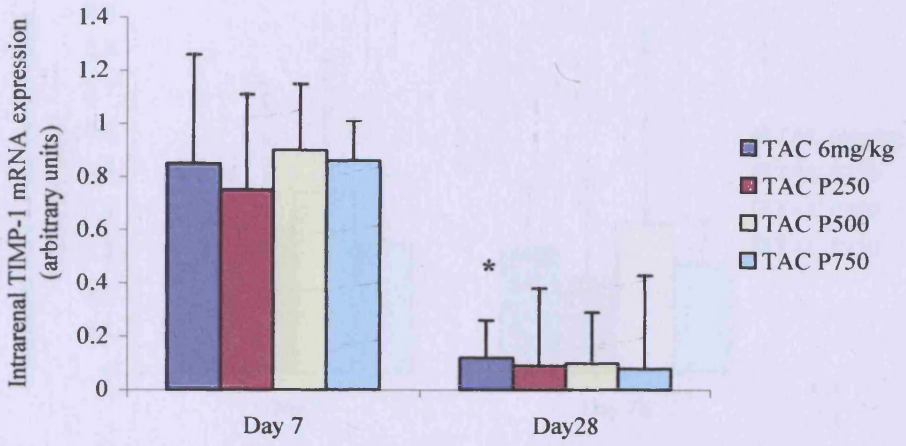


Figure 4.51 Intrarenal TIMP-1 mRNA expression. See Figure 4.49 legend for description.
* P=0.006 vs TAC at Day 7

Although addition of pirfenidone to tacrolimus produced a non-dose dependent numerical reduction in MMP-2 expression (see Figure 4.52) at 28 days, this was of borderline significance for 250mg pirfenidone (0.21 ± 0.08 vs. 0.36 ± 0.17, P=0.079), and 500mg (0.2 ± 0.1 vs. 0.36 ± 0.17, P=0.075), and non-significant for 750mg pirfenidone (0.22 ± 0.13 vs. 0.36 ± 0.17, P=0.14).

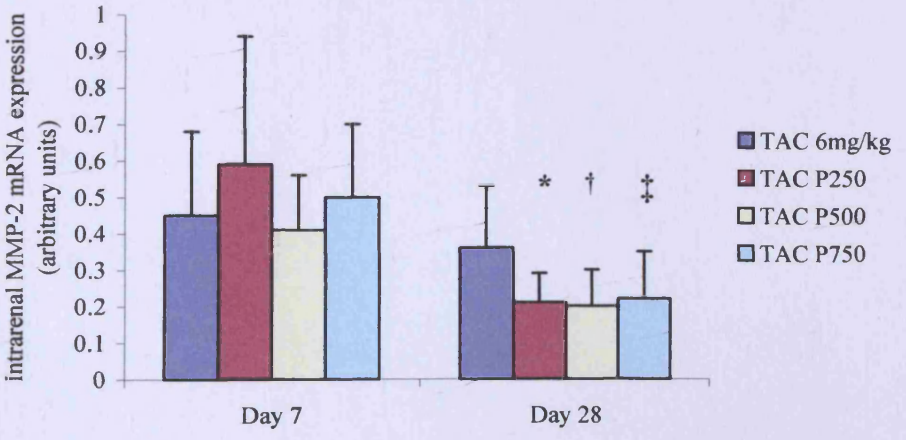


Figure 4.52 Intrarenal MMP-2 mRNA expression. See Figure 4.49 legend for description.
* P=0.079 vs TAC at same time point
† P=0.075 vs TAC at same time point
‡ P=0.14 vs TAC at same time point

MMP-9 expression was unaltered by the addition of pirfenidone to tacrolimus at either 7 or 28 days (Figure 4.53).

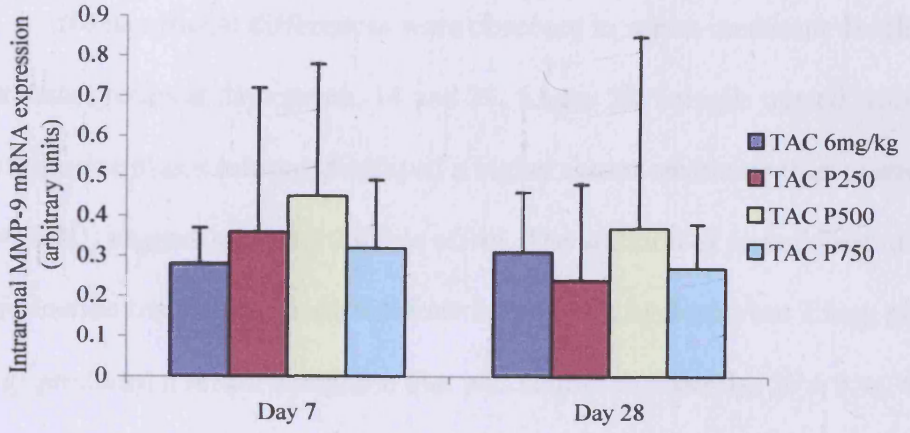


Figure 4.53 Intrarenal MMP-9 mRNA expression. See Figure 4.49 legend for description.

4.5 Section E: The effect of pirfenidone when added to a combination of calcineurin inhibitor plus sirolimus.

4.5.1 Serum creatinine

No significant differences were observed in serum creatinine levels between treatment groups at days seven, 14 and 21. At day 28, animals treated with cyclosporine plus sirolimus displayed a higher serum creatinine than control animals (P=0.002), suggesting a nephrotoxic effect. The addition of pirfenidone to combination treatment (i.e. pirfenidone 500mg plus cyclosporine 7.5mg plus sirolimus 1mg) produced a serum creatinine that was similar to controls (56 ± 9 vs. 61 ± 7 respectively, P=0.378), but significantly lower than combination treatment alone (56 ± 10 vs. 82 ± 5, P=0.002) suggesting pirfenidone abets the nephrotoxic effect of combination treatment. See Figure 4.54. An identical pattern was seen for combination treatment with tacrolimus plus sirolimus with or without the addition of pirfenidone (see Figure 4.55).

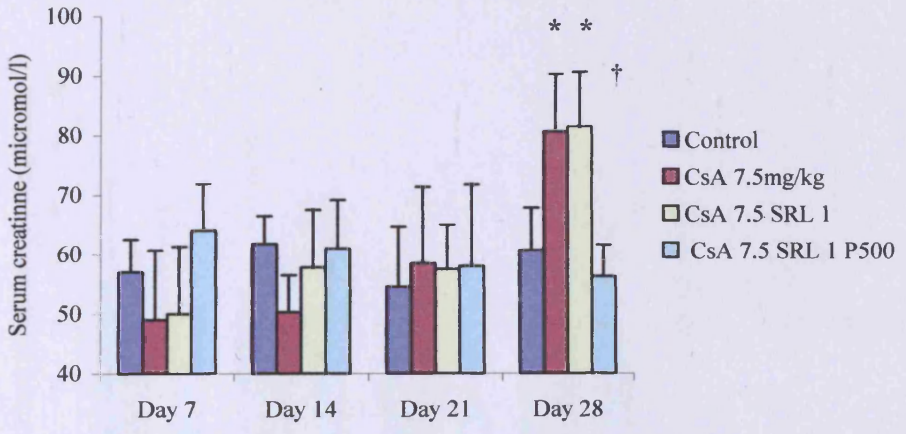


Figure 4.54 Serum creatinine (μmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, CsA = cyclosporine, SRL = sirolimus, P500 = pirfenidone. Doses are mg/kg/day.
* P<0.05 vs Control at same time point
† P=0.002 vs CsA 7.5 + SRL 1 mg/kg/day at same time point

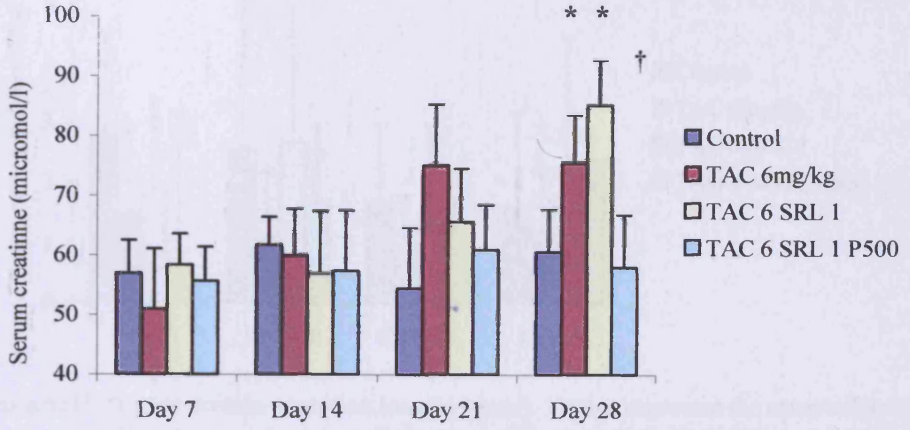


Figure 4.55 Serum creatinine (μmol/litre) for various drug treatments. Each point represents the mean value for six rats. Error bars represent one standard deviation of the mean. Control = low salt diet, TAC = tacrolimus, SRL = sirolimus, P500 = pirfenidone. Doses are mg/kg/day.
* $P<0.05$ vs Control at same time point
† $P<0.01$ vs TAC 6 + SRL 1 mg/kg/day at same time point

4.5.2 Urinary protein

Urinary protein excretion was unchanged amongst all groups (one-way analysis of variance). See Figures 4.56 and 4.57.

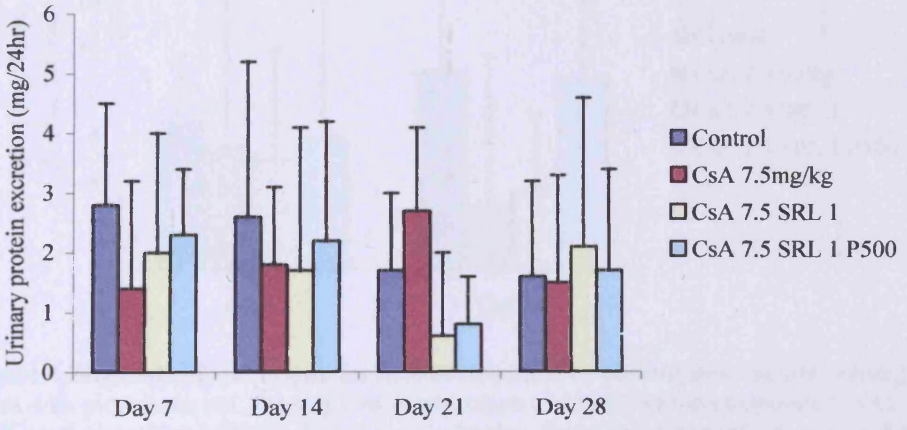


Figure 4.56 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, CsA = cyclosporine, SRL = sirolimus, P500 = pirfenidone. Doses are mg/kg/day.

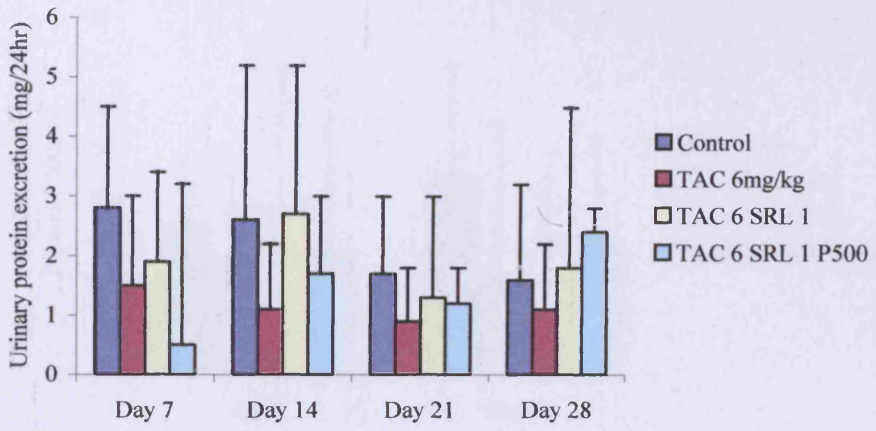


Figure 4.57 Urinary protein excretion (mg/24 hours). Values represent the mean of six animals. Error bars represent one standard deviation of the mean. One-way analysis of variance revealed no difference between groups. Control = low salt diet, TAC = tacrolimus, SRL = sirolimus, P500 = pirfenidone. Doses are mg/kg/day.

4.5.3 Interstitial fibrosis

Interstitial fibrosis evaluation using Sirius red staining revealed no differences across groups, one-way analysis of variance. See Figures 4.58 and 4.59.

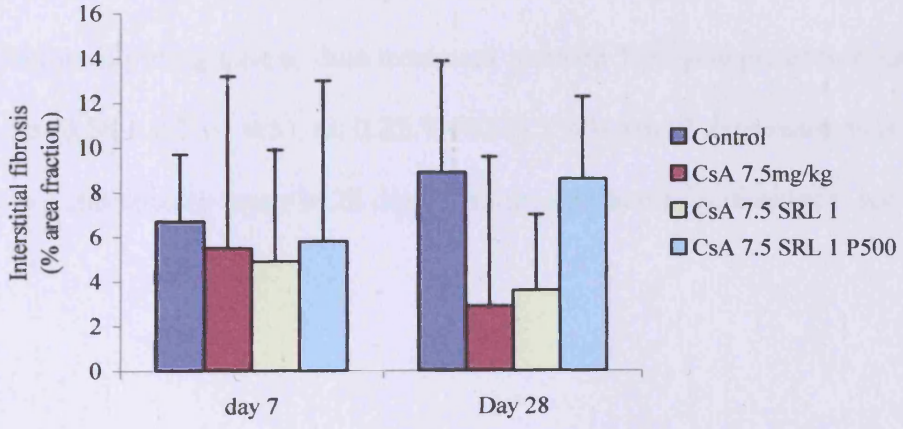


Figure 4.58 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with cyclosporine (CsA), sirolimus (SRL) and pirfenidone (P500). Doses are mg/kg/day. Error bars represent one standard deviation of the mean.

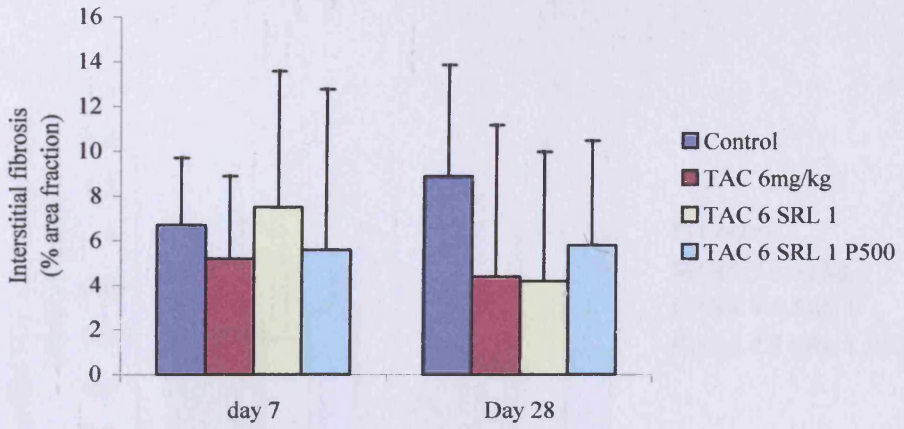


Figure 4.59 Renal cortical interstitial fibrosis measured by percent area fraction staining of cortical slices with pico-sirius red, for single or combination treatment with tacrolimus (TAC), sirolimus (SRL) and pirfenidone (P500). Doses are mg/kg/day. Error bars represent one standard deviation of the mean.

4.5.4 RT-PCR messenger RNA expression

TGF-β expression in kidney samples taken from animals sacrificed after 7 days of treatment was unaltered in those groups tested, displayed in Figure 4.60. At 28 days TGF-β expression in the group subjected to combination treatment was higher than in those treated with cyclosporine alone (1.02 ± 0.38 vs. 0.51 ± 0.22, P=0.021). Addition of pirfenidone to dual treatment reduced TGF-β expression back to control levels (0.54 ± 0.2 vs. 0.51 vs. 0.22, P=0.81). Collagen III expression was numerically but not statistically lower at 28 days with the addition of pirfenidone, see Figure 4.61.

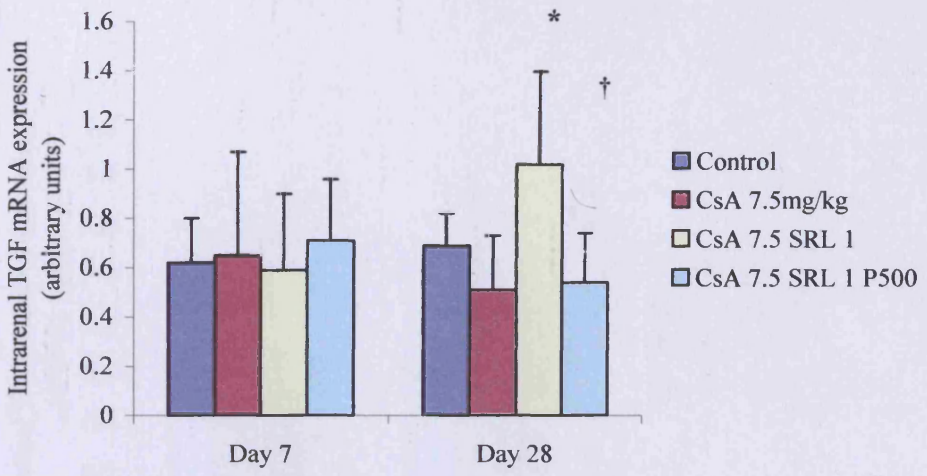


Figure 4.60 Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. CsA – cyclosporine, SRL – sirolimus, P500 - pirfenidone. Doses are mg/kg/day. Error bars represent one standard deviation of the mean.
* P=0.021 vs CsA 7.5mg/kg/day at same time point
† P=0.81 vs CsA 7.5mg/kg/day at same time point

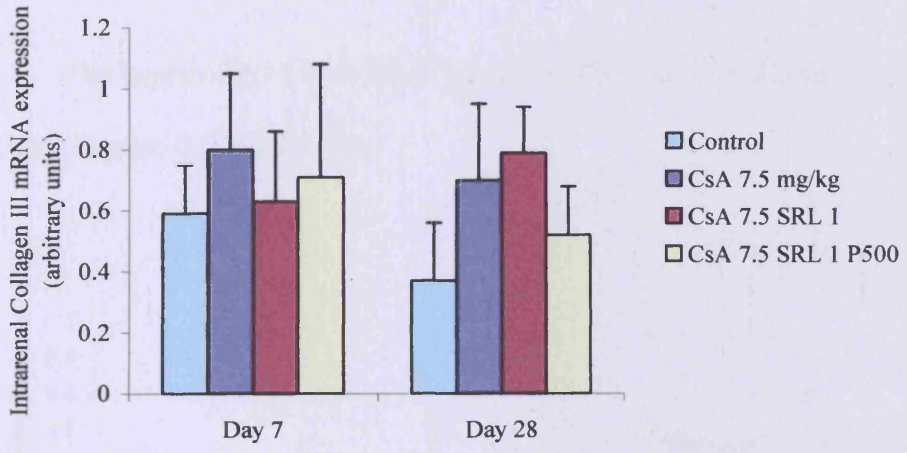


Figure 4.61 Intrarenal Collagen III mRNA expression. See Figure 4.60 legend for description.

Seven days after commencement of treatment, the addition of sirolimus or sirolimus plus pirfenidone to cyclosporine caused no change in the expression of TIMP-1. By day 28, both cyclosporine and cyclosporine plus sirolimus treatment had produced a similar marked rise in TIMP-1 expression. The addition of pirfenidone significantly reduced TIMP-1 expression compared to cyclosporine alone (0.45 ± 0.27

vs. 1.04 ± 0.3, P=0.006) or cyclosporine plus sirolimus (0.45 ± 0.27 vs. 0.87 ± 0.16, P=0.011). See Figure 4.62.

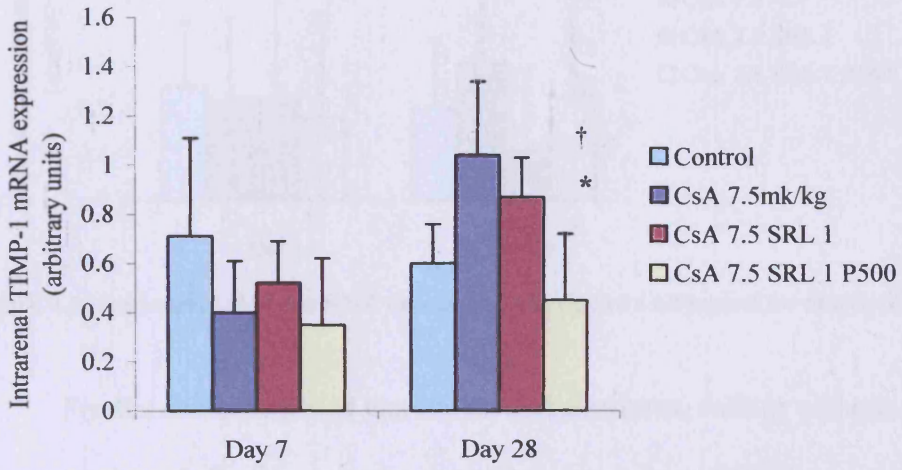


Figure 4.62 Intrarenal TIMP-1 mRNA expression. See Figure 4.60 legend for description.
* P=0.006 vs CsA 7.5mg/kg/day at same time point
† P=0.011 vs CsA 7.5 + SRL 1mg/kg/day at same time point

The expression of both MMP-2 and MMP-9 were unchanged for the treatment groups (Figures 4.63 and 4.64).

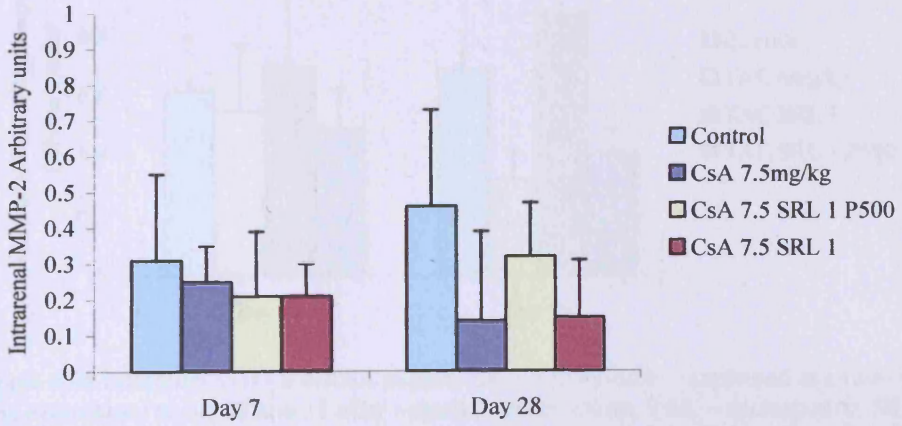
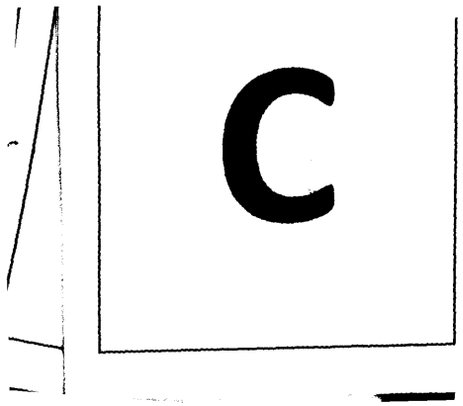


Figure 4.63 Intrarenal MMP-2 mRNA expression. See Figure 4.60 legend for description.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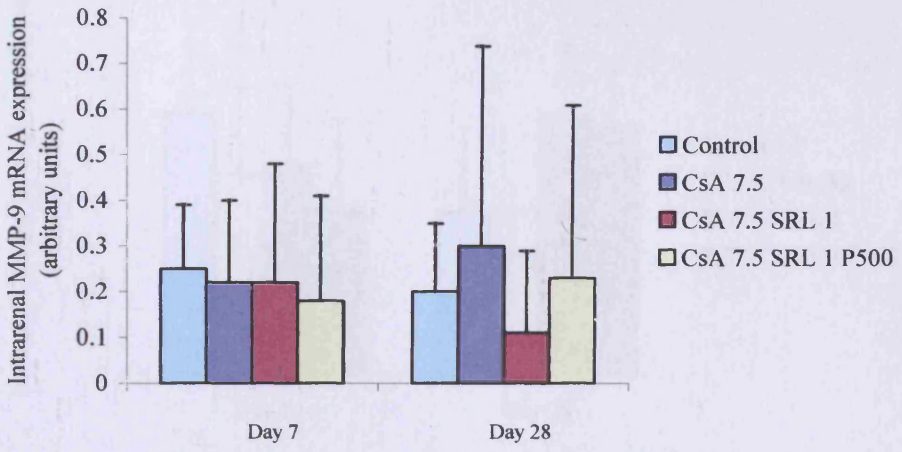

Figure 4.64 Intrarenal MMP-9 mRNA expression. See Figure 4.60 legend for description.

For the combination of tacrolimus and sirolimus, with or without pirfenidone, the expression of all genes tested was unaltered at 7 days. TGF-β expression at 28 days was significantly greater with combined tacrolimus/sirolimus treatment compared to tacrolimus alone (0.88 ± 0.25 vs. 0.32 ± 0.1, P=0.002). Addition of pirfenidone reduced expression back to the level of tacrolimus treatment alone (0.41 ± 0.08 vs. 0.32 ± 0.1, P=0.005), see Figure 4.65.

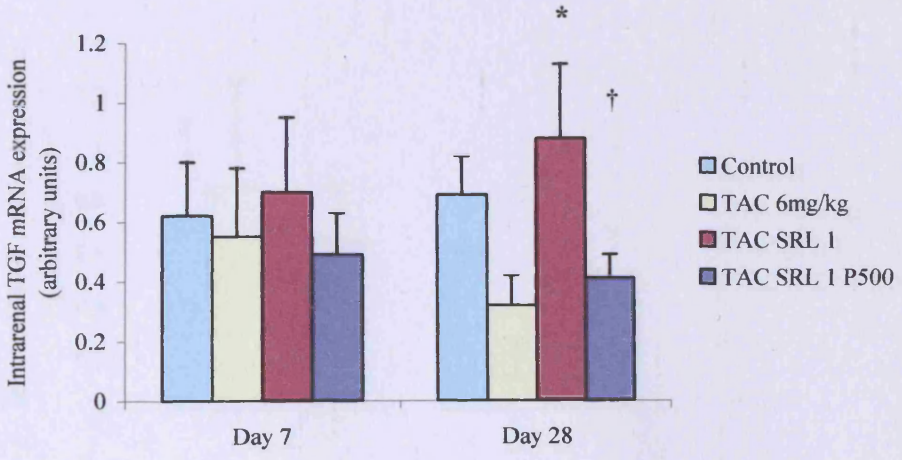

Figure 4.65 Intrarenal TGF- β mRNA expression (arbitrary units – expressed as a ratio of TGF-β to β-actin expression) at days 7 and 28 after commencing treatment. TAC – cyclosporine, SRL – sirolimus, P500 - pirfenidone. Doses are mg/kg/day. Error bars represent one standard deviation of the mean.
* P=0.02 vs TAC 6 mg/kg/day at same time point
† P=0.005 vs TAC 6 + SRL 1mg/kg/day at same time point

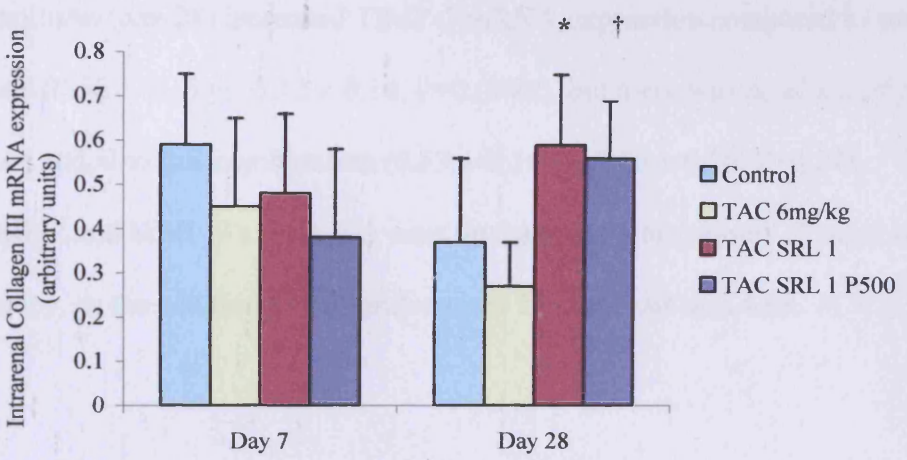

Figure 4.66 Intrarenal Collagen III mRNA expression. See Figure 4.65 legend for description
* P=0.02 vs TAC 6 mg/kg/day at same time point
† P=0.73 vs TAC 6 + SRL 1mg/kg/day at same time point

Tacrolimus suppressed collagen III expression at 28 days. The addition of sirolimus to tacrolimus reversed this effect (0.59 ± 0.16 vs. 0.27 ± 0.1, P=0.002, see Figure 4.66), and the addition of pirfenidone had no further effect (0.56 ± 0.13 vs. 0.59 ± 0.16, P=0.73).

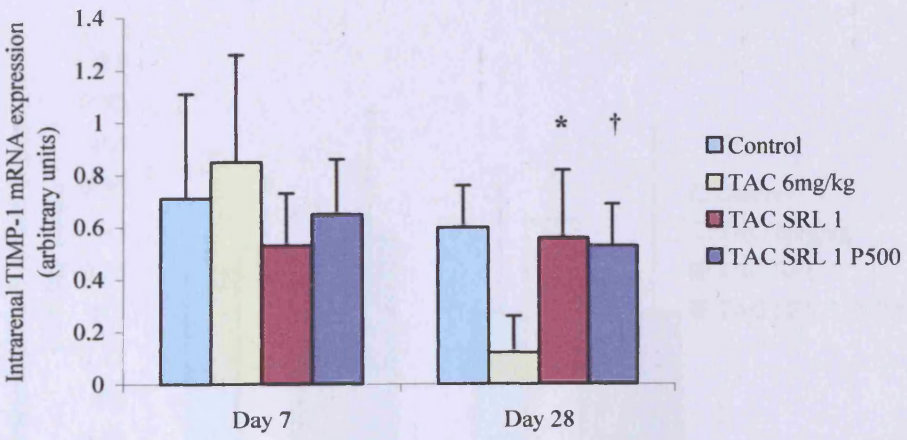

Figure 4.67 Intrarenal TIMP-1 mRNA expression. See Figure 4.65 legend for description
* P=0.008 vs TAC 6 mg/kg/day at same time point
† P=0.24 vs TAC 6 + SRL 1mg/kg/day at same time point

At day 28 tacrolimus reduced the expression of TIMP-1 compared to the expression elicited at day 7 (see Figure 4.67). Sirolimus in combination with

tacrolimus (day 28) increased TIMP-1 mRNA expression compared to tacrolimus alone (0.56 ± 0.26 vs. 0.12 ± 0.14, P=0.0082), but there was no effect of pirfenidone when added to this combination (0.53 ± 0.16 vs. 0.56 ± 0.26, P=0.24).

MMP-2 and MMP-9 expression were unchanged by tacrolimus alone or combination therapy, or the addition of pirfenidone, see Figures 4.68 and 4.69.

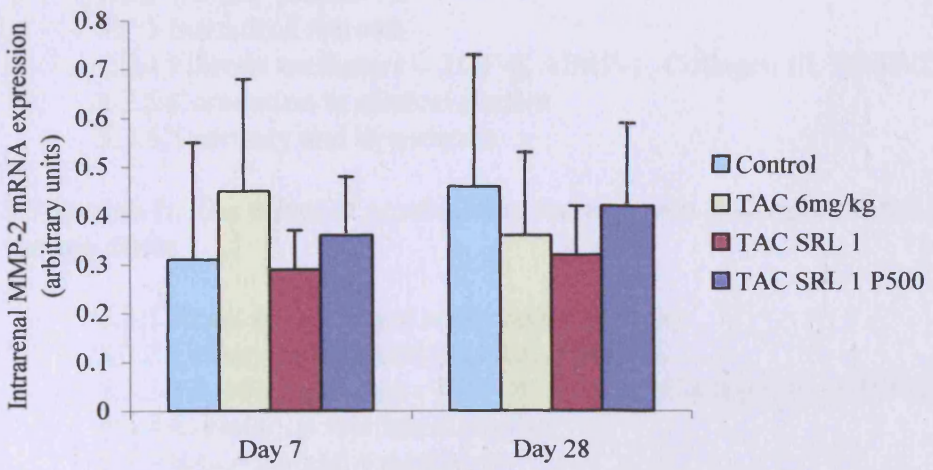

Figure 4.68 Intrarenal MMP-2 mRNA expression. See Figure 4.65 legend for description

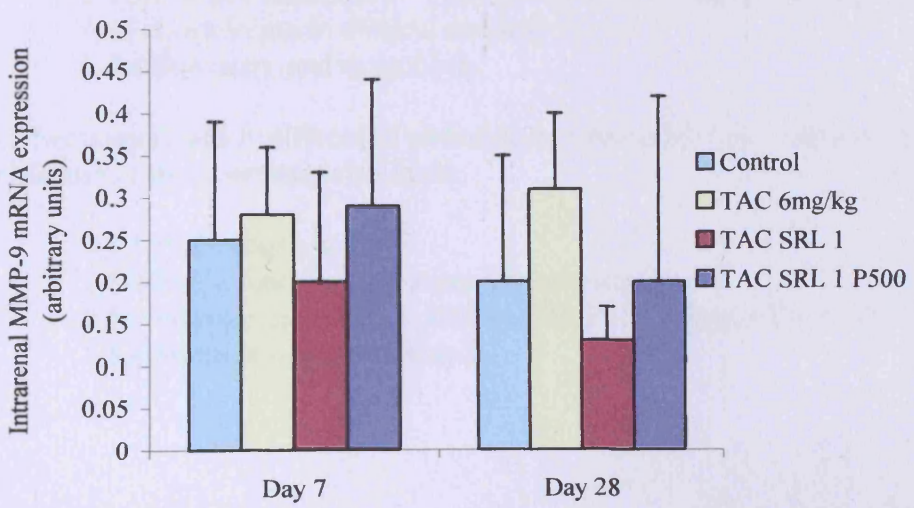

Figure 4.69 Intrarenal MMP-9 mRNA expression. See Figure 4.65 legend for description

CHAPTER 5 – DISCUSSION

5.1 Introduction

5.2 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

- 5.2.1 Renal function and acute nephrotoxicity
- 5.2.2 Urinary protein
- 5.2.3 Interstitial fibrosis
- 5.2.4 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and -9
- 5.2.5 Correlation to clinical studies
- 5.2.6 Summary and hypotheses

5.3 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

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- 5.3.3 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and –9
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- 5.3.5 Summary and hypotheses

5.4 Section C: The effect of the combination of tacrolimus and sirolimus

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- 5.5.1 Pirfenidone dosing
- 5.5.2 Renal function and acute nephrotoxicity
- 5.5.3 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and –9
- 5.5.4 Summary and hypotheses

5.1 Introduction

As a disease process, chronic allograft nephropathy is set about with questions and uncertainties. It accounts for the loss of a considerable number of renal allografts every year. This is particularly important because donor supply is increasingly unable to meet demand. Extensive research has been applied to various aspects of CAN, but presently treatment options are limited for established disease. This is partly because it is a complex process, with many alloantigen dependent and independent contributing factors. Kreis and Ponticelli stated:

> "We are almost certain that alloantigen-dependent and alloantigen-independent factors work in concert to increase stress on the transplanted kidney" [8].

This stress is expressed as the biological and clinical changes comprising CAN.

A central aim of renal transplant programmes is to provide organs in the best possible functional condition. Long-term results have been improved by minimising the physiological trauma to organs after donor brain death, during retrieval and storage, and by reducing the exposure of the graft to damaging processes in the recipient such as hypertension. Because immunosuppressive therapy itself has deleterious effects on allografts, an important approach is the use of the most potent but least toxic agents, with the most favourable side effect profiles, at the lowest possible doses. The calcineurin inhibitors are pivotal for immunological modulation of allograft recipients because of their potency as prophylactics for acute allograft rejection. The number of episodes and extent of acute rejection is a major predictor of CAN[64;65], and in the absence of acute rejection, graft failure due to CAN is uncommon[73]. Despite reductions in acute rejection associated with the introduction of calcineurin inhibitors, there has so far been no measurable increase in graft half-life consequent on their introduction. This is because their anti-rejection potency is countered by their acute and chronic nephrotoxicity. Additionally, some of their side

effects (diabetes, hypertension and hyperlipidaemia) are damaging to the allograft and decrease long-term survival[15;89;95]. All of these factors contribute to tubulointerstitial fibrosis, characterised by disruption of the three dimensional extracellular matrix structures that determine and maintain the organisation of renal tissues. The normal interstitium consists of a loose matrix of collagens, proteoglycans, matrix producing resident fibroblasts, macrophages, dendritic and endothelial cells. Disruption of the ECM framework results in an inability to maintain or restore graft parenchymal architecture[16]. Multiple and sustained insults to graft cells leads to their senescence and consequent failure to control fibrosis[21]. A number of cellular signals (e.g. TGF-β, the matrix metalloproteinases, and their tissue inhibitors) control the balance of matrix accumulation/degradation. Calcineurin inhibitors are known to alter the balance of these signals and this is how they produce graft fibrosis, which leads to shortened graft half-life.

Attempts to reduce calcineurin-inhibitor induced graft damage have focused on reduced CNI exposure by the addition of adjuvant agents (sirolimus and mycophenolate), or neoadjuvant treatment with non-CNI induction immunosuppression (IL-2 antagonists). Other techniques include CNI withdrawal after the graft has become established in the recipient, or total avoidance. Treatment of established graft fibrosis is particularly challenging. The complexity of the aetiology, the multiple and progressive sites of cellular and molecular corruption, and the heterogeneous nature of transformed infiltrating inflammatory cells, all challenge attempts at therapeutic intervention for calcineurin-inhibitor toxicity and CAN. Despite the multiple causative mechanisms, renal fibrosis is effected through a final common pathway. Therapeutic intervention in this final pathway may be the required approach. This is the remit of antifibrotic agents, of which pirfenidone is a promising

example. It has demonstrated potent histological and molecular antifibrotic action in renal and non-renal models of animal disease, and acts by inhibition of profibrotic signals at the level of both transcription and translation.

In humans, it is difficult to clearly dissect out the proportional contribution of the calcineurin inhibitors in CAN. Resorting to animal models allows many of the extraneous and additional damaging factors to be eliminated, providing a clearer picture of the true effect of CNIs. However, it has been very difficult to construct models of human CNI-induced renal changes. Many older animal models failed to produce changes in the short-term. It was not until the development of the rat salt-depletion model that a representative system was constructed. An important component of this model is that it allows the demonstration of the dissociation of structural and functional changes observed in human allograft disease in a short time period.

5.2 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

5.2.1 Renal function and acute nephrotoxicity

This study demonstrated a rise in serum creatinine for animals treated with the calcineurin inhibitors. CsA had a more marked effect than TAC. An increase in serum creatinine and concurrent decrease in creatinine clearance was also noted by Shihab et al.[436] in their experiments with CsA in salt depletion. They concluded that the impairment of renal function with cyclosporine was not a direct consequence of tubulointerstitial abnormalities, because Cr clearance also decreased in the cyclosporine-treated rats on normal (salt-replete) diet, in which the structural changes were trivial. In that study and the present one, the observed changes occurred at 28 days. The functional toxicity of cyclosporine is due to vasoconstriction of preglomerular arterioles and arteries, leading to reduced renal blood flow, reduced glomerular filtration rate, and increased renal vascular resistance[229]. These effects are mediated by an imbalance between renal vasoconstrictors (angiotensin II, thromboxane, endothelin, platelet-activating factor, catecholamines) and vasodilators (prostaglandins, nitric oxide)[230]; a shift in balance that favours vasoconstriction contributes to acute downstream renal ischaemia. Cyclosporine may also directly stimulate vascular smooth muscle or mesangial cell contraction processes dependent on influx of calcium; CsA increases intracellular calcium in these cells[231] and causes vasoconstriction directly in isolated arterial rings[232]. The functional changes are not dependent on structural disruption, but structural changes are linked to worsening renal function, decreased concentrating ability and enzymuria. Despite this link, acute toxicity is thought to be a separate entity from chronic toxicity.

TAC has a different chemical structure and binding immunophilin than CsA, but does potently inhibit the phosphatase activity of calcineurin[254]. Some studies

suggest tacrolimus is toxic to vasculature, affects smooth muscle relaxation and alters vascular haemodynamics in a similar manner to cyclosporine. Tacrolimus increased the responsiveness of rat and human resistance artery to NAd and decreased response to ACh[287], and peripheral and renal renin concentrations are elevated in experimental tacrolimus toxicity[37]. The similarities in the effect of cyclosporine and tacrolimus on RBF, GFR, urinary excretion of nitric oxide, high fractional excretion of magnesium and resultant hypomagnesaemia[254], further support a common mechanism of action and toxic effect.

Differences are apparent in the ability of CsA and TAC to cause vasoconstriction in humans and rats[450-453]. In chronic dosing studies in normal healthy subjects and paediatric renal transplant recipients, CsA but not TAC decreased GFR and renal plasma flow[450]. In a small (CsA n=7, TAC n=7) but well constructed study, instantaneous intrarenal transplant haemodynamics were assessed with real time colour Doppler imaging after dosing with one or other of the CNIs. Cyclosporine, but not TAC, induced phasic hypoperfusion within small to medium sized intrarenal arteries one to two hours after dosing[454]. These data support a superior acute therapeutic profile of TAC.

Sirolimus produced no measurable acute nephrotoxic effect in this study. Whilst SRL has a similar structure and binds the same immunophilin as TAC, it does not inhibit calcineurin. It shows considerably less nephrotoxicity than TAC or CsA, or in some reports, no nephrotoxicity[233;357;373]. SRL does not decrease GFR or RBF, and does not increase urinary excretion of nitric oxide or inhibition of tubular Na/K ATPase, in contrast to the effects of CsA and TAC[254]. Andoh et al.[254] found that CsA and TAC decreased urinary excretion of nitric oxide, but SRL did not. Nitric oxide synthase is an *in vitro* substrate of calcineurin, and this may partly explain the renal

dysfunction seen with the calcineurin inhibitors. It would appear that reduced GFR and renal blood flow are prerequisites for acute renal dysfunction rather than altered NO metabolism because all three drugs produce renal tubular dysfunction; they generate increased urine volume, and decreased urine osmolality and free water absorption by affecting urine concentrating ability[254]. Sirolimus does have other effects in common with the calcineurin inhibitors, namely profound hypomagnesaemia, with a high fractional excretion of magnesium, and some studies demonstrate the same parenchymal lesions as those caused by CsA and TAC[254;455]. Although most studies with SRL suggest a lack of *acute* nephrotoxicity, there is evidence that it may prolong recovery in kidneys after ischaemic insult, and extend the duration of delayed graft function, albeit through an unknown mechanism[381;456]. In Lewis renal allografts, both TAC and SRL displayed acute nephrotoxicity. The dose of TAC used was similar to the present study, but higher doses (up to 6.5mg/kg/day) of SRL were employed[457].

5.2.2 Urinary protein

Tubular disease causes loss of low molecular weight proteins (<40kD) with no albuminuria. Glomerular disease damages various barriers to protein filtration and generally results in albuminuria, with or without globulinuria. For protein loss, this study concentrated on total protein excretion, but it anticipated only tubular protein loss with CNI (and possibly SRL) treatment. None was observed in any of the groups tested across the entire study. Measurement of tubular and/or glomerular enzyme excretion may have been a more sensitive way to measure renal damage. Andoh et al.[254] measured excretion of the proximal tubular enzyme alanine aminopeptidase, finding that CsA, TAC and SRL all increased excretion (by roughly the same order) compared to controls in the salt-depletion model. Proteinuria, with afferent and

efferent vasoconstriction and glomerular hypertension was also noted in rats with angiotensin-induced salt-sensitive hypertension[458]. The lack of total proteinuria in the present study may be related to the lack of tubulointersitial fibrosis.

5.2.3 Interstitial fibrosis

This study failed to display histological fibrosis with the addition of CsA, TAC or SRL. The dissociation of functional and structural abnormalities in the kidney is a well-recognised phenomenon[159;276]. That tubular dysfunction can be dissociated from altered renal haemodynamics challenges the cause and effect theory of chronic renal vasoconstriction producing renal tubular damage. Experimental studies have shown that CsA withdrawal leads to improved GFR, but continued progression of tubular atrophy and tubulointerstitial fibrosis[276]. Thus, long-term exposure to CsA (and perhaps TAC) can produce morphological changes that may not be reversible with reduction or cessation of the drug [59]. Conversely, CsA-induced fibrosis can be reversed by the addition of inhibitors of the renin-angiotensin system, without improvements in GFR[239]. As previously mentioned, the nitric oxide system is an important facilitator of acute cyclosporine-induced vasoconstriction. A link between the nitric oxide system and chronic, structural changes associated with calcineurin inhibitors has been demonstrated; adding nitric oxide donors (decreased nephrotoxicity) or inhibitors of nitric oxide synthase (increased nephrotoxicity) to models of cyclosporine-induced renal dysfunction results in modulation of renal structure and mRNA expression of matrix proteins[256-258]. Therefore, acute toxicity in the absence of structural change can be explained, but we need to consider why there were no CNI-associated structural changes in this study. The lack of interstitial fibrosis in this study does limit the power of the results, despite the significant changes in mRNA expression of genes controlling matrix. Some of the possible

reasons for the lack of fibrosis have been alluded to above. First is the anatomical location of the first signs of fibrosis in (human) kidneys. The outer medulla is the first area to demonstrate calcineurin-inhibitor related changes, rather than the cortex, and this area was not examined. However, other studies using the salt-depleted model have found fibrosis in the cortex.

The length of salt-depletion may not have been adequate to prime the kidneys for damage by the calcineurin-inhibitors, but various other studies utilising the seven-day period of salt-depletion have been successful in producing renal interstitial fibrosis[37;258;436;459]. In the slightly different setting of the model of post-cyclosporine nephropathy and hypertension, Kang et al. [460] used 45 days of salt-depletion along with CsA before the commencement of treatment regimens. It is not clear whether a longer period of salt depletion may have been beneficial in the present study.

We have no clear evidence that there were sufficient levels of the drugs to produce fibrosis, apart from indirect evidence of acute toxicity and the changes in mRNA expression; immunosuppressant blood concentration was not measured. At the outset, this variable was not factored into the study. It had previously been demonstrated by other authors that identical doses and drug administration techniques produce adequate levels (*vide infra*). Furthermore, the relevance of drug levels (and not just doses) in animals, and especially rodents must be treated with caution, as they do not necessarily bear a resemblance to levels in humans because of species or strain-specific differences with relation to drug toxicity[457]. However, in hindsight it would have been useful to know drug levels when it was clear that fibrosis did not occur. There are, however, some major limitations of blood concentrations as indicators of drug action on the kidney. Podder *et al.*[339] have produced evidence showing that there is not necessarily a close correlation between blood and tissue concentrations of

immunosuppressive drugs. Measurement of tissue concentrations may therefore be necessary in future studies.

Whether or not SRL causes morphological changes is dependent not only its dose but also on the model to which it is applied. Ryffel et al.[233] described no renal histopathologic changes with administration of low dose SRL in Wistar rats, as they did for the CNIs. At a dose of 1mg/kg, SRL altered neither function nor histology in Sprague-Dawley rats; at 10mg/kg it produced only minor functional abnormalities but no morphological effects[373]. In the spontaneously hypertensive rat model, 0.8mg/kg/day SRL caused functional changes, with marked vasculopathy and tubular atrophy. Andoh et al.[254] showed that sirolimus (3mg/kg/day) produced similar morphological changes (tubular injury and nephrocalcinosis) to the calcineurin inhibitors in salt-depleted rats. Later studies from the same group using $1/10^{th}$ of the dose in the same model found that SRL did not cause morphological change[461]. Whiting et al. [338;372] reported that 14 days of SRL treatment (1.5mg/kg/day) produced a slight rise in serum creatinine and a significant increase in urinary flow rate, whilst kidney morphology was unchanged in Sprague-Dawley rats. Animals treated with CsA displayed a wider range of functional disturbance, and marked acute tubular necrosis.

Because none of the drugs used in our study demonstrated interstitial fibrosis, it is difficult to know exactly how SRL was acting in this setting. However, it is fairly clear that the stimulation of pro-and anti-fibrotic genes was dissimilar to that produced by cyclosporine, and for some mediators was different to that produced by tacrolimus. Isolated human glomeruli exposed to cyclosporine and tacrolimus demonstrate differential expression of matrix mediators. Cyclosporine increased collagen IV, TIMP-2 and TGF-β mRNA expression, whilst tacrolimus did not. The levels of

collagen IV protein were unaltered, and there were no differences in renal histology with the two drugs[462]. Overall, these findings were similar to the present study, and differences in the effects of the two agents may explain the marked mesangial expansion and glomerulosclerotic change in chronic CsA (but not TAC) toxicity reported in other studies.

5.2.4 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and -9

Transforming growth factor-β mRNA expression is a key signal in fibrosis. It acts directly to promote fibrosis, and indirectly by altering the expression of other signals such as MMPs (decreased mRNA) and TIMPs (increased). Elevated TGF-β may well be a contributory factor to acute as well as chronic renal damage, since there is evidence that TGF-β plays a role in vascular dysfunction. Sharma et al.[463] demonstrated that TGF-β inhibits calcium transients in isolated rat preglomerular vascular smooth muscle cells. Although data remains limited, there is no *de facto* reason that TGF-β cannot act as a direct mediator of vasoconstriction.

The rise in TGF-β mRNA expression with CsA treatment in the present study is in agreement with other reports in the same model[168;436]. Previous studies in salt-depletion describe TAC-associated rises in both TGF-β and collagen I mRNA levels, and effects on basement membrane collagen IV. The present study demonstrated a fall in TGF-β with tacrolimus treatment, but the literature is at odds over the effect of tacrolimus on TGF-β expression in the setting of renal injury. Increased renal TGF-β expression is associated with TAC[212] as well as CsA treatment in human renal and non-renal allografts, although TGF-β mRNA expression and renal structural injury may be less than that caused by cyclosporine[464]. Some authors report that tacrolimus does not stimulate TGF-β in renal transplant recipients[169;171;465].

The marked elevation of TIMP-1 by CsA underlines the profibrotic effect of CsA, and may be an indirect reflection of increased TGF-β expression, or a direct effect of the drug. A study of renal transplant glomeruli demonstrated higher expression of TIMP-1 in CsA- than TAC-treated recipients[169]. Jain et al.[292] demonstrated a tacrolimus-induced *reduction* in TGF-β expression in rat renal ischaemia/reperfusion injury, along with a reduction in TIMP-1 mRNA, suggesting a limited pro-fibrogenic (or antifibrotic) action of tacrolimus at the molecular level. Tacrolimus caused TIMP-1 mRNA levels to become depressed in the present study. Most other models of renal fibrosis have demonstrated an *increase* in TIMP-1 activity[34;36;37], but Mo et al.[39] found a similar reduction in TIMP-1 (and 2) in the model of bromoethylamine-induced papillary necrosis. They reasoned that this decrease might have represented an attempt by tissue to increase collagenolytic activity in response to injury. The Leicester unit have previously demonstrated that tacrolimus[40] inhibits TIMP-1 mRNA expression in a mechanically-injured rat carotid artery model, with resultant inhibition of smooth muscle cell proliferation. In the setting of renal transplant biopsies, TIMP-1 levels correlate with graft fibrosis (measured by collagen III immunohistochemical staining)[38]. Rat studies of glomerulosclerosis and tubulointerstitial fibrosis with tacrolimus demonstrate reduced MMP and enhanced TIMP levels; these alterations impair proteolysis and enhance the accumulation of extracellular matrix[117]. A marked and significant decrease in TIMP-1 by TAC is a further important finding, demonstrating that tacrolimus has contrasting effects on some mediators.

The powerful stimulatory effect of CsA on collagen III expression (late effect at 28 days) supports a fibrotic action of this immunosuppressant. Collagen III is the

main component of the extracellular matrix, and therefore increased mRNA expression is an indication of a profibrotic effect at the transcriptional level. That tacrolimus had no effect on collagen III production indicates at least a non-profibrotic role, if not an antifibrotic effect.

Reports of direct comparisons of the effect of the CNIs effects on fibrosis-associated genes are few. Bicknell et al.[169] found that collagen III mRNA and TIMP-1 expression was significantly less in tacrolimus-treated post-transplant glomeruli. In a rat model of ischaemia reperfusion injury, Jain et al.[292] report that tacrolimus significantly reduced the expression of TGF-β and TIMP-1. Although cyclosporine treatment reduced levels of MMP-2 and -9, this was not statistically significant.

In this report, sirolimus had no effect on TGF-β or collagen III expression, and produced a marginal fall in TIMP-1 mRNA expression compared to controls. Likewise, it had no effect on matrix metalloproteinase expression. These findings suggest no effect on matrix mediators. Using Northern blotting, Shihab et al. [461] reported significant rises in TGF-β, biglycan and collagen I mRNA expression in salt depletion, but expression was lower than that caused by CsA.
In renal ischaemia reperfusion injury, Jain et al.[385] showed that SRL reduced TGF-β and TIMP-1, -2 & -3 mRNA expression compared to the effect of cyclosporine. Further, Waller[40] demonstrated SRL-induced falls in profibrotic gene expression in the carotid balloon angioplasty model. Comparison between models is difficult, and it seems that the effects of SRL may vary. Sirolimus treatment may also lead to a different *pattern* of gene expression in the kidney compared to other immunosuppressants[466]. Ninova et al., using a rat allograft model, demonstrated that TAC produced distal tubular TGF-β staining, but SRL produced staining in the proximal tubules[457], suggesting different mechanisms of nephrotoxicity.

In the present study, there were no significant effects of the three drugs on matrix metalloproteinase expression.

5.2.5 Correlation to clinical studies

Clinical data concerning the acute nephrotoxicity of CsA and TAC are few and conflicting. In patients (n=22) receiving kidneys from the same donor, (one treated with CsA and one with TAC), renal function and creatinine clearance were better in the tacrolimus group[467]. The same authors examined ten patients converted from cyclosporine to tacrolimus, finding improvements in renal function after conversion. In one single centre randomised study of renal transplant recipients, increased interstitial fibrosis was observed at 12 months with CsA + azathioprine compared to TAC + azathioprine[468]. Contrary to this, no difference in histopathological findings on 2-year protocol biopsies was demonstrated between TAC and CsA in the US multicentre kidney study[300]. Four large multicentre studies[182;469-471]and a meta-analysis[472]of tacrolimus versus cyclosporine primary therapy in renal transplantation all demonstrate lower incidence and severity of acute rejection with tacrolimus. Long term studies demonstrate that tacrolimus is associated with improved five-year graft survival[473] and lower rates of chronic rejection[474] compared to CsA without an increase in the incidence of adverse events associated with long-term immunosuppression. The intent-to-treat analysis of Vincenti's report[473] revealed that serum creatinine was significantly lower in tacrolimus treated patients, and GFR was greater. Treatment failure was also higher in the cyclosporine group. In a randomised trial of tacrolimus versus cyclosporine in paediatric renal transplantation, one-year GFR was significantly greater in the TAC (62 ± 20 ml/min) than in the CSA group (56 ± 21 ml/min, P=0.03). Graft survival was similar, but acute rejection rates were significantly lower with tacrolimus[471]. Similarly, in black recipients of cadaveric renal

transplants randomised to CsA (n=21) or TAC (n=14) lower acute rejection and serum creatinine was demonstrated in the TAC group[475]. Whilst these benefits may be directly linked to the reduction in acute rejection, it may also be because TAC produces less chronic nephrotoxicity in a manner unrelated to AR.

There are conflicting studies. Margreiter et al.[470] reported that although acute rejection episodes were significantly less common in tacrolimus than cyclosporine treated patients, graft survival and renal function were no different. Crossover only occurred in 1 tacrolimus treated patient (0.3%) but in 10% of cyclosporine-intent to treat patients. In a comparison of 66 patients on CsA and 75 on tacrolimus, Muirhead et al.[476] found that there were no differences in serum creatinine between the CsA and TAC groups for up to 5 years post-renal transplant.

Unfortunately, many of these studies use historical data for the cyclosporine groups and there are few well-constructed randomised trials of TAC versus CsA. In primary simultaneous pancreas-kidney transplantation, one-year results indicated that renal (and pancreatic) function was no different in patients randomised to TAC (n=103) or CsA (n=102)[477]. In the setting of a steroid-withdrawal trial in living donor renal transplant recipients (6 month data), again there was no difference in plasma creatinine for CsA and TAC treated patients[478] (however, in these grafts serum creatinine at six months was essentially normal). Four-year follow-up of a randomised trial of CsA vs. TAC (both with MMF) indicated similar renal function between groups. However, further analysis revealed a significant difference in the number of patients whose creatinine had increased two-fold since renal transplant (CsA – 63%, TAC – 38%, P=0.04)[214]. A large, randomised European multicentre trial of tacrolimus versus cyclosporine in paediatric liver transplantation demonstrated no difference in

calculated GFR at twelve months, and equal numbers of patients developed ARF in both groups[479].

Conversion trials (for situations such as 'chronic rejection', CsA toxicity, and steroid-resistant rejection) have demonstrated improved renal function when CsA is converted to TAC[480;481]. Again, some of these results may be due to the greater efficacy of tacrolimus in preventing acute rejection. However, several reports[225;482] have demonstrated a fall in serum creatinine after conversion from CsA to TAC in heart and liver transplant patients, in whom impairment of renal function by acute rejection could not have had an impact. Problems such as hyperglycaemia[480] after conversion to TAC have been noted and need close attention, but there may be improvements in overall cardiovascular risk factors after conversion[481;483].

Ahsan et al.[484] reported the 2-year results of a multicentre trial comparing TAC+MMF. CsA+MMF and TAC+AZA in 223 primary cadaveric kidney allografts, and found that renal function was best in patients receiving TAC+MMF. In patients who experienced delayed graft function, those treated with TAC+MMF had a 23% higher graft survival rate than the CsA+MMF group. The inference is that TAC is less toxic to the kidney, at least in the setting of delayed graft function. Three-year follow-up data confirmed superior graft function and survival in all patients on TAC compared to CsA[485].

Stoves et al.[486] demonstrated that cyclosporine dose reduction (permitted by the addition of MMF) is superior in terms of resultant renal function, compared to conversion from cyclosporine to tacrolimus in renal transplant patients with evidence of established CAN. This indicates that both the CNIs may be equivalently toxic, and the key to preserving renal function is CNI dose reduction, although the numbers in each group were small (n=13). Indeed, reduction (n=20) or withdrawal (n=18) of CsA

or TAC, with the addition of mycophenolate mofetil, from patients with established CAN and declining renal function demonstrated improved renal function with no episodes of acute rejection[487]. The greatest improvement was seen in those patients in whom the CNI was withdrawn. All grafts in this study were >1 year post-transplant. This study highlights an element of recovery from renal damage with CNI withdrawal (perhaps by removal of the acute vasoconstrictor effects of CNI). Another (retrospective) study has shown that 50% CsA dose reduction results in improved renal function, although the greatest benefit was obtained by total withdrawal[488]. Some of these effects may be indirect i.e. reduction in blood pressure with reduction or removal of the CNI. Certainly, higher blood pressure is correlated with faster decline in renal function[80].

This is some evidence from randomised trials for a beneficial profile of sirolimus on renal function. Groth *et al.*[349] (cyclosporine or sirolimus, plus azathioprine and prednisolone) and Kries *et al.*[353] (cyclosporine or sirolimus, plus mycophenolate and prednisolone) reported lower mean serum creatinine from patients randomised to primary sirolimus treatment. Renal function at 6 months is a powerful predictor of CAN and long-term graft survival[354], thus there are theoretical benefits of sirolimus, which could translate into the reduction of late allograft failure. Two important calcineurin inhibitor withdrawal trials are the '212'[363] and the Rapamune® Maintenance Regimen (RMR) studies[364]. In both, patients were randomised to receive either triple therapy (sirolimus/cyclosporine/prednisolone), or to have cyclosporine eliminated after 2 months (212) or 3 months (RMR). In both studies, measures of renal function were significantly improved at 6 and 12 months in the groups with cyclosporine withdrawn, whilst graft and patient survival were comparable. In RMR,

mean serum creatinine was 1.25mg/dl for the withdrawal group vs. 1.4mg/dl for continued cyclosporine (P<0.001).

Pre-clinical and clinical studies suggest that sirolimus may reduce the progression of established chronic renal allograft dysfunction. Sirolimus inhibits growth-factor-mediated proliferation of vascular smooth muscle cells, and disrupts signal transduction by a variety of cytokines [4]. Both of these mechanisms contribute to the development of chronic allograft nephropathy, so the absence of these effects is likely to be beneficial.

Trial	Follow-up	Graft survival		Biopsy-proven acute rejection		Serum creatinine (mg/dl)	
		TAC	CsA	TAC	CsA	TAC	CsA
Margreiter et al.[489]	6 months	94.8	91.9	**19.6**	**37.3** *	1.57	1.66
Murphy et al.[302]	1 year	96	90	35	36	1.78	1.92
Vincenti et al.[161]	1 year	93	89	33	32	1.7	1.9
Mayer et al.[182]	1 year	82.5	86.2	**25.9**	**45.7** *	1.87	1.89
Pirsch et al.[469]	1 year	91.2	87.9	30.7	46.4	1.66	1.64
Shapiro et al.[490]	1 year	82	79	n/a	n/a	1.8	1.8
Jensik et al.[491]	3 years	81.9	77.8	n/a	n/a	1.6	1.63
Mayer et al.[307]	4 years	72	71.3	**26.2**	**48.5** *	1.98	2.23
Vincenti et al.[473]	5 years	64.3	61.6	n/a	n/a	**1.4**	**1.7** *
Jurewicz et al.[468]	6 years	**81**	**60** *	n/a	n/a	n/a	n/a

Clinical outcome variables in prospective randomised trials comparing TAC with CsA in combination with azathioprine and corticosteroids (Adapted from Maes & Vanrenterghem[492]).
* P<0.05

Trial	Follow-up	Graft survival		Biopsy-proven acute rejection		Serum creatinine (mg/dl)	
		TAC	CsA	TAC	CsA	TAC	CsA
Liu et al.[493]	6 months	100	91.7	7	8	1.25	1.33
Johnson et al.[494]	1 year	89	88	15.3	20	1.3	1.6
Yang et al.[495]	1 year	90	96.6	13.3	13.3	n/a	n/a
Ashan et al.[215]	2 years	82.8	76.7	16.7	22.7	**1.3**	**1.57** *
Gonwa et al.[485]	3 years	80.6	73.3	16.7	25.3	1.4	1.6

Clinical outcome variables in prospective randomised trials comparing TAC with CsA in combination with mycophenolate and corticosteroids (Adapted from Maes & Vanrenterghem[492]).
* $P<0.05$

5.2.6 Summary and hypotheses

The hypothesis for this section was CsA, TAC and SRL vary in their effects on structural, functional and molecular indices of renal injury. This has been demonstrated for acute toxicity, with cyclosporine more damaging than tacrolimus. Sirolimus did not display a functional effect. No structural changes were observed, but differences in the expression of fibrosis-associated genes were observed with the different agents. Changes in gene expression are likely to be surrogate early markers of fibrosis. The interactions between mediators are complex, and there are other systems involved apart from those examined here. It is therefore difficult to draw comparisons to other basic science findings that use different drug doses and different models, let alone to draw connections between these findings and those from clinical studies. Nonetheless, overall tacrolimus appears to display less acute nephrotoxicity both in this study and in clinical reports. The tables above highlight that there are a few studies demonstrating long-term benefits for tacrolimus compared to cyclosporine

(long-term renal function and graft survival) that may reflect a lower fibrotic potential of tacrolimus in transplant recipients. Evidence suggests sirolimus does not alter renal haemodynamics, suggested by a lack of clinical deleterious effect on early renal function (except in delayed graft function).

Daily drug doses used in this model were higher than those doses used in humans (for CsA, typically 4 to 8 mg/kg). Although blood cyclosporine levels were not measured in the present study, they were higher in Andoh's rat experiments (approximately 3000ng/ml)[254] than target levels in humans (approx 500ng/ml or less). These authors explain that higher levels are necessary to overcome allograft rejection in studies with rodents, and thus the relative therapeutic window between efficacy and side effects is likely to be similar to that in humans. Likewise, TAC doses were about 10 times higher than doses used in humans (0.1 to 0.3 mg/kg/day). This dose was chosen because the bioavailability of TAC in rodents is lower than that in humans[496]. Again, Andoh demonstrated that tacrolimus levels (for a dose of 6mg/kg/day) in the salt depleted model (approx 10ng/ml) were in the therapeutic range for humans[254].

5.3 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

Combining a calcineurin inhibitor with an mTOR inhibitor offers practical and theoretical therapeutic advantages, principally CNI dose reduction and reduced nephrotoxic exposure. Furthermore, exposure to the other extrarenal deleterious effects of CNIs that contribute to graft damage, recipient morbidity and poor compliance, is limited.

The different mechanisms of action of CsA and SRL on the cell cycle mean they have complimentary, additive effects on T-lymphocyte inhibition. In addition, a synergistic effect is derived from cross-inhibition and competition for metabolic enzymes and the p-glycoprotein transporter. Concomitant oral administration of SRL and CsA produces a marked alteration in drug bioavailability as initially observed in rats. CsA increases the bioavailability of SRL by 2- to 11-fold, while SRL increases CsA bioavailability 2- to 3-fold. Tissue concentrations of SRL increase 6-fold in the spleen and 17-fold in the liver upon concomitant administration[340]. Therefore, one of the main challenges of using this combination is correct dosing. It is not sufficient just to reduce the CsA dose; both drug doses must be carefully tailored.

5.3.1 Renal function and acute nephrotoxicity

Six dose combinations of sirolimus plus cyclosporine were tested, all at seven, 14, 21 and 28 days. For 15mg/kg/day CsA, addition of sirolimus caused a trend towards increased serum creatinine at 7 days. Addition of 1mg/kg/day sirolimus to 15mg/kg/day cyclosporine caused animal death midway through the study period. This was preceded by a large rise in serum creatinine, which may indicate direct nephrotoxicity or renal failure secondary to distant organ dysfunction. Regardless of the exact cause, this was an effect of the combination of the two agents; either agent

used as lone therapy at these doses did not cause animal illness or death. At the other dose combinations, the trend for increased creatinine was maintained to 28 days. Therefore, any dose of SRL added to CsA 15mg/kg produces deterioration in renal function compared to the use of CsA alone, in this model.

The combination of 7.5mg/kg/day CsA plus any dose of SRL resulted in a lower serum creatinine compared to 15mg CsA with matched doses of SRL. This confirms the interaction between the two agents in terms of acute toxicity.

Whiting et al.[338] showed that the combination of SRL (1.5mg/kg/day) and CsA (15mg/kg/day) produced a four-fold increase in rat urinary flow rate compared to either drug alone, and that the drug combination exacerbated CsA-induced renal impairment. Kidneys of SRL-treated rats appeared normal but combination-treated animals demonstrated mild, focal acute tubular necrosis. Importantly, SRL administration did not affect whole blood CsA concentration, although a pharmacokinetic interaction cannot be ruled out. Podder et al. described that an important component of CsA-sirolimus toxicity is the interactions that increase *intrarenal* CsA concentrations[339], so the *blood* concentration may not be a true representation. In salt-depleted Wistar rats, ascending doses of CsA in the presence of SRL doubled the blood concentrations of CsA compared to CsA administered alone. Additionally, for a given CsA dose, a higher SRL dose caused a disproportionate rise in renal tissue CsA levels. These findings were also associated with dose-dependent elevations in creatinine, most marked at CsA doses of 2.5mg/kg/day or greater[339].

The importance of correct dosing of these drugs was demonstrated in 12 lung transplant recipients treated with a combination of CNI, SRL and steroid. Despite CNI dose reduction to maintain appropriate trough concentrations, serum creatinine still rose in 75% of patients[497]. Analysis of renal transplant recipients receiving SRL and

CsA showed the greatest reduction in risk of rejection for SRL levels between 5 and 12ng/ml regardless of the CsA level. In the pivotal trials it was recommended that oral CsA and SRL should be separated by at least four hours because of the significant pharmacokinetic interactions that occur with co-administration[498]. Low dose CsA (down to 50ng/ml) plus relatively high-dose SRL (10-15ng/ml) was a safe approach in renal allografts (n=29) in one study[499] (followed to 18 months), with a low (10%) risk of acute rejection. Serum creatinine was equivalent to a 'control' group taking MMF plus CsA, and both groups had a low and equivalent level of acute rejection. In the USA Rapamune Phase III study, those patients treated with SRL plus CsA had higher serum creatinine at one-year, despite lower rates of acute rejection, and this is likely to be due to increased exposure to CsA because of drug interactions[500].

5.3.2 Urinary protein and interstitial fibrosis

Protein excretion and interstitial fibrosis were unchanged compared to controls for all doses and combinations of SRL and CsA, so the effects on structure could not be assessed for these combinations. However, there were interesting changes for the molecular studies.

5.3.3 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and -9

Whilst 1mg SRL added to 15mg CSA numerically increased TGF-β expression compared to CSA alone, the lower doses of 0.5 and 0.1mg SRL caused a significant decrease in TGF-β expression. A similar trend was observed for collagen III expression, but not for the other profibrotic mediator, TIMP-1. Thus for TGF-β and collagen III, the addition of low dose sirolimus to high dose cyclosporine confers a beneficial effect at the molecular level. In parallel the antifibrotic signal of MMP-2

was increased when low doses of SRL were used in combination with CsA, compared to CsA alone. A similarly beneficial effect was observed on MMP-2 expression for the lower dose (7.5mg) of CsA + the lower dose of SRL (0.1mg), compared to CsA alone; i.e. MMP-2 expression was raised. Overall, a careful balance of SRL dose when added to high dose CsA can have potentially beneficial effects on matrix-related gene expression.

Andoh et al.[440] were the first to test the combination of CsA (2, 4 and 8mg/kg/day) and SRL (0.01 and 0.1 mg/kg/day) in the salt-depleted model. Broadly, their findings agree with those on acute renal dysfunction in this study; whilst SRL (0.1mg) alone had no effect, when added to CsA (8mg), a marked rise in plasma creatinine and a fall in GFR were noted. Molecular changes were not reported, but semi-quantitative fibrosis score was greater than with either treatment alone. Dose reduction of both agents to 0.01 mg SRL and 2mg CsA was required to minimise these functional and structural changes. The drugs were given by subcutaneous injection in Andoh's report, which may account for the differences in toxic dose in that and the present study. SRL did not affect CsA plasma concentration in their study. Of course, there may be a pharmacokinetic interaction.

A later study from the same group[461] used 0.3mg SRL plus 5mg CsA, finding that nephrotoxicity was similar to 10mgCsA, as was the expression of TGF-β mRNA and protein, and the expression of the ECM proteins biglycan and types I and IV collagen. SRL alone had minimal nephrotoxic or molecular effects[461]. PAI-1 mRNA expression (a marker of ECM degradation) was decreased. This contrasts with the present study's findings on MMPs, where the addition of 0.5 or 0.1 mg SRL to 15mg CsA produced an increase in the expression of the matrix degrading MMP-2.

The synergistic action of these two drugs in combination has been harnessed in animal studies. Using sub-therapeutic doses of sirolimus plus cyclosporine to treat heart- and kidney-allografted rats[357], cardiac allografts in mice[358], renal allografts in dogs[359], and rat lung allografts[360], survival has been prolonged compared to treatment with either agent used alone. As drug concentrations were increased in the present study, there was a switch from a beneficial to a deleterious effect. This phenomenon has been observed in other models. The combination of SRL and CsA led to sequential synergistic effects on CD25 expression in rats, which became antagonistic at higher doses. Similar findings are reported in isolated mononuclear cells or purified cell lines from mice or humans[501].

5.3.4 Correlation to clinical studies

A number of clinical trials (see also Chapter 3.2) have been constructed to examine the potential benefits of combining these two drugs. Two phase III, multicentre trials comparing cyclosporine/azathioprine/prednisolone with cyclosporine/sirolimus (2mg or 5mg) /prednisolone demonstrated significantly reduced acute rejection rates for the sirolimus-treated group. Mean serum creatinine was higher, and creatinine clearance was lower, in those patients treated with sirolimus. The augmented toxicity was related to increased renal *tissue* concentrations of cyclosporine[339]. The phase III randomised study from the Rapamune Study Group compared two different doses of sirolimus (6mg loading + 2mg maintenance, or 15mg loading + 5mg maintenance) plus cyclosporine[502]. Renal function at one year was better in the low dose group, whilst the incidence of acute rejection was similar between the two dose-groups. Cyclosporine trough levels were high-normal in the

sirolimus high dose group throughout, and this may account for the poorer renal function.

CsA-treated Taiwanese patients with clinically-defined or biopsy-proven CAN were dose-reduced by the addition of sirolimus (average dose 1.8mg)[503]. Fifty percent demonstrated improved renal function (secondary to a beneficial effect of SRL or reduced exposure to CsA, or both). Those patients who failed to benefit generally had higher pre-trial baseline serum creatinine. In these patients, it is reasonable to speculate either that the potential benefits of SRL are masked by the continued exposure to CsA, or that the graft has exceeded a threshold of damage, after which it cannot regain or regenerate function.

Using careful attention to drug doses, clinical trials have demonstrated that with sirolimus-based regimens, early reduction/elimination of CsA is safe and leads to better renal function[355;504;505]. Contrary findings were reported by Saunders et al.[506], who randomised cyclosporine-treated patients with biopsy-confirmed CAN to a 40% dose reduction either with or without (control) the addition of sirolimus 2mg/day. Despite no significant differences in drug trough levels throughout the study, glomerular filtration rate fell by 10% over six months in sirolimus treated patients, but remained stable in the controls. TGF-β mRNA expression in glomeruli from six-month biopsies fell in the control but not in the sirolimus group, whilst collagen III and TIMP-2 increased in the sirolimus group. Interstitial fibrosis fell in the control but not the sirolimus group.

These findings highlight the fact that the addition of sirolimus with CsA dose reduction may not necessarily be advantageous and supports the present study's findings of unfavourable molecular changes for some of the doses tested.

A further theoretical benefit of the addition of sirolimus to treatment schedules is its antiproliferative effect. This has certainly been demonstrated in a number of non-renal systems. SRL confers a low incidence of malignancy when it is used in immunosuppressive regimens, particularly a reduction in post-transplant lymphoproliferative disorder. This may partly be accounted for by a greatly reduced incidence of CMV, but may also be related to antiproliferation. Sirolimus inhibits growth factor-stimulated smooth muscle cell proliferation and intimal hyperplasia in various settings[334;377;379;380]. Clinical data demonstrate amelioration of the progression of coronary arteriosclerosis in recipients of heart transplants treated with SRL[507]. In the carbon-tetrachloride model of hepatic fibrosis, sirolimus inhibited both PDGF-induced proliferation of hepatic stellate cells and extracellular matrix deposition[383]. Furthermore, *in vitro* cultured human fibroblast proliferation in response to PDGF and bFGF is inhibited by sirolimus[508]. It is still unclear whether sirolimus can act in an antifibrotic manner in renal disease. At the molecular level, sirolimus reduces the expression of fibrosis-associated genes in the rat renal ischaemia-reperfusion model[385]. There is no evidence yet of an histological effect.

5.3.5 Summary and hypotheses

The hypotheses for this section were that dose-manipulation of the CsA/SRL combination produces variable effects on indices of renal injury, and at the correct doses, produces favourable outcomes for the end-points measured. These hypotheses are confirmed: variation in effects was seen, and low-dose sirolimus plus low-dose cyclosporine conferred benefits at functional and molecular levels. High doses of the two drugs produced toxicity.

The potentiation of CsA toxicity by SRL could be considered surprising given its known antiproliferative action in certain settings. In fact, it may be that this antiproliferation (if targeted at normal cells) is disadvantageous, rather than advantageous, as one would expect if targeted at fibroblasts and other inflammatory cells. Pharmacokinetic and pharmacodynamic explanations are possible. Drug concentrations were not measured, but other studies using salt-depletion[461] have demonstrated that SRL does not necessarily augment blood cyclosporine levels. That study used subcutaneous administration, and the situation may be different with the oral co-administration employed in the present report. Barten et al.[501] demonstrated that SRL blood concentrations do not predict sirolimus' effects on immune cells. Further, tissue rather than blood concentrations may be the important determinant. The exact role of drug levels will be an important extension of this study.

Some of the clinical studies on the use of SRL for CsA reduction show improved renal function with relatively short follow-up. The effect of this drug combination on long-term graft survival remains unknown, and there are no detailed chronic nephrotoxicity studies on the clinical combination of CsA and SRL.

5.4 Section C: The effect of the combination of tacrolimus and sirolimus

Emerging evidence suggests that tacrolimus may offer favourable graft outcomes compared to cyclosporine. Data point to improved graft function[471;473;484], reduced severity[469] and frequency[471] of biopsy-confirmed acute rejection and better graft survival[473;484]. These benefits may be especially marked in grafts with delayed graft function[484]. The potential benefits of adding SRL to a CNI have already been discussed. This section of the study was designed to examine the effect of the combination of the least nephrotoxic CNI tacrolimus, with sirolimus in the salt depleted model.

5.4.1 Renal function and acute nephrotoxicity

The earlier results of the present study demonstrate that sirolimus alone does not produce acute toxicity, whilst tacrolimus does. Combination treatment with the two drugs produced a higher early serum creatinine than tacrolimus alone (7 and 14 days) but there was no difference later in the study period (21 and 28 days).

5.4.2 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and -9

It will be recalled that whilst tacrolimus alone produced a decrease in TGF-β expression, sirolimus did not, and the addition of sirolimus abolished this beneficial effect of tacrolimus. A similar amelioration of the antifibrotic effect of TAC was noted when it was used in combination with SRL for collagen III and TIMP-1 expression. Again, there was no alteration in the expression of the matrix metalloproteinases with TAC + SRL treatment.

Studies of the effects of combined tacrolimus and sirolimus are sparse, particularly concerning the molecular effects of interactions between the two drugs. Shihab's study[461] of CsA + SRL is the closest report, but the way in which TAC and SRL interact may be very different from that of CsA + SRL. Certainly, the reversal of some of TAC's beneficial effects warrants further investigation. Alteration in drug concentration needs to be examined in future, although this does not seem to be as critical for TAC+ SRL as it is for CsA + SRL.

Renal function and fibrosis is rats treated with TAC (3mg/kg/day) + SRL (0.4mg) was essentially the same as for those treated with monotherapy in one study, suggesting that this combination is minimally nephrotoxic (acute and chronic)[509]. Although molecular markers were not examined, this is a different effect from that observed in the present study, where sirolimus *reverses* some of the implied beneficial molecular effects of tacrolimus. This effect bears some similarity to that in two other reports. Cao et al.[510] reported the ability of tacrolimus to reverse sirolimus-induced inhibition of bFGF- induced vascular smooth muscle cell DNA synthesis. Waller et al.[511] reported that the TAC + SRL combination reduced expression of pro-and antifibrotic mediator mRNA in rat carotid after balloon injury compared to controls, but did not report the effect of tacrolimus alone for comparison.

Although dose variation was not studied in the present report, Barten et al.[501] reported that increasing the drugs' concentrations for co-administered SRL and TAC led from synergistic to antagonistic effects on inhibition of lymphocyte function (in much the same way as it did for CsA + TAC).

The pharmacokinetic interaction between TAC and SRL is less well defined than that between CsA and SRL. Nonetheless, there is clear evidence that CYP3A4 is primarily responsible for the metabolism of TAC[512] so similarities in the interactions

of the CNIs with SRL are probable. However, the doses of cyclosporine used in combination with sirolimus are about 50 times higher than those of tacrolimus, which may therefore have a lesser effect than CsA on shared enzyme systems[346]. A different pattern of drug interactions (effects on intestinal and hepatic CYP3A4 and P-glycoprotein) is seen in renal transplant patients treated with CsA vs. TAC plus SRL[513]. With SRL and CSA, a 4-hour interval between dosing of the two drugs is recommended, even though it is inconvenient for patients and may affect compliance. In 25 liver and kidney-pancreas transplant recipients treated with a combination of SRL and low-dose TAC, neither PK profiles of SRL nor those of TAC were altered by simultaneous administration. It is thought that simultaneous dosing of TAC and SRL after transplantation is safe[346], and trough level monitoring is adequate to control therapy.

Overall, reports of the nature of the interaction between tacrolimus and sirolimus are inconsistent. *In vitro* assays have suggested an antagonistic action between the compounds[361], but an *in vivo* study in rat heart allografts suggested a synergistic action[343]. Various animal models have demonstrated extended graft survival with the combination of TAC and SRL compared to treatment with either agent alone[343;514], suggesting a synergistic effect. Likewise, combined TAC and SRL produced augmented suppression of rat autoimmune uveoretinitis compared to treatment with either drug in isolation[515]. It remains to be explained why these synergistic effects contrast with the antagonistic effects at the molecular level, reported in the present study.

5.4.3 Correlation to clinical studies

The clinical use of combination SRL and TAC was delayed by fears of competition for available cytoplasmic FKBP12[516], and therefore the potential for antagonism. TAC and SRL are both macrolides, binding the same immunophilin, but their downstream cellular actions are different. In contrast to the TAC-FKBP12 complex, the SRL-FKBP12 complex does not inhibit calcineurin activity. Rather, it binds and inhibits the mTOR kinase, inhibiting T-cell progression in G_1-S phase. SRL therefore acts at a later stage in the cell cycle than the CNIs. Hence the additive effect on the suppression of lymphocyte proliferation and IL-2 expression[517]. In contrast to the synergistic nephrotoxic effect of CsA and SRL, TAC plus sirolimus seems to be better tolerated[370;500;509].

Randomised clinical trials involving the combination of tacrolimus and sirolimus are scarce. Van Hooff et al.[370] report a study comparing TAC plus steroids, with TAC plus SRL plus steroids in three different dose combinations. Tacrolimus dose was initially adjusted for a trough concentration of 10-20ng/ml, tailoring down to 5-15ng/ml thereafter. The three groups taking sirolimus received doses of 0.5mg, 1mg or 2mg/day. The rise in SRL trough levels precipitated by tacrolimus was less than in studies with cyclosporine[346;355]. In all groups, renal function was similar, but acute rejection episodes occurred less frequently in sirolimus-treated patients; these rates were lower than in other studies utilising CsA-SRL regimens. Side effects were not significantly increased by the addition of sirolimus. This only reports 6-month results of this therapeutic strategy, but indicates that a) TAC + SRL may be a very effective combination for immunosuppression, b) relatively high doses of SRL can be used safely.

Wu's study[503] of CNI reduction after introduction of SRL in biopsy-proven (75%) or clinically-defined (25%) CAN included 17 patients on TAC-based immunosuppression. In terms of renal function, there was only a 50% response, seen in those patients with lower baseline serum creatinine. Formica et al. randomised 33 patients to SRL+ TAC or TAC+MMF ("controls"). In the treatment group, the concentrations used were SRL 10-15ng/ml & TAC 5ng/ml. Serum creatinine and acute rejection rates were equivalent in both groups.[499]

The one-year interim results of a study that compares (amongst other groups) tacrolimus/sirolimus with cyclosporine/sirolimus (in both groups a protocol of CNI reduction was employed) points to improved renal function at one year with TAC treatment[518]. However, this benefit was of marginal significance only for serum creatinine, and there was no difference when creatinine clearance was considered.

5.4.4 Summary and hypothesis

The hypothesis was that the addition of SRL to TAC is favourable compared to TAC alone. This is rejected because sirolimus worsened early renal function, and reversed the beneficial molecular effects of TAC. The interactions between the drugs in this potentially important combination require more investigation. Whilst evidence suggests that TAC is less nephrotoxic than CsA, minimisation of exposure is clearly desirable. Addition of SRL may allow such minimisation, but the danger is amelioration of the beneficial effects of tacrolimus.

5.5 Sections D and E: Effects of pirfenidone when added to treatment with calcineurin inhibitors, with and without sirolimus

As previously discussed, one strategy to reduce calcineurin inhibitor toxicity is the use of tacrolimus rather than cyclosporine. However, the presence of a CNI still confers an element of toxicity and resultant allograft fibrotic change. A new approach to this nephropathy is reduction or prevention of fibrosis by antifibrotic agents. The rationale for the use of pirfenidone in the setting of CNI and SRL dual therapy is that although the use of concomitant mTOR inhibition with CNIs allows CNI dose-reduction, there is still evidence for the toxic effects of these agents at low doses.

Pirfenidone demonstrates resolution of fibrotic lesions by preventing or reversing ECM accumulation[519-521] and acts via alterations in the balance of forces acting on matrix[41;522;523]. Its mechanism of action appears to be related to inhibition of signals that stimulate inflammation and fibrosis. Because these signals are altered in the presence of nephrotoxic immunosuppressants, it was logical to apply pirfenidone to this model of CNI-induced renal damage.

5.5.1 Pirfenidone dosing

The 500mg/kg/day dose of pirfenidone was chosen because earlier reports had demonstrated its efficacy, with apparent lack of side effects. The Leicester laboratory has shown that 500mg/kg/day is effective in the model of carotid artery intimal hyperplasia[41]. Given orally, this dose is efficacious in rat asbestos-induced lung fibrosis[519], bleomycin-induced hamster lung fibrosis[524;525], prevention of progression renal sclerotic lesions in rats, and in the 5/6 nephrectomised model[526]. Most recently, Leh et al.[527] used 500mg/kg/day to exhibit pirfenidone's amelioration of structural damage in rat anti-glomerular basement membrane nephritis. In models that measured changes in molecular messengers or growth factors, 500mg/kg/day proved effective.

Furthermore, in the few studies that have been performed in humans, similar doses were employed[426;528;529]. In the present study, pirfenidone at doses of 250, 500 and 750mg/kg/day acted in a statistically non-dose-dependent manner.

5.5.2 Renal function and acute nephrotoxicity

The serum creatinine rises observed with CsA, CsA + SRL, TAC and TAC + SRL were decreased by the addition of pirfenidone. This attenuation of cyclosporine-induced serum creatinine rise by pirfenidone in the present study contrasts with the findings in streptozotocin-diabetic rats, where pirfenidone failed to improve renal blood flow or GFR[530]. Previous studies in the salt-depleted model demonstrated a clear improvement in CsA-induced decline in GFR with pirfenidone, but this did not reach statistical significance[459]. It is not clear how the functional effect of pirfenidone in the present study was mediated.

Because pirfenidone is capable of reducing the expression of proinflammatory cytokines, it can at least be deduced that a chemical signal involved in vasoconstriction might be inhibited by pirfenidone. This may or may not involve one of the established pathways controlling afferent glomerular arterial tone. Leh et al.[527] demonstrated that pirfenidone reduced plasma renin-activity in rats with glomerulonephritis (to the same extent as an angiotensin II receptor blocker). The potential role for TGF-β in acute toxicity has previously been discussed. Briefly, Sharma et al.[463] demonstrated reduction of calcium currents in vascular smooth muscle by TGF-β. As pirfenidone inhibits TGF-β, this is a putative mechanism for its action in the reduction of CNI-induced functional toxicity. Obviously there are complex second messenger systems involved in renal hypoperfusion, and it is reasonable to suggest that pirfenidone might act directly or indirectly through one or

more of these pathways to reduce the acute CNI toxicity. This is a potential mode of action for pirfenidone's functional effect.

5.5.3 Fibrotic mediators – TGF-β, TIMP-1, Collagen III, MMP-2 and -9

In agreement with this study, other salt-depleted studies demonstrate a rise in TGF-β mRNA expression with CsA-treatment[168;436]. It was unsurprising that TGF-β expression was suppressed back to control levels by the addition of pirfenidone for cyclosporine plus sirolimus treatment. It is clear from previous sections of this study that treatment with TAC reduces TGF-β expression, and that addition of sirolimus reverses this effect. Pirfenidone added to the TAC + SRL combination reduced TGF-β levels to those of tacrolimus treatment alone, i.e. to lower levels than controls. It may be that co-administration of sirolimus and tacrolimus promotes a signal that is inhibited by pirfenidone. Shihab's study[459] demonstrated that pirfenidone attenuates the rise in TGF-β produced by CsA. This is consistent with what is known about pirfenidone's mechanism of action, as is this study's finding that the elevated expression of collagen III (stimulated by CsA) is reduced by pirfenidone. Leh et al. found that collagen Iα mRNA expression was decreased with pirfenidone treatment in nephritic rats[527], and other animal models have demonstrated the ability of pirfenidone to decrease collagen deposition and/or mRNA expression[41;523;531-536], most likely to be an effect at both the transcriptional and translational levels. In support of a transcriptional effect, pirfenidone inhibits proline hydroxylase levels and therefore might reduce the availability of hydroxyproline, required for collagen synthesis[525;531].

These prior studies have generally suggested an effect of pirfenidone only on excess collagen deposition; this study found a reduction in collagen III beyond control levels with the highest dose of pirfenidone. Actually, Garcia et al.[531] demonstrated that

prifenidone significantly suppresses steady-state levels of interstitial and basement membrane collagen mRNAs, and suggested that this may be either at the transcription level, or due to diminished mRNA life span. Caution may therefore be required in human studies of pirfenidone – disruption of normal collagen homeostasis may not be desirable.

Collagen III expression, stimulated by combined treatment for both CSA + SRL and TAC + SRL, was not significantly inhibited by pirfenidone. This is slightly at odds with the studies stated above. Indeed, it is at odds with earlier findings in this study for the effect of pirfenidone on collagen III. All that can be deduced is that there is an effect of the CNI + SRL combination that is not regulated by pirfenidone, although there is no published evidence that these combinations produce a different cytokine or signal.

Whilst much work has been directed at collagen (the principal matrix protein) and TGF-β, little is known about the effect of pirfenidone on other effectors of matrix remodelling such as MMPs and TIMPs. The focus on the effect of TGF-β is cogent because 1) TGF-β is the central point in the initiation of pro-fibrogenic events, and 2) TGF-β up-regulates collagens and TIMP-1. The alteration of these other mediators by prifenidone may well be mediated through suppression of TGF-β. The attenuating effect of pirfenidone on TIMP-1 demonstrated here, and conversely, the ability of pirfenidone to reverse the depressant effect of cyclosporine on MMP2 mRNA levels, further supports evidence that pirfenidone affects genes determining matrix remodelling. The Leicester unit[41] has previously demonstrated that pirfenidone inhibits excess expression of MMP-2 and -9, and TIMP-1, in a mechanically-injured rat carotid artery model, thereby reducing vascular smooth muscle cell proliferation and migration. TIMP-1 levels are also decreased by pirfenidone in experimental liver

fibrosis[531]. Recently, Di Sario et al.[537] found that addition of pirfenidone after chemically-induced rat liver injury reduced biochemical markers of liver injury, and downregulated elevated levels of TIMP-1, TGF-β and procollagen α1. They also found that MMP-2 levels were decreased by pirfenidone. Pirfenidone has previously been shown to inhibit lipopolysaccaride-stimulated MMP expression[538] and to inhibit MMP-2 expression in the cortex of the post-obstructed ureter model[523].

When applied to tacrolimus-treated animals, pirfenidone inhibited neither TGF-β nor TMIP-1, although this may be because tacrolimus itself had suppressed these mediators below control levels. Pirfenidone did reduce TIMP-1 expression to control levels when added to the CSA + SRL combination. For TAC + SRL, prifenidone had no effect on the expression of TIMP-1. The Leicester unit have also previously demonstrated that both tacrolimus[40] and pirfenidone[41] separately inhibit TIMP-1 mRNA expression, in rat carotid artery, with resulting inhibition of proliferation of smooth muscle cells.

Levels of the lytic enzymes MMP-2 and 9 were statistically unchanged by tacrolimus treatment, and therefore any inhibitory effect of pirfenidone on stimulated MMP expression could not be observed.

5.5.4 Summary and hypotheses

The hypotheses for this section were:

- Pirfenidone has dose-dependent effects,
- Pirfenidone reduces markers of CNI-induced injury in the salt-depleted model,
- Pirfenidone confers benefits on markers of renal injury when added to the combination of CNI + SRL.

Pirfenidone's actions were non-dose dependent when tested against CsA + TAC. It appears to be relatively free of side effects in animals, and is well tolerated in humans. Dose adjustment according to GFR may be required in pirfenidone-treated patients in any future clinical studies[424]. With lone CsA therapy and CsA + SRL, pirfenidone reduced functional and molecular markers of renal injury. The action of TAC on markers of injury suggested less acute toxicity and an antifibrotic effect. Reversal of these effects by SRL was abrogated by pirfenidone.

When considering any effect of pirfenidone, there is the possibility that it altered the metabolism, distribution or pharmacodynamics of the immunosuppressive agents, and this was responsible for its effect. However, Shihab's study did not demonstrate alterations in drug levels with concomitant pirfenidone administration[459]. Furthermore, pirfenidone has effects on matrix proteins in the kidney [522;534] and other organs[41;531;539;540] in the absence of other drugs, so probably has a direct, specific effect. With regards the exact mechanism of action of pirfenidone, a recent finding in endotoxin-induced liver injury suggested that one mechanism of action may be its ability to inhibit neutrophil infiltration into the liver[541]. This raises the possibility that as well as an effect on matrix genes, prifenidone may inhibit the influx of other effector cells involved in the inflammatory process in the kidney. Whether this is a direct inhibitory effect or the inhibition of a chemoattractant signal is not clear. The inhibition of TNF-α and subsequent endotoxin shock in mice by pirfenidone[542] indicates that this effect may be mediated through inhibition of chemical signals. Regardless, a beneficial effect at the molecular level has been demonstrated in the present study. If these results can be translated to human studies, pirfenidone may offer an avenue for treatment of renal transplant fibrosis.

Other inflammatory models suggest that pirfenidone can prevent the accumulation of, limit the progression of, and reverse established fibrotic lesions. Pirfenidone was administered at the same time as the calcineurin inhibitors in this model. Another approach may be to include pirfenidone dosing before or after use of CNIs, to prevent or reverse renal allograft fibrosis. Di Sario et al.[537] found that addition of pirfenidone 3 weeks into a 5 week course of dimethylnitrosamine reduced the degree of liver injury in rats, downregulating the transcription of TGF-β, procollagen α1, TIMP-1 and MMP-2. This indicates that pirfenidone is effective when administered after the induction of damage.

CHAPTER 6 – CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

6.1.1 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

6.1.2 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

6.1.3 Section C: The effect of the combination of tacrolimus and sirolimus

6.1.4 Sections D and E: Effects of pirfenidone when added to treatment with calcineurin inhibitors, with and without sirolimus

6.2 FUTURE STUDIES

6.2.1 Clarification of fibrosis and proteinuria

6.2.2 Molecular changes as an early marker of fibrosis

6.2.3 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

6.2.4 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses, and Section C: The effect of the combination of tacrolimus and sirolimus

6.2.5 Sections D and E: Effects of pirfenidone when added to treatment with calcineurin inhibitors, with and without sirolimus

6.1 CONCLUSIONS

The central tenet of the investigations presented here is that calcineurin inhibitors, whilst a mainstay for immunosuppression after transplantation, produce a cascade of acute and chronic injurious events that contribute to chronic allograft nephropathy. Alterations in molecular signals are caused by this injury, and functional and (eventually) structural changes in the kidney ensue. Most, if not all, transplant patients treated with calcineurin inhibitors will express some degree of renal injury. The literature reports various approaches for reducing the damage caused by cyclosporine and tacrolimus. These include use of non-calcineurin inhibitor adjuvant agents (e.g. sirolimus) to allow calcineurin-inhibitor dose-reduction and therefore exposure. There is a relative paucity of work examining the role of antifibrotic agents such as pirfenidone for halting or reversing fibrosis.

This study has utilised the rat salt depletion model of calcineurin inhibitor toxicity to examine the effects on renal functional, structural and molecular markers for clinically relevant combinations of cyclosporine, tacrolimus and sirolimus. Further, the effect of pirfenidone, when added to these drug combinations has been examined.

6.1.1 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

As proposed by the hypothesis for this section (see 2.2.1), there are differences in the effects of cyclosporine and tacrolimus on functional and molecular variables, with tacrolimus displaying results that are more favourable. A small number of (but certainly not all) clinical studies describe a measurable benefit from the use of tacrolimus in place of cyclosporine. This may reflect a lesser fibrotic potential of tacrolimus in transplant recipients. As sole therapy, sirolimus had no effect on renal

function or mRNA expression. Thus, the use of tacrolimus in place of cyclosporine, and sirolimus to allow cyclosporine or tacrolimus dose-reduction, may represent strategies for lowering the prevalence and severity of chronic allograft nephropathy. There were no differences in urinary protein or interstitial fibrosis measurements across the groups.

This section of the study also served as a baseline for the following sections, when drug combinations were used.

6.1.2 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses

The hypotheses for this section of the study (firstly that dose manipulation of the two drugs produces variable effects, and second that at the correct doses, SRL added to CsA is beneficial compared to CsA alone – see section 2.2.2) were both confirmed. Deterioration in renal function and a deleterious effect on molecular markers of fibrosis were seen when the drugs were combined at high doses; at lower doses, favourable outcomes for these end-points were elicited. There were no differences in urinary protein or interstitial fibrosis measurements across the groups.

Some of the clinical studies of the use of SRL for CsA dose reduction show improved renal function with relatively short follow-up. The effect of this drug combination on long-term graft survival remains unknown, and there are no detailed chronic nephrotoxicity studies examining the clinical combination of CsA and SRL.

6.1.3 Section C: The effect of the combination of tacrolimus and sirolimus

The hypothesis (that the combination of TAC plus SRL was favourable compared to TAC alone – see section 2.2.3) was rejected, based on the results from this section. Sirolimus worsened early renal function, and reversed the beneficial

molecular effects of tacrolimus. There were no differences in urinary protein or interstitial fibrosis measurements across the groups. Although TAC is possibly less nephrotoxic than CsA in the clinical setting, dose minimisation of TAC may be desirable. Adjuvant use of SRL may allow this, but the present findings suggest that amelioration of the beneficial effects of tacrolimus may be a hazard.

6.1.4 Sections D and E: Effects of pirfenidone when added to treatment with calcineurin inhibitors, with and without sirolimus

The hypotheses for this final section were threefold: i) that pirfenidone has dose dependent effects, ii) that pirfenidone reduces markers of calcineurin inhibitor-induced renal injury, and iii) that when added to the clinically important combination of CNI and SRL, pirfenidone has beneficial effects. Pirfenidone's actions were non-dose dependent, and beneficial effects for renal function and molecular markers were demonstrated. There were no differences in urinary protein or interstitial fibrosis measurements across the groups. Without fibrosis it is impossible to say whether pirfenidone acted in a *histologically* antifibrotic manner. However, the molecular changes suggest such an effect may have been developing. The effect of pirfenidone on renal function has not previously been described.

6.2 FUTURE STUDIES

This animal study has examined questions involving some of the functional and molecular effects of commonly employed immunosuppressants, alone and in combination. The overall purposes & outcomes of the work are summarised above. A number of points for further study have been raised, and are highlighted below.

6.2.1 Clarification of fibrosis and proteinuria

Although fibrosis was not demonstrated in this study, the observed changes in gene expression are likely to represent a precursor of fibrosis. The lack of fibrosis is discussed in the body of the text (see 5.2.3). Briefly, possible reasons include an inadequate length of salt-depletion, and insufficient renal drug concentration. The lack of proteinuria is discussed in section 5.2.2, and may reflect the absence of structural damage. Rather than quantification of total protein, a more sensitive marker of tubular damage such as alanine aminopeptidase could be measured. Future studies in this model would benefit from trials of longer periods of salt depletion, and from measurement of drug concentrations in both serum and renal tissue.

6.2.2 Molecular changes as an early marker of fibrosis

One of the important points raised in this study, and emphasised in others, is that changes in the expression of mediators of ECM turnover probably serve as interim markers for subsequent fibrosis. If these early changes can be clearly linked to later fibrosis, protocol biopsies in renal transplant recipients may allow risk stratification for CAN, and early intervention for patients at risk.

6.2.3 Section A: The effect of single agent therapy with cyclosporine, tacrolimus or sirolimus

The key area for future investigation under this heading is elucidation of the effect of TAC in *de novo* renal transplants and in established CAN. Tacrolimus is assuming a prominent role in immunosuppressive strategy because it is thought to be less nephrotoxic than CsA. However, this and a small number of other studies suggest TAC may actually demonstrate an antifibrotic action.

Sirolimus is a relatively new introduction to the field. Its apparent lack of overt renal toxicity (although there is evidence for prolongation of delayed graft function, and for acute toxicity in terms of magnesium loss) explains its place in current clinical trials.

6.2.4 Section B: The effect of combination therapy with cyclosporine and sirolimus at varying doses, and Section C: The effect of the combination of tacrolimus and sirolimus

The use of sirolimus as adjuvant therapy with the calcineurin-inhibitors is one approach for reducing nephrotoxic exposure in renal transplants. Because of complex drug interactions, it will be important to tailor drug doses to maximise immunosuppression, whilst minimising adverse functional and molecular effects. Further clinical studies with CNIs and sirolimus, looking at the endpoints of graft function and survival, will be helpful especially if such investigations examine protocol biopsies for molecular markers of fibrosis. Clinical studies with tacrolimus (emerging as the least nephrotoxic of the CNIs) and sirolimus (to allow minimisation of nephrotoxic exposure) will be particularly important. Little is known about the clinical effects of this combination on renal haemodynamics and structure. It will be interesting to investigate whether SRL cancels any beneficial effects of TAC.

6.2.5 Sections D and E: Effects of pirfenidone when added to treatment with calcineurin inhibitors, with and without sirolimus

The beneficial effects of pirfenidone on renal function are interesting and merit further consideration. It is not clear how the acute, functional effect of pirfenidone in the present study was mediated, but it is likely that a chemical signal involved in vasoconstriction is inhibited by prifenidone. This may or may not involve one of the established pathways controlling afferent glomerular arterial tone, such as the RAS[527]. TGF-β has recently been implicated in the control of arterial tone[463]. As pirfenidone

inhibits TGF-β, this is a possible mechanism for its action in the reduction of CNI-induced functional toxicity. The addition of a TGF-β antagonist to the model may prove useful when investigating this point.

Clinical studies of pirfenidone (see section V.3.5, Chapter 1) have demonstrated that it is relatively free of side effects when administered orally, and a study in glomerulosclerosis suggested that pirfenidone slows renal functional decline[424](see V.3.5). The encouraging results of the present animal study suggest that pirfenidone may have a beneficial effect in human chronic allograft nephropathy. The Leicester group is preparing a clinical trial of pirfenidone in calcineurin inhibitor-treated patients with biopsy-proven CAN. The trial will investigate the ability of this antifibrotic to attenuate disease progression. Endpoints will be allograft function and survival. The study of pirfenidone in glomerulosclerosis[424] concluded that dose-adjustment according to GFR is required to avoid gastrointestinal side effects; this will be pertinent for patients with CAN taking immunosuppression.

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