The Effect of Rapamycin after Cyclosporin dose reduction on Chronic Allograft Nephropathy

By

R N Saunders MA, MB, BChir, MRCS(Eng).

A thesis submitted for the degree of
Doctor of Medicine

From

The Department of Transplant Surgery, University of Leicester, 2002

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

I dedicate this dissertation to my late father who inspired me to pursue a career in surgery and my wife Diane who has patiently supported me throughout.

The work on which this dissertation is based was performed by myself except where acknowledged.

R N Saunders June 2002

Synopsis

Chronic allograft nephropathy (CAN) is the commonest cause of late decline in renal allograft function and subsequent failure. Histopathologically it is underpinned by the accumulation of extracellular matrix. The first chapter provides a thorough review of the current opinions regarding the aetiology, pathophysiology and management of this complex condition. Overexposure to Cyclosporin is a major risk factor for chronic allograft nephropathy and thus Cyclosporin dose reduction has been advocated in some reports. Rapamycin is a relatively new immunosuppressant, recently introduced in renal transplantation. The second chapter reviews this new agent and discusses experimental evidence supporting its use in patients with chronic allograft nephropathy. The aim of this work was to determine the impact of the addition of Rapamycin after Cyclosporin dose reduction in renal allograft recipients with chronic allograft nephropathy and thus to ascertain whether such a regimen was beneficial.

In order to achieve this 31 renal transplant recipients with biopsy confirmed CAN were prospectively randomised to receive either a 40% dose reduction in Cyclosporin (control), or a 40% dose reduction in Cyclosporin with the addition of Rapamycin 2mg/day (Rapa). Renal function and side effect parameters were assessed at 1,2,4,6,8 weeks, 3 and 6 months. The third chapter presents the clinical results. Proteinuria, serum creatinine and calculated GFR were similar in both groups. However the rate of decline of the calculated GFR was reduced over the study in control but not Rapa patients. Furthermore radio-isotope GFR fell in those in the Rapa group but not controls. The use of Rapamycin was safe with only relatively minor side effects and some temporary haematological and hyperlipidaemic changes.

The patients above had a renal allograft biopsy on recruitment and again at 6 months. Glomeruli were plucked from the surface of each biopsy core and these as well as a small sample of interstitium underwent total mRNA extraction. Complementary DNA was synthesized by reverse transcription and polymerase chain reactions used to amplify specific genes involved in the turnover of extracellular matrix in CAN. These were quantified using an ELISA technique. The fourth chapter details the changes in expression of some of these genes in. In glomeruli, TGFβ-1 remained constant in Rapa patients but fell in controls. Collagen III and TIMP-2 increased in those taking

Rapamycin but not in controls. TIMP-1 and MMP-2 expression increased in a similar fashion in both groups. Glomerular TGFβ-1, TIMP-1 and -2 expression appeared to be related to calculated GFR. There were fewer molecular changes within the interstitium but collagen III expression increased in Rapa patients.

The fifth chapter discusses the use of Sirius red staining and computerised histomorphometry in order to obtain an accurate assessment of the impact of this regimen on the amount of collagen present in the biopsies taken above. The interstitial volume fraction of biopsy cores stained with Sirius Red fell over the study in controls but a similar effect did not occur in Rapa patients.

The final chapter concludes that the addition of Rapamycin (2 mg/day) after Cyclosporin dose reduction in patients with CAN did not improve functional outcome or molecular and histological markers of CAN. Possible explanations are discussed and the need for a larger multicentre trial emphasised in order to substantiate these findings. Studies utilising complete Cyclosporin elimination with the addition of Rapamycin may have better prospects for the future.

Acknowledgements

The work for this thesis was performed in the Department of Transplant Surgery at Leicester General Hospital under the supervision of Professor M L Nicholson. His enthusiasm and drive was a constant inspiration and I am very appreciative of his support.

My thanks are also extended to Dr G R Bicknell, who taught me the molecular biological techniques used, as well as Dr R Sandford and Dr P N Furness for their assistance with Sirius red histomorphometry. I am similarly grateful to to Professor D R Jones at the Leicester University Department of Epidemiology and Public Health for his statistical advice and Wyeth-Ayerst for their sponsorship of the clinical trial.

Prizes / Commendations arising from this work

- International young investigator award, American Society of Transplantation, Chicago, USA, 2001
- Best abstract award, European Society of Organ Transplantation, Lisbon, Portugal, 2001
- Best poster prize, British Transplant Society, Oxford, UK, 2001

Presentations arising from this work

- **Saunders R N**, Carr S J, Nicholson M L. A randomised controlled trial of Cyclosporin dose reduction with or without the addition of Rapamycin for renal transplant recipients with chronic allograft nephropathy.
  British Transplant Society, 2000.
  The Association of Surgeons of Great Britain & Ireland, 2000.
  American Society of Transplantation, 2000

- **Saunders R N**, Murphy G J, Metcalfe M S, Carr S J, Nicholson M L. The side effect profile of Rapamycin in renal transplants recipients with chronic allograft nephropathy.
  The Association of Surgeons of Great Britain & Ireland, 2000

- **Saunders R N**, Metcalfe M S, Murphy G J, Bicknell G, White S A, Nicholson M L. The effects of Rapamycin after Cyclosporin dose reduction on profibrotic gene expression in both glomeruli and interstitium from renal transplant recipients with chronic allograft nephropathy.
  British Transplant Society, 2001 *-Best Poster Prize-*

- **Saunders R N**, Metcalfe M S, Murphy G J, Bicknell G, White S A, Nicholson M L. The effects of Rapamycin after Cyclosporin dose reduction on glomerular profibrotic gene expression in renal transplant recipients with chronic allograft nephropathy.
  European Society of Organ Transplantation, 2001 - *Best Abstract Award* -

- **Saunders R N**, Metcalfe M S, Murphy G J, Bicknell G, White S A, Nicholson M L. Rapamycin does not improve renal function or reduce profibrotic gene expression in chronic allograft nephropathy.
  The Association of Surgeons of Great Britain & Ireland, 2001 - *Moynihan Prize Session* -
  American Society of Transplantation, 2001 - *International Young Investigators Award* -

Publications arising from this work

- **Saunders R N**, Metcalfe M S, Nicholson M L. Rapamycin in transplantation. A review of the evidence.
  Kidney International 59: 3-17, 2001

- **Saunders R N**, Nicholson M L. A randomised controlled trial of Cyclosporin dose reduction with or without the addition of Rapamycin for renal transplant recipients with chronic allograft nephropathy.
  Transplantation accepted, May 2002.

- **Saunders R N**, Nicholson M L. Causes of late allograft loss in otherwise well renal transplant recipients.
  Medicine Matters 30: 2-4, 2001

- **Saunders R N**, Carr S J, Nicholson M L. Chronic allograft nephropathy: A randomised controlled trial of Cyclosporin dose reduction with or without the addition of Rapamycin.
  Transplantation, 2000; 69(8S): 432 (Published abstract)

- **Saunders R N**, Carr S J, Nicholson M L. The side effect profile of Rapamycin in renal transplants recipients with chronic allograft nephropathy.
  British Journal of Surgery, 2000; 87(S1): 82 (Published abstract)

- **Saunders R N**, Metcalfe M S, Murphy G J, Bicknell G , White S A, Nicholson M L. Rapamycin increases glomerular profibrotic gene expression after Cyclosporin dose reduction in patients with chronic allograft nephropathy.
  American Journal of Transplantation, 2001; 1(S1): 477 (Published abstract)

- **Saunders R N**, Metcalfe M S, Murphy G J, Bicknell G , White S A, Nicholson M L. Rapamycin does not improve renal function or reduce profibrotic gene expression in chronic allograft nephropathy.
  British Journal of Surgery, 2001; 88 (S1): 26 (Published abstract)

Abbreviations

A	Adenosine
ACE	Angiotensin converting enzyme
AJ 10X	Alec Jeffries 10X Buffer
AMVRT 5X	Avian Myeloblastosis Virus Reverse Transcriptase 5X Buffer
AR	Acute Rejection
ATN	Acute tubular necrosis
Aza	Azathioprine
bFGF	Basic fibroblast growth factor
BP	Blood pressure
BSA	Bovine serum albumin
C	Cytosine
CAN	Chronic allograft nephropathy
CADI	Chronic allograft damage index
CAV	Chronic allograft vasculopathy
Chol	Cholesterol
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CR	Chronic Rejection
^{51}Cr	Chromium-51
CTS	Collaborative transplant study
CVR	Chronic vascular rejection
CyA	Cyclosporin
DEA	Diethanolamine
DEPC	Diethylpirocarbonate
DNA	Deoxy-ribonucleic acid
DGF	Delayed graft function
DM	Diabetes mellitus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMBL	European molecular biology laboratories
eNOS	Endothelial nitric oxide synthetase
ESRF	End stage chronic renal failure
ET-1	Endothelin-1
FBC	Full blood count
FKBP-12	FK506 binding protein-12
FK506	Tacrolimus
G	Guanine
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCG	Genetics computer group
GFR	Glomerular filtration rate
GVD	Graft vascular disease
HDL	High density lipoprotein
HLA	Human leucocyte antigen
HLA-DR	Human leucocyte antigen type DR
HMGCoA	Hydroxy-3-methylglutaryl coenzyme A
H_20_2	Hydrogen peroxide

Ig-	Immmunoglobulin type-
IGF-1	Insulin growth factor-1
IFN-γ	Interferon-γ
IL-	Interleukin (type-)
iNOS	Inducible nitric oxide synthase
IVF	Interstitial volume fraction
kg	Kilogram
LiDS	Lithium dodecylsulphate
LDL	Low density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
M/mol	Molar / moles
mM/mmol	millimolar / millimoles
m^2	$metre^2$
MCP-	Macrophage chemotactic protein (type-)
MHC	Major histocompatibility complex
mg	Milligram
ml	Millilitre
min	Minute
MMF	Mycophenolate Mofetil
MMP-	Matrix metalloproteinase (type-)
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of Rapamycin
ng	Nannogram
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
PAI-1	Platelet activating inhitior-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PHA	Phyto-haemaglutinin
PNPP	Paranitrophenyl phosphate
Pred	Prednisolone
PTLD	Post transplant lymphoproliferative disease
R_p	Pearson's correlation coefficient
R_s	Spearman's correlation coefficient
RANTES	Regulated upon activation of normal T cell expressed and secreted
Rapa	Rapamycin
RCT	Randomised controlled trial
Rnase	Ribonuclease
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
T	Thymidine
Taq	Thermophilus Aquaticus
TG	Triglyceride
TGFβ1	Transforming growth factor beta-1
TH 1/2	T Lymphocyte helper type 1/2
TIMP-1/2	Tissue inhibitor of matrix metalloproteinases-type1/2
TL	Trough level
TNF-α	Tumour necrosis factor-α

TOR	Target of Rapamycin
TXB2	Thromboxane-B2
UKTSSA	United Kingdom transplant support services authority
UNOS	United States organ sharing network
URTI	Upper respiratory tract infection
UTI	Urinary tract infection
VCAM-1	Vascular cell adhesion molecule-1
µl	Microlitre
µmol	Micromole

Contents

Chapter 1

Chronic allograft nephropathy

1.1 Introduction

1.1a History
1.1b Histology
1.1c Clinical presentation and diagnosis
1.1d Epidemiology

1.2 Aetiology

1.2a Immune factors
1.2b Non-immune factors
1.2c Cyclosporin nephrotoxicity

1.3 Pathogenesis

1.3a Pathophysiology
1.3b Molecular basis

1.4 Management strategies

1.4a ACE inhibitors and HMG CoA reductase inhibitors
1.4b Immunosuppressants in denovo allografts
1.4c Immunosuppressants in established CAN
1.4d Surrogate markers in CAN

1.5 Conclusion

1.1 Introduction

There have been significant improvements in short-term renal allograft survival over the last decade and currently ≥80-90% of allografts survive 1 year[1]. Advances in both immunological and surgical practices were originally responsible for this improvement and have been sustained by the advent of new immunosuppressive drugs that have reduced both the frequency and severity of acute rejection. Until recently these early benefits did not appear to influence long-term graft survival and this remained static throughout the 1970s and 80s. However a recent analysis of over 93,000 renal transplants performed in the USA between 1988 and 1996 has documented an increasing half-life from 7.9 to 13.7 and 12.7 and 21.6 years in cadaveric and live donor allografts respectively[2].

Despite these statistics, renal allograft loss following the first year of transplantation remains a significant problem. CAN remains the leading cause of late transplant loss and is responsible for a 3-4% annual decline in functioning allografts[3]. The deterioration in renal function and ultimate return to dialysis is often associated with significant morbidity. A well functioning renal transplant not only improves quality of life but more importantly a recent American analysis of over 220,000 patients has shown a significant survival advantage over dialysis for some patients, particularly those who are young, caucasian or diabetic[4]. Thus considerable research is aimed at potential treatments for CAN in order to prolong long-term graft survival. This has obvious benefits for the individual but will also attenuate both the financial and physical pressures placed on nephrology units as well as reducing the number of patients requiring re-transplantation and thus demand on a limited supply of donor organs.

1.1a History

The first successful human kidney transplants were performed by Hume et al at The Peter Bent Brigham Hospital, Boston, USA (1951-54)[5]. Four out of nine patients developed renal function that in the case of 1 patient lasted 6 months before death. In this individual renal function had begun to deteriorate in the month before death. The allograft was found to be pale and mottled and histological examination showed striking atherosclerosis with marked intimal thickening as well as sclerosed glomeruli and tubular atrophy. These features are consistent with those of CAN although it was some time before this was appreciated.

In the following years there were a number of isolated reports of vascular changes in allografts with longer-term survival[6,7]. The true significance of these lesions however was first appreciated by Porter (1963) who observed that 11 of the 29 published transplants surviving beyond 6 months had obliterative vascular changes[8]. Furthermore he noted that hypertension occurred after the development of these changes and so was unlikely to be the initial cause of them as originally postulated by Hume. The editorial in the same issue of the British Medical Journal used the term *'chronic rejection'* (CR) to describe these changes for the very first time. At a similar time Hamburger (1964, Paris) described 2 patients who had developed marked proteinuria, hypertension and deteriorating renal function and had lobular glomerular changes on allograft biopsy of uncertain origin that did not resemble recurrent glomerular disease[9].

Progress in the recognition of CR was initially slow due to the small number of allografts surviving the early months post-transplantation. Up to 1963 only 9 of the 211 transplants recorded in the world literature had survived more than 1 year.

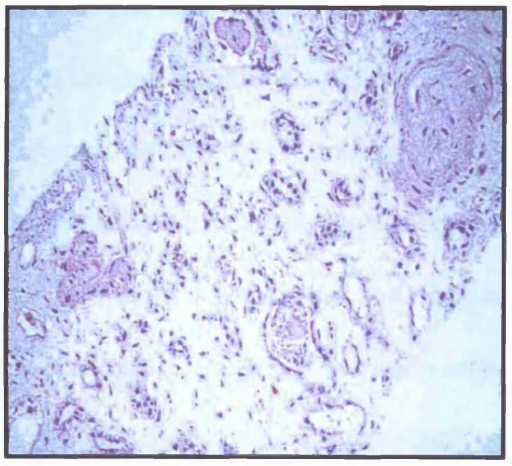
However by 1969 Najarian recognised that the arterial lesions described by Porter and the glomerular lesions described by Hamburger were part of the same process[10]. Thus CR was distinguished from acute rejection (AR) by both histology and time course. However the major challenge facing physicians in the pre-cyclosporin era remained allograft loss secondary to AR and thus there was relatively little interest in CR at this time. With the advent of Cyclosporin and subsequently lower rates of AR, longer-term graft survival became the goal and there has been much work in the late 1980s and 1990s in an attempt to optimise this.

The term CR implies an immune mediated aetiology and whilst immunological factors are undoubtly important in the development of chronic transplant damage it has become appreciated over the last decade that non-immunological factors also play a prominent role in this process. Thus the term 'chronic allograft nephropathy' has evolved, implying a chronic allograft injury secondary to a variety of insults occurring over a sustained time period.

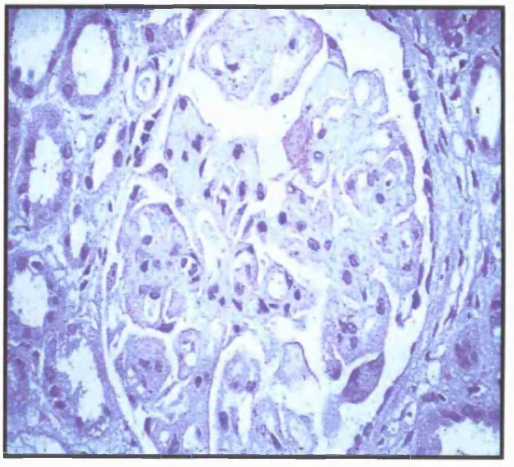
1.1b Histology of CAN

The diverse aetiologies involved in the pathogenesis of CAN (see later) result in relatively non-specific sometimes patchy histopathological changes in the interstitium, glomeruli and vasculature of allografts (Fig 1.1)[11]. Pathologists have long-recognised that CAN was a useful descriptive term as it is often not possible to distinguish between chronic rejection, chronic cyclosporin nephrotoxicity, hypertensive vascular disease and chronic infection or reflux and in many instances such aetiologies co-exist[12].

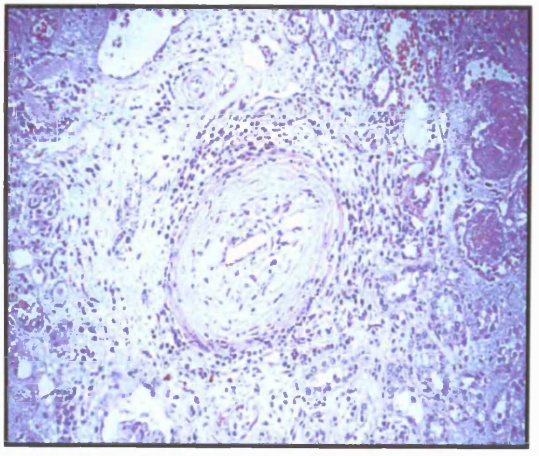
Fig 1.1 Histology of chronic allograft nephropathy



Interstitial Fibrosis



Glomerulosclerosis



Intimal Hyperplasia

The vascular changes observed in CAN are dominated by a vasculopathy reported to occur in 7.5% of protocol renal allograft biopsies at 3 months and 36% at 2 years[13,14]. This is characterised by progressive concentric intimal thickening that may become eccentric at arterial branch points and is similar to atherosclerosis. However it may also involve smaller vessels not usually affected by atherosclerosis[15]. As intimal hyperplasia develops it becomes less cellular and more fibrous, extending into the lumen of vessels causing partial and sometimes complete occlusion. A similar vasculopathy is seen in cardiac, lung and pancreatic allografts with CR, implying that a mechanism common to all solid organ transplants may underpin its development[16]. In CAN, allograft vasculopathy has been shown to be an adverse prognostic feature and is associated with significant reductions in long-term graft survival[13].

Microvascular lesions such as peritubular capillary basement membrane splitting and lamination have also been identified in CAN. These were originally described by Monga et al[17] using electron microscopy and have been confirmed by other studies that have identified these changes in 60% of individuals with CAN and noted that that they increase in severity with time following transplantation[18,19].

A number of glomerular lesions are seen in CAN including wrinkling and collapse of the glomerular tuft, glomerular hypertrophy and hypercellularity and mesangial matrix expansion[20]. Collectively these changes are known as transplant glomerulopathy a relatively specific but infrequent lesion in CAN (5-13%)[21,22], initially reported by Hamburger et al in 1964[9]. They are accompanied by a progressive usually focal glomerulosclerosis documented in 42% protocol biopsies 2 years post-transplantation (Fig 1.1)[14]. These glomerular changes co-exist with the vascular pathology discussed above in many instances[23].

The most prevalent changes in CAN are interstitial fibrosis and tubular atrophy (Fig 1.1) present in almost two thirds of protocol biopsies 2 years post- transplantation[14]. The tubulointerstitium occupies approximately 80% of the renal volume[24] and thus is less prone to biopsy sampling error than more patchy vascular or glomerular allograft structures. Therefore it has been used to assess the overall severity of CAN (I-III, mild, moderate, severe) in the Banff classification of kidney transplant pathology[12]. Each of the 4 types of lesion in CAN can also be graded from 0-3 (absent, mild, moderate or severe), independent of the overall classification. The purpose of this was to standardise the interpretation of changes and to provide more objective reporting of CAN in order to guide patient management and help establish objective end-points for clinical trials. The Banff criteria were updated in 1997 but the assessment and grading of CAN remained similar to the original classification[25]. Some investigators have criticised this suggesting that it places too much emphasis on the severity of interstitial fibrosis when assessing CAN. However accurate histomorphometric assessment of such changes has been shown to correlate with long-term allograft survival[26]. Although more complex scoring systems that take into account other factors exist e.g. Chronic allograft damage index (CADI)[27], these are quite cumbersome for use in routine clinical practice and have not been widely adopted.

1.1c Clinical presentation and diagnosis of CAN

Clinically CAN is characterised by a progressive decline in renal allograft function resulting in an inevitable return to dialysis. The time-span over which this occurs is unpredictable with some patients having a slow insidious decline over several years whilst others have very rapid deteriorations, resulting in allograft failure within a few months[28]. Proteinuria and hypertension are often associated with both the uraemic and

endocrine signs of impaired renal function. The development of proteinuria may reflect the onset of CAN and a positive correlation between the level of proteinuria and severity of CAN has been reported[29]. In one study 20-28% of patients with CAN had proteinuria greater than 0.5g/day and occasionally this can increase into the nephrotic range[30]. Hypertension is very common in all renal allograft recipients including those with CAN and thus it has little diagnostic significance[31]. Nevertheless an association between the degree of hypertension and the severity of both functional and histological changes in CAN has been reported[32,33].

It has proven difficult to get a worldwide consensus on the definition of CAN. However in 1989 Foster proposed a definition for chronic rejection in renal allografts that was adopted with only minor variation by the 'Fourth Alexis Carrel conference on chronic rejection and accelerated arteriosclerosis in transplanted organs' (1992)[34,35].

1. Patients must be at least 3 months post-transplantation
2. The regression of the reciprocal of plasma creatinine over time must be significantly different from zero
3. Graft histology should show changes compatible with chronic rejection
4. Other causes of allograft failure such as vascular and urological complications must be excluded

Despite some criticism (discussed below), these diagnostic criteria have not been changed substantially over the last decade.

1. The proposal to limit the diagnosis of chronic rejection to allografts at least 3 months post-transplantation aimed to ensure that a period of stable renal function had occurred prior to the onset of 'chronic changes' i.e. that grafts seriously damaged for

other reasons such as severe acute rejection that would inevitably fail, were not labelled as having chronic rejection. However Foster originally proposed that allografts should be at least 6 months post-transplantation. This was changed to 3 months by the conference in an attempt to apply a standard time for the diagnosis of chronic rejection across both cardiac and renal transplantation. However whilst a diagnosis of chronic rejection can be confidently distinguished from acute rejection at 3 months in cardiac transplantation (using angiography), renal allograft histology can be ambiguous and sometimes frankly misleading at this time. Within the transplant community this 3 month period is accepted as being an arbitrary one and thus many centres including our own have adopted Foster's more conservative 6 month time scale. This does not exclude the possibility that changes of chronic rejection are developing from 3 months, but avoids inclusion of a small number of patients whose allograft decline may be due to alternative pathology.

2. The presence of acute rejection can usually be distinguished from CAN relatively easily in older allografts. However recurrent or denovo renal disease is more difficult to discriminate and additional electron microscopy and immunofluorescence is often required to differentiate between the two. This should be considered in any diagnostic criteria for CAN.

1.1d Epidemiology of CAN

Transplant registries have confirmed significant improvements in 1 year renal allograft survival in the 1980s but suggested that this had little impact on long term survival with half-lives remaining static at 8-10 years[1]. A more recent UNOS analysis (1988-1996) is more encouraging and has documented an increasing half-life from 7.9 to 13.7 and 12.7 and 21.6 years in cadaveric and live donor allografts respectively[2].

Although such registry data are extremely powerful tools in the retrospective study of such epidemiological trends, they have been unable to collect data specifically relating to CAN[36]. Therefore our current knowledge of the epidemiology of CAN is based on reports from single or small multicentre studies with considerable variation in patient groups studied and the criteria used to define CAN. The majority are retrospective and analyse the results of diagnostic allograft biopsies performed to investigate deteriorations in renal function. These occur at various time intervals following transplantation and give no information on allografts with stable renal function that are not biopsied. Nevertheless such studies have suggested that CAN is rarely seen in the 3 months immediately post-transplantation and occurred in only 2.4-10% of diagnostic biopsies performed in the first 6 months[37,38]. As length of follow-up increased so does the prevalence of CAN with between 5-71% of individuals reported to have this condition on the basis of diagnostic biopsies performed 3-6 years following transplantation[38-41]. CAN was thought to be the cause of allograft failure in 6-58% of patients after 4-5 years[42,43] and as many as 86% after 10 years[44].

The prevalence of CAN may be more accurately estimated in prospective analyses of protocol biopsies performed at defined time points post-transplantation. Two such studies have reported histological evidence of CAN in 25% (n=42) and 39% (n=280) of patients despite stable renal function as early 3 months post-transplantation contradicting data from retrospective work[13,45]. Isoniemi et al looked at 128 two year protocol biopsies in patients taking either triple therapy CyA / Aza / Pred or various dual therapy regimens[14]. Despite normal or near normal renal function in 77% of patients, interstitial fibrosis and tubular atrophy were present in 62 and 64% of allografts respectively although other histological features of CAN such as glomerulosclerosis (43%) and vascular intimal proliferation (36%) occurred less

frequently. These findings suggested that many of the retrospective analyses discussed above were probably under-estimating the prevalence of CAN. This argument was supported by more recent protocol biopsies performed at 2 years in 144 patients on either CyA or Tacrolimus that documented CAN in 62% of those on Tacrolimus and 73% on CyA[46].

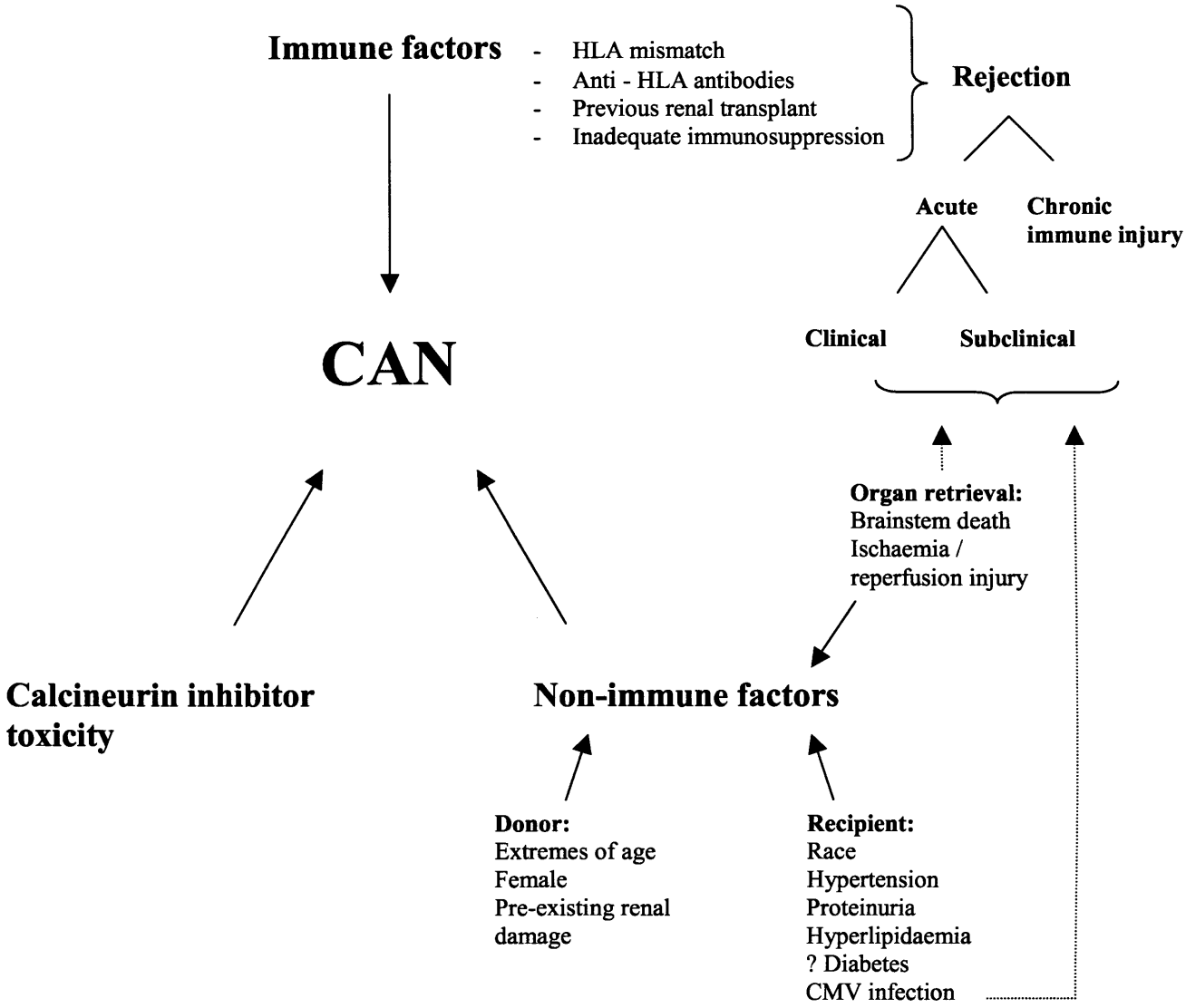
1.2 Aetiology of chronic allograft nephropathy

The aetiology of CAN (Fig 1.2) involves a number of diverse immune and non-immune factors, discussed below. In addition Cyclosporin nephrotoxicity is now thought to play an important role.

1.2a Immune factors

Human Leucocyte Antigen (HLA) matching has clearly enhanced both the short and long term survival of renal allografts[47,48]. The best results are observed from HLA identical-sibling donors with 2 year protocol biopsies in such individuals confirming a complete absence of CAN[45] and projected half-lives exceeding 30 years compared to half-lives of 16-18 years for recipients of 1 and 2 haplotype mismatched allografts[1]. Similar benefits for HLA matching have been demonstrated in cadaveric allografts with graft half-life deteriorating in a stepwise manner as the number of HLA mismatches increases[48]. However because currently registry data collection uses allograft and patient survival as its primary end points and not all late graft loss is due to CAN, these are unable to determine whether HLA matching has had a specific impact on the development and progression of CAN[36]. Similarly there are relatively few single centre analyses that have examined this relationship. However one study found that the number of HLA-DR mis-matches correlated with rate of

Fig 1.2 Aetiology of Chronic allograft nephropathy



graft loss secondary to CAN (confirmed in 85% of cases by histology) in a univariate analysis, but when acute rejection episodes were taken into account in a multivariate model this was no longer statistically significant[29]. A more recent multivariate analysis in over 1500 patients by Matas et al showed similar results[49].

Inadequate immunosuppression either as a result of patient non-compliance or secondary to over-enthusiastic reductions in immunosuppression may predispose to CAN[50]. Patients with CAN have been reported to have a lower overall exposure to CyA than patients without[51] and CyA doses of less than 3 and 5 mg/kg/day were identified as risk factors for CAN in large series of both adult and paediatric recipients[52-53]. Protocol biopsies from patients taking triple immunosuppression (CyA / Aza / Pred) were found to have lower CADI scores compared to those taking dual immunosuppression (CyA/Pred, Aza/Pred and CyA/Aza)[14]. The consistency of administration as well as the overall level of immunossuppression is thought to be important. Kahan et al have identified individuals with a high co-efficient of variation in blood CyA levels as a result of differential gastrointestinal absorption who are at increased risk of CAN[54]. This is exacerbated in non-compliant individuals presumably explaining their increased frequency of late allograft loss secondary to either acute or accelerated chronic rejection.

A high prevalence of preformed anti-HLA antibodies has also been linked with a decline in allograft half-life[55], as well as increased risk for the development of chronic vascular rejection[56]. This may help to explain the impaired survival of re-transplants compared to primary allografts although the exact mechanisms underpinning this process are currently unclear.

Each of the points discussed above can cause upregulations in allo-immune activity and predispose to AR. Episodes of AR are thought to be one of the most important risk factors for the development of CAN[57-60]. It is widely acknowledged that severity of AR has a significant impact on both short and long term allograft survival. Acute vascular rejection is a highly statistically adverse prognostic feature compared with tubulointerstitial rejection[61,62] and lack of response to steroid therapy has been reported to increase the risk of CAN[63]. In keeping with this, the density of graft infiltration with mononuclear cells and Class II lymphocytes has been noted to correlate with the severity of future CAN[64]. Furthermore studies have reported that patients with multiple episodes of AR had a significantly higher prevalence of CAN than those experiencing only a single episode[65].

The relative importance of the timing and reversibility of episodes of AR is more controversial. Isonemi et al have suggested that if AR occurred during the first 3 months post-transplantation it did not predict the development of CAN[66]. However others have disputed this[60]. Nevertheless when early and late episodes of are compared, late AR (> 1 year post-transplant) tended to increase the risk of CAN and graft loss to a greater extent, with one study demonstrating an increased risk of at least 3-fold[65,67]. Possibly a more important variable in determining long term outcome is the reversibility of each episode of AR. Vereerstraeten et al subdivided patients in the first year post-transplant following an episode of AR and completion of antirejection therapy on the basis of serum creatinine values[68]. Recipients with serum creatinines less than pre-rejection values (implying complete resolution of AR) had 5 year graft survival similar to those who had never had an episode of AR. Furthermore a serum creatinine within the normal range at 6 or 12 months post-transplantation despite an episode of AR have been shown by others to have a similar impact[69,70]. Although

some work has not been able to show a relationship between renal function during or following an episode of AR and the development of CAN[71], it is not unreasonable to suggest that patients who recover from AR with little loss of renal function may have a better long term outcome than those where the immune injury has been so severe that renal function is irreversibly impaired.

Whilst there is little doubt that clinically overt AR can cause significant allograft injury and thus predispose to CAN, some investigators have also hypothesised that a lower grade immune injury termed 'sub-clinical' rejection may also play an important role. This can occur because of relative under-immunosuppression or following a successfully treated episode of AR that still has histopathological evidence of ongoing immune activity. Despite normalisation of renal function, 10 days after treatment with methyl-prednisolone such changes were documented in 26.7% of cases[72]. Identification of an interstitial infiltrate of mononuclear cells in allograft biopsies is thought to be diagnostic of sub-clinical rejection[73] and this finding in 3 month protocol biopsies was associated with an increased incidence of CAN at 2 years despite no episodes of AR in these patients[74]. On the basis of this Rush et al performed a small (n=36) randomised trial to determine the merits of treating subclinical rejection identified on protocol biopsies 1, 2, or 3 months after transplantation with steroids[75]. Treatment reduced early and late (>6 months) AR, lowered the chronic tubulointerstitial damage score at 6 months and improved graft function at 2 years as well as graft survival at 4 years (p=0.076).

Although acute and possibly subacute rejections are important aetiological factors in the development of CAN, persistent low grade immune injury associated with both B cell antibody mediated allograft injury and T cell factors may also be an important[76].

Lymphocytes and macrophages are commonly found intermingled in the vascular endothelium of chronically rejecting allografts[76]. In addition the interstitial infiltrate in chronic rejection contains an abundance of plasma cells[77] and immunofluorescence studies show non-specific pattern of antibody deposition particularly in glomerular capillaries and basement membranes[78]. However neither the relative influence of B and T cell mechanisms nor their relationship with respect to acute or subacute rejection is fully appreciated[79].

1.2b Non-immune risk factors

The non-immune aetiologies important in the pathogenesis of CAN can be considered as pre-existing donor factors, allograft retrieval factors and problems relating to the recipient both before and after transplantation.

1.2b(i) Pre-existing donor factors

Kidneys and thus renal allografts are not created equal[80]. Males tend to have larger kidneys with approximately 20% more nephrons than females of the same body weight[81]. The number of glomeruli per kidney as well as the mean glomerular volume are closely correlated with the kidney weight and negatively correlated with subject age beyond 60 years[82]. Clinical data link cadaveric kidneys from young (<10 years), old (>50 years) and female donors with decreased long-term graft survival[83-85]. Experimentally, mass reduced rat kidney allografts develop accelerated functional and morphological deteriorations similar to CAN[86]. The potential mechanism to explain these findings (discussed later) suggests that both the number of nephrons in allografts as well as the metabolic demands upon them may have an impact on the development and progression of CAN[80].

1.2b(ii) Allograft retrieval factors

Cold and warm ischaemia as well as post-ischaemic reperfusion injury contribute to the insult suffered by allografts at organ retrieval and implantation[87]. A study of over 70,000 patients by Opelz et al found that duration of warm ischaemia had no significant influence on long term graft outcome. Furthermore although cold ischaemia of greater than 48 hours was associated with significantly impaired outcome, intervals less than this did not produce consistent effects on allograft survival[88]. Other investigators concur with this finding[89] although one smaller clinical study has documented a convincing relationship between increasing cold ischaemia and the development of CAN[90]. Clinically de novo allograft ischaemia is manifested as delayed graft function (DGF). Some single centre studies have reported that DGF followed by full recovery in the absence of acute rejection is not detrimental to long term allograft outcome[91,92]. However other work suggests that DGF increased the risk of late allograft loss[93,94]. Yokoyama et al (excluding primary non-function) noted a 5 year graft survival rate of 89% in immediate functioning kidneys, 85% in those on dialysis for less than 1 week and 50% in those on dialysis for greater than 1 week[95]. Similar findings were confirmed by Cacciarelli et al implying that the duration of DGF may be important in this respect[96].

Ischaemic damage may increase levels of pro-inflammatory cytokines and adhesion molecules[97] that in conjunction with free radical injury following reperfusion may stimulate the development of both AR and chronic vascular damage. Administration of the anti-oxidant recombinant super-oxide dismutase reduced the frequency of both reversible and irreversible AR as well as improving allograft survival compared to untreated controls after 4 years follow-up[98]. Several studies have shown an increased rate of AR in patients with DGF and it has been suggested that this may explain the

association between DGF and CAN[99,100]. However although the combination of AR and DGF has poor prognostic implications, analysis of registry data has suggested that DGF exerts an independent effect on long term allograft survival[94,100].

Despite our incomplete understanding of the pathophysiological events surrounding brainstem death, some investigators have suggested that it may be an further factor affecting long-term allograft survival[101]. It can cause disruption of the hypothalamic-pituitary axis and endogenous increases in catecholamine release resulting in labile blood pressures. Periods of warm ischaemia may occur and histopathological damage has been reported in heart, liver and kidneys[102]. In addition massive release of cytokines from the injured brain into the peripheral circulation have been noted[103] and these may increase the immunogenicity of allografts. Indeed, in a rat model of brainstem death organs from such animals are rejected in an accelerated fashion by the 'normal' host compared to controls[104]. However investigation into its impact on human allograft immunogenicity is at a very early stage and there is currently no firm evidence linking brainstem death to the development of CAN.

1.2b(iii) Recipient factors

In the 1960s and 70s, Afro-Caribbean renal transplant recipients were at greater risk of early failure than Caucasians but since the introduction of CyA in 1985 this has no longer been the case[105]. Nevertheless they do continue to have worse long-term outcomes. Poor HLA matching in Afro-Caribbean communities has been blamed for this[106]. However 5 year survival in HLA matched ethnic groups was found to be similar for Caucasians, Hispanics and Indo-Asians but significantly worse for Afro-Caribbeans[107] suggesting that other factors such as higher rates of non-compliance and an increased severity of post transplant hypertension are also highly relevant.

Calcineurin inhibition, steroids, native renal disease, transplant renal artery stenosis and CAN all contribute to the high prevalence of hypertension (70-80%) in renal transplant recipients. Hypertension is a well documented risk factor for the progression of renal disease in native kidneys[108]. In renal transplant recipients severity of recipient hypertension at 1 year had a highly significant relationship with late graft loss[109] and strongly predicted allograft survival irrespective of renal function[110]. Furthermore the degree of hypertension has been shown to correlate with both the functional and morphological severity of CAN[111] and appears particularly detrimental in Afro-Caribbeans[112]. However it remains unclear whether hypertension is a cause or a consequence of CAN, particularly as many of clinical studies do not take baseline renal function into account. Experimental studies in chronically rejecting rat allografts have shown that antihypertensive agents reduce elevated glomerular capillary pressure resulting in attenuation of proteinuria, amelioration of glomerulosclerosis and improvements in allograft survival[113]. However although antihypertensive treatment has potential benefits from the cardiac and cerebrovascular perspectives, there is currently little evidence in humans that it has a beneficial impact on CAN.

Proteinuria like hypertension is both a marker of renal disease and a factor in its progression[114]. In addition one study has shown it to be a significant risk factor for the development of CAN[29]. It has been suggested that reabsorption of excessive amounts of protein by proximal tubular cells may lead to release of inflammatory and vasoactive substances that contribute to tubulointerstitial injury[114]. A low protein diet can slow the progression of renal dysfunction in chronic 'nephropathies' [115] and a similar benefit has been shown in a small study in renal allografts where this caused a reduction in the rate of decline of the slope of the reciprocal serum creatinine against

time[116]. Angiotensin converting enzyme (ACE) inhibitors may have similar benefits and have been the subject of several recent studies (see later).

The vasculopathy in CAN has features in common with systemic atherosclerosis (AS) and in view of the high prevalence of hyperlipidaemia in the renal transplant community (≥50%)[117], lipoprotein abnormalities have been implicated in the aetiology of CAN. In vitro, oxidized low density lipoprotein (LDL) cholesterol causes intracellular changes to endothelial cells, disruption of vascular integrity[118] and induction of macrophages to form 'foam' cells. Furthermore it stimulates production of profibrotic cytokines and extracellular matrix proteins in cultured mesangial cells[119,120]. In vivo, diet induced hypercholesterolaemia in a rat cardiac allograft model produced marked lipid deposition in areas of intimal thickening but little smooth muscle cell proliferation[121]. However in other models whilst hypercholesterolaemia alone only increased eicosanoid metabolism with little effect on arteriosclerotic intimal changes[122], when combined with hypertriglyceridaemia there was increased expression of epidermal and insulin like growth factors as well as doubling of the intimal thickness[123]. Clinically there remains uncertainty as to whether hyperlipidaemia represents a cause or a consequence of CAN. Nevertheless patients with deteriorating renal function secondary to CVR have a more pronounced, proatherogenic pattern of hyperlipidaemia compared to those with stable allografts[124]. Hypertriglyceridaemia hypercholesterolaemia and elevated LDL cholesterol have all been advocated as risk factors for CAN[124,125], although not all clinical studies concur with this[126]. Furthermore hypercholesterolaemia both before[127] and after[125] transplantation may predict late graft loss although some investigators have suggested that only hypertriglyceridaemia was significant in this respect[29].

Diabetes Mellitus (DM) causes both macro and microvascular damage and it would seem logical that this could contribute to the development of CAN. However there are relatively few investigations into this association and those that exist appear contradictory. Two and a half percent of patients (33/1325) developed post-transplantation DM in one such study. When these were compared with paired-control allograft recipients although 6 year patient survival was similar, allograft survival tended to be better in controls[128]. A different centre found that there was no significant difference in 5 year graft survival between diabetic and non-diabetic patients. However the major cause of graft loss was death with a functioning graft secondary to cardiovascular disease in diabetics compared to CAN in non-diabetics[129]. Thus the role of DM in CAN remains unclear.

Acute Cytomegalovirus (CMV) infection causes sub-endothelial inflammation and induces chronic allograft vasculopathy in vivo[130,131]. In rat renal allograft models, CMV infection increased collagen III mRNA expression and histological evidence of CAN as well as augmenting the CADI index[132-4]. Homology and cross reactivity of CMV proteins with allograft MHC I and II domains, increased expression of vascular adhesion molecules e.g.VCAM-1 and endothelial MHC II complexes as well as upregulations in cytokines such as TGFβ / PDGF increase the immunogenicity of allografts during CMV infection, with implications for both acute and chronic immune injury[135-139]. In humans prolonged CMV viraemia is associated with the development of cardiac allograft vasculopathy[140]and moreover CMV nucleic acids have been identified in association with severe accelerated cardiac allograft arteriosclerosis[141]. Several clinical studies in renal allograft recipients clearly demonstrate that long term graft survival is decreased in patients treated for CMV disease[142,143] . However a small study in renal transplant recipients with obliterative

vasculopathy failed to demonstrate CMV by immunohistochemistry, in situ hybridisation or PCR[144]. CMV infection is linked to acute rejection[145] presumably because the increased immunosuppression required to treat such episodes enhances the risk of both primary CMV infection as well as re-activation of latent disease[146]. A major criticism of many clinical studies is that they are univariate analyses that have not controlled for acute rejection. When this was undertaken in a recent multi-variate analysis it appeared that CMV disease was only a risk factor for CAN in the presence of acute rejection, not in isolation[147]. Thus the direct impact of CMV on human CAN is unclear. Although the prophylaxis and treatment of CMV are beneficial in terms of patient morbidity and mortality their impact with respect to CAN is currently unknown.

1.2c Cyclosporin nephrotoxicity

The introduction of CyA revolutionised renal transplantation producing significant improvements in 1-year graft survival over the last 20 years[148]. The paradox of this agent is that it is highly nephrotoxic and may play a significant role in the development of CAN, thus limiting long-term allograft survival.

CyA nephrotoxicity is common in patients with psoriasis and other inflammatory / autoimmune diseases in addition to individuals after transplantation. A meta-analysis in patients with auto-immune disease demonstrated a risk difference of 21% for developing nephrotoxicity when CyA and alternative therapies were compared[149]. Even low dose CyA (≤ 5mg/kg/day) for 12 months resulted in the development of non-specific morphological changes consistent with denovo renal injury. Serum creatinine values > 150μmol/L were seen in 12-52% of cardiac allograft recipients

treated with CyA 2 years after surgery and by 4 years these were >250 μmol/L in 13-17% of individuals[150,151]. Furthermore 2/3 bone marrow transplant recipients demonstrated histological changes of chronic nephrotoxicty[152]. The prevalence of CyA nephrotoxicity in renal allografts is thought to be quite high but has proved difficult to estimate. This is because the diagnosis is based on histopathological changes that are often indistinguishable from other causes of CAN.

CyA nephrotoxicity can be categorised into an acute 'functional' and a chronic 'morphological' problem. These are often considered separately, but are actually inter-related processes with the former often leading to the latter over the lifetime of the graft. Administration of CyA causes a rapid intense vasoconstriction of preglomerular afferent arterioles resulting in an acute reduction in renal blood flow and glomerular filtration rate (GFR), manifested clinically as an increase in serum creatinine. This generally occurs in the early weeks post-transplantation, is often associated with only mild histopathological changes and responds to CyA dose reduction[153]. The proximal tubules seem especially prone to toxicity and non-specific reversible lesions such as isometric vacuolisation, inclusion bodies, microcalcification and acute tubular necrosis (ATN) may develop soon after initiation of CyA therapy[154]. Vascular lesions particularly in afferent arterioles[155] develop later (1-2 months+) and produce more permanent morphological damage. A combination of vasoconstriction and direct endothelial cell injury cause vacuolation followed by necrosis of endothelial and smooth muscle cells. Fibrin, platelets and plasma protein deposits cover these denuded areas producing areas of hyalinisation. These are frequently interspersed with a second form of CyA associated arteriolopathy characterised by areas of intimal thickening. Although remodelling has been reported, particularly after CyA dose reduction, partial as well as complete vascular occlusion can occur[154,156].

The downstream effects of this are seen in both glomeruli and tubulointerstitium. The prevalence of glomerular lesions can be related to the severity of the vasculopathy: 65% vs 45% vs 25% in severe, medium and mild vasculopathy respectively[157]. Morphologically these include thrombi, haemolytic uraemic syndrome-like lesions, thickening or wrinkling of glomerular basement membranes and ultimately glomerulosclerosis[158]. Such changes occur relatively late and are associated with 'stripes' of tubular atrophy and interstitial fibrosis interspersed between areas of normal or hypertrophied tubules[158]. Such histopathological changes can be clearly visualised in native kidneys, but in renal allografts the changes of chronic CyA toxicity occur against a background of other profibrotic insults in CAN making them difficult to distinguish, particularly later in the life of the allograft. However CyA toxicity can sometimes be identified on the basis of the type of vasculopathy present[154,155,158]. Hyaline arteriolar changes tend to occur in CyA toxicity and arterial intimal infiltration and proliferation tends to occur in CAN, but neither size of vessel nor histology is diagnostic. In addition both are patchy processes that may be missed or exaggerated due to biopsy sampling error.

The relationship between the vasoconstriction underpinning acute nephrotoxicity and the accumulation of extracellular matrix (ECM) seen in the longer term is not well understood. However a number of mediators including angiotensin II[159-161], thromboxanes[162,163], endothelins[164,165] and nitric oxide[166-168] are thought to have both vasoactive and profibrotic properties thus linking these 2 processes. In addition CyA exerts a direct profibrotic effect irrespective of its haemodynamic properties. This has been identified both in vitro and in vivo where an interstitial infiltrate and elevated levels of TGFβ1 still occurred despite low doses of CyA that produced normal haemodynamic and blood pressure parameters[169]. Thus the development of chronic

CyA toxicity in renal allografts may occur in response to ischaemia or as a result of direct CyA toxicity causing cell cycle arrest and death of renal epithelial cells[170]. Apoptosis of tubulointerstitial cells plays a role in this respect. CyA increased expression of pro-apoptotic molecules both in vitro and in vivo and such apoptotic positive cells have been correlated with the degree of tubular atrophy and interstitial fibrosis[171-173]. However the major histopathological process underpinning the long-term effects of CyA is the accumulation of ECM.

In vitro, CyA increased collagen synthesis in endothelial, epithelial cells and fibroblasts as well as upregulating TIMP-1 and downregulating MMP-2 and 9 expression from fibroblasts; changes that potentially augment accumulation of ECM [165,167]. CyA increased TGFβ expression in proximal tubule cells, mesangial cells (genotype permitting), T lymphocytes and fibroblasts[174-178]. In vivo CyA increased renal TGFβ expression in both murine and rat models of chronic CyA nephrotoxicty[179] and antibodies to TGFβ blocked CyA induced tubular swelling and vacuolisation as well as the expression of collagen, fibronectin and TGFβ genes[180]. In humans, administration of CyA increased the expression of TGFβ1 in the serum of patients with ESRF[181]. The dose reduction of CyA and the addition of MMF is associated with a decline in plasma TGFβ1 expression as well as improvements in renal function and blood pressure[182]. However a recent study contradicts these findings stating that plasma TGFβ1 expression is unaffected by either the initiation or cessation of CyA[183]. Probably of more relevance is the intra-graft expression of profibrotic molecules. Immunohistochemistry has suggested that the expression of TGFβ1 is greatest in graft biopsies with CyA toxicity, less in those with acute rejection and almost absent in ATN[184]. CyA has been shown to increase the levels of

active TGFβ1 compared to Tacrolimus although this study was performed in diagnostic biopsies that may have prejudiced the findings somewhat[185]. In protocol renal biopsies, CyA increased glomerular collagen III and TIMP-1 expression at 1 week post-transplantation more than Tacrolimus, although in this study TGFβ1 levels were similar[186]. Although TGFβ expression is important in the development of allograft fibrosis, CyA can induce collagen III expression independent of TGFβ mediated pathways in vivo[187]. Alternative profibrotic cytokines upregulated by CyA in vitro, such as PDGF and IGF-1 may be responsible for this effect[174].

1.3 Pathogenesis of chronic allograft nephropathy

1.3a Pathophysiology

Clearly multiple diverse aetiological factors are implicated in the pathogenesis of CAN. In view of this it has been proposed that initial allograft injury associated with pre-existing damage, brain death and ischaemia reperfusion injury, is augmented by immunological damage and acute calcineurin inhibitor toxicity in the early stages after transplantation and that this is sustained by low grade immune injury, chronic drug toxicity and other non-immunological factors later in the life of the allograft. Isolated episodes of allograft injury particularly if relatively minor result in a stereotypical inflammatory response that usually resolves with few sequelae. However severe injury or the cumulative effect of multiple insults from different aetiologies cause the development of CAN, a process characterised by the accumulation of extracellular matrix and cellular apoptosis throughout the allograft. This hypothesis is supported by the work of Tullius et al in a rat model of CAN[188]. These experiments suggest that 'a point of no return' is reached beyond which, despite removing immune stimuli, the development of CAN propagated by non-immune factors is inevitable. It

is not known in humans at what stage CAN becomes irreversible but it presumably varies between individuals and depends on the overall level of injury that the allograft has sustained.

The development of intimal hyperplasia throughout the allograft vasculature is thought to be central to this process[16,189]. This is initiated by damage to the endothelium from both immune / non-immune insults and causes subendothelial accumulation of inflammatory cells as well as upregulation of inflammatory cytokines, growth factors and vasoactive mediators involved in tissue repair e.g. TGFβ, PDGF, bFGF, IL-1, IFNγ, TNFα and angiotensin II. Vascular smooth muscle cells are stimulated via paracrine and autocrine mechanisms to proliferate and change from contractile to secretory phenotypes[189-190]. Proteolytic enzymes such as matrix metalloproteinase inhibitors[191] are up-regulated and disrupt surrounding extracellular matrix allowing migration of smooth muscle cells from the media to the intima. Once within the intima, smooth muscle cells stimulate the accumulation of extracellular matrix resulting in remodelling of the vascular wall and the development of intimal hyperplasia[192]. It has been suggested that these changes cause the exposure of novel 'cryptic' antigens that activate the immune system, perpetuating this response[193]. Whether this is the case or not, repetitive cycles of endothelial injury, smooth muscle cell migration and subsequent intimal hyperplasia gradually produce luminal obliteration causing hypoxic damage to the parenchyma[194]. Increased expression of cytokines and growth factors following vascular injury or direct tubulointerstitial cell damage cause recruitment / activation of inflammatory and renal tubular cells. This stimulates remodelling and accumulation of ECM in the interstitium[195]. Enhanced apoptosis[196] and premature senescence of graft endothelial or tubular cells secondary to multiple insults upon them may also play a role[197]. The subsequent failure to

regulate processes such as tissue repair in a normal manner can cause inappropriate responses that permit the accumulation of ECM.

Mesangial cells have features in common with vascular smooth muscle cells and it has been suggested that similar mechanisms cause both allograft vasculopathy and glomerulosclerosis[198]. However an additional explanation has also been proposed[199]. Allograft injury can cause a reduction in functional renal mass, and this is associated with increases in glomerular capillary blood flow and pressure resulting in increased glomerular permeability[200], glomerular hypertrophy[201], mesangial expansion and ultimately the development of glomerulosclerosis. In addition the loss of glomerular selectivity results in the delivery of large amounts of protein to the glomerular filtrate. This in turn is reabsorbed by proximal tubule cells resulting in protein overload and cellular damage that may contribute to the development of interstitial fibrosis[202]. Glomerular hypertension has been demonstrated in CAN micropuncture studies in vivo and reducing this improved graft survival, reduced proteinuria and preserved graft structure [113,203]. Furthermore this theory conveniently explains the clinical observations that a mismatch between recipient metabolic demands and functional allograft mass results in worse long term graft survival. Studies directly examining this relationship have been inconclusive mainly due to problems with measuring nephron mass. Nevertheless, 3 year survival from allografts donated by female, very young or very old donors is reduced[80] and recipients with high body mass also tend to have worse allograft survival[204], although inadequate immunosuppression may play a role in this respect. However not all work supports a role for glomerular hypertension in the development of CAN. Glomerular hypertension in rat isografts does not result in CAN[205]. In addition glomerulosclerosis the pathological hallmark of glomerular hypertension is not seen in all allografts. Thus it would appear that whilst this may

enhance the development of CAN, it is only one mechanism amongst several that may contribute to this process.

1.3b Molecular basis of CAN

1.3b(i) Extracellular matrix

The ECM is a highly complex structure consisting of collagens, glycoproteins such as laminin, fibronectin and tenascin, proteoglycans such as decorin and biglycan as well as glycosaminoglycans. In the kidney the fibrillar collagen types I and III and the non-fibrillar type IV (the principal component of the basement membrane) make up the bulk of the ECM[206]. The tubulointerstitium contains all three collagens whereas glomeruli contain only collagen types III and IV[206]. However collagen I is sparse being deposited relatively late in fibrosis and is probably the least important type. ECM matrix can be broken down by four main families of proteinases (Serine-, metallo- cysteine and aspartic-proteinases) as well as a number of endo- and exo-glycosidases. The most widely appreciated with respect to renal fibrosis are the matrix metalloproteinases (MMPs).

Over 15 MMPs have now been identified[207]. These zinc-containing metalloendopeptidases have a number of conserved amino acid sequences in common and are initially secreted in zymogen form. They can be divided into 5 main classes (Interstitial collagenases: MMP-1, 8, 13; Gelatinases: MMP-2, 9; Stromelysins: MMP-3, 7, 10, 11; Elastinases: MMP-12 and Membrane type: MMP-14) that differ widely in their substrate specificity for ECM proteins[208]. With the exception of MMP-9 they are produced constitutively but their expression can be regulated at both transcriptional and post-translational levels. IL-1 activates a common promoter

sequence in collagenases, stromelysins and gelatinase B and TGFβ has a similar influence on gelatinase A, thus upregulating transcription[209,210]. Post-translational control involves either the activation or inhibition of MMPs. All MMPs except those that are bound to cell membranes are secreted in an inactive form and require activation by cleavage or disruption of specific domains and both plasmin and activated MMPs themselves have been implicated in this process[211,212]. The inhibition of MMP activity occurs through the activity of four tissue inhibitors of metalloproteinases (TIMPs 1,2,3 and 4)[213]. These form a 1:1 stoichiometric complex with MMPs inhibiting both latent and activated forms[214]. Most renal research has focused on TIMP-1; undetectable in healthy kidney but upregulated in several models of renal fibrosis as well as human CAN[215,216]. In contrast both TIMP-2 and TIMP-3 expression are detectable in healthy kidney with TIMP-2 expression mainly constitutive[217] and TIMP-3 involved in cell cycle regulation[218].

Intimal hyperplasia, glomerulosclerosis and interstitial fibrosis are characterised by the excessive accumulation of ECM. Under normal physiological conditions ECM provides a framework around which cells are organised, but in pathological states accumulation can disrupt the normal architecture of tissues resulting in loss of function e.g. tubular epithelial cells need intact basement membranes to proliferate and organise their polarity but if these are disturbed then cells may undergo apoptotis[219] or alternatively differentiate into fibroblasts resulting in further ECM production[220]. There have been very few studies looking at the turnover of ECM in renal allografts. Nevertheless work in native renal fibrosis suggests that the accumulation of ECM is a relatively stereotyped process dominated by both increased matrix synthesis and decreased matrix degradation[24]. The proteins discussed above play an integral role in this process but how they interact with one another as well as

other molecular species such as antibasement membrane antibodies, often seen in CAN[221], is not known. Furthermore a number of cytokines and growth factors have been implicated in the turnover of ECM including PDGF, IGF, EGF and TNFα. Probably the most widely recognised of these is TGFβ.

1.3b(ii) Transforming Growth Factor β (TGFβ)

There are 3 isoforms of TGFβ in humans, although TGFβ-1 has been the focus of most research. Under normal physiological conditions it can be seen as a regulatory molecule acting to restore balance after deviations from the norm by either stimulating or inhibiting cell growth and proliferation. TGFβ is secreted as an inactive precursor in a complex bound with a latency-associated peptide[222]. Various factors including changes in pH, several proteolytic enzymes and thrombospondin can release the active form from this complex[223] which can in turn bind to one of 3 types of TGFβ receptor. The function of active TGFβ can also be controlled by the binding of a number of 'local' proteins such as decorin that inhibit its activity[224].

Over-expression of TGFβ has been linked with the development of CAN. In vitro TGFβ administration stimulates fibroblasts, mesangial cells and glomerular epithelial cells to produce extracellular matrix proteins including collagen, fibronectin and proteoglycans[225-227]. In murine and rat models of chronic immune injury the development of CAN was associated with increases in TGFβ mRNA identified by Northern blotting and immunohistochemistry[228-230]. Most human work has shown an upregulation of TGFβ in allografts after renal transplantation. Furthermore immunohistochemistry has shown that whereas in native kidneys only the renal tubular compartment stained strongly for TGFβ, in allografts not only did this stain

more strongly but TGFβ was also clearly identified in both glomeruli and blood vessels[231-232]. In CAN, Sharma et al using RT-PCR have shown an increase in the expression of TGFβ in allograft biopsies compared to those without CAN (26/36 (72%) vs 43/91 (47%) biopsies, P=0.01)[233]. Suthanthiran et al using similar techniques identified a strong positive correlation between the expression of TGF-β1 mRNA in human allograft biopsies and histological evidence of CAN[234]. Nicholson et al provided further evidence supporting the profibrotic nature of TGFβ by positively correlating TGFβ mRNA with immunohistochemical staining for collagen III in 16 stable renal transplant patients[235]. TGFβ expression has also been tentatively linked with renal function in CAN. An immunohistochemical study (n=40) found that 72% of patients with allograft fibrosis who expressed high levels of TGFβ had a much steeper decline in renal function than those with minimal or low expression of TGFβ[236]. However whether TGFβ expression represented a cause for declining renal function or an effect of such changes is not known.

1.4 Management strategies in CAN

The optimal management of CAN is to prevent it from occurring by the use of high quality organs, optimal immunosuppression and the appropriate prevention of risk factors. Once CAN is established management has traditionally been supportive involving treatment of fluid overload, hypertension and proteinuria as well as ensuring adequate control of diabetes and hyperlipidaemia. Two agents that may have benefits with respect to CAN in addition to their supportive primary roles are the ACE inhibitors / Angiotensin II receptor antagonists and the HMG CoA reductase

inhibitors. In addition changes to patients' immunosuppressive regimens have been advocated in attempts to improve renal function.

1.4a ACE inhibitors and HMG CoA reductase inhibitors

The renin-angiotensin system has been linked to upregulations of TGFβ both in vitro and in vivo models of renal fibrosis and chronic CyA nephrotoxicity[159,237]. ACE inhibitors might be expected to have a beneficial effect in CAN with evidence suggesting a reduction in both systemic and glomerular hypertension and a decrease in fibrosis in native chronic renal disease[238]. Serum TGFβ levels are reduced in patients with CAN given angiotensin II receptor antagonists alluding to a similar antifibrotic effect[239]. In a rat model of chronic renal allograft rejection treatment with enalapril attenuated the development of proteinuria, ameliorated morphological damage, decreased leucocyte infiltration and prevented a rise in renal mRNA levels of profibrotic growth factors and cytokines[240]. However there are only a few small studies using these agents in patients with renal allograft fibrosis. Renal transplant recipients with chronic graft impairment have reductions in proteinuria after administration of ACE inhibitors and some small uncontrolled studies have also documented a stabilisation of renal function in many patients[241,242]. However larger studies are needed to confirm these preliminary findings.

Experimental evidence suggests that hypercholesterolaemia may accelerate the development and progression of intimal lesions in CAN. There have been no clinical studies in renal allograft recipients examining the impact of HMG CoA reductase inhibitors in CAN. However a randomised trial in cardiac allograft recipients using Pravastatin showed that this reduced coronary artery intimal thickening at 1 year[243]. In a further study Wenke et al showed that Simvastatin produced nearly a 3-fold

reduction in graft vessel disease 4 years after transplantation[244]. HMGCoA reductase inhibitors have been shown to have immunosuppressive properties in addition to their lipid lowering effects but it is currently unclear to what extent these contribute to the results[245]. Furthermore it is unknown whether these agents will have a similar impact in renal transplantation.

1.4b Immunosuppressants in denovo renal allografts

Optimal management of renal allograft recipients should entail strategies to limit the development of CAN. Clearly the earlier this is considered the greater the chances of success and thus the type of immunosuppressive regimen used in denovo allografts and its impact on the development of CAN has been investigated. Most modern denovo immunosuppressive regimens use CyA or Tacrolimus based therapy.

A recent meta-analysis of trials comparing the efficacy of these agents confirms results from both European and US multicentre trials reporting that Tacrolimus is more effective than CyA in preventing acute and steroid resistant rejections in primary renal allografts[246-248]. Both CyA and Tacrolimus are nephrotoxic and can cause the development of allograft fibrosis although some work suggests that Tacrolimus may not upregulate profibrotic cytokines such as TGFβ as much as CyA[249]. In view of these findings one may expect the incidence of CAN to be lower for patients taking Tacrolimus but protocol biopsies from a US multicentre trial have suggested that this is similar for both[46]. Nevertheless there have been anecdotal reports of an improvement of renal function in some patients with CAN (5/14) switched from CyA to Tacrolimus[250]. It is worth noting that there is stronger evidence from large multicentre trials in liver transplantation that the use of Tacrolimus reduces the incidence of chronic rejection[251]. In a smaller study in liver recipients with

established CR, 11/13 patients had a partial response or improvement in their liver function after switching to Tacrolimus compared to 3/15 controls[252].

Mycophenolate Mofetil (MMF, Cellcept, Roche) is a non-nephrotoxic immunosuppressant with a similar mechanism of action and utilisation as Aza. It has an antiproliferative effect on vascular smooth muscle / mesangial cells in vitro[253]. Furthermore it inhibited early CR in rat aortic allografts[254] and inhibited the release of profibrotic cytokines such as TGFβ as well as reducing histological evidence of CAN in rat renal allografts[255]. Three large RCTs have shown that MMF reduced the rate of acute rejection by 50% compared to placebo or Aza in denovo renal transplant recipients receiving CyA[256]. However only one has shown a statistically significant reduction in allograft loss censored for death after 3 years follow-up[257]. Thus despite promising experimental evidence, the impact of MMF on the development of CAN in denovo allografts is uncertain at the present time.

1.4c Immunosuppressants in established CAN

In view of the importance of immune aetiologies in CAN some investigators have attempted to treat established CAN by increasing levels of immunosuppression by the addition of Aza or MMF. Pascual et al added Aza (1-2mg/kg/day) and noted improvements in chronic graft dysfunction in patients with both chronic rejection (n=8) and CyA toxicity (n=8)[258]. They suggested that this needed to be performed in the initial stages as response tended to be worse in those who had high baseline serum creatinines. Rocher et al reported similar improvements in renal function in such a setting[259]. Campistol et al have added MMF 1g bd instead of Aza to 31 patients with biopsy confirmed CAN[260]. They observed a stabilisation in the rate of decline of renal function compared to the pre-trial period and an actual improvement of serum

creatinine in 61% of individuals after 6 months follow-up. The addition of MMF produced similar findings in 44 patients retrospectively analysed by Fritsche et al[261]. However this failed to provide any clear benefits in 1 small study and only achieved minor improvements in renal function in approximately half the patients in another[262,263]. Clearly larger RCTs are required to investigate these findings further.

More recently the profibrotic nature of CyA and its potential contribution to the development and progression of CAN has been highlighted resulting in the introduction of regimens that reduce exposure to this agent whilst maintaining adequate immunosuppression. Complete CyA withdrawal has been investigated in a number of studies but unfortunately there have been major differences in their designs, results and conclusions. Some have been controlled[264,265] but many were not[266,267]. Many reported CyA withdrawal to be safe and effective[264,267] but others reported problems severe enough to prompt the interruption and termination of the study[268,269]. A meta-analysis of these studies has been performed by Kasiske et al[270] and has suggested that increased rates of acute rejection followed CyA withdrawal but that this did not affect short-term allograft or patient survival. Outcome was unaffected by the manner of CyA withdrawal. In a subgroup analysis of the results of the randomised trials that compared CyA withdrawal to patients who had never received CyA, graft survival was better in the former group[270].

Relatively few studies have performed CyA withdrawal with the addition of MMF. The largest of these is a recently published randomised study comparing the addition of Aza (2 mg/kg) with that of MMF (1g/bd) in 64 stable renal transplant recipients[271]. Serum creatinine improved to a similar extent in both groups after CyA withdrawal

but significantly less rejection was encountered in patients converted to MMF confirming the results of smaller uncontrolled pilot studies[272].

Because of concerns relating to increased rates of acute rejection with complete CyA withdrawal, some investigators have concentrated their efforts more towards CyA dose reduction with the addition other non-nephrotoxic immunosuppressants. In 23 patients with biopsy confirmed CyA toxicity, 18 patients underwent CyA dose reduction and 5 complete elimination with the addition of 1-2 mg/kg/day Aza[273]. CyA dose was reduced to 50-70% over a 1 month period resulting in a mean CyA dose of 2.2 mg/kg/day and trough levels between 50-60 ng/ml at 6 months and 1 year. At 2 year follow-up GFR had increased, mean arterial pressure had decreased and at 5 years serum creatinine had also decreased with only 1 patient having an episode of acute rejection. Similar findings are reported by Kliem et al who performed almost a 40% CyA dose reduction with the addition of Aza producing mean trough levels of 74 ng/ml[274]. Once again there was a long term improvement in graft function mostly within the first 6 months of conversion that was subsequently sustained over 4-6 years follow-up without any episodes of acute rejection.

Enthusiasm for CyA reduction regimens increased with the introduction of MMF to renal transplantation. Several studies have shown a clear benefit for CyA dose reduction with the addition of MMF[182,275,276] (Table1.4) although only one randomised controlled trial has been performed[277]. Critical evaluation of these studies suggests that 50-75mg of CyA twice daily in combination with MMF (2g/day) is probably the optimal combination for allografts with established chronic damage although a large RCT is necessary to confirm this.

Table 1.4 Summary of trials of CyA dose reduction with the addition of MMF

Authors	Study Type	Number Patients	CyA dose Reduction (%)	CyA levels post-reduction (ng/ml)	Length follow-up (months)	Results
Weir et al[275]	Prospective, non-randomised	28	50	nr	7.2	1.Reduction in rate of loss of renal function with actual improvement in 21/28 patients 2.No reduction in BP 3.No AR
Hueso et al[182]	Prospective, non randomised	16	42	53	6	1.Improved serum creatinine and BP 2. Increased GFR and renal blood flow 3.Reduction in plasma TGFβ levels 4. No AR
Islam et al[276]	Prospective, non randomised	35	40	50	15	1.Improved serum creatinine and reduction in rate of decline of renal function 2. No AR
Nicholson et al[277]	RCT	10 MMF vs 11 Aza	40	nr	6	1. MMF improved the rate of decline in renal function compared to controls 2. One episode of AR and 2 graft failures in controls

*MMF 2g/day unless stated, nr = not recorded

1.4d Surrogate markers in CAN

Allograft loss and return to dialysis or re-transplantation constitutes a clinically relevant end-point for therapeutic intervention studies. However late allograft loss occurs at the rate of 4-6% annually with 20-30% occurring secondary to death with a functioning graft in situ. Therefore studies designed to test the efficacy of interventions to prevent graft loss from CAN will have to follow large numbers of patients for an extended period of time. The drop out rate and non-compliance even with short (1-2 year) clinical studies is often significant and thus as demonstrated above, no large definitive interventional studies have been performed. Therefore the use of appropriate surrogate end-points has been suggested in order to allow the design of trials using smaller numbers of patients followed up for shorter periods. Although acute rejection is probably the most important risk factor for CAN, data from the UNOS database suggest that it neither paralleled nor predicted late graft loss and thus is not a suitable surrogate marker[278]. Proteinuria is a risk factor for CAN[29] and may cause as well as signify renal damage. It is more common in patients with CAN than those without but the relatively low prevalence (20-28% in CAN) makes it useful in only a minority of patients[279]. Clearly deterioration in renal function itself can be used as a surrogate marker. Although several patterns of declining renal function have been reported in CAN, once a decline has been established it has been shown to be unrelenting in over 85% of patients[28].

More recently it has been suggested that biopsies may be used to predict allograft loss. Isoniemi et al examined biopsies taken 2 years post-transplantation and found a significant correlation between the chronic allograft damage index (CADI) and serum creatinine between 3 and 6 years as well as long term outcome[27]. Other histological studies using 6 month biopsies to predict 3 year graft survival[280]as well as

histomorphometric analysis of interstitial fibrosis concur with these findings[281]. Although the possibility of sampling error using such techniques has been raised, it appears that graft histology obtained between 6 months and 2 years may act as a reliable surrogate marker for CAN. Several studies have taken this further and studied the expression of extracellular matrix and cytokine mRNA responsible for the development of the histopathological changes in CAN. TGFβ expression has been correlated with the degree of allograft fibrosis[235] but the complexity of the molecular mechanisms underlying CAN has made this a difficult area to study and further work is necessary to determine if changes in gene expression can predict graft survival per se.

1.5 Conclusion

CAN remains a major challenge for the transplant community. It develops as a result of multiple varied immune and non-immune factors resulting in the accumulation of ECM in the allograft. Although early graft survival has been significantly improved with the use of CyA, paradoxically the nephrotoxic and profibrotic properties of this agent may significantly contribute to the development and progression of CAN thus limiting long-term graft survival. A number of management strategies have been attempted in order to limit CAN with varied success, but several small trials of CyA dose reduction or elimination have improved renal function in such patients. However few of these have looked at other surrogate markers of outcome after CyA dose reduction or the addition of new agents in established CAN.

Chapter 2

Rapamycin

2.1 Introduction

2.2 Rapamycin in denovo renal transplantation

2.2a Mechanism of action
2.2b Immunosuppressive properties
2.2c Nephrotoxicity
2.2d Side effect profile

2.3 Rapamycin in CAN

2.3a In vitro
2.3b In vivo models of vascular injury and fibrosis
2.3c In vivo allografts
2.3d Evidence from human studies

2.4 Conclusion

2.5 Aims of thesis

2.1 Introduction

Rapamycin (Rapa, Rapamune, Sirolimus; Wyeth-Ayerst Pharmaceuticals) is a non-nephrotoxic immunosuppressant with antiproliferative properties that has recently been introduced into the clinical arena. It originated as a macrocyclic fermentation product of *Streptomyces hygroscopicus*, an actinomycete originally isolated from a soil sample on Rapa Nui (Easter Island; 1975)[282,283]. This was initially investigated as an antifungal / antitumour agent and several years past before it's lymphopaenic properties were appreciated, heralding its role as an immunosuppressant.

2.2 Rapamycin in denovo renal transplant recipients

2.2a Mechanism of action

The mainstays of modern immunosuppression CyA and Tacrolimus, bind to the intracellular cytosolic immunophilins Cyclophilin and FK Binding Protein 12 (FKBP12) respectively, inhibiting calcineurin phosphatase. This prevents transcription of cytokines (e.g. IL-2) and progression of the T cell cycle from G0 to G1[284]. Rapa has a similar molecular structure to Tacrolimus and also binds to FKBP12[285]. However the Rapa-FKBP12 complex has no effect on calcineurin phosphatase (Fig 2.1a). Instead it binds to one or more proteins known as 'Targets of Rapamycin' (TOR)[286]. These effector proteins were originally demonstrated as TOR1 & TOR2 in yeast[287], but a mammalian homologue given various acronyms but now known as, 'Mammalian target of Rapamycin' (mTOR) was subsequently identified[288-291]. Both cytokines e.g. IL-2 and the CD28/B7 costimulatory pathway activate mTOR resulting in downstream events critical for cell cycle regulation (Fig 2.1b). This is

Fig 2.1a Mechanism of action of Rapamycin is distinct from calcineurin inhibition

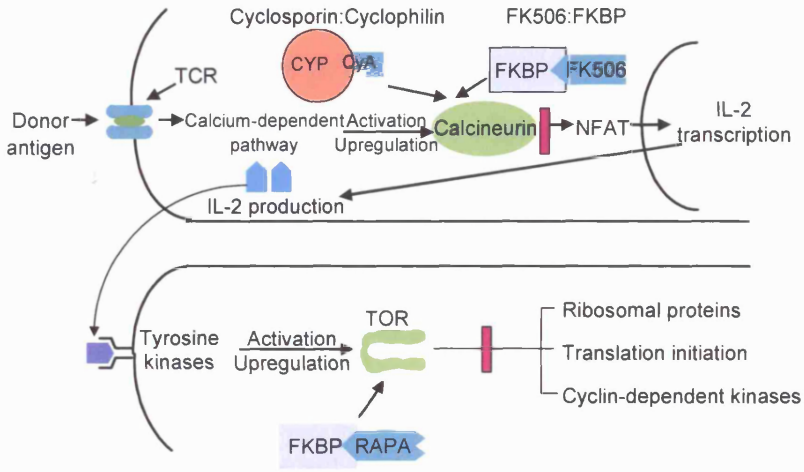
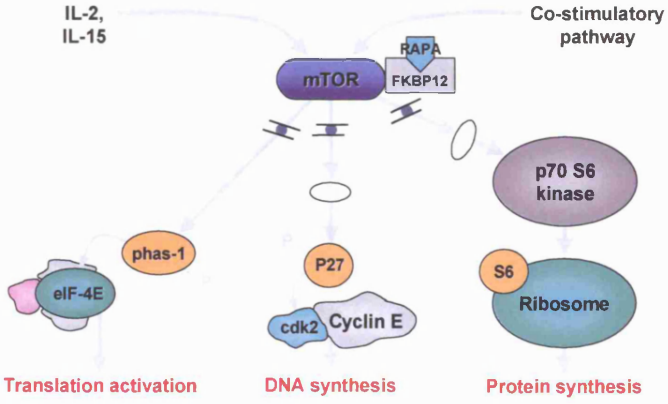


Fig 2.1b mTOR is a critical Kinase in cell cycle progression



mTOR = Mammalian target of Rapamycin. IL= Interleukin. Rapa inhibits 1. Phosphorylation of 4E-BP1 preventing release of eIF-4E and initiation of translation[293,294] 2.p27 mediated activation of cdk2-cyclin E and synthesis of proteins important for cell cycle progression[295] 3. p70 S6 kinase activation limiting ribosomal protein S6 phosphorylation and reducing synthesis of ribosomal / translational proteins[296,297].

complex and the underlying metabolic pathways have not been fully characterized[292-296]. However the Rapa-FKBP12 complex binds mTOR and subsequently inhibits both DNA and protein synthesis resulting in arrest of the cell cycle between late G1 and the S phase[297].

2.2b Immunosuppressive properties

2.2b(i) In vitro

The immunosuppressive properties of Rapa result from inhibition of leucocyte activity. It blocks T cell proliferation induced by cytokines (IL1, 2, 3, 4, 6, 7, 12 and 15), alloantigen and mitogens in a dose dependent manner[298-300]. Rapa acts on B cells independently of its effects on T helper cells causing an inhibition of antigen and cytokine driven B cell proliferation[301]. In addition it has been shown to inhibit cytokine dependent (IL-2 & IL-6) differentiation into antibody producing cells, thus decreasing immunoglobulin synthesis[302].

In contrast to the calcineurin inhibitors, it has been claimed that Rapa has limited effects on cytokine expression in vitro. It does not inhibit the transcription of IL2, 3, 4 or TNF-α in mitogen activated T cells[298] and has been shown to have variable effects on IFN-γ transcription dependent on the stimulus[303]. However work comparing the expression of TH1 and TH2 cytokines using RT-PCR in Concanavalin A stimulated splenic cells suggested that Rapa inhibited the expression of IL2, IFN-γ, IL4 and IL10 more than non-immunosuppressed controls[304]. Indeed Rapa inhibited IL10 expression more effectively than CyA (100% vs 65% inhibition). Rapa's main mode of action is the inhibition of cytokine activated signal transduction but clearly any inhibitory effect on proinflammatory cytokine transcription (however minor) would complement its immunosuppressive efficacy.

The concept of inhibiting cytokine transcription and simultaneously blocking cytokine mediated signal transduction has been investigated in vitro using CyA/Rapa combinations. Rapa augmented the inhibitory effects of CyA upon antibody and phytohaemaglutinin stimulated peripheral blood leukocyte activation[305]. Furthermore relatively high doses of Rapa and CyA alone were required to inhibit cell-mediated lympholysis in vitro but the same degree of inhibition was produced by a combination of both at much lower doses. In a further study the IC50s required to inhibit the proliferation of an IL-2 dependent cell line for Rapa and CyA alone were 90.9 and $2602x10^{-9}$M respectively, compared to 65 and $260x10^{-9}$M when combined[303]. This work has shown that the combination of Rapa and CyA is highly complimentary. Reduced doses of both drugs achieve a response much greater than one would expect from a purely additive effect suggesting a synergistic interaction. The pharmacokinetic/pharmacodynamic mechanisms underlying this are not clear. However this combination ensures that only small amounts of cytokine are produced causing reduced activation of cytokine receptors and an attenuated downstream signal that is more easily inhibited by Rapa.

2.2b(ii) Animal Studies

The first in vivo studies using Rapa as an immunosuppressant were published by groups led by R Y Calne and R E Morris (1989)[306,307]. Both gave Rapa at various doses to rats receiving heterotopic cardiac allografts and noted longer graft and animal survival rates in comparison to non-immunosuppressed controls. Similar benefits have been demonstrated for skin, renal, small bowel, pancreatic and pancreaticoduodenal allografts in a number of species including mice, rats, rabbits, pigs, dogs and primates; although in dogs and primates fatal gastrointestinal side effects frequently

occurred[306-321]. It is difficult to compare the immunosuppressive efficacy of Rapa between studies as various doses, routes of administration and allograft models have been used. However Rapa is 20-100 times more potent than CyA and acted in a dose dependent manner to prevent acute allograft rejection. Subsequently Rapa (0.8 mg/kg/day iv) was investigated as a potential treatment for ongoing acute rejection and was found to prolong the survival of presensitized rat skin and cardiac allograft recipients[322]. This dose dependent effect (Rapa 0.08-0.8mg/kg) was confirmed in cardiac, renal and pancreas allografts[323,324].

The synergy between Rapa and CyA suggested in vitro was also observed in animal work. Subtherapeutic doses of Rapa (0.01-0.04 mg/kg/day) and CyA (0.5-2.0 mg/kg/day) prolonged rat cardiac and kidney allograft survival compared to either drug alone or the additive effect of each combination[325]. Similar results were observed in studies using rat lung[326] and mouse kidney allografts[327] as well as a mongrel canine model[317].

2.2b(iii) Human studies

In September 1999, Rapa received approval from the US Food and Drug Administration for marketing as an agent for the prevention of acute rejection in renal transplant recipients. This was based on early results from several multicentre prospective randomised trials (RCT) that are currently ongoing. These include a US (n=719, 38 centres), a Global (n=576, 34 centres), a combined European-US (n=247) and two European studies (n=83 & 78, 11 & 14 centres respectively).
Both US and Global studies administered combinations of Rapa (fixed dose 2 or 5 mg/day), CyA (and Prednisolone (Pred)) in an attempt to take advantage of the immunosuppressive synergy between these agents[328,329]. The US study gave

azathioprine (Aza, 2-3mg/kg) to controls where as the global study gave a placebo. Two-year follow-up data from both Global and US groups showed that there was comparable patient and graft survival for patients taking Rapa compared to controls. The incidence of AR was reduced in both trials when patients received either 2 or 5 mg/day of Rapa[330,331]. Similarly good results were observed from a single centre (Houston)[332]. This group also investigated the efficacy of a Rapa (0.5-7mg/kg/m^2)/ CyA/Pred regimen (n=43) versus CyA/Pred (n=126) in patients with IgG anti-HLA panel reactive antibodies (PRA) > 10% (i.e. individuals who have an increased risk of acute rejection and poor graft survival). There was a statistically significant reduction in the frequency of acute rejection in those patients on Rapa (11.6 vs 67%, $p<0.001$), supporting the immunosuppressive efficacy of CyA/Rapa combinations in high-risk recipients[333]. However an analysis of the effect of race on the efficacy of Rapa in the US study demonstrated that African-American patients receiving Rapa 2 mg/day had significantly higher rates of acute rejection compared to non-African-Americans (30.2 vs 13.1 %)[334]. Rapa 5 mg/day produced similarly low rates of acute rejection that were less than controls in both racial groups (14.8 vs 11.3 vs 28.6-30.3%), suggesting that this dose of Rapa should be used in 'higher risk' African-American patients.

The success of work combining full dose CyA and Rapa encouraged the introduction of regimens that maintained the synergy between these agents, yet minimised exposure to CyA. One hundred and forty-nine denovo renal allograft recipients were randomised to receive Rapa or placebo with the addition of either full or half dose CyA (Month 1 trough levels ~300 or ~200 ng/ml respectively) and steroids[335]. The incidence of acute rejection within 6 months of transplantation in patients receiving full dose CyA was reduced from 32% to 8.5% in patients receiving Rapa (1-3 mg/m^2/day). Similarly low rates of acute rejection (10.7%) occurred in Caucasians

treated with Rapa (1-5 mg/m^2/day) and reduced dose CyA but not in African-Americans (39%) suggesting that this type of regimen is satisfactory for 'low risk' recipients but is less acceptable for those at 'higher risk'. Those receiving reduced dose CyA tended to have better renal function compared to full dose CyA recipients but this was only statistically significant at a Rapa dose of 1 mg/m^2/day, 3 months after transplantation. A similar European/US prospective multicentre study (n=247) currently ongoing compares full dose CyA and fixed dose Rapa (2mg/day) with reduced dose CyA and concentration controlled Rapa (10-20 ng/ml)[336,337]. The incidence of acute rejection at 2 months was equally low (13.5 vs 10.9%). However the aim of this study was to withdraw reduced dose CyA from patients who had not rejected in the first 3 months post-transplantation. This was achieved in 51/77 (66%) patients and after 6 months and 1 year of follow-up the incidence of acute rejection in both groups was similar with significantly improved renal function in those who had CyA reduction/withdrawal.

Two major European prospective randomised multicentre studies have adopted a different approach to the use of Rapa[338,339]. Both compare Rapa triple therapy directly against CyA triple therapy in an attempt to determine if Rapa could replace CyA as the primary immunosuppressant for renal allograft recipients. Doses of both drugs were adjusted according to trough levels (CyA: 200-400 ng/ml for 2 months and 100-200ng/ml thereafter; Rapa: 30 ng/ml for 2 months and15 ng/ml thereafter. The first study used Aza (2mg/kg) and Pred whilst the second used Mycophenolate Mofetil 1g twice daily (MMF) and Pred as additional agents. At 1-year follow-up graft survival, patient survival and incidence of acute rejection showed no statistically significant differences when CyA was compared to Rapa. MMF combined with either CyA or Rapa produced lower rates of acute rejection than these agents and Aza. However

even patients on MMF and Rapa had a tendency to require treatment for acute rejection with bolus steroids more often than those on CyA and MMF (20 vs 11, p=0.068). This suggests that there may be a small price to pay for completely avoiding the nephrotoxicity of CyA. However the clinical significance of this is debatable as renal function tended to be better in Rapa treated patients over follow-up in both studies.

Rapa has also been used to treat refractory acute rejection not responding to either conventional pulsed steroid or antilymphocyte preparations[340]. Patients (n=21) were given Rapa (7mg/m^2 for 5 days, followed by 5mg/m^2 thereafter) with no change in CyA dose[341]. Ninety percent of patients had successful reversal of rejection producing an actuarial 1-year graft survival of 81%. These individuals had a mean serum creatinine of 2.4mg/dl (160 μmol/L) at 1 year. Refractory rejection often has a poor prognosis with many patients requiring dialysis. These results provide reason for optimism but larger studies are necessary to confirm this.

2.2c Nephrotoxicity

Calcineurin inhibition is responsible for both the therapeutic benefits and nephrotoxicity of CyA. Rapa has a different mechanism of action and thus the nephrotoxicity seen with calcineurin inhibition does not occur. Work in both pigs (0.1-0.4 mg/kg) and rats (5 mg/kg/day) has shown that Rapa has no deleterious effects on GFR or renal blood flow and caused minimal morphological signs of toxicity[342,343]. However supratherapeutic doses of Rapa (1.5-10 mg/kg/day) may cause small reductions in creatinine clearance in rat native kidneys[342,344,345]. Rapa reduced medullary concentrating ability and increased tubular enzymuria suggesting that mild tubular injury may occur[346]. In humans pooled data from both European trials

comparing Rapa with CyA triple therapy showed a statistically significant improvement in both serum creatinine and calculated GFR at 2 years follow-up in Rapa patients (mean creatinine < 120μmol/L and mean GFR > 65ml/min)[347]. This suggested that even if Rapa caused mild tubular damage at high doses, it had little clinical significance.

In humans Rapa (1-13 mg/m^2/day) was given to 30 stable renal transplant recipients taking CyA at normal therapeutic doses with no significant effects on GFR or creatinine clearance over a 14 day period[348]. Therefore it was rather surprising that the combination of Rapa and CyA caused an impairment in the renal function of patients compared to controls in the recent US multicentre study[328]. It has been suggested that Rapa may in some way potentiate CyA nephrotoxicity although the mechanism underlying such an occurrence is unclear. Some preliminary evidence supporting this has noted that Rapa increased CyA area under the concentration-time curves compared to controls in this study despite the fact that Rapa was given 4 hours after CyA in an attempt to prevent any interaction between these agents at the level of p-glycoprotein or P450 enzymes in the gastrointestinal tract[328]. In a salt depleted rat model of CyA toxicity the Rapa / CyA combination produced a functional and morphological deterioration[349]. However it is difficult to know the relevance of such findings when CyA trough levels were as high as 2850ng/ml and Rapa trough levels were not recorded. A study in the Wistar-Furth rat showed that the combination of oral Rapa 0.4mg/kg and CyA 2.5mg/kg produced a moderate reduction in GFR compared to either drug alone. However when the doses Rapa and CyA were doubled (0.8 and 5 mg/kg/day respectively) this produced an extremely marked decline. Although low doses of Rapa had no effect on CyA trough levels higher doses (>0.4mg/kg) increased them and this was offered as an explanation for these

findings[350]. Furthermore it has also been observed that Rapa increases CyA levels in renal tissue to a greater extent than in whole blood concentrations[351]. In humans CyA average concentrations (C_{AV}) and Rapa trough levels (TL) (655 samples, 96 patients) were compared with the calculated GFR at each timepoint[352]. Increasing Rapa exposure was not associated with decreased renal function. However as Rapa TLs increased the CyA C_{AV} necessary to achieve optimal renal function fell. Further prospective studies are clearly required to determine the pharmacokinetic relationship between Rapa and CyA and its impact on nephrotoxicity.

2.2d Side effects

Rapa is poorly tolerated by certain species. Dogs given Rapa at low doses (0.05 mg/kg/day) develop anorexia, fever, vomiting, leucocytosis and hyperamylasaemia. Higher doses (0.3-2.0 mg/kg/day) are often fatal and autopsy findings suggest that Rapa produces a submucosal vasculitis with mucosal erosion/ulceration[309,317,318]. A similar but less frequent syndrome occurs in baboons[321] but is not seen in all species. Regular administration of Rapa produces a number of side effects in humans. These can occur at low daily doses (1-2+ mg/day) and include headaches, polyarthralgia, mild stomatitis, epistaxis, diarrhoea and skin complaints e.g. mild acne. However myelosuppression, hyperlipidaemia and problems related to over-immunosuppression remain the major concerns.

2.2d(i) Myelosuppression

This was first observed in mice when Rapa delayed recovery from 5-fluorouracil induced leucopaenia and thrombocytopaenia in a reversible manner[353]. The underlying mechanism is unknown, but may relate to inhibition of signal transduction

from haematological growth factor receptors that have sequence homology with the cytokine receptors whose action is inhibited by Rapa e.g. IL-11 receptor that stimulates platelet production has the same gp130 β chain as the IL-2 receptor [354,355]. Rapa produced a dose dependent reversible thrombocytopaenia and leucopaenia in stable renal transplant recipients within 2 weeks that persisted but improved over treatment. Rapa doses of 1-3, 5-6 & 7-13 mg/m^2/day reduced mean platelet counts by 14, 80 & 97 cells/mm^3 respectively. A reversible leucopaenia also occurred, with a mean reduction of ~2 x 10^9/L for all Rapa doses[348]. The European multicentre (Aza/MMF) studies documented thrombocytopaenia in 37 and 45% of Rapa patients (Controls 0 and 8% respectively, $p<0.05$). Thirty-nine percent on Rapa/Aza/Pred had leucopaenia (Control 14%, $p<0.05$) but in those receiving MMF there was no significant difference between Rapa and controls (28 vs 18%). CyA/Rapa combinations produced a similar incidence and severity of leucopaenia / thrombocytopaenia that correlated with Rapa TLs[332]. Although quite common myelosuppression is usually mild at the doses used in current studies. In the minority of individuals where it is significant or causing a clinical problem Rapa dose reduction/discontinuation causes signs of recovery usually apparent within 5 days.

2.2d(ii) Hyperlipidaemia

Both calcineurin inhibitors and steroids promote hyperlipidaemia[356], glucose intolerance[357], and hypertension[358] well-recognised risk factors for the development of cardiovascular disease. In contrast Rapa has little influence on blood pressure or serum glucose concentrations and an early study in pigs reported that hyperlipidaemia was mild and similar to that observed with CyA[319]. Human Phase I studies were also encouraging with only higher Rapa doses (5-13 mg/m^2/day) causing statistically

significant hypercholesterolaemia and no significant effects on triglycerides[348]. However this data only reflected a 15-day course and longer administration has shown a different picture.

The European (Aza) study comparing Rapa and CyA triple therapy noted that Rapa caused significantly more frequent and severe hypercholesterolaemia and hypertriglyceridaemia than CyA (44 vs 14% and 51 vs 12% respectively)[357]. Hyperlipidaemia was maximal after 2 months (triglycerides (TG) 5.3 vs 2.1 mmol/L $p<0.001$, cholesterol (Chol) 9.2 vs 6.4 mmol/L $p<0.001$) when Rapa target levels were high (30ng/ml) but as these were reduced (15ng/ml) this improved (6 month TG: 3.6 vs 1.6 mmol/L $p=0.007$, 6 month Chol: 6.9 vs 6.1 mmol/L $p=0.15$)[338]. This improvement was sustained at 2 years follow-up when a combined analysis of lipid parameters for both Aza and MMF European multicentre studies was performed (TG: 2.40 vs 1.82 mmol/L $p=0.051$, Chol: 6.46 vs 5.93 mmol/L $p=0.087$)[359]. Lipid lowering agents were administered to 53% of those taking Rapa compared to 24% in controls. An analysis of both US and Global studies has shown a similar picture. Rapa produced a dose related increase in Chol and TGs by 3 months that was persistent but reduced at 1 year follow-up[360]. The use of statins/fibrates was effective in the majority, causing significant decreases in Chol/TG levels respectively with few serious clinical consequences. Hypertriglyceridaemia can be severe with CyA/Rapa regimens (11.7-42 mmol/L) particularly at higher Rapa doses, but is reversible after dose reduction or cessation of Rapa[361]. However discontinuation of Rapa in the Global/US trials was rare (0.4% Rapa 2mg, 2.5% Rapa 5 mg). Analysis of 1-year cholesterol values using The Framingham model suggested that Rapa would cause only a small increased incidence of ischaemic heart disease in renal transplant recipients (2 and 3 new cases/1000 persons/year; Rapa 2 and 5 mg respectively)[360].

Dose reduction or elimination of CyA from Rapa patients may reduce this risk further and results are awaited with interest.

2.2d (iii) Over-immunosuppression

This predisposes patients to both typical / atypical infections as well as increasing the risk of neoplasia and post-transplant lymphoproliferative disease (PTLD). The European (Aza) study noted a higher incidence of herpes simplex (24 vs 10%, p=0.08), and pneumonia (17 vs 2%, p=0.03) in Rapa patients compared to controls[338]. However when MMF replaced Aza as the secondary agent the incidence of herpes simplex was similar in both Rapa and control patients and although an increased incidence of pneumonia occurred it was not statistically significant (15 vs 5%)[339]. The multicentre trials combining CyA and Rapa reported similar significant increases in herpes simplex infections (Global) and pneumonia (US). However the incidence of life threatening infections and CMV were not increased despite the immunosuppressive synergism between these drugs.

Clinical use of Rapa is still at an early stage and its impact on post transplant lymphoproliferative disease is currently unknown. Kahan has followed up 250 patients treated with Rapa (3-48 months) and noted only 2 cases of PTLD (0.8%)[362], a statistic comparable to the general transplant population[363]. Multiple small bowel segments were involved in one and nuchal lymph nodes in the other. Rapa withdrawl and CyA dose reduction resulted in remission with both patients alive and well 6 and 36 months later. Two year follow-up of 1295 patients from the Global and US trials has shown that only 2 patients (0.7%) have died as a result of neoplasia. Rapa has antiproliferative properties and has been investigated as an antitumour agent[364]. The

impact of this with respect to neoplasia / PTLD will become apparent over long-term follow-up.

2.3 The effect of Rapamycin on CAN

The histology of CAN is dominated by the growth factor driven accumulation of extracellular matrix in response to persistent or repetitive insults to the allograft. A number of lines of evidence suggest that Rapa may theoretically influence this

2.3a In vitro

Rapa inhibits growth factor-mediated proliferation of the major cell types involved in the pathogenesis of CAN. The development of intimal hyperplasia is associated with proliferation of endothelial and more importantly vascular smooth muscle cells. Rapa exerted an antiproliferative effect in non-stimulated as well as bFGF, IGF-1 and PDGF driven vascular smooth muscle cells[365-367] that was greater than either MMF or Tacrolimus[368]. A similar effect was encountered in bFGF induced bovine aortic and human umbilical vein endothelial cells[369]. The pivotal cell type involved in the development of interstitial fibrosis is likely to be the fibroblast and both PDGF/bFGF stimulated rat cardiac and human lung fibroblast proliferation are inhibited by Rapa[370,371]. These antiproliferative properties were antagonised by combination with Tacrolimus in molar excess, suggesting that the formation of the Rapa/FKBP12 complex is an important underlying mechanism[372]. Proliferation of mesangial cells is one of the central features of glomerulosclerosis. Rapa has been shown to inhibit both mitogen independent and PDGF stimulated mesangial cell proliferation (via ^{3}H-thymidine uptake) in a dose dependent manner[373]. However Rapa does not uniformly

inhibit all cell proliferation as some haematopoietic and epidermoid cell lines are unaffected by its administration[374,375].

Although such in vitro evidence supports the use of Rapa in CAN, a recent study by Dodge et al has introduced an element of caution[376]. It has been noted that under certain conditions Rapa and TGFβ have similar immunoregulatory effects. Thus splenic leucocytes from BALB/c mice were stimulated in vitro with anti-CD3 or in vivo with allogeneic cells in the presence or absence of Rapa. Rapa had an antiproliferative effect on leucocytes stimulated both in vitro and in vivo and was associated with an increased TGFβ production in both instances compared to controls. Thus it is possible that the antiproliferative effect of Rapa is in part mediated by this increased TGFβ production. In the early phases of allograft rejection TGFβ production may be beneficial in inhibiting acute immune activation. However sustained expression of TGFβ may promote fibrosis and thus CAN. A similar upregulation of TGFβ expression occurs with CyA administration and is thought to contribute to the profibrotic properties of this agent (discussed earlier). A further study has examined the effect of combining Rapa and either CyA or Tacrolimus on the proliferation of PHA activated lymphocytes and quantified the presence of both IL-2 and TGFβ using RT-PCR and an ELISA technique[377]. In this work Rapa in combination with either CyA or Tacrolimus significantly inhibited lymphocyte proliferation, inhibited IL-2 expression and induced TGFβ compared with any single drug alone. Furthermore the levels of TGFβ and IL-2 correlated positively and negatively respectively with inhibition of leucocyte proliferation thus supporting this as an important mechanism by which such combination regimens exert their powerful immunosuppressive effects. However because several current clinical trials use Rapa

in combination with CyA and TGFβ is such an important profibrotic cytokine there is concern that both agents may synergise not only with respect to immunosuppression but also fibrogenesis.

2.3b In vivo models of vascular injury and fibrosis

Intimal injury and subsequent hyperplasia is common to most solid organ allografts undergoing CR. Although it only represents one histological aspect of CAN it is thought to play a pivotal role in the development of this condition. Both immune and non-immune models have been used to recreate this in vivo. Rapa treatment reduced the degree of intimal thickening and associated local cytokine expression at doses of 1.5 mg/kg/day after mechanical vascular injury (angioplasty balloon catheter)[378]. A similar dose reduced intimal hyperplasia in the central part of femoral allografts[379]. Rapa also reduced the severity of established intimal hyperplasia in rat femoral artery allografts when given 14, 21 and even 30 days after the onset of alloimmune injury[379]. However larger Rapa doses were required (3-6 mg/kg/day) and intimal hyperplasia was most successfully reduced when Rapa was added at an early stage i.e. when intimal hyperplasia was relatively immature. Rapa (1.5 mg/kg/day) in combination with MMF was also highly effective after balloon catheter damage. Administration of Rapa and MMF for 14 days after injury inhibited intimal hyperplasia at day 14, but by day 40 intimal hyperplasia had returned and was similar to untreated controls. However if administered 3 days before and 14 days after insult there was little intimal hyperplasia observed at day 40[380], presumably as sufficient concentrations were present to dampen the initial cytokine cascade immediately after injury, generating a smaller intimal response that resolved with few sequelae.

At the molecular level, rat aortic allografts treated with Rapa (0.5 mg/kg/day) had little intimal hyperplasia but significant inducible nitric oxide synthase (iNOS) expression at 30 days. In contrast CyA treatment was associated with marked intimal hyperplasia but no detectable iNOS expression[381]. Nitric oxide (NO) inhibited the proliferation of vascular smooth muscle cells in both balloon injured and aortic allograft models and also prevented the development of arteriosclerosis[382,383]. Thus one possible explanation for Rapa's actions is that iNOS expression is maintained, causing high local NO levels that limit vascular smooth muscle responses to injury. Future work combining Rapa with selective iNOS inhibitors will delineate this process further.

These studies show that the vascular response to both immune and non-immune injury is inhibited by Rapa but the interplay between the antiproliferative and immunosuppressive properties that contribute to this is unclear. The balloon injury model lends support to an antiproliferative effect. However, Rapa (3 mg/kg/3x/week) prevented both acute rejection and longer-term arteriosclerotic changes in rat aortic allografts[384]. The positive correlation noted between these processes suggested that the immunosuppressive properties of this agent also play an important role.
Rapa has also been shown to inhibit the development of fibrotic changes in other non-vascular models. Wistar rats underwent clamping of the right renal pedicle and left nephrectomy, a model of ischaemia-reperfusion injury known to be associated with an upregulation of profibrotic gene expression. The addition of Rapa (1mg/kg/day) over a 4 month period was associated with a decrease in TIMP 1,2 and 3 and TGFβ mRNA expression (detected using RT-PCR) compared to controls as well as improved renal function / proteinuria. However it should be noted that the lower dose of Rapa used (0.5mg/kg/day) did not show such benefits[385]. In a model of obliterative bronchiolitis,

a larger dose of Rapa (6 mg/kg/day) prevented the development of luminal fibrosis or loss of respiratory mucosa in rat tracheal allografts more effectively than CyA or MMF[386]. The effects of Rapa (1.5 mg/kg/day) have also been investigated in a carbon tetrachloride model of rat hepatic fibrosis in vivo and on hepatic stellate proliferation, an important component of the hepatic fibrogenic process, in vitro[387]. Rapa inhibited histological evidence of extracellular matrix deposition compared to untreated controls and a decrease in collagen deposition was confirmed using a hydroxyproline assay. Northern blot hybridisation analysis was performed on this tissue and mRNA levels of procollagen and TGFβ1 were significantly decreased following administration of Rapa. However although Rapa inhibited PDGF induced proliferation of hepatocytes and fibroblasts, it had no significant effect on procollagen III or TGFβ mRNA production in vitro.

The models discussed above are very different and none of them completely reflect the pathological changes occurring in CAN. However they provide molecular and histological support that Rapa inhibits the accumulation of extracellular matrix and fibrosis in both a vascular and extravascular setting. This has important implications with respect to CAN although clearly additional work in models of CAN is required before this can be confirmed.

2.3c In vivo allografts

The effect Rapa on CR in whole organs has been investigated in rat cardiac allografts where it is manifested as graft vessel disease (GVD). Meiser et al were the first to observe that Rapa inhibited GVD in such circumstances[388]. Further work has shown that inhibition of transplant vasculopathy and graft vessel luminal obstruction secondary to myointimal changes (100 days postoperatively) was most effective when

Rapa was given at high doses (5 mg/kg/day for 14 days followed by 2.5 mg/kg/day thereafter)[389]. Lower Rapa doses (0.5 and 2 mg/kg/day) were less effective but did still produce a significant reduction in vasculopathy (Control 59±7% vs Rapa 0.5 mg/kg/day 25±15% vs Rapa 2 mg/kg/day 22±11%). The addition of Rapa (0.5 mg/kg/day) to low dose CyA (1.5 mg/kg/day) for 14 days reduced lymphocytic infiltration but did not cause an additional reduction in vasculopathy. The immunosuppressive synergism between these agents appeared to have little beneficial impact on GVD at the low doses used, over this short time period. However the combination of CyA (15 mg/kg/day) and Rapa (1 mg/kg/alternate days) for 12 weeks after rat cardiac allografting reduced the frequency and severity of both acute rejection and GVD compared to CyA alone[390]. Rapa/CyA allografts showed sparse infiltration of inflammatory cells, little expression of adhesion molecules, no expression of IFNγ/TNFα and little GVD. These findings suggest that the synergism between Rapa and CyA plays an important role in the inhibition of chronic rejection, contradicting earlier work. Wasowska et al confirmed these findings[391] and also investigated the expression of monocyte associated chemokines involved in the development of arteriosclerosis[392]. The expression of IL-12, MCP-1 and RANTES were significantly decreased by CyA/Rapa therapy, providing further evidence that the synergism between these drugs may be beneficial in GVD.

Rats with established cardiac GVD received Rapa 3mg/kg/day over a 30-day period 2 months after transplantation in order to determine if this process was reversible[393]. Rapa compared to an equipotent dose of CyA led to a significant reduction in GVD although the overall level of graft inflammation and perivascular infiltrate was similar implying analogous levels of immunosuppression. Presumably Rapa had an antiproliferative effect causing the reduction in GVD. As with models of vascular

injury both the antiproliferative and immunosuppressive properties of Rapa seem important in the prevention of CR in cardiac allografts.

2.3d Evidence from human studies

Rapa has been clearly shown in vitro, in vivo and in humans to be a powerful and effective immunosuppressant, particularly in combination with calcineurin inhibitors where it reduces both the rate and severity of episodes of acute rejection compared to controls. Under-immunosuppression and severe, recurrent or late episodes of acute rejection are all risk factors for the development and progression of CAN. Thus the addition of Rapa may have a significant impact on the incidence and severity of CAN in the future. However long term follow-up data is required to determine this.

Cyclosporin produces chronic nephrotoxicity, a further risk factor for CAN, and thus the advent of Rapa may allow safe reduction or even elimination of CyA potentially limiting the contribution of such nephrotoxicity to CAN. Multicentre trials in denovo allograft recipients utilising such regimens have recently been instituted (discussed earlier). However it will be several years before meaningful data with respect to CAN becomes available and thus the use of surrogate markers of CAN such as profibrotic gene expression and quantitative assessment of interstitial fibrosis have been advocated.

In response to in vitro studies suggesting that Rapa increased TGFβ production, serum TGFβ1 levels in 20 renal allograft recipients receiving either Rapa and CyA (n=10) or Rapa alone (n=10) over a mean of 15.2 months were analysed[394]. No histological evidence of CAN was observed and only 1 patient taking Rapa alone had any detectable (ELISA) TGFβ1 in their plasma, contrasting significantly to the

authors previously published findings of detectable TGFβ1 in 75% of 103 patients on CyA mono or triple therapy with Aza. This study appears to contradict the findings relating to TGFβ in vitro and lends further support to a beneficial effect of Rapa on CAN. However it only assesses serum TGFβ and this may not reflect levels within the allograft that are obviously more relevant in the development of CAN. A study that goes some way to addressing this issue has been performed by Xavier et al, who looked at fine needle aspiration biopsies from renal allografts that were cultured for 48 hours before assessment of the culture supernatants using an ELISA technique[395]. TGFβ1 levels were significantly greater in those patients taking Rapa compared to those taking a conventional triple therapy regimen of CyA, Aza and prednisolone suggesting that the changes in TGFβ observed in vitro may also occur in vivo. However further studies using alternative techniques to quantify TGFβ expression are required to confirm these findings.

A further small human study has attempted to assess the impact of the combination of Rapa and CyA on histological evidence of chronic CyA toxicity found in protocol biopsies one year after transplantation[396]. There was no difference in either the percentage change in interstitial fibrosis or arteriolopathy over this time when 8 patients on Rapa / CyA therapy were compared to 12 taking CyA / Aza / Pred. Although these results are encouraging they must be treated with caution as the number of patients in this study is small.

Currently only 1 or 2 studies have investigated the influence of Rapa on patients with established CAN and it is thus unclear whether CyA should simply be reduced in dose or completely eliminated in this setting. The more cautious approach with respect to concerns about the development of late episodes of AR is CyA dose reduction. One

such report of 19 patients who received a 47% CyA dose reduction and conversion from MMF to Rapa was disappointing. There were 7 allograft failures, 4 patients withdrawn from Rapa due to side effects and little change in renal function in the remainder over a mean 7 month follow-up period[397]. However this was a small non-randomised study and remains unconfirmed. On a more promising note a further non-randomised series of 23 patients taking either CyA or Tacrolimus based immunosuppression a median time of 54.9 months after renal transplantation had their calcineurin inhibitors stopped and started Rapa (although the dose and Rapa trough levels are not stated)[398]. Over 8 months follow-up median serum creatinine improved from 211μmol/L to 174μmol/L ($p<0.05$). Proteinuria did not alter and although anaemia, thrombocytopaenia and dyslipidaemia occurred the most worrying complication was seen in 8 patients who developed an interstitial pneumonitis. These results are encouraging, particularly as no acute rejection occurred during the switch between immunosuppressants and renal function improved. However the safety of this regimen remains in question because of the frequency of pneumonitis (35%) and it would be useful to know the trough levels of Rapa in these patients as these may have been quite high. Ultimately, prospective randomised controlled trials will be necessary in order to determine the optimal approach.

Outside the field of renal transplantation Rapa has been used in a sequential series of 16 liver transplant recipients with chronic rejection either with or without Tacrolimus[399]. Rapa levels were maintained at 10-15 ng/ml resulting in 50% of these patients having resolution of CR after a median follow-up of nearly 3 years, with responders having significant improvements in liver function. Similarly in cardiac transplantation successful salvage of 2 allografts with declining function secondary to graft vascular disease has been published[400]. Although these reports lend some weight

to Rapa as an agent that may be beneficial in chronic rejection, they are anecdotal and thus larger randomised studies are necessary in order to confirm their validity.

Despite the experimental evidence that Rapa may limit the development and progression of cellular and histological changes seen in CAN, several confounding factors need to be considered. As previously discussed although Rapa is not itself nephrotoxic, used in combination with CyA in denovo allografts renal function was impaired when compared with controls. Furthermore Rapa may augment the expression of TGFβ, a profibrotic cytokine also upregualted by CyA. Rapa also causes hyperlipidaemia, a risk factor for the development of CAN. Any potential therapeutic benefit with respect to CAN may be reduced by such a tendency. Although hyperlipidaemia can usually be corrected by appropriate medication, starting patients on one drug to counteract the effects of another can produce significant side effects as well as incurring substantial costs that may or may not be justified by improved long-term outcome. Arguably a more important issue is whether the relatively low doses of Rapa given to humans (0.2-0.3 mg/kg/day Rapa alone and 0.01-0.17 mg/kg/day in Rapa/CyA combinations) are large enough to effectively inhibit CR. In rat models the inhibition of intimal hyperplasia occurs at 1.5 mg/kg/day and increases significantly as Rapa doses are elevated. If such doses were administered to humans side effects and over-immunosuppression would be poorly tolerated.

2.4 Conclusion

There is good evidence supporting the use of Rapa in denovo renal transplant recipients. It reduces the incidence of acute rejection when given alone but most

effectively in combination with CyA. However such combination regimens may enhance nephrotoxicity and thus regimens utilising CyA reduction or elimination are currently under investigation and should reduce both toxicity and side effects. Although there is substantial in vitro and in vivo work suggesting that Rapa could also be beneficial for patients with respect to CAN, several factors discussed above may limit it's therapeutic potential. Long-term follow-up from the current multicentre trials should determine whether the use of Rapa can inhibit the development of CAN in de novo recipients. However at the present time there have been very few human studies using Rapa in patients with established CAN. Thus there several unknowns including whether this agent at human doses can be effective in such an arduous setting and secondly whether the use of reduced dose CyA with the addition of Rapa, or CyA substitution with Rapa will obtain the best results.

Clearly a large multicentre study with long-term follow-up would be the ideal solution to address these issues but the practicalities of such a study in patients with CAN is daunting. Furthermore the length of follow-up does not provide us with evidence to guide treatment of patients at the present time. Thus in this thesis surrogate markers such as renal function, gene expression and histomorphometry have been utilised in an attempt to predict the impact of CyA dose reduction with the addition of Rapa on established CAN.

2.5 Aims of thesis

1. To investigate the safety and efficacy of CyA dose reduction with or without the addition of Rapa in patients with established CAN.

2. To investigate the molecular impact of CyA dose reduction with or without the addition of Rapa in this setting.

3. To investigate the effect of CyA dose reduction with or without the addition of Rapa on established allograft fibrosis.

Chapter 3

A prospective randomised controlled trial of Cyclosporin dose reduction with or without the addition of Rapamycin in patients with chronic allograft nephropathy

3.1 Study Design

A prospective randomised open label single centre study comparing the effects of CyA reduction alone to CyA reduction with the addition of Rapa in patients with established CAN was performed. Local ethical committee approval was granted and written informed consent was obtained from each patient recruited. Enrolment took place between August 1999 and January 2000. Inclusion criteria required that patients were aged 18-70 years old and had received an allograft more than 6 months before study entry. Each individual had a renal transplant biopsy prior to randomisation and only those with histopathological evidence of CAN were enrolled. Patients were considered ineligible if the biopsy showed evidence of acute rejection or recurrent renal disease. In addition, if a normalised single shot radioisotope (^{51}Cr labelled EDTA) glomerular filtration rate (GFR) was less than 15 ml/min/1.73m^2 patients were excluded. Other exclusion criteria were radiological evidence of renal artery stenosis or ureteric obstruction, multi-organ transplants, pregnant or breast feeding women, a history of non-compliance with immunosuppressive therapy, patients who had taken part in other trials using alternative immunosuppressive agents and a documented hypersensitivity to macrolide antibiotics or their derivatives.

Power calculations were performed based on a previous study comparing GFR and immunohistochemical staining for collagen III, six months post-transplantation in protocol renal allograft biopsies[26]. In order to detect a difference in collagen III immunostaining difference of 12.5 % with a power of 80% and an $\alpha = 0.05$, fifteen patients would need to be entered into each arm of the study (i.e. A total of 30 patients). A similar calculation using 6 month GFR as the end point suggested that in order to detect a difference in GFR of 10 ml/min with a power of a 80% and an $\alpha =$

0.05, fourteen patients would be required in each group. In view of these calculations it was initially hoped that recruitment of 32 patients could be achieved but due to the strict inclusion and exclusion criteria this was not possible and 31 patients were enrolled. Blocked randomisation using a series of computer generated random numbers was performed in order to ensure similar numbers were randomly allocated to either a control or Rapamycin treated group. Patients were recruited in blocks of 4. The first two unique numbers between 1 and 4 from the computer generated series of random numbers represented the position of those individuals randomised to Rapa in that block of four patients e.g. if these numbers were 1 and 3, then the first and third patients out of that set of four individuals were allocated to Rapa whilst the second and fourth were allocated to the control group. This was repeated using a different row of random numbers for each block of 4 patients ultimately randomising 15 patients to the control group and 16 to the Rapa group.

Before recruitment, patients received 1.8-6.5 mg/kg/day CyA (Neoral™, Novartis) , 5mg/day or 10mg/alternate day Pred and 1-2 mg/kg/day Aza (19 individuals). The number of patients originally taking Aza was similar in both control (n=9) and Rapa groups (n=10) after randomisation. At study entry Aza was stopped and patient's immunosuppressive regimens were amended as shown below:-

1. **Control group: 40% CyA dose reduction adjusted to maintain trough levels at 50-75 ng/ml**
2. **Rapa group: 40% CyA dose reduction adjusted to maintain trough levels at 50-75 ng/ml with the addition of Rapa 2mg/day**

Rapa was dispensed as a single loading dose of 6mg, followed by a daily maintenance dose of 2 mg adjusted to maintain trough levels of 5-15ng/ml. CyA was taken twice

daily with doses administered 12 hours apart in both groups. Rapa oral suspension was diluted with water or orange juice and taken 4 hours after the first daily dose of CyA. Patients continued Pred and all other routine medication without interruption. The primary outcome measure was the change in renal function over a 6 month treatment period. In addition the safety / tolerability of each regimen was assessed.

3.2 Assessment of renal function

Both clinical and biochemical assessments of renal function were undertaken as described below:-

1. Blood pressure and weight pre-trial and then at weeks 1,2,4,6,8, 3 and 6 months.
2. Proteinuria indicated by early morning urinary albumin / creatinine ratio at study entry, 3 and 6 months.
3. Serum urea, electrolytes and creatinine pre-trial and then at weeks 1,2,4,6,8, 3 and 6 months.
4. Cockroft and Gault estimated creatinine clearance was used as a surrogate marker for GFR at these time points as well as over the 6 month pre-trial period. This estimate is based on patient age, sex and body mass according to the following equation[401].

   $$\text{Cockroft \& Gault equation for males} = \frac{1.23\,(140-\text{age (years)}) \times \text{body mass (kg)}}{\text{Serum creatinine } (\mu\text{mol/L})}$$

   $$\text{Cockroft \& Gault equation for females} = \frac{1.04\,(140-\text{age (years)}) \times \text{body mass (kg)}}{\text{Serum creatinine } (\mu\text{mol/L})}$$

5. Normalised single shot radioisotope (^{51}Cr labelled EDTA) GFR at study entry and 6 months. This provides an actual measurement rather than an estimation of GFR and is thus considered to be a 'gold standard' technique to assess renal function.

6. Renal allograft failure with return to dialysis or re-transplantation.

3.3 Assessment of Safety Profile

CyA whole blood trough concentrations were determined locally using an enzyme immunoassay technique (Behring Diagnostics, Milton Keynes, UK) and assessed at 1, 2, 4, 6, 8 weeks, 3 and 6 months. CyA dose reduction has been shown to be safe in several previous studies[273-277]. Nevertheless there is the potential for patients to develop late episodes of acute rejection as a result of a reduction in immunosuppression. Therefore any patient who developed clinical evidence of acute allograft dysfunction or had a sustained rise in 10% or greater in their serum creatinine in the absence of a urinary tract infection or evidence of obstruction, underwent a diagnostic renal allograft biopsy.

Rapa has a number of both clinical and metabolic side effects that are related to its serum trough level and have been discussed previously. Both control and Rapa patients were asked to complete a simple questionnaire at study entry and 6 months in order to determine the prevalence of these in individuals with CAN. In view of the haematological and hyperlipidaemic side effects of Rapa a FBC and fasting serum lipids / lipoproteins were measured at 1, 2, 4, 6, 8 weeks, 3 and 6 months. Rapa blood trough concentrations were determined at a central laboratory (Analytical Unit, Cardiological Sciences, St George's Hospital Medical School, London) at the same time periods. Initially the Abbott IMx immunoassay was used but 6 months into the study this was changed to a high performance liquid chromatography assay with mass spectrometric detection. Based on a cross validation analysis between these

techniques a conversion formula was calculated, (HPLC-MS value = 0.8 x IMx value) in order to provide comparable results using this new technique.

3.4 Statistical analysis

Patients included in the study were analysed on an intention to treat basis. All continuous data was assessed for normality of distribution and analysed using t-tests. On the occasions that only 2 measurements were assessed a p-value < 0.05 was considered significant. When multiple measurements of a single variable were assessed a Bonferroni correction was made based on the number of consecutive variables assessed (8 in this study). A p value < 0.00625 was considered significant. Categorical variables were compared using Fisher's exact test.

3.5 Results

Both groups had similar patient demographics (Table 3.5(a)). Mean (±sd) CyA doses before and after dose reduction were not statistically different in either group (PreCyA: 3.8 (±1.5) vs PreRapa:3.3 (±0.9), p=0.19 and PostCyA: 2.5 (±0.9) vs PostRapa 2.0 (±0.6) mg/kg/day, p=0.08). As expected CyA trough levels fell in both groups after dose reduction and remained stable thereafter with similar levels in both groups throughout the study (Figure 3.5(i)). Rapa trough levels were maintained within the target range (5-15 ng/ml) throughout (Figure 3.5(i)).

One control patient died 4 months into the study after an episode of acute pulmonary oedema secondary to left ventricular dysfunction. There were no episodes of acute rejection or allograft failures in either group. Two patients taking Rapa discontinued the drug. The first developed jaundice 2 weeks after starting Rapa. This was stopped

because of concerns regarding Rapa induced cholestasis and the jaundice resolved without sequelae over the next 5 days. A subsequent ultrasound scan confirmed the presence of gallstones in the gallbladder and the patient underwent laparoscopic cholycystectomy. The second reported a maculo-papular rash over both upper and lower limbs after 4 weeks that resolved without treatment within a week of stopping Rapa. Both individuals were subsequently maintained on reduced dose CyA without Rapa.

3.5(i) Renal function

Body mass remained stable in both groups over the 6 month treatment period (Fig3.5(ii)). Likewise neither systolic nor diastolic BP varied significantly (Fig 3.5(iii)). Eleven (69%) Rapa and 9 (60%) Control patients required an increase in antihypertensive treatment over this time but ACE inhibitors were only initiated in one of these (Control).

Early morning urinary albumin: creatinine ratio increased in 10/15 (67%) control patients and 11/16 (69%) Rapa patients over the study. However there was wide inter-individual variation in the values obtained with proteinuria being minimal in some patients but so great in others that it exceeded the assay range. Thus although the mean urinary albumin : creatinine ratio increased over the first 3 months of treatment in both groups, this was not statistically significant (Fig 3.5(iv)).

Serum creatinine showed a similar pattern in both groups with an initial significant improvement compared to pretrial values (Fig 3.5(v)). This was sustained for 2 weeks in Rapa and 8 weeks in control patients. Subsequently a small non-significant increase occurred in both groups.

At entry into the study the estimated GFR was similar in both groups. After dose reduction no significant differences between groups were noted. However there was a significant increase compared to pretrial values at week 4 in controls that did not reach significance in the Rapa group. If the mean (sd) linear trend in GFR over the pre-trial and trial 6 month periods were compared within each group, this remained unchanged for Rapa patients (-0.03 (0.13) vs -0.16 (0.27) ml/min/wk, p=ns) but showed an improvement in controls that was of marginal statistical significance (-0.24 (0.20) vs -0.04 (0.27) ml/min/wk, p=0.07) with 12 controls compared to only 6 Rapa patients (p=0.03) reducing the rate of decline in estimated GFR over time (Fig 3.5(vii).

Normalised single shot radioisotope (^{51}Cr labelled EDTA) GFR was similar in both groups initially and decreased over the 6 month study period. However this fall was only statistically significant in Rapa patients (Fig 3.5(viii), p=0.003).

3.5(ii) Adverse Effects

The new onset of adverse effects over the study is shown in Table 3.5(b). Arthralgia, mild gastrointestinal symptoms and hypertriglyceridaemia occurred more frequently in patients taking Rapa ($p \leq 0.05$).

A significant reduction in haemoglobin compared to pretrial values occurred in Rapa patients at weeks 8 and 12, however this subsequently resolved. Rapamycin caused a significant fall in white cell count at week 2 compared to pretrial values that also resolved. These haematological changes were minor and were of little clinical significance (Table 3.5(c)). Serum cholesterol remained relatively stable in both groups (Fig 3.5(ix)) and only 3 patients, 1 control and 2 Rapa, developed

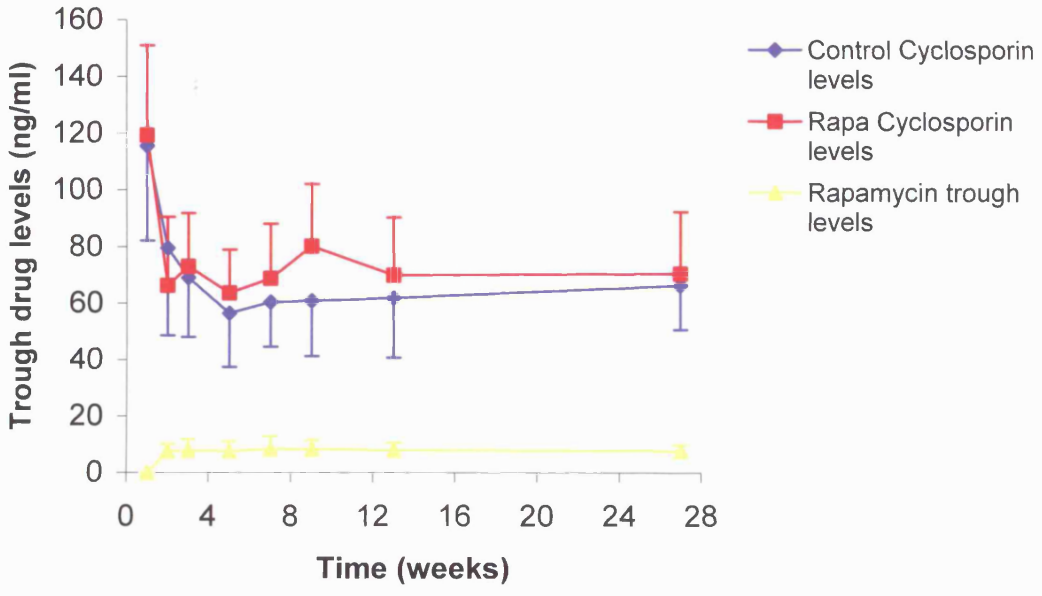
hypercholesterolaemia requiring the initiation of HMG CoA reductase inhibitor therapy over the study. There was an increase in serum triglycerides compared to pretrial values in the Rapa group that was significant at 6, 8 and 12 weeks although as above this was not dramatic or sustained. There were no significant changes in serum LDL or HDL (Fig3.5(x)). There were no myocardial infarctions, cerebrovascular events or episodes of acute pancreatitis.

Table 3.5a Patient characteristics

	Group	
	Rapa (n=16)	**Control (n=15)**
Patient Age (years)	43 (12)	47 (11)
Sex (male / female)	11 / 5	8 / 7
Race (Caucasian / Afro-Caribbean / Asian)	14 / 0 / 2	12 / 2 / 1
Months post-transplant	84 (40)	69 (30)
Donor type (cadaveric / non-heart beating / live)	14 / 1 / 1	11 / 2 / 2
HLA mismatches	2.7 (1.2)	2.3 (1.2)
Previous delayed graft function	5	5
Previous acute rejection	7	7
Diabetic patients	0	2
Pre-trial Azathioprine	10	9
Pre-trial HMG CoA reductase inhibitor	2	2
Pre-trial ACE inhibitor	0	2

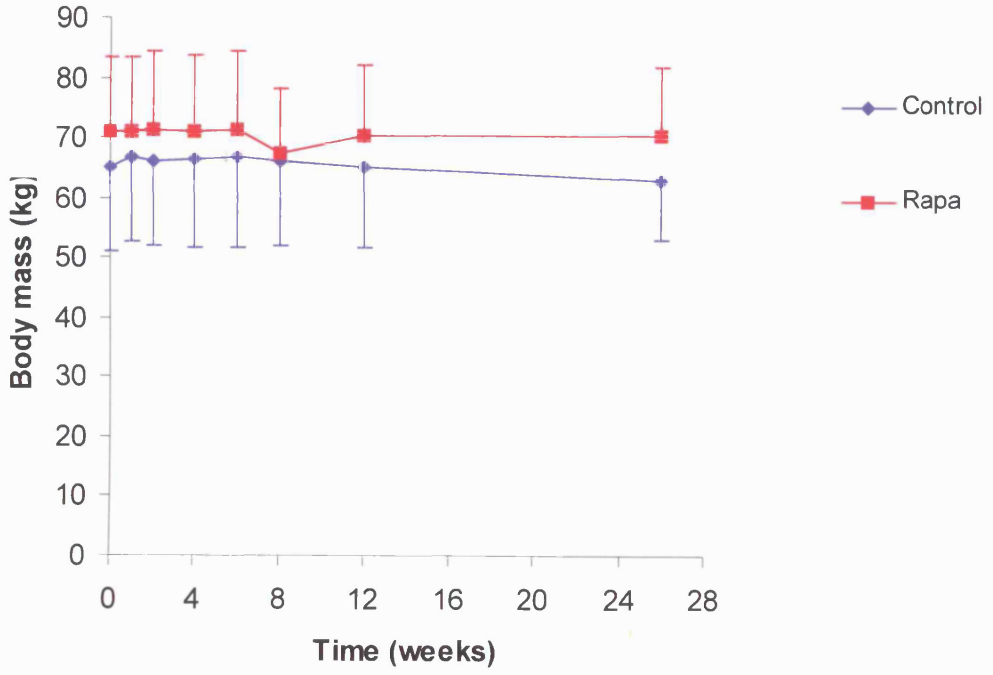
Values expressed as raw data or mean (±sd). No significant differences noted.

Fig 3.5(i) Variation in Cyclosporin and Rapamycin trough levels



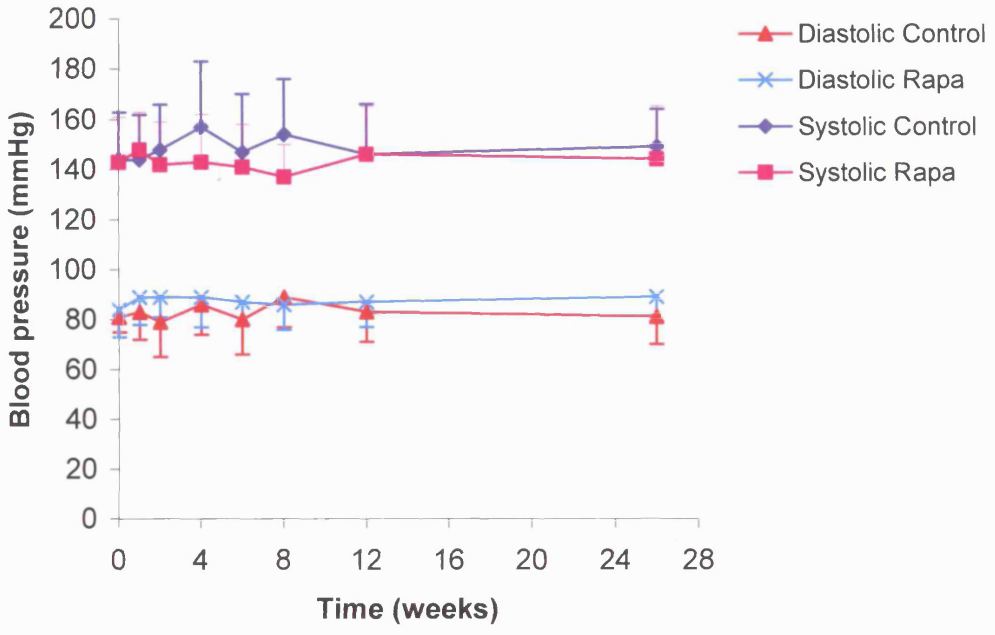
Values shown are mean ±sd (error bars). No significant differences noted.

Fig 3.5(ii) Variation in body mass



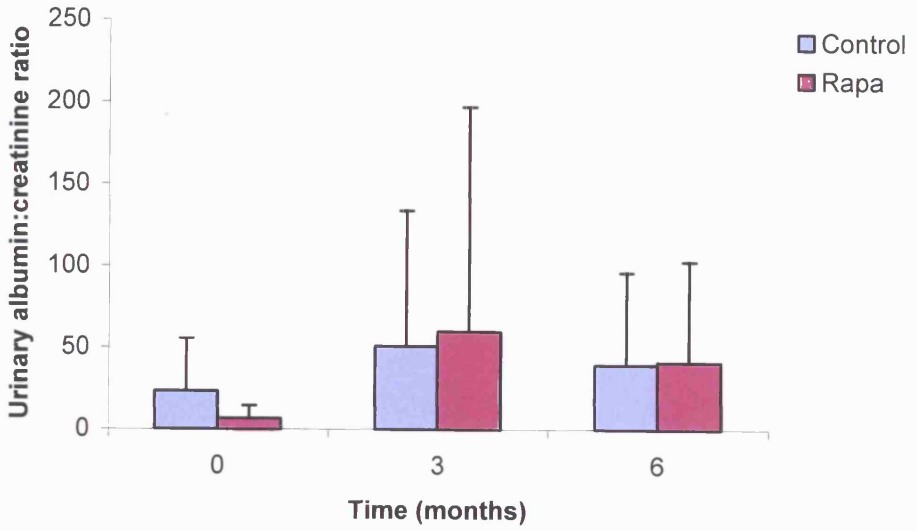
Values expressed as mean ±sd (error bars). No statistically significant differences noted.

Fig 3.5(iii) Variation in blood pressure



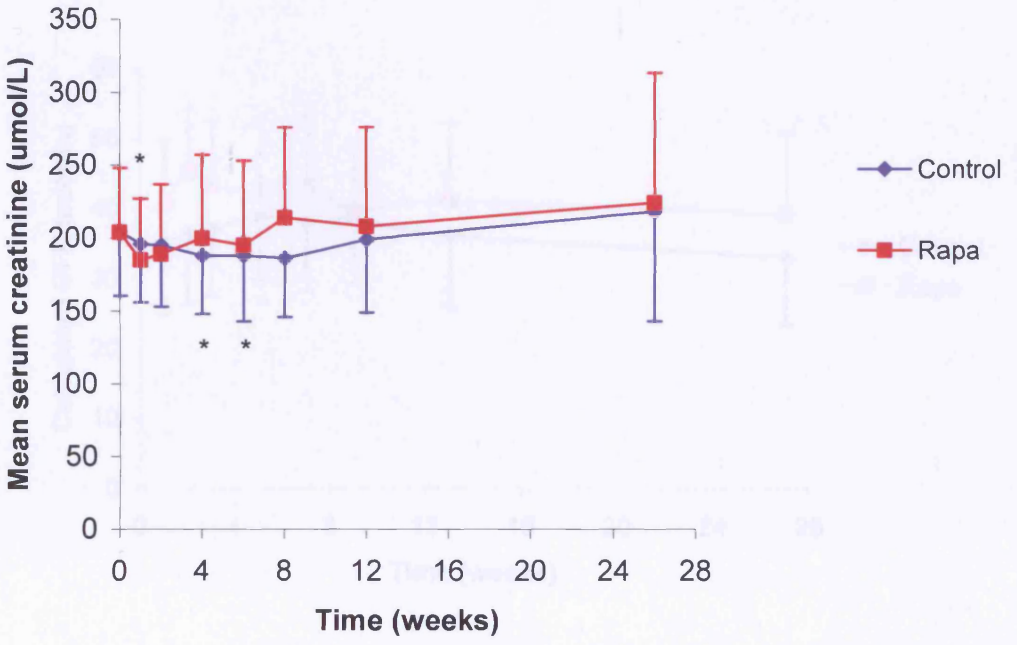
Values expressed as mean ±sd (error bars). No statistically significant differences noted

Fig 3.5(iv) Variation in urinary albumin: creatinine ratio



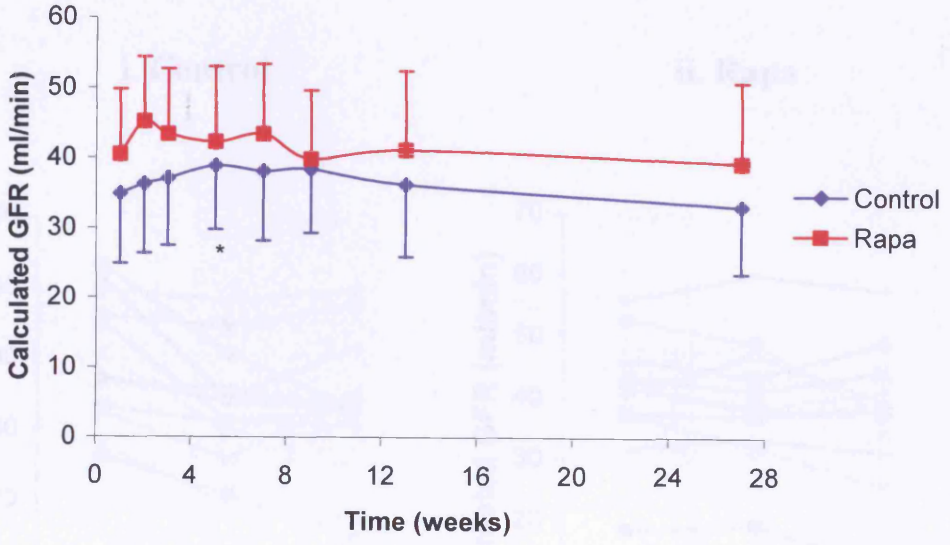
Values expressed as mean ±sd (error bars). No statistically significant differences noted.

Fig 3.5(v) Variation in serum creatinine



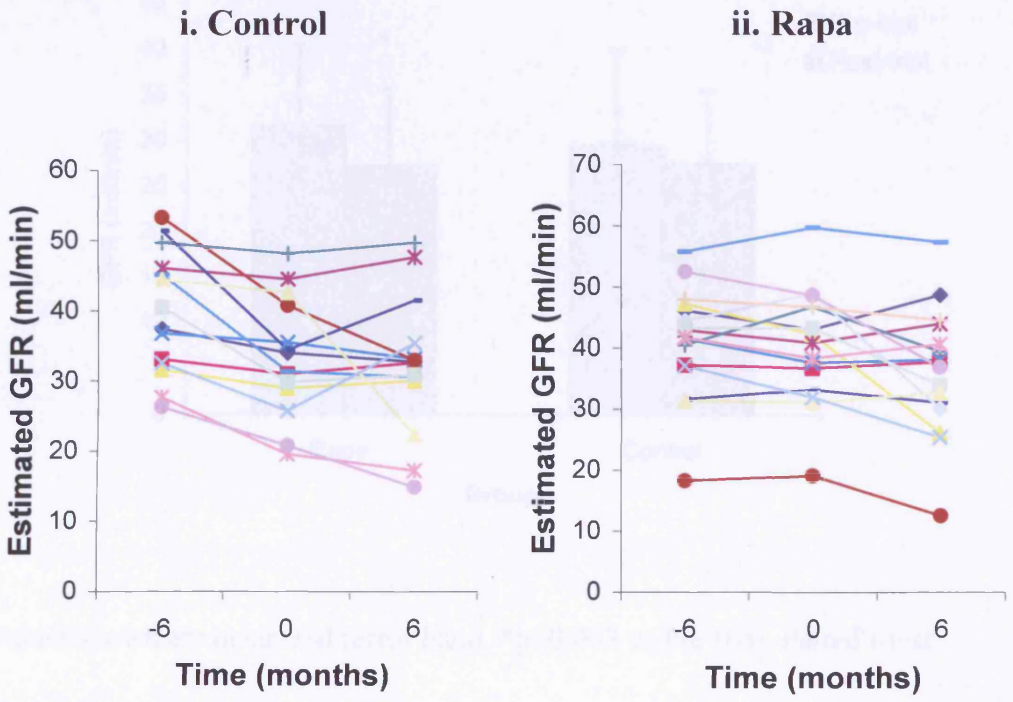
Values shown are mean ±sd (error bars). * Statistically significant vs pretrial value, Paired t-test with Bonferroni correction ($p<0.00625$).

Fig 3.5(vi) Variation in Cockroft and Gault estimated GFR



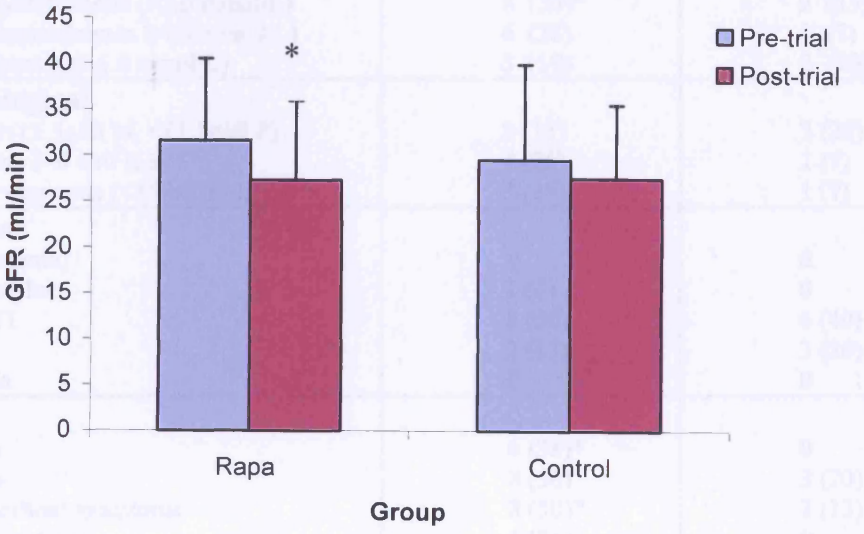
Values shown are mean ±sd (error bars). * Statistically significant vs pretrial value, Paired t-test with Bonferroni correction $p<0.00625$.

Fig 3.5(vii) Comparison of individual trends in estimated GFR in the 6 months preceding and 6 months after dose reduction



12 Control patients vs 6 Rapa patients showed an improvement or reversal in the rate of decline GFR when changes seen in the pretrial and trial 6 months were analysed, p=0.03, Fishers' exact test.

Fig 3.5(viii) Variation in normalised single shot radioisotope (^{51}Cr labelled EDTA) GFR



Values shown are mean ±sd (error bars), *p=0.003 vs Pre-trial, Paired t-test.

Table 3.5(b) New onset of adverse events over study

	Group	
	Rapa (n=16)	**Control (n=15)**
Metabolic-fasting		
Hypertriglyceridaemia (>2.0 mmol/L)	8 (50)*	2 (13)
Hypercholesterolaemia (>6.6 mmol/L)	6 (38)	1 (7)
Hyperglycaemia (>6.0 mmol/L)	3 (19)	3 (20)
Haematological		
Anaemia (<13.5g/dl M, <11.5g/dl F)	2 (13)	3 (20)
Leucopaenia (<4 x10^9/L)	4 (25)	1 (7)
Thrombocytopaenia (<150x10^9/ L)	3 (19)	1 (7)
Infections		
CMV (viraemia)	0	0
Herpes simplex	1 (6)	0
Viral/URTI	8 (50)	6 (40)
UTI	2 (13)	3 (20)
Pneumonia	0	0
Other		
Arthralgia	6 (38)*	0
Headaches	8 (50)	3 (20)
Gastro-intestinal symptoms	8 (50)*	2 (13)
Peripheral oedema	4 (25)	0
Increased antihypertensive drugs	11 (69)	9 (60)
Malignancy	0	0

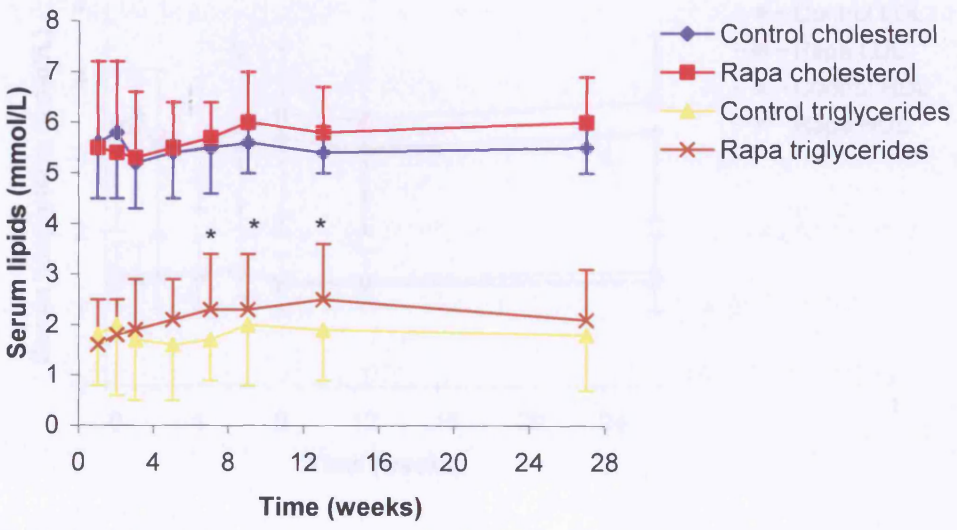
Values expressed as raw data (% of group). *$p \leq 0.05$ vs Control, Fisher's exact test.

Table 3.5(c) Haemoglobin, leukocyte and platelet counts

	Group	
	Rapa (n=16)	**Control (n=15)**
Haemoglobin (g/dl)		
Pretrial	12.3 (1.6)	12.1 (1.8)
Wk1	12.0 (1.7)	12.4 (1.9)
Wk2	11.8 (1.5)	12.1 (2.0)
Wk 4	11.7 (1.6)	11.9 (1.9)
Wk 8	10.8 (1.1)	12.0 (1.9)
Wk 12	11.2 (1.8)*	12.2 (1.7)
Wk 26	11.6 (1.8)	12.2 (1.5)
White cell count ($x10^9$/L)		
Pretrial	7.5 (2.6)	7.8 (2.5)
Wk1	6.8 (1.9)	7.4 (1.8)
Wk2	5.9 (2.0)*	8.0 (3.0)
Wk 4	6.4 (1.8)	7.4 (2.5)
Wk 8	7.1 (2.5)	7.8 (2.9)
Wk 12	6.7 (2.0)	7.9 (2.2)
Wk 26	7.4 (1.7)	7.5 (2.4)
Platelet count ($x10^9$/L)		
Pretrial	215 (66)	248 (95)
Wk1	193 (71)	266 (101)
Wk2	218 (77)	254 (100)
Wk 4	227 (81)	244 (88)
Wk 8	237 (70)	248 (92)
Wk 12	244 (73)	277 (178)
Wk 26	224 (77)	242 (94)

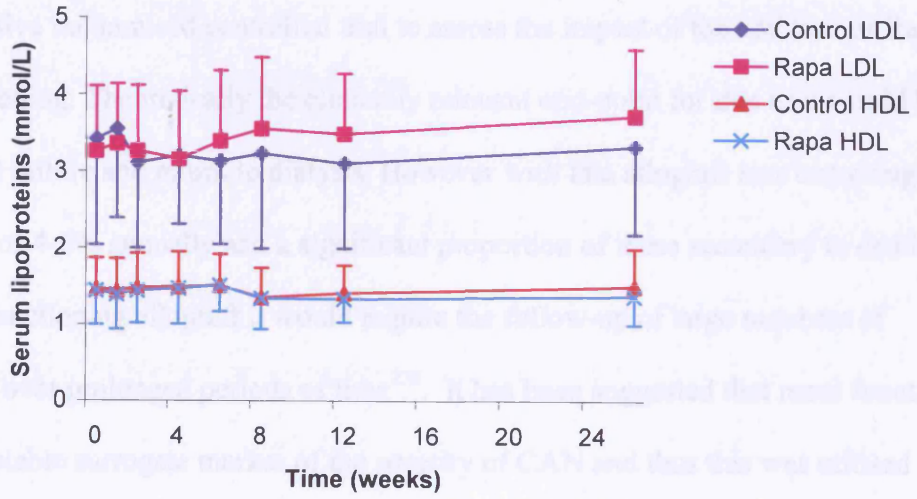
Data expressed as mean ±sd (error bars).* Statistically significant vs pretrial value, Paired t-test with Bonferroni correction $p<0.00625$.

Fig 3.5(ix) Fasting serum cholesterol and triglycerides



Values shown are mean ±sd (error bars). * Statistically significant vs pretrial value, Paired t-test with Bonferroni correction ($p<0.00625$).

Fig 3.5(x) Fasting serum lipoproteins



Values shown are mean ±sd (error bars). No statistically significant differences noted.

3.6 Discussion

Although CyA dose reduction between 40% and 50% with the addition of Aza or MMF has beneficial effects on renal function in patients with CAN with few episodes of acute rejection reported[273-275], this is one of the first studies and the only prospective randomised controlled trial to assess the impact of the addition of Rapa in such a setting. Theoretically the clinically relevant end-point for this trial would be allograft failure and return to dialysis. However with late allograft loss occurring at the rate of 4-6% annually and a significant proportion of these secondary to death with a functioning allograft it would require the follow-up of large numbers of patients over prolonged periods of time[278]. It has been suggested that renal function is an acceptable surrogate marker of the severity of CAN and thus this was utilised in order to assess the efficacy of Rapa in this setting[279]. Although the number of patients is relatively small and results therefore subject to type II statistical error, this data suggests that the addition of Rapa 2 mg/day to a regimen of reduced dose CyA produced no additional benefits in terms of renal function compared to CyA dose reduction alone. Indeed, in some respects patients given Rapa have actually fared worse than their 'control' counterparts.

Renal function was assessed in a number of ways including proteinuria, serum creatinine, calculated and measured GFR. Proteinuria measured as the urinary albumin:creatinine ratio showed massive variation between patients with some having minimal (lowest = 0.5) and others having very high values (highest >400). Thus the mean values obtained at 0,3 and 6 months were impossible to interpret in a meaningful manner. This concurs with previous studies noting significant proteinuria in less than 1/3 of patients with CAN[29] and suggests that this is not a good surrogate marker of outcome when looking at groups of individuals with CAN.

The most commonly used measurement of renal function in clinical practice is serum creatinine. However this does not take into account variations in creatinine production due to age, sex or body mass and thus can be misleading. Therefore calculated GFR has been advocated, the most widely used of which is that of Cockroft and Gault[401]. This reduces the variability seen with serum creatinine values in populations of males and females of different ages. Furthermore by comparing change in estimated GFR in the pretrial and post-trial periods, each patient can effectively act as their own control. Thus although there were no sustained differences in estimated GFR in either group, control patients demonstrated a reduction in the rate of decline in their GFR when the pre and post-trial periods were compared, that was not present in Rapa patients. However the Cockroft and Gault formula consistently overestimates GFR in obese or oedematous patients and does not take into account extrarenal elimination of creatinine, tubule absorption / secretion or inaccuracies in laboratory assessments of serum creatinine[402]. Furthermore it does not correlate well with measured GFR in renal transplant recipients[403]. Therefore actual measurement of the GFR was also undertaken in order to confirm these findings. Although inulin clearance has long been considered the gold standard substance for this technique it is both expensive and relatively scarce and radionucleotide bound substances have been advocated, the most popular of which has been ^{51}Cr labelled EDTA[402]. This fell significantly in patients taking Rapa over time but not in controls. Neither group had an allograft failure presumably reflecting the relatively short follow-up and the exclusion of allografts that had normalised ^{51}Cr labelled EDTA GFR < 15ml/min/1.73m^2.

The lack of stabilisation or improvement in renal function in Rapa patients was disappointing as it was hoped that Rapa would have similar or better efficacy than MMF after CyA dose reduction. However these results compare favourably to a series

of 19 patients with CAN who received a 47% CyA dose reduction and conversion from MMF to Rapa. This reported 7 allograft failures, 4 patients withdrawn from Rapa due to side effects with the remainder showing little change in renal function (31 vs 30 ml/min) over a mean 7 month follow-up period[397].

The safety of CyA dose reduction in patients with CAN with or without the addition of Rapa 2 mg/day was investigated in this current study. Rapa had an acceptable side effect profile in patients with CAN. There were no episodes of pneumonitis, contrasting significantly with the findings of a previous study where CyA was eliminated and replaced with Rapa[398]. Arthralgia and mild gastrointestinal upset occurred more frequently but these had few clinical consequences and resolved over time. Although haematological and lipid abnormalities occurred these were not marked and had little clinical significance. The relatively minor side effect profile was not unexpected as the Rapa dose used produced relatively low mean trough levels. This was clearly an important factor in ensuring long-term compliance in a group of patients who were several years post-transplantation and had grown accustomed to a stable immunosuppressive regimen. CyA dose reduction has been reported to increase renal plasma flow and GFR with subsequent improvements in blood pressure[182]. However these were not seen in either group.

Several factors (discussed briefly in Chapter 2) may explain the lack of therapeutic benefit following the addition of Rapa:-

1. In order to give the therapeutic changes instituted a realistic chance of improving renal function in humans, individuals with a normalised GFR < 15ml/min/1.73m^2 were excluded as it was felt that such allografts had sustained potentially irreversible damage and would be likely to fail irrespective of intervention. Nevertheless

allografts had been in situ for long periods (mean 69-84 months) and thus the pathological changes of CAN were severe in many cases. It is well recognised that the histopathological changes of CAN often precede a deterioration in renal function[14]. Thus it is quite possible that many of the allografts in this study had reached a point of no return at and beyond which outcome was unlikely to be altered significantly by a pharmacological intervention despite only moderate impairment in renal function.

2. A further factor is the relatively low 2mg daily dose of Rapa (~0.03 mg/kg/day) used in this study. Rapa has been shown to be a powerful immunosuppressant in its own right, having similar efficacy to CyA in triple therapy regimens in de novo renal transplant recipients. Experience in combining Rapa and CyA in vitro, in vivo and in humans suggests that there is a potent immunosuppressive synergy between these agents and even 2 mg / day Rapa combined with CyA produced low rates of acute rejection in de novo allograft recipients[328,329]. Therefore this relatively low dose was chosen in the current study in order to limit the possibility of over-immunosuppression particularly as the majority of patients have had allografts in situ for 5 years or more. The oral bioavailability of Rapa is low (~15%) but it exhibits significant inter and intra-individual variability suggesting blood levels are more important than actual dose given[404]. In this study 2mg/day Rapamycin produced trough levels of 7-8 ng/ml. These are within the recommended range for the prophylaxis of acute rejection in humans (5-15 ng/ml). However in rat models of vascular injury and chronic cardiac allograft rejection, larger doses (≥1.5 mg/kg/day) were required to inhibit intimal hyperplasia / GVD with efficacy increasing in a dose dependent manner[379,392]. Differences in the route of administration as well as between different species may alter the bioavailability of Rapa in these models. As Rapa trough levels have not been assessed in this in vivo work, the significance that should

be attached to these large doses is not known. However if such dosages are required to inhibit CAN in humans, then the subsequent over-immunosuppression and side effect profile would be poorly tolerated making such regimens unusable in this context.

3. Although the pathogenesis of CAN is multi-factorial, hyperlipidaemia may play a role (chapter1). Rapa caused an increased incidence of hypertriglyceridaemia with only a mild transitory increase in mean TG levels at 6, 8 and 12 weeks but no significant changes in serum cholesterol. Thus Rapa induced hyperlipidaemia is unlikely to have played a major role in the progression of CAN in this study. Hyperlipidaemia was less severe than has been reported in de-novo allograft trials presumably because both CyA and steroid doses were relatively low in comparison to those given to new transplant recipients. However it should be noted that Rapa causes hyperlipidaemia in a dose dependent manner[360] and thus in studies using higher doses the therapeutic benefits of Rapa with respect to CAN may be reduced.

4. The final point that should be considered is the possibility that Rapa may augment CyA induced nephrotoxicity. The European multicentre studies comparing Rapa with CyA triple therapy in denovo allograft recipients have confirmed that Rapa is non-nephrotoxic at human doses[338,339]. However in a large multicentre trial combining Rapa and CyA, renal function was impaired at both 6 and 12 months follow-up compared to controls[328]. It has been suggested that Rapa may in some way potentiate CyA nephrotoxicity although the mechanism underlying this is unclear. A study in the Wistar-Furth rat suggested that decreases in GFR when CyA and Rapa are combined could be attributed to an increase in serum CyA levels[350]. It has been noted previously that Rapa and CyA can interact via both p-glycoproteins and CYP P450 enzymes in

the gastrointestinal tract, potentially affecting the absorption of both drugs. Thus in both the denovo multicentre studies, and our trial, Rapa and CyA were administered 4 hours apart in order to reduce the chances of such an occurrence. After CyA dose reduction trough levels were low in both groups with no statistically significant differences between groups. However CyA trough levels do not have a very strong correlation with total exposure to CyA determined by area under the concentration versus time curve[328]. Some preliminary evidence has noted that Rapa increased CyA area under the concentration-time curves compared to controls[328], but further studies will be required to investigate whether this occurs across the spectrum of CyA / Rapa dosages. Although the serum exposure of drugs often reflects tissue exposure (and thus the beneficial / toxic effects of an agent) this is not always the case. It is therefore worth noting that a recent study in rats has suggested that Rapa may increase tissue levels of CyA more than in plasma[351], although there has been no comparable work in humans to date. Clearly the interaction between CyA and Rapa is not well understood at the present time and additional studies are necessary to characterise this further. However in CAN the functional reserve of organs is often minimal resulting in particular sensitivity to any increases in CyA exposure associated with Rapa administration even if these were quite small and this may help to explain the lack of a beneficial response to Rapa observed in this study.

A recently published Tri-continental multicentre trial in denovo renal allograft recipients who had taken CyA, Rapa (TLs> 5 ng/ml) and Pred for immunosuppression for 3 months, randomised patients to remain on this therapy or to have CyA withdrawn and immunosuppression maintained with Rapa (TLs 20-30 ng/ml) and Pred. At 12 months patient and graft survival were similar in both groups but despite a slightly higher rate of acute rejection in those withdrawn from CyA (9.8 vs 4.2%,

p=0.035), both renal function and blood pressure had significantly improved[405]. A similar large study (discussed in chapter 2) has reported equally promising improvements in renal function following CyA withdrawal without an increase in the incidence of acute rejection[336,337]. This approach has been attempted on a smaller scale in patients with established chronic allograft damage. A non-randomised series of 23 patients taking either CyA or Tacrolimus based immunosuppression a median of 54.9 months after renal transplantation had their calcineurin inhibitors eliminated and replaced with Rapa. Over 8 months follow-up median serum creatinine improved from 211μmol/L to 174μmol/L ($p<0.05$) although proteinuria did not alter[398]. Similarly improvements in renal function have been reported in a series of 12 patients with chronic calcineurin inhibitor toxicity switched from CyA to Rapa 5mg /day after 6 months follow-up[406]. These preliminary results are encouraging, particularly as acute rejection was not a problem in either study. The improvements in renal function suggest that future regimens introducing Rapa in patients with CAN should perform CyA elimination rather than dose reduction as this appears safe, abolishes CyA nephrotoxicity and avoids any potential interaction between CyA and Rapa. Currently none of the trials that have attempted this approach are very large and their results need to be confirmed in a multicentre study. Furthermore none have had control groups and it is thus unclear whether the use of Rapa produces any additional benefit over agents such as Aza or MMF in such a setting.

Chapter 4

Does the addition of Rapamycin after Cyclosporin dose reduction influence profibrotic gene expression?

4.1 Acquisition of renal allograft tissue and isolation of glomeruli

4.2 Principles of RT-PCR

4.3 Investigation of profibrotic mRNA expression

4.4 Principles of ELISA

4.5 Semi quantitative assessment of PCR products using ELISA

4.6 Data analysis

4.7 Glomerular profibrotic gene expression

4.8 Interstitial profibrotic gene expression

4.9 Discussion

4.1 Acquisition of renal allograft tissue and isolation of glomeruli

The renal allografts of the patients studied in Chapter 3 were biopsied at enrolment and after 6 months (Fig 4.1(i)). On each occasion, 10 mls 1% lignocaine with adrenaline was infiltrated around the upper pole of the renal allograft under aseptic conditions. A 16 gauge trucut® needle mounted on a spring loaded biopsy gun was introduced percutaneously and advanced onto the capsule of the allograft using real time ultrasound guidance (Fig 4.1(ii)). Two biopsy cores of renal cortex (Fig 4.1(iii)) were taken and haemostasis obtained using direct pressure for 5-10 minutes followed by bed rest for 4 hours.

Immediately after the first biopsy core was taken 3 glomeruli were plucked from this by hand, using ultra-fine forceps under a stereoscopic microscope (Fig 4.1(iv)). These glomeruli and a small section of interstitium were placed into separate Eppendorf tubes containing 100μl of lysis-binding buffer (an inhibitor of mRNA breakdown, *Appendix A*) and frozen at -20°C until required. The remaining core was placed in 4% formaldehyde and subsequently mounted and sectioned for histological examination.

4.2 Principles of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The genetic template in all human cells consists of double stranded DNA configured to form chromosomes. Within each chromosome small fragments of DNA, 'genes,' encode for the various intra and extracellular proteins. If there is an appropriate signal these undergo transcription producing mRNA species specific for each gene that in turn can be translated to produce a final protein product. Proteins often accumulate within cells and if measured may not reflect the current state of protein synthesis

Fig 4.1(i) Renal allograft biopsy under ultrasound guidance

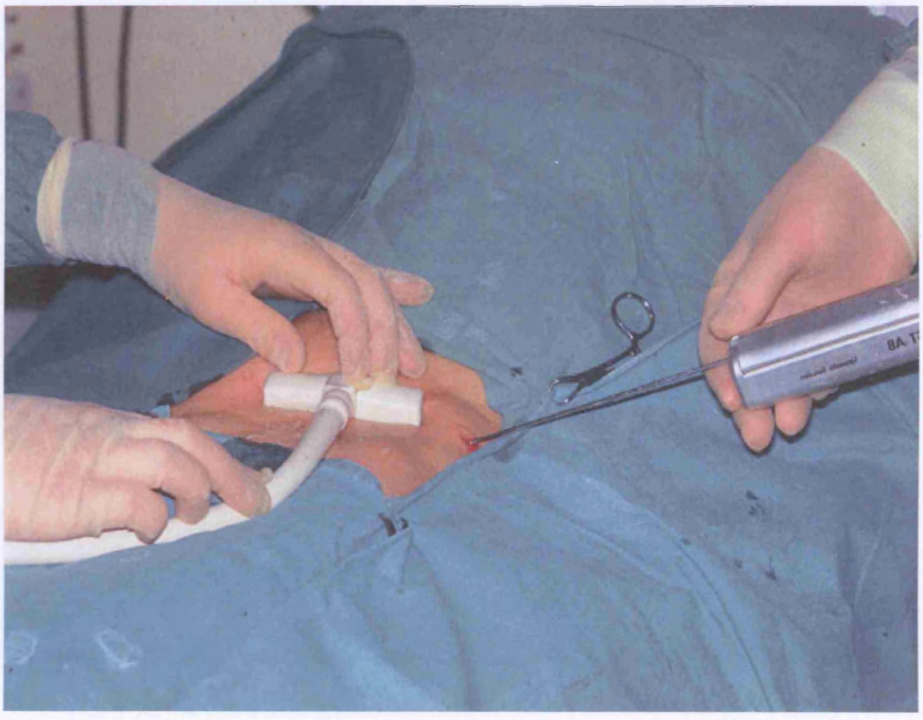


Fig 4.1(ii) Ultrasound image of a renal allograft biopsy

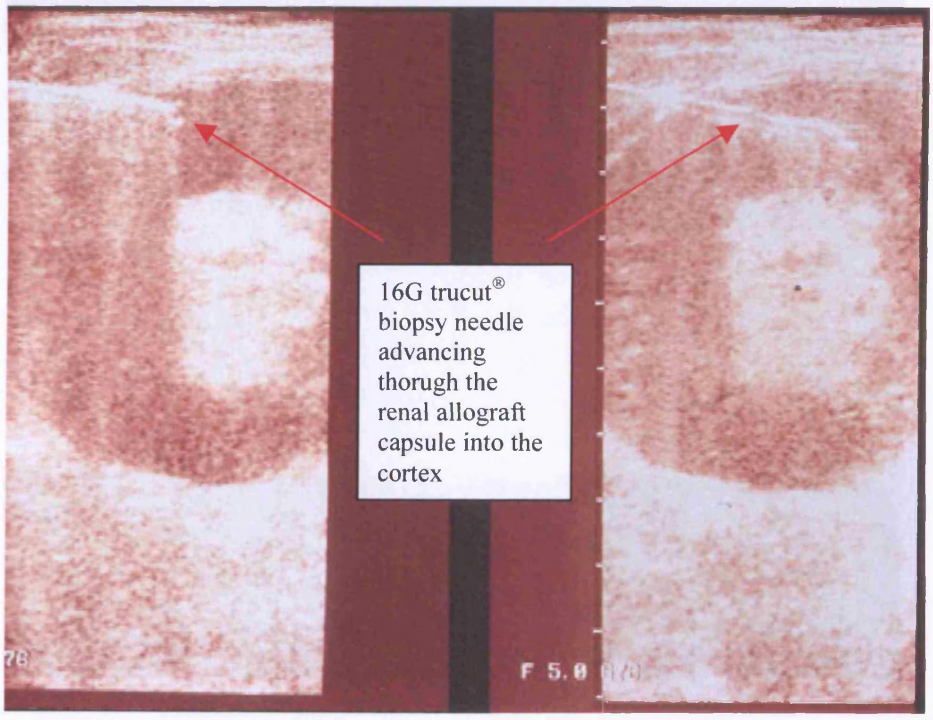


Fig 4.1(iii) **Core biopsy from renal allograft**

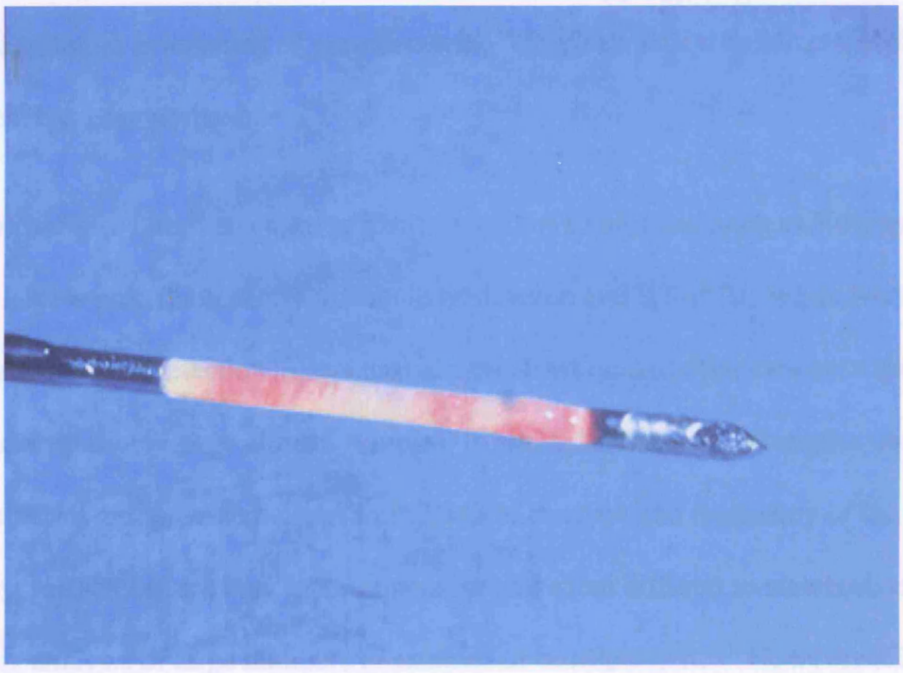
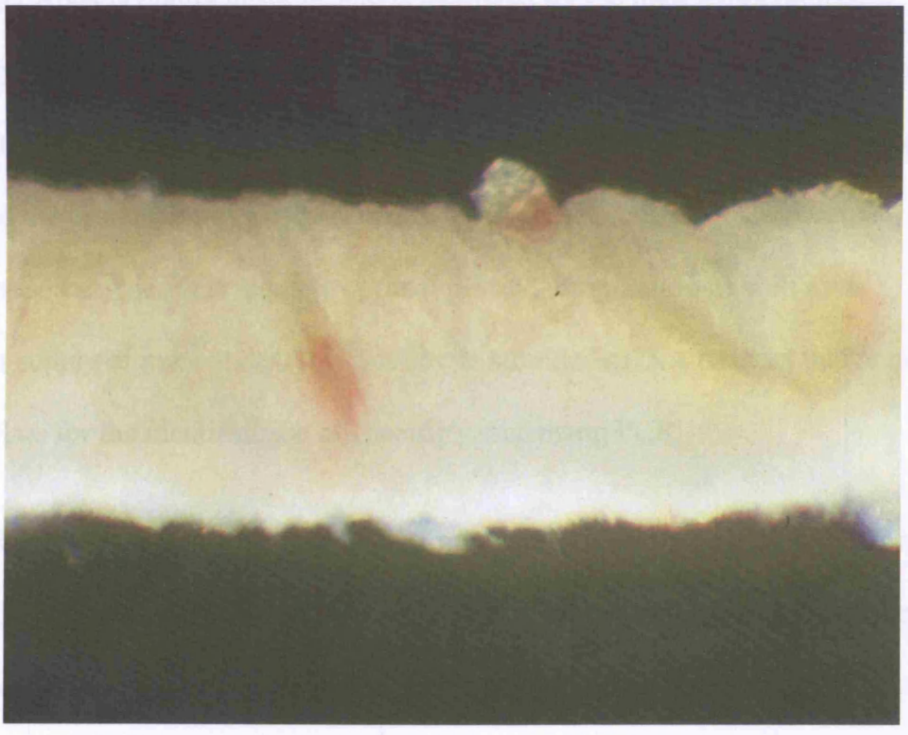


Fig 4.1(iv) **A single glomerulus in a biopsy core (x25)**



merely what has happened in the past. In addition the combination of identification and isolation of a specific proteins and the small sizes of samples such as glomeruli, makes quantitative assessment of protein composition impractical and thus mRNA assessment has been proposed.

Several techniques have been used to identify mRNA expression such as Northern blotting, RNase protection assays, in situ hybridisation and RT-PCR. While Northern blotting and RNase protection assays provide excellent quantitative measures they both require relatively large clinical samples. In situ hybridisation techniques use smaller samples and provide a direct evaluation of the type and frequency of the cells expressing mRNA but are very labour intensive and often difficult to standardise precluding analysis of large numbers of samples or multiple genes. However RT-PCR allows the evaluation of large numbers of samples and multiple genes with high sensitivity[407].

Messenger RNA is highly unstable and is degraded by the high temperatures necessary for PCR. Furthermore the enzyme integral to PCR, Taq polymerase, cannot amplify mRNA. Thus RT-PCR relies upon the isolation of mRNA from genomic DNA and the production of a complementary cDNA sequence using a reverse transcriptase enzyme. This requires optimal pH and temperature as well as an abundant supply of nucleotides. It is this single stranded cDNA product that is used as the template for the identification of specific genes using PCR.

The PCR reaction is an in vitro technique for the selective exponential amplification of a single fragment of DNA that is so massive that when performed in a complex mixture of DNAs results in the relative purification of the DNA fragment of interest. The reaction takes place in a single tube and requires a 'target' sequence of DNA to

be amplified, an excess of nucleotides, a DNA polymerase, a buffer solution and both a 'forward' and 'reverse' oligonucleotide 'primer' complementary to a small sequence of DNA (typically at least 20 nucleotides) at the 5′ of each DNA strand. These 'primer' sequences bind to their respective complementary DNA fragment and provide the focal point at which DNA polymerase activity is initiated. It is important to ensure that both primers are specific to the gene of interest in order to minimise the chances of non-specific DNA amplification. Both the composition and length of primer can affect this[408,409]. Primers containing a majority of Cytosine-Guanine base pairs reduce non-specific binding as they can withstand higher annealing temperatures[408]. If short primers are used the risk of non-specific binding is increased. However if excessively long sequences are used it becomes possible for the non specific binding to occur within the primer itself, producing a tertiary structure that prevents binding to the original template DNA[409]. In addition complimentarity between the forward and reverse primers should be avoided as this encourages binding to one another rather than the template DNA thereby producing 'primer dimers' and reducing the efficiency of the PCR reaction[408]. In order to minimise these potential problems computer software is now available to design primer sequences for target genes.

The first step of the PCR reaction involves separation of double stranded DNA into 2 single stranded DNA templates by heating to 95°C. The temperature is then reduced to 50-60°C allowing the 2 oligonucleotide primers to bind or 'anneal' to their respective target sequences. The optimum annealing temperature varies from primer to primer but should be identified as it reduces non-specific binding thus ensuring maximum yield from each cycle of the PCR reaction. DNA polymerase binds to the gene of interest adjacent to each primer sequence and when the temperature reaches

72°C synthesises a new strand of DNA in the 5′ to 3′ direction along each single stranded DNA template. The new strands of DNA extend past the site to which the complementary primer attaches such that when the new strand is separated from its template by heating to 95°C a complementary site for each primer is exposed thus enabling the synthesis of the next new DNA strand in the opposite direction. This cycle of steps can be performed repeatedly with an effective doubling of the PCR product on each occasion such that only 20 cycles produces a theoretical million-fold amplification (2^{20} copies) assuming that each reaction is 100% efficient[410]. Although automated thermal cyclers and the use of Taq polymerase from the thermostable organism *Thermus aquaticus* have revolutionised the speed and efficiency of modern PCR the principles of this technique have not changed since the initial description by Mullis et al[411].

4.3 Investigation of profibrotic mRNA expression

The use of RT-PCR to identify mRNA expression in renal tissue has been previously described[407,412]. In the present study this technique was learnt using samples obtained from nephrectomy specimens. Subsequently both glomeruli and interstitium from the 15 control and 16 Rapa patients taken at study entry and after 6 months were assessed. Because of the limited material available reverse transcription was performed in small batches of 3 samples at a time in order to limit any losses as a result of technical error. Full details of the reagents and protocols used are given in *Appendices A and B.*

4.3a. mRNA extraction

All equipment was immersed in 3% H_2O_2 for 30 minutes and then washed with DEPC treated water in order to ensure it was free from RNases. Each sample was defrosted,

homogenised with a 'Pellet pestle' and then incubated with 10µl of Proteinase K at 37°C for 1 hour. The homogenate is often viscous due to released DNA and as this can impair the extraction of mRNA, a DNA shearing step was performed. This was achieved by pressing the homogenate 3 times through a 21-gauge needle using a 1 ml syringe. Foaming was reduced by centrifugation at 10000 rpm for 1 minute and the supernatant removed, taking great care to avoid visible contamination from the cellular debris and DNA that has been forced to the bottom of the centrifuge tube. The mRNA rich supernatant was then added to 10µl of oligo-dt-linked Dynabeads® (Dynal®) for isolation and purification. Each mRNA species has a repetitive sequence of adenine nucleotides at its 3′ end known as a 'poly-A tail'. Dynabeads® are small paramagnetic beads that each has a repetitive sequence of thymidine nucleotides known as a 'poly-T tail'. Adding the supernatant to the Dynabeads® allows the poly-A mRNA tails to anneal to the poly-T Dynabead® tail thus isolating mRNA. After a 10 minute annealing period the Dynabeads® undergo a series of purification steps in order to minimise DNA contamination. This involves the thorough mixing of 50 µl of washing buffer, initially with and then without LiDS (twice and 3 times respectively) with each Dynabead® sample. The Dynabeads® are placed in a magnetic field and form a pellet separate from the contaminated wash buffer allowing this to be discarded with minimal loss of Dynabeads®. When this has been completed the purified mRNA linked Dynabeads® are re-suspended in 10µl of DEPC labelled water in preparation for reverse transcription.

4.3b. Reverse Transcription

This step was always performed immediately after mRNA extraction. A 'mastermix' containing 5µl of AMV RT5X buffer, 2.5µl of 10mM DEPC treated nucleotides,

8.4µl of DEPC treated water and 0.6µl of 40U/µl RNasin to prevent degradation of mRNA, was made for each reaction. Eight micro-litres of Dynabeads® in DEPC treated water were added to 16.5µl of 'mastermix' followed by 0.5µl of 10U/µl Avian Myeloblastosis Viral Reverse Transcriptase (RT+ sample) and incubated at 42°C for 1 hour. The 'poly-T tails' of the Dynabeads® act as the primer for reverse transcriptase and thus allow the synthesis of cDNA directly onto the beads. In order to control for genomic DNA contamination, the remaining 2µl of Dynabead® DEPC treated water mixture from each mRNA extraction was included in a similar reaction without reverse transcriptase (RT- sample). A PCR reaction using GAPDH primers was subsequently performed for each RT+ and RT- sample and the PCR product was visualised by agarose gel electrophoresis *(Appendix D).* If the mRNA extraction was of poor quality or there was significant DNA contamination the sample was discarded.

The Dynabead® 'poly-T tails' are non-specific primers for the reverse transcription of all species of mRNA. Theoretically each mRNA molecule is primed equally and therefore this method enables production of a cDNA library, the size and composition of which accurately reflects the quantity of mRNA in each sample at the time of biopsy. Twenty-five micro-litres of cDNA-Dynabead® linked library is produced each time and can be stored at 4°C indefinitely. Thus the activity of multiple genes can be assessed, as only 1-2µl of cDNA is required for each PCR reaction.

4.3c. Design and work-up of Primers for PCR

As discussed earlier each gene has specific forward and reverse primers required that affect the efficiency and selectivity of the PCR reaction. These were designed using

the Wisconsin computer software package from the Genetics Computer Group. The original oligonucleotide sequences were obtained from the European Molecular Biology Laboratories database and each was tested for homology with up to 2 mismatches against all human sequences in the database. The forward primer was biotinylated to enable capture and quantification of each PCR product using an ELISA technique (discussed later). The optimum annealing temperature for each primer was determined by performing sequential PCRs at different annealing temperatures and the temperature that yielded the PCR product with the best ELISA result was used. The optimum number of PCR cycles was determined in a similar fashion for each gene of interest. Details of the forward and reverse primer sequences, annealing temperatures and number of PCR cycles used for each gene studied are given in Tables 4.3(i) and 4.3(ii).

4.3d. Polymerase Chain reaction

Effective mRNA extractions and reverse transcription were obtained from 27 pre-study and 23 six-month glomerular samples. Likewise this was successful in 27 pre-study and 28 six-month interstitial samples. Large PCRs for each of the 9 genes studied were performed using all pre-study or all 6-month cDNA samples simultaneously. Glomeruli and interstitium were analysed separately. GAPDH, a glycolytic enzyme constitutively expressed in all cells was the first gene assessed. The expression of this 'housekeeping' gene was regarded as an indicator the quality of mRNA extraction and subsequent reverse transcription for each sample. Thus if relatively small amounts of an abundant gene such as GAPDH were identified, then the expression of other less common mRNA species would be significantly reduced and double the amount of cDNA was used as the initial PCR template in such circumstances.

Table 4.3(i) Forward and reverse PCR primer sequences

Primer	Primer Oligonucleotide Sequence (5′ - 3′)
GAPDH (Forward)	AGA ACA TCA TCC CTG CCT C*
GAPDH (Reverse)	GCC AAA TTC GTT GTC ATA CC
TIMP-1 (Forward)	TGG GGA CAC CAG AAG TCA AC*
TIMP-1 (Reverse)	CAG GGG ATG GAT AAA CAG GG
TIMP-2 (Forward)	AAC GAC ATT TAT GGC AAC CC*
TIMP-2 (Reverse)	ACC TGT GGT TCA GGC TCT TC
Collagen III (Forward)	CCT GGT ACA TCT GGT CAT CC*
Collagen III (Reverse)	CCA TTT TCA CCC TTT AAT CC
TGFβ-1 (Forward)	AAG TTA AAA GTG GAG CAG C*
TGFβ-1 (Reverse)	CAC TTG CAG TGT GTT ATC C
MMP-2 (Forward)	ATT GAT GCG GTA TAC GAG GC*
MMP-2 (Reverse)	GGC ACC CTT GAA GAA GTA GC
MMP-9 (Forward)	TTC TAC GGC CAC TAC TGT GC*
MMP-9 (Reverse)	CGC CCA GAG AAG AAG AAA AG
iNOS (Forward)	GCC CAA GGT CTA TGT TCA GG*
iNOS (Reverse)	GTC CTT CTT CGC CTC GTA AG
Tenascin (Forward)	TGA ACA AAA TCA CAG CCC AG*
Tenascin (Reverse)	CAG TGG AAC CAG TTA ACG CC

*Biotylinated

Table 4.3(ii) Optimum annealing temperatures and number of PCR cycles

Gene	Annealing temperature (°C)	Number of PCR cycles
GAPDH	59	35
TIMP-1	59	40
TIMP-2	59	40
Collagen III	59	40
TGFβ-1	59	40
MMP-2	59	40
MMP-9	59	40
iNOS	59	40
Tenascin	59	40

The PCR protocol is described in *Appendix C*. In summary a 'mastermix' containing all the reagents required for the sample batch to be assessed was produced. Frozen AJ 10X buffer was thawed and then centrifuged at 10,000 rpm for 1minute in order to pellet the precipitated BSA present. Precipitated BSA inhibits the PCR reaction but in solution BSA promotes the efficiency of PCR by preventing *Taq polymerase* from coating the plastic Eppendorf tubes used for each reaction. AJ 10X buffer was carefully separated from any precipitated BSA and 5μl was combined with 2μl of 5pmol/μl forward and reverse primer mix, 41.6μl of sterile distilled water and 0.4μl of 2.5U/μl Jump-start Taq Polymerase for each sample. The addition of Jump-start Taq to the 'mastermix' reduces any methodological errors introduced by adding Taq individually to each reaction tube. Despite this it retains the advantages of the 'hotstart' technique used with conventional Taq. This involves adding Taq at the primer annealing temperature, after DNA denaturation in order to reduce the non-specific DNA amplification obtained. The Jump Taq polymerase binding site is masked by an antibody that is tightly bound to it preventing premature activation. This is cleaved during the first DNA denaturation at 95°C thus initiating Taq activity thereafter in a similar fashion to the 'hot start' technique.

Forty-nine μl of 'mastermix' were added to the Eppendorf tubes containing each individual's bead linked cDNA. One drop of mineral oil was added to the meniscus of each tube in order to prevent evaporation of the reagents and the Eppendorfs were transferred to the thermocycler and the annealing temperature and cycle number set. A water blank containing only the components in the 'mastermix' but no bead-linked cDNA was also prepared for each PCR. This allowed the identification of cDNA contamination in any of the components of the 'mastermix' that if unappreciated

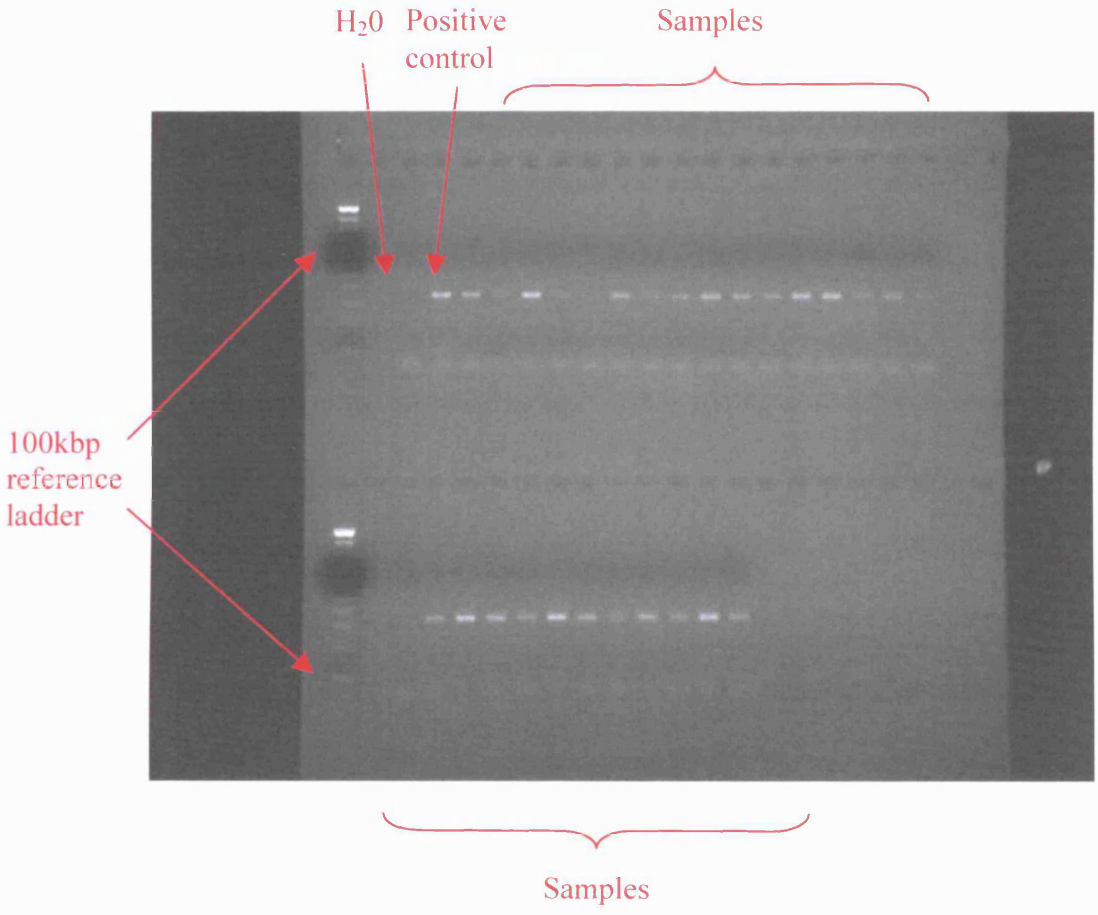
would result in false elevation of the PCR product. In addition a positive DNA control containing 49μl of 'mastermix' and 1μl of 1:100,000 concentration of the DNA species of interest was included in each PCR. This not only confirmed the efficiency of PCRs but also provided a means to correct for variability between the pre-trial and 6 month reactions when these were compared for each gene.

Following completion of each PCR, the amplified PCR product underwent agarose gel electrophoresis *(Appendix D)* for 45 minutes prior to visualisation of the bands under UV light using a computerised gel documentation system. The cDNA in each well migrates towards the positive electrode when an electric current is passed through the gel at a velocity determined by its mass. By inclusion of a reference 'base pair' ladder the size of the DNA species can be determined according to its position on the gel and thus its identity confirmed. This technique was performed prior to ELISA for each PCR in order to ensure the absence of DNA contamination and the efficiency of the PCR (Fig 4.3(i)). Subsequently, each Eppendorf containing the remaining PCR product was stored at 4°C prior to quantitation.

4.4 Principles of Enzyme Linked Immunosorbent Assay (ELISA)

The method of detecting and measuring the products of RT-PCR is important. Many studies have used densitometry to analyse the bands seen after gel electrophoresis. This has several disadvantages including variability introduced by differences in gel thickness and staining from gel to gel, as well as the use of radioisotopes. The use of 'phosphor' imaging devices can compensate for differences between gels but is highly specialized and expensive. The application and reproducibility of ELISA for the quantitation of PCR products has been previously described[407,412].

Fig 4.3(i) GAPDH cDNA viewed by Agarose gel electrophoresis



A schematic representation of the ELISA technique is shown in Fig 4.4(i). The biotinylated forward strand of each cDNA molecule is attached to the ELISA plate by covalently bonding to a biotin-avidin protein complex on the surface of the plate. A digoxygenin labelled probe specific for the DNA species of interest is then attached to each cDNA molecule and this in turn is bound by an anti-digoxygenin antibody conjugated with alkaline phosphatase. Surpluses of both 'unbound' probe and antibody are removed from each ELISA well using a thorough wash protocol after each of these steps. The addition of PNPP allows the alkaline phosphatase present to catalyse a colorimetric reaction producing one unit of yellow colour for each bound probe. As only one probe can bind to each cDNA molecule and this is the only probe remaining at this stage, the number of units of colour generated reflects the number of cDNA molecules present. This can be accurately quantified using an optical colorimeter producing results that range from zero to over four for each individual well on the ELISA plate. The relationship between the number of units of colour detected and time must be linear in order to ensure the accuracy of this technique. In order to confirm this, ELISA reactions were performed on a series of 16 samples and the expression of TGFβ-1 and Collagen III cDNA was determined at a number of timepoints and then plotted against time for each sample (Fig 4.4(ii-iii)). Gene expression increased in a reasonably linear fashion with time below ELISA values of 3.0. However at values greater than 3.0, expression tended to plateau off. Thus values greater than 3.0 were ignored when pre and post-study gene expression was analysed.

Fig 4.4(i) Schematic representation of the detection of a single cDNA molecule using ELISA

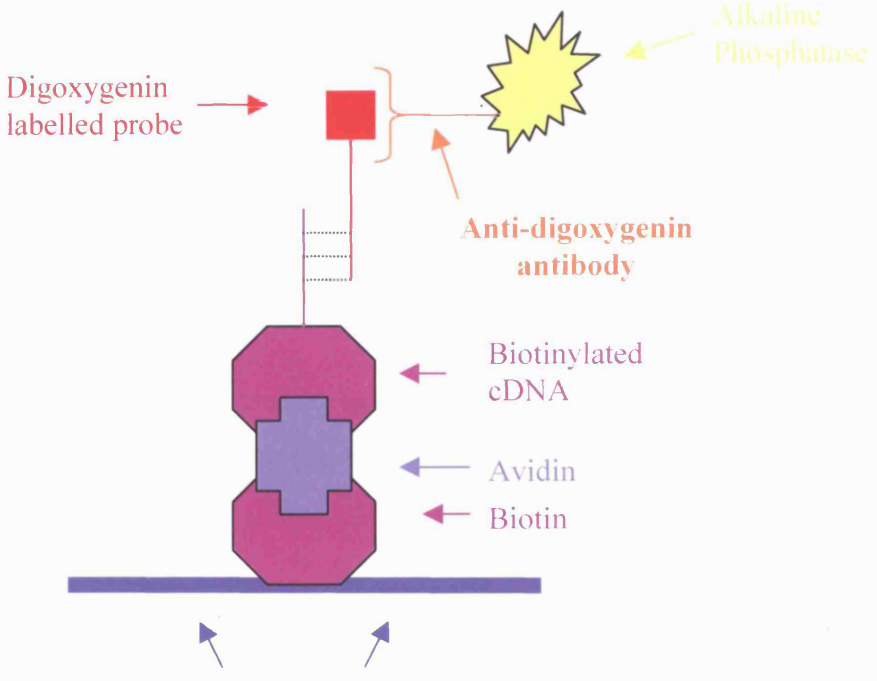


Fig 4.4(ii) TGF-β ELISA values over time for each sample

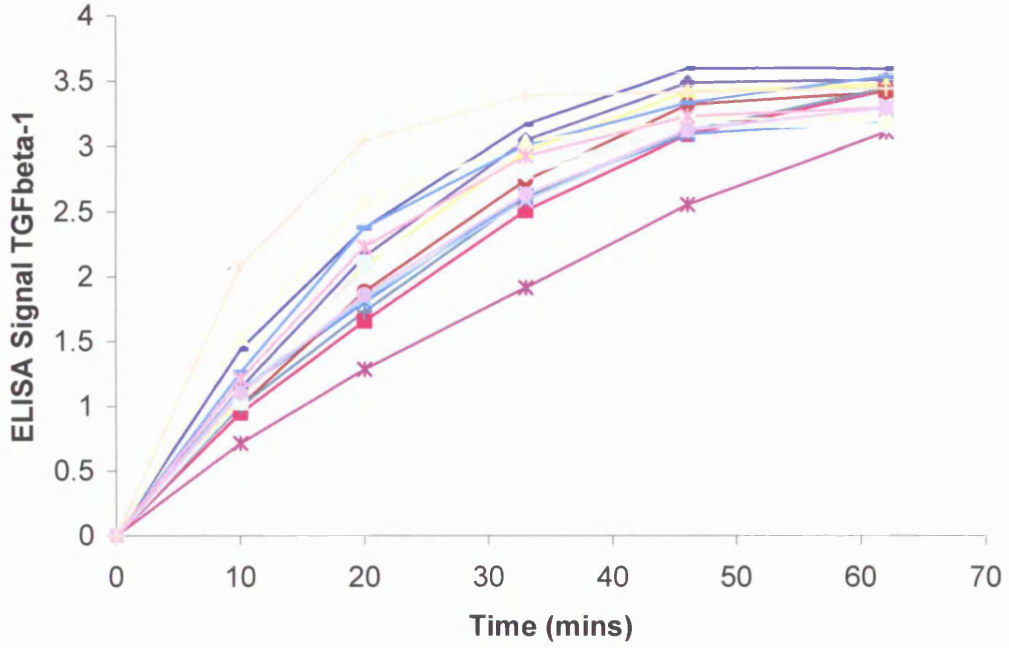
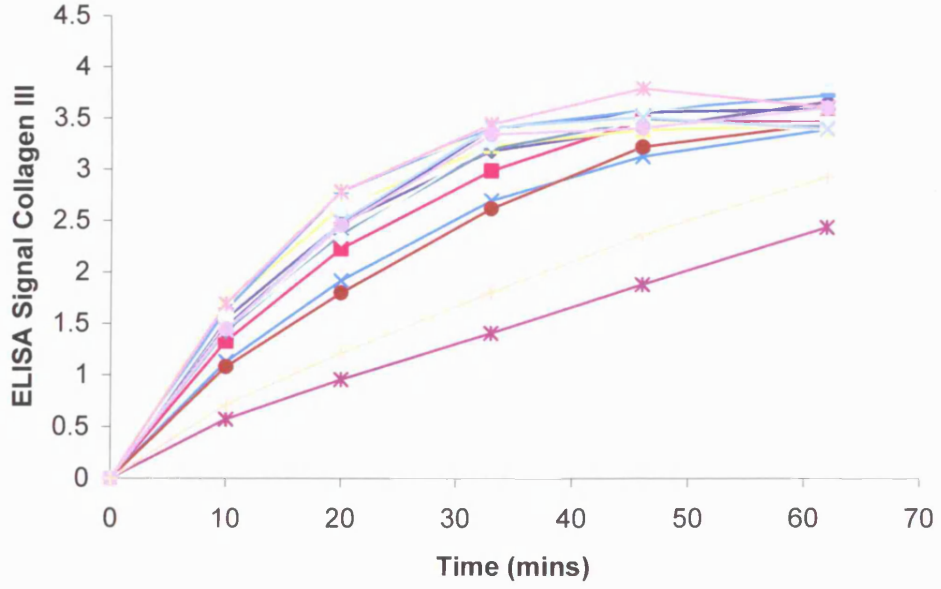


Fig 4.4(iii) Collagen III ELISA values for each sample



4.5 Assessment of PCR product using ELISA

The PCR products from all pre and post study samples for each mRNA species of interest were quantified using the same ELISA in order eliminate any variability between different ELISA assays. The probes required to detect the forward mRNA strand for each gene of interest were designed using the Wisconsin computer package from the Genetics Computer Group and oligonucleotide sequences obtained from the European Molecular Biology Laboratories database. These are shown in Table 4.5. Each probe was labelled with digoxygenin and full details of this and the ELISA reagents and techniques used are described in *Appendix E.*

In summary, each well on the streptavidin coated ELISA plate was treated with 100µl of 1% solution of BSA in PBS for 15 minutes. This was aspirated and a further 25µl 1% BSA/PBS was added to each well into which 2µl of PCR product was also dispensed. A further 75µl of BSA/PBS was added and the biotinylated forward strand cDNA were allowed to bind to the plate for 30 minutes. Unbiotinylated reverse stranded cDNA was denatured from the forward strand by the addition of 100µl of 0.25M sodium hydroxide for 10 minutes. Each well was subsequently washed three times in Amersham wash buffer leaving only forward strand cDNA bound to the ELISA plate. One hundred microlitres of digoxygenin labelled probe in 0.2pmol/100µl Rapid-Hyb buffer was then added and incubated at 42°C for 2 hours. Following this each well was re-washed with Amersham buffer before the addition of a 1:500 dilution of anti-digoxygenin antibody in 1%BSA/PBS for 30 minutes. A final wash at this time was followed by the addition of 100µl of 1mg/ml solution of PNPP in DEA that in turn required incubation at 37°C for 1-5 hours. Subsequently ELISA plates were analysed using the Labsystems Multiskan EX colorimeter that detects

colour at 405nm with a differential reading at 630nm. The results were recorded using the Genesis software package from Syngene®. Controls were included for each plate in order to confirm the quality of the ELISA (*Appendix E*).

4.6 Data and Statistical Analysis

Each sample was assessed in duplicate and the mean ELISA value used in data analysis. A number of steps were undertaken in order to allow accurate comparison between samples using the RT-PCR-ELISA technique described.

1. Intra-batch variability was limited by the use of master-mixes so that the only difference between RT-PCR reactions was the amount of mRNA / cDNA present in each sample [412]. Two large PCR batches utilising all the pre and all the post trial samples respectively were performed for each gene. A correction for inter-batch PCR variation caused by changes in the efficiency of reactions performed at different times was made in order to allow comparison between these PCRs. Each PCR performed included 1µl of 1:100,000 concentration of the DNA species of interest as a positive control. The efficiency of each PCR reaction was reflected in the ELISA value obtained for this positive control and when this was compared for pre and post trial PCR batches it allowed an appropriate correction for PCR efficiency.
2. The volume of cDNA-linked dynabeads used (1µl or 2µl) for each PCR was taken into account.
3. Non-specific probe binding was measured for each ELISA by assessing a non-specific binding control; *i.e.* an equivalent dilution of unrelated DNA

instead of the target DNA (*e.g.* β-actin instead of GAPDH) was used. The ELISA result from this control was subtracted from the ELISA value obtained from the target DNA in order to correct for non-specific probe binding.

4. GAPDH is a constitutively expressed housekeeping gene. As such its expression can provide an assessment of cellularity for each biopsy sample[407]. Results were therefore presented as a ratio to GAPDH expression (in arbitrary units), in order to apply a correction for the variation in cell numbers seen between biopsy samples.

Patients included in the study were analysed on an intention to treat basis using the SPSS v 8.0 software package. Messenger RNA expression for each gene studied from both glomeruli and interstitium at entry and 6 months follow-up was normally distributed and thus results were expressed as means (±sd). Statistical analysis between groups and within each group over the study was performed using student's t-tests and a p-value < 0.05 was considered significant, with p-values $\geq 0.05 - 0.10$ considered marginally significant. The relationship of the different mRNA species with one another, with renal function and with interstitial volume fraction of Sirius red staining was assessed using either Pearson's correlation (r_p) or Spearman's rank correlation (r_s) according to the normality of the data.

Table 4.5 Probe oligonucleotide sequences identifying each forward cDNA strand of interest

Probe	Oligonucleotide sequence (5'-3')
GAPDH	GTT GAA GTC AGA GGA GAC C
TIMP-1	GTA GTG ATG TGC AAG AGT CC
TIMP-2	TCT ATA TCC TTC TCA GGC CC
Collagen III	ACC AGA TGG ACC TAT AGC AC
TGFβ-1	CGG TGA CAT CAA AAG ATA AC
MMP-2	CTC CAG AAT TTG TCT CCA GC
MMP-9	GTT GCA GGC ATC GTC CAC CGG ACT CAA AGG
iNOS	GCG CTC GTA AGG AAA TAC AG
Tenascin	TGA TGG CTG AAT CTG TGT CC

4.7 Glomerular profibrotic gene expression

4.7a Results

Mean glomerular collagen III cDNA expression (Fig 4.7(i)) was initially similar in both groups. After 6 months there was a significant increase compared to the pretrial value in Rapa patients that did not occur in Controls. However when 6 month collagen III expression was compared between groups there was no significant difference.

Mean glomerular TIMP-1 and TIMP-2 cDNA expression (Fig 4.7(ii) & Fig 4.7(iii)) was similar in both groups at the onset of the study. There was a significant increase in TIMP-1 cDNA expression in both Control and Rapa patients over 6 months that was comparable for both groups. However only Rapa patients developed a significant increase in TIMP-2 cDNA expression over time.

Mean glomerular MMP-2 cDNA expression (Fig 4.7(iv)) was initially low but increased in an equivalent fashion in both groups over the study. MMP-9 expression was only detected in 2 pretrial and 6 post-trial samples. These numbers were too small to warrant further analysis.

Mean glomerular TGFβ cDNA expression (Fig 4.7(v)) was similar in both groups initially. This fell over time in the Control group but remained constant in patients on Rapa, although the 6 month values were not statistically different when compared. Mean iNOS cDNA expression (Fig 4.7(vi)) was once again similar in both groups initially. It increased markedly in a comparable manner in both groups over the study. Mean glomerular tenascin cDNA expression (Fig 4.7(vii)) showed no significant change over time or between groups.

4.7b Relationship between different glomerular profibrotic cDNA species and their correlation with GFR

All pretrial samples were compared in order to determine whether the expression of different genes was related and also if gene expression correlated with renal function. The relationships between different glomerular cDNA species are shown in Table 4.7(viii). Statistically significant positive correlations were found between TIMP-1 vs TIMP-2, TIMP-1 vs TGFβ, TIMP-2 vs TGFβ, TGFβ vs Collagen III, TGFβ vs MMP-2 and MMP-2 and Collagen III. No significant correlation was observed when the glomerular and interstitial cDNA expression for each gene studied was compared (data not shown).

Estimated GFR showed a significant negative correlation with a number of profibrotic genes. This included TGFβ, TIMP-1 and TIMP-2 (Fig 4.7 (ix), Table 4.7(x)). However no significant correlation was found between estimated GFR and the other genes studied (Table 4.7(x)). In addition there were no significant relationships between radio-isotope (^{51}Cr labelled EDTA) GFR and glomerular profibrotic gene expression (data not shown).

Fig 4.7(i) Glomerular collagen III cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 15 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in collagen III cDNA in Rapa patients (p=0.01) not observed in controls. However expression at 6 months was similar in both groups.

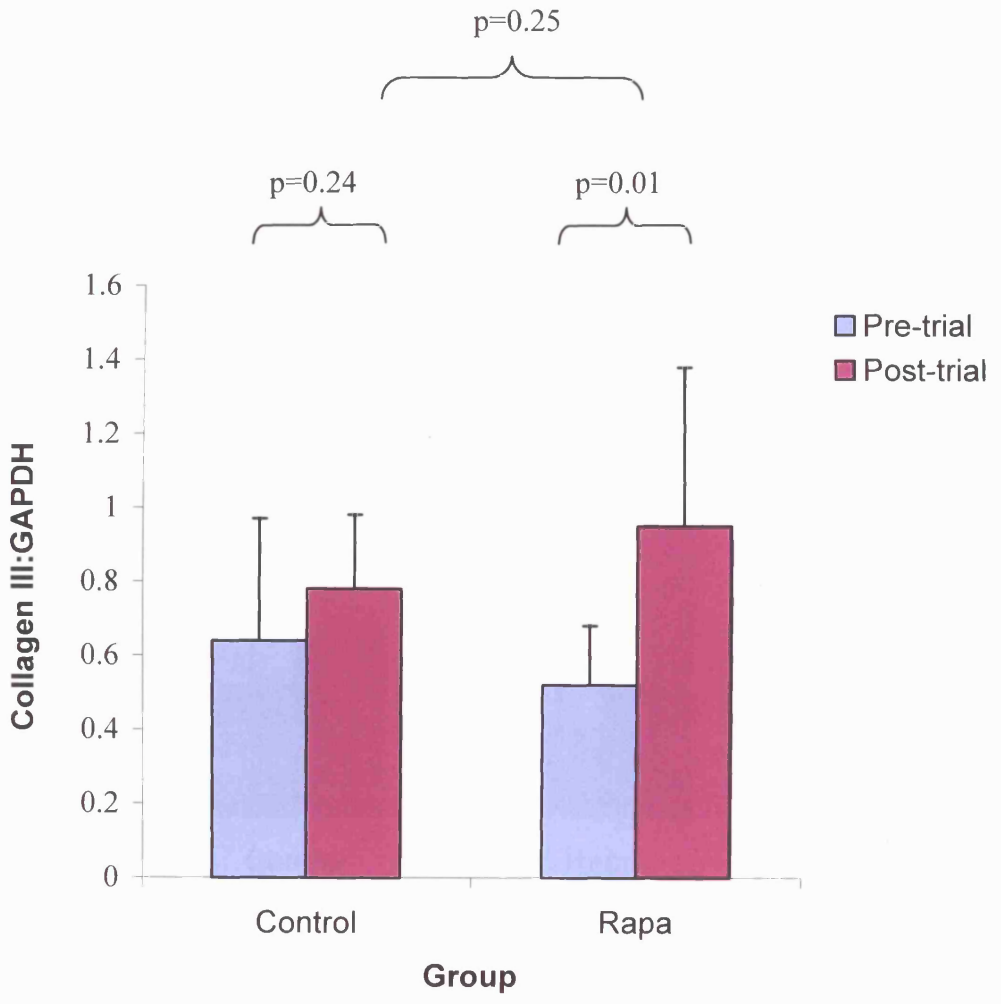


Fig 4.7(ii) Glomerular TIMP-1 cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 15 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in TIMP-1 cDNA in both control (p=0.003) and Rapa (p=0.004) groups over the study with similar levels of expression at 6 months.

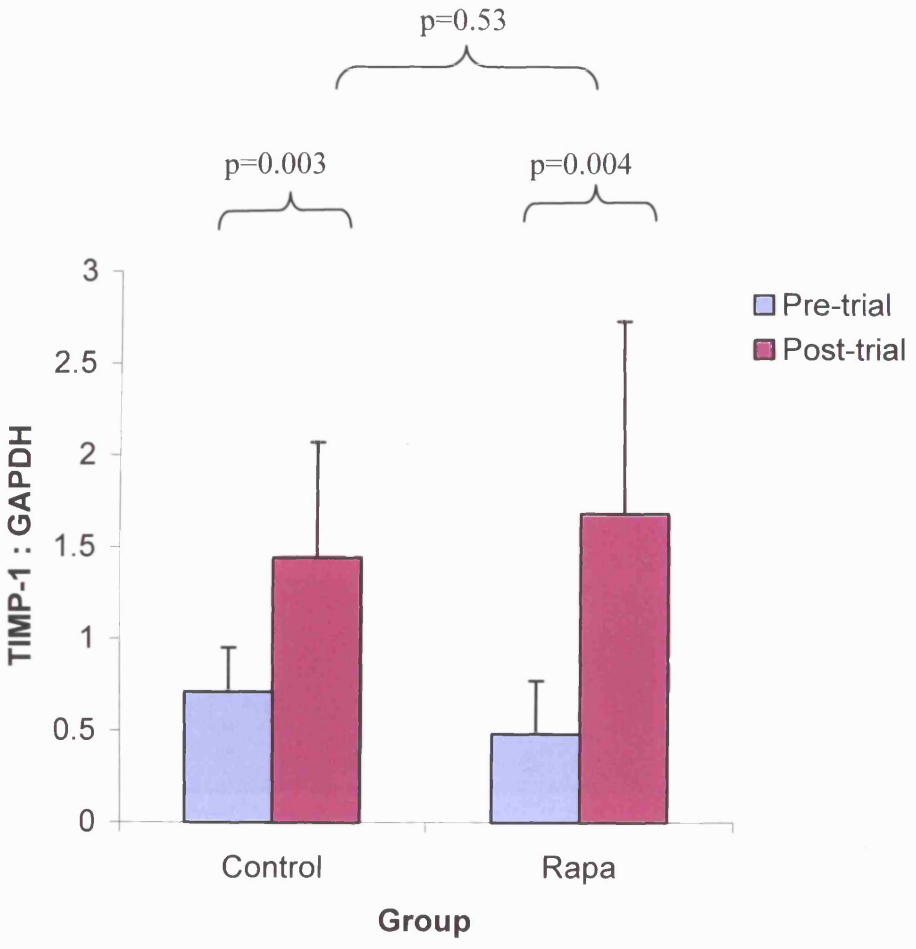


Fig 4.7(iii) Glomerular TIMP-2 cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 10 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in TIMP-2 cDNA in Rapa patients (p=0.04) not observed in controls. Expression at 6 months was similar in both groups.

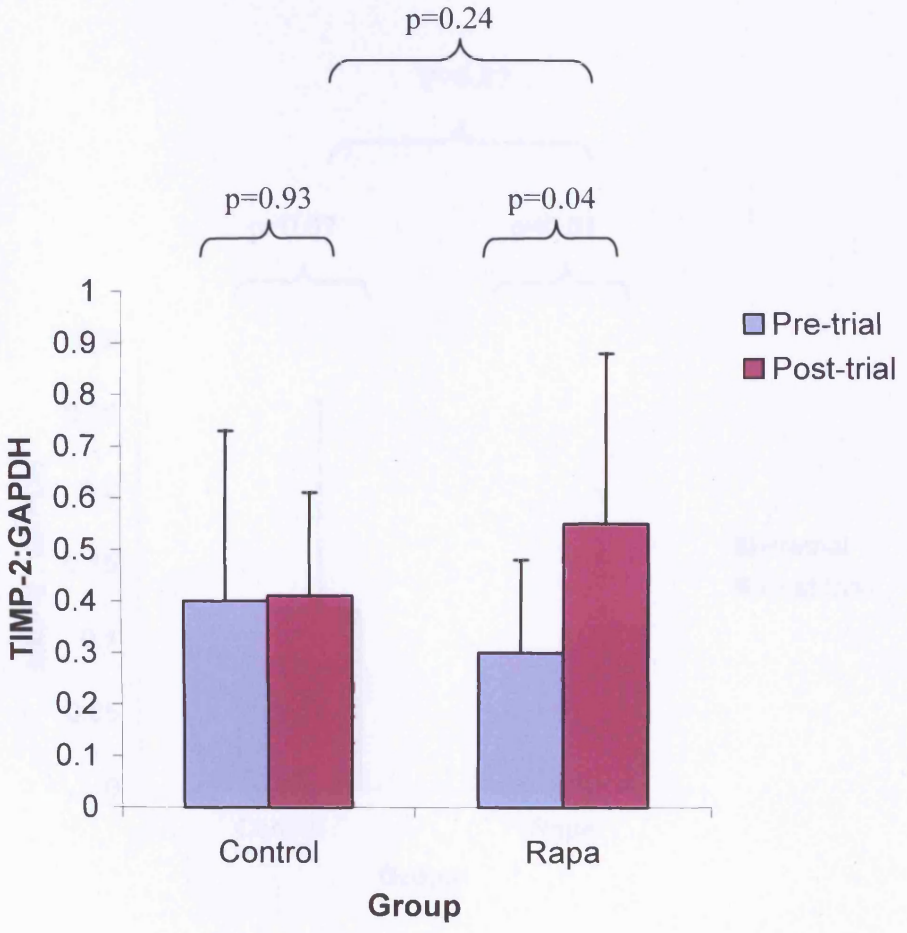


Fig 4.7(iv) Glomerular MMP-2 cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 10 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in MMP-2 cDNA in Rapa patients (p=0.01) and a similar trend (p=0.07) in controls. Expression at 6 months was similar in both groups.

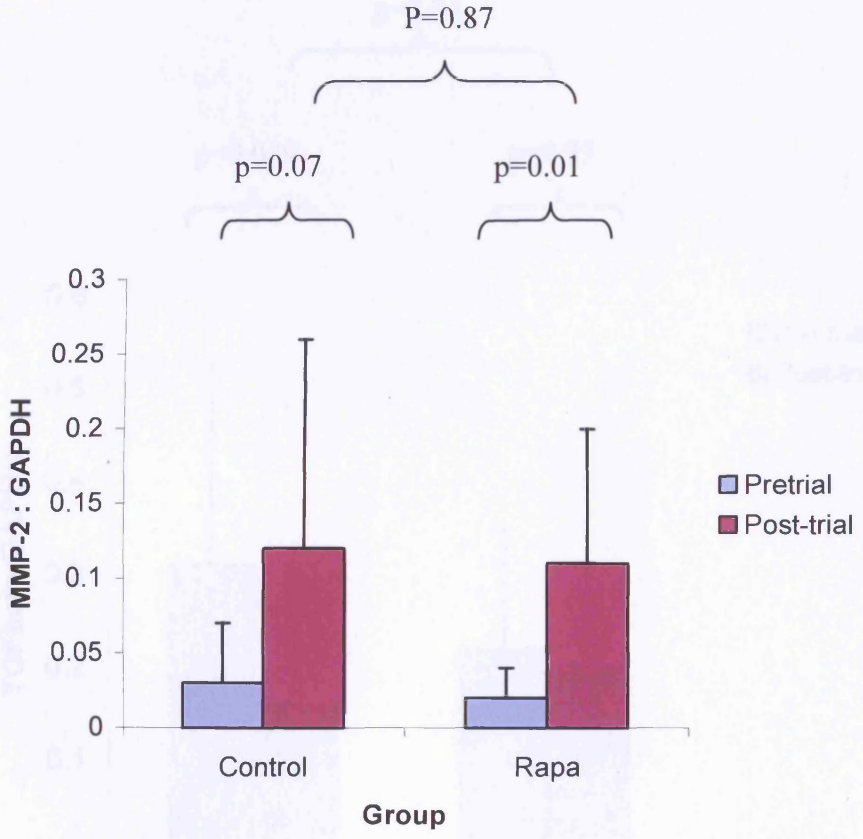


Fig 4.7(v) Glomerular TGF-β1 cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 15 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was a decrease in TGF-β1 cDNA in controls (p=0.049) not observed in Rapa patients. Expression at 6 months was similar in both groups.

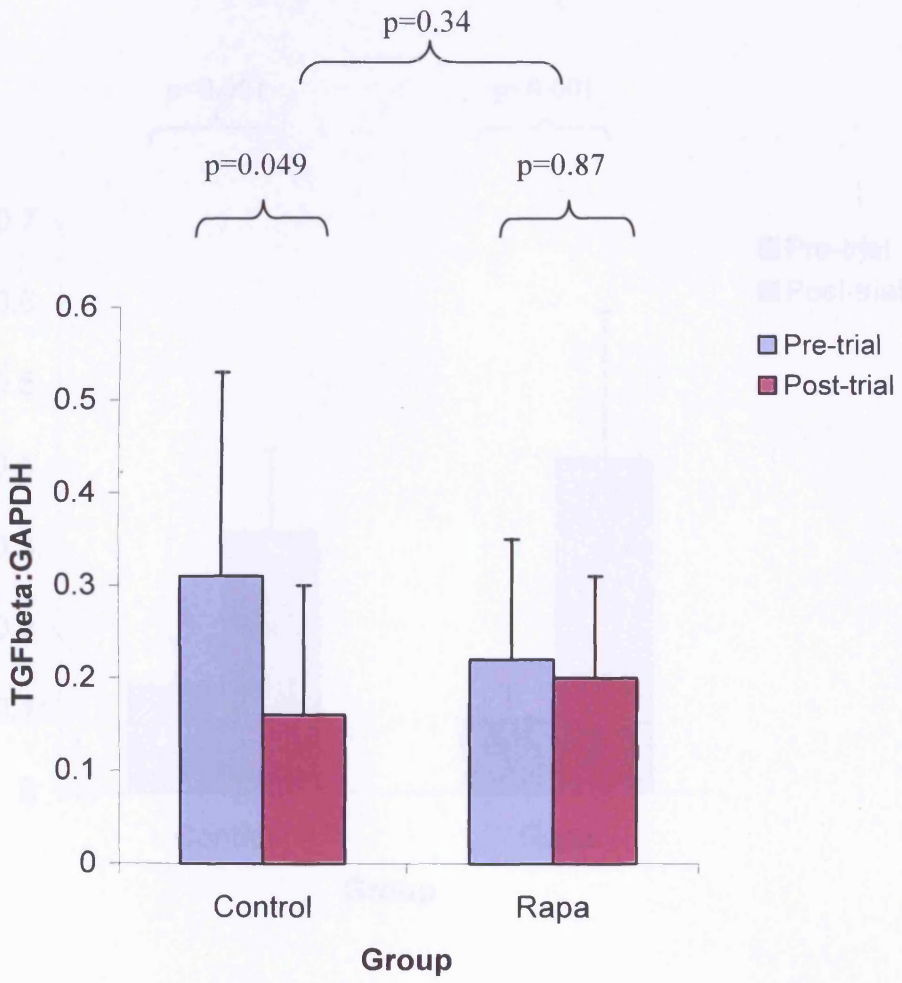


Fig 4.7(vi) Glomerular iNOS cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 15 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in iNOS cDNA in both control ($p<0.001$) and Rapa ($p<0.001$) groups over the study with similar levels of expression at 6 months.

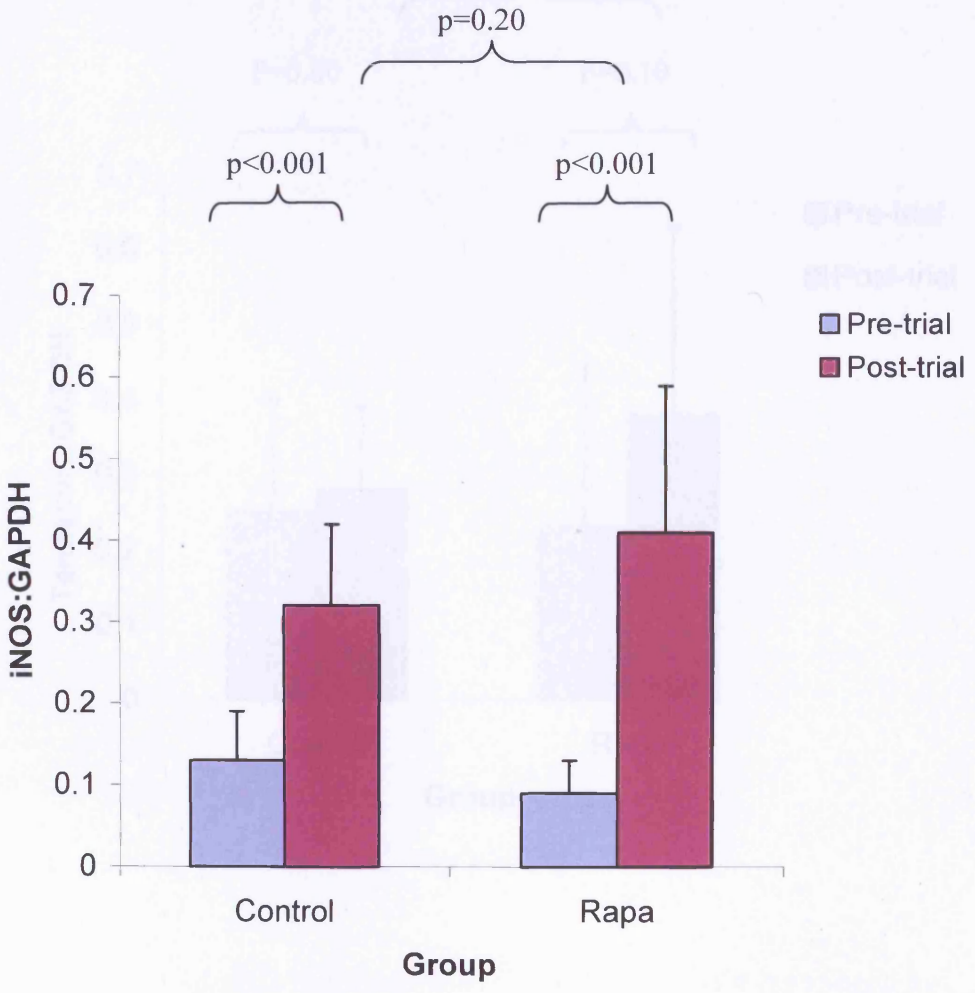


Fig 4.7(vii) Glomerular tenascin cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 15 Rapa patients. Post-trial results were obtained in 11 Control and 11 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. No significant changes in tenascin expression were noted.

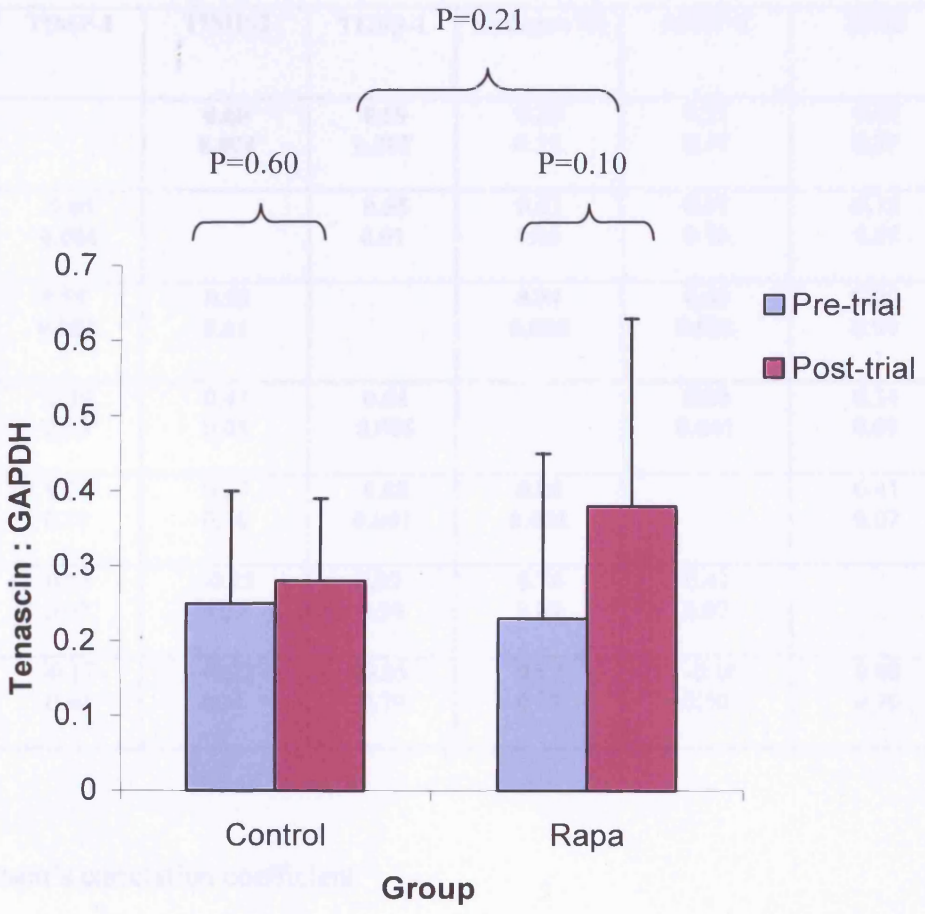


Fig 4.7(viii) Relationship between different glomerular cDNA species

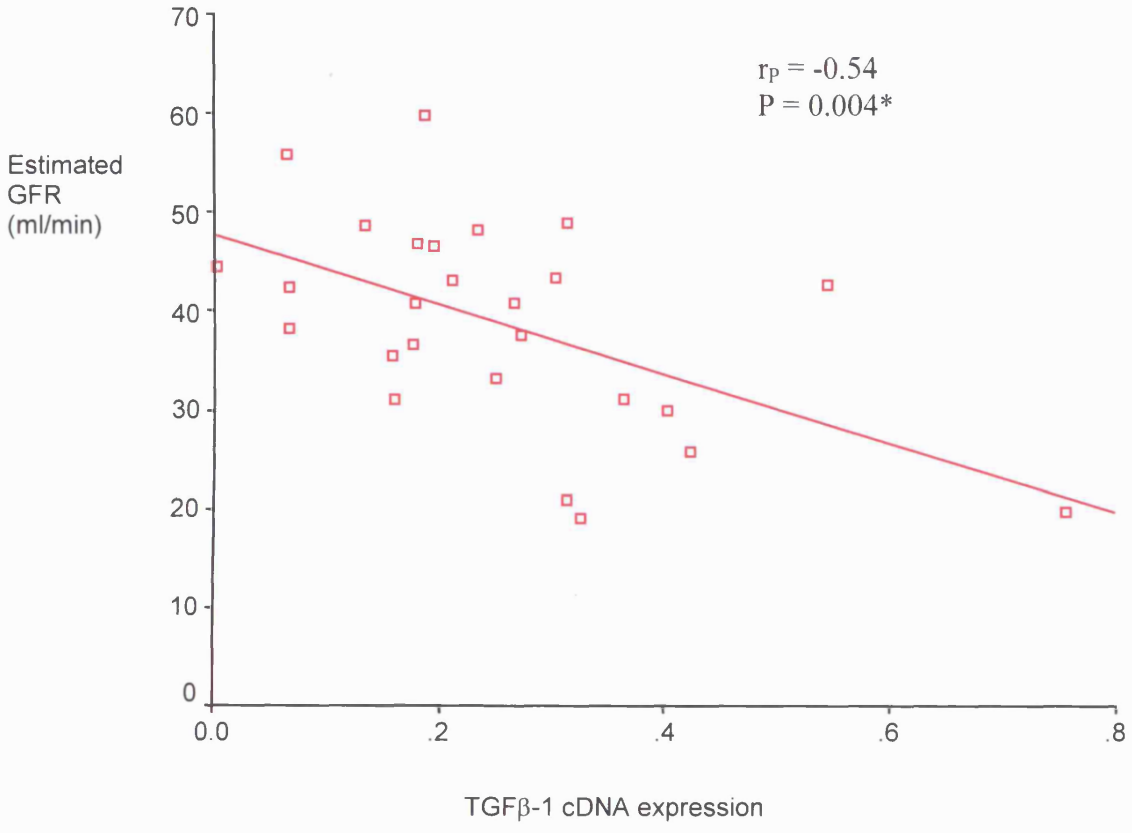
cDNA species		TIMP-1	TIMP-2	TGFβ-1	Collagen III	MMP-2	iNOS	Tenascin
TIMP-1	**- r_P**		**0.60**	**0.59**	0.29	0.31	0.01	-0.17
	- P		**0.004**	**0.002**	0.15	0.17	0.97	0.40
TIMP-2	**- r_P**	**0.60**		**0.55**	0.43	0.37	-0.13	-0.23
	- P	**0.004**		**0.01**	0.05	0.10	0.57	0.31
TGFβ-1	**- r_P**	**0.59**	**0.55**		**0.54**	**0.65**	0.00	0.05
	- P	**0.002**	**0.01**		**0.005**	**0.001**	0.99	0.79
Collagen III	**- r_P**	0.29	0.43	**0.54**		**0.80**	0.34	0.12
	- P	0.15	0.05	**0.005**		**0.001**	0.09	0.57
MMP-2	**- r_P**	0.31	0.37	**0.65**	**0.80**		0.41	-0.16
	- P	0.17	0.10	**0.001**	**0.001**		0.07	0.50
iNOS	**- r_P**	0.01	-0.13	0.00	0.34	0.41		0.08
	- P	0.97	0.57	0.99	0.09	0.07		0.70
Tenascin	**- r_P**	-0.17	-0.23	0.05	0.12	-0.16	0.08	
	- P	0.40	0.31	0.79	0.57	0.50	0.70	

r_P - Pearson's correlation coefficient

P - p-value generated via Pearson's correlation

Significant positive correlations (highlighted in bold print) were found between TIMP-1 vs TIMP-2, TIMP-1 vs TGFβ, TIMP-2 vs TGFβ, TGFβ vs Collagen III, TGFβ vs MMP-2 and MMP-2 and Collagen III.

Fig 4.7(ix) Relationship between Cockroft and Gault estimated GFR and TGFβ-1 cDNA expression



*Pearson's Correlation

4.7(x) Relationship between Cockroft and Gault estimated GFR and other glomerular cDNA species

Glomerular cDNA	'r_P value'	p-value*
TIMP-1	-0.51	0.008
TIMP-2	-0.57	0.007
Collagen III	-0.29	0.15
iNOS	0.32	0.11
MMP-2	-0.25	0.26
Tenascin	0.07	0.73

*Pearson's correlation

4.8 Interstitial profibrotic gene expression

4.8a Results

Mean interstitial collagen III cDNA expression (Fig 4.8(i)) was initially similar in both groups. After 6 months there was a significant increase compared to the pretrial value in Rapa patients that did not occur in Controls. However when 6 month collagen III expression was compared between groups there was no significant difference.

Mean interstitial iNOS cDNA expression (Fig 4.8(ii)) showed a similar pattern with a highly significant increase over time in Rapa patients that was not present in controls, but no significant difference was noted when 6 month values were compared. However neither Cyclosporin dose reduction alone or with the addition of Rapa had any significant effect on the mean interstitial TIMP-1, TIMP-2, MMP2, MMP-9, TGF-β1 and tenascin cDNA expression over the time period assessed. These results are summarised in Table 4.8(iii).

4.8b Relationship between different interstitial profibrotic cDNA species and their correlation with GFR

The relationships between different interstitial cDNA species are shown in Table 4.8(iv). Statistically significant positive correlations were found between TIMP-1 vs TIMP-2, TIMP-1 vs TGFβ, TIMP-1 vs MMP-2, TIMP-2 vs TGFβ, TIMP-2 vs MMP-2, TGFβ vs MMP-2, TGFβ vs iNOS, MMP-2 and Collagen III vs iNOS. There were no significant correlations between interstitial cDNA expression and either estimated or measured GFR (data not shown).

Fig 4.8(i) Interstitial Collagen III cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 16 Rapa patients. Post-trial results were obtained in 12 Control and 15 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in collagen III cDNA in Rapa patients (p=0.02) not observed in controls. However expression at 6 months was similar in both groups.

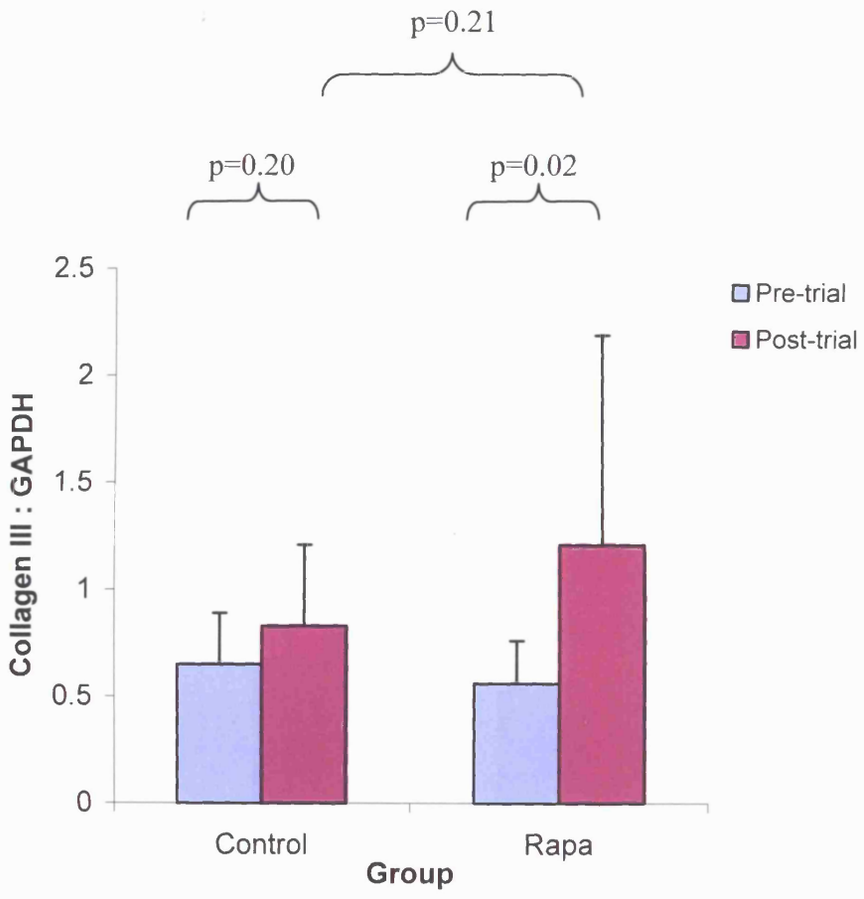


Fig 4.8(ii) Interstitial iNOS cDNA expression

Values expressed as mean ±sd (error bars). Pre-trial results were obtained in 11 Control and 16 Rapa patients. Post-trial results were obtained in 12 Control and 15 Rapa patients. Statistical analysis was performed using unpaired student's t-tests. There was an increase in iNOS cDNA in Rapa patients (p=0.006) not observed in controls. However expression at 6 months was similar in both groups.

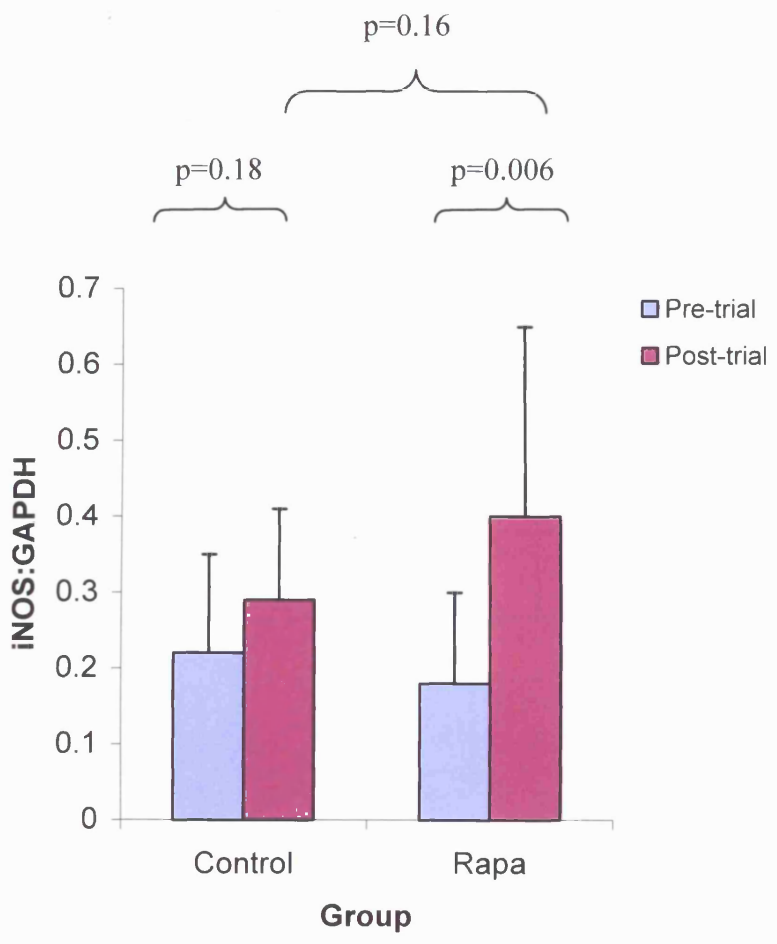


Table 4.8(iii) Interstitial TIMP-1, TIMP-2, MMP-2, MMP-9, TGF-β1 and tenascin cDNA expression

Values expressed as mean (±sd). Statistical analysis performed using unpaired student's t-tests with no significant differences between or within groups noted.

Interstitial cDNA species	Control		Rapa	
	Pre	Post	Pre	Post
TIMP-1	0.55 (0.37)	0.68 (0.39)	0.47 (0.38)	0.66 (0.43)
TIMP-2	0.30 (0.17)	0.27 (0.26)	0.18 (0.21)	0.21 (0.19)
TGF-β1	0.13 (0.12)	0.22 (0.11)	0.10 (0.12)	0.16 (0.12)
MMP-2	0.02 (0.04)	0.08 (0.07)	0.02 (0.04)	0.09 (0.11)
MMP-9	0.15 (0.16)	0.19 (0.19)	0.09 (0.14)	0.16 (0.14)
Tenascin	0.40 (0.45)	0.57 (0.42)	0.31 (0.40)	0.59 (0.65)

Fig 4.8(iv) Relationship between different interstitial cDNA species

cDNA species		TIMP-1	TIMP-2	TGFβ-1	Collagen III	MMP-2	MMP-9	iNOS
TIMP-1	**- r_P**		**0.42**	**0.39**	-0.33	**0.46**	-0.26	-0.07
	- P		**0.04**	**0.04**	0. 10	**0.02**	0.25	0.72
TIMP-2	**- r_P**	**0.42**		**0.60**	0.08	**0.61**	-0.06	-0.20
	- P	**0.04**		**0.001**	0.70	**0.001**	0.80	0.32
TGFβ-1	**- r_P**	0.39	**0.60**		0.03	**0.43**	-0.24	**0.55**
	- P	0.04	**0.001**		0.87	**0.03**	0.29	**0.003**
Collagen III	**- r_P**	-0.33	-0.08	0.03		-0.15	0.39	**0.41**
	- P	0.15	0.69	0.87		0.47	0.08	**0.03**
MMP-2	**- r_P**	**0.46**	**0.61**	**0.43**	-0.15		0.08	0.07
	- P	**0.02**	**0.001**	**0.03**	0.47		0.73	0.74
MMP-9	**- r_P**	-0.26	-0.06	-0.24	0.39	0.08		0.22
	- P	0.25	0.80	0.29	0.08	0.73		0.33
iNOS	**- r_P**	-0.07	0.20	**0.55**	**0.41**	0.07	0.22	
	- P	0.72	0.32	**0.003**	**0.03**	0.74	0.33	

r_P - Pearson's correlation coefficient

P – p-value

Significant positive correlations (highlighted in bold print) were found between TIMP-1 vs TIMP-2, TIMP-1 vs TGFβ, TIMP-1 vs MMP-2, TIMP-2 vs TGFβ, TIMP-2 vs MMP-2, TGFβ vs MMP-2, TGFβ vs iNOS, MMP-2 and Collagen III vs iNOS.

4.9 Discussion

This is the first prospective randomised human study that has attempted to look at the effect of CyA dose reduction with or without the addition of Rapa (2 mg/day) on molecular markers thought to be involved in the pathogenesis of CAN. It has been suggested in earlier work that such molecular analysis would prove to be a useful tool in the investigation of CAN and furthermore could provide sensitive measures of the efficacy of different treatment strategies[235]. The process of ECM remodelling that underpins the histological changes in CAN is complex, being determined by a balance between the rate of matrix synthesis and the rate of its degradation. This study assessed genes involved in both the synthesis (TGF-β1, Collagen III, Tenascin mRNA) and degradation (TIMP-1, TIMP-2, MMP-2, MMP-9 mRNA) of extracellular matrix. The main findings were that CyA reduction alone reduced glomerular TGF-β1 expression and stabilised collagen III expression over the study. The addition of Rapa attenuated the reduction in TGF-β1 expression and was associated with increased expression of collagen III in both glomeruli and interstitium. CyA dose reduction with or without Rapa was associated with increases in the transcription of genes that both activate and inhibit the degradation of ECM in glomeruli but not in interstitium. The expression of iNOS increased in both groups.

Expression of TGF-β1, a profibrotic cytokine thought to be upregulated in human CAN[233,234], was initially assessed. Glomerular but not interstitial expression fell over time in patients receiving a CyA dose reduction alone. CyA is a profibrotic agent that increases TGFβ expression both in vitro and in vivo[174,175,179,180] and in humans has been shown to increase the expression of TGFβ1 in patients with ESRF[181]. Thus the reduction in glomerular TGFβ expression observed was not unexpected and concurred

with the decline in plasma TGFβ1 seen by Hueso et al following CyA dose reduction with the addition of MMF in a recent study[182]. The addition of Rapa after CyA dose reduction was not associated with a reduction of TGFβ1 expression. These findings are supported by recent in vitro and in vivo studies. In vitro, it has been suggested that Rapa increased TGFβ expression[376]. Such increases may be augmented significantly when CyA and Rapa are combined[377]. In vivo, TGFβ1 levels from human renal allograft fine needle aspirates cultured for 48 hours before ELISA assessment were significantly greater in those patients taking Rapa compared to those taking a conventional triple therapy regimen of CyA, Aza and Pred[395]. Furthermore, a recent study from our unit in Leicester using rat aortic interposition allografts has suggested that the combination of Rapa and CyA significantly increased the expression of the profibrotic genes TGFβ and collagen III compared to Rapa alone[413]. It is possible that upregulation of TGFβ expression may contribute to the immunoregulatory properties of Rapa in the short term but clearly in the longer term this remains a profibrotic cytokine and persistent elevation may be linked to the progression of CAN.

Collagen III is a major constituent of the ECM in both glomeruli and interstitium. It has been observed in previous work that levels of TGFβ1mRNA have a strong positive correlation with collagen III protein identified by immunohistochemistry[235]. After CyA dose reduction alone expression of both glomerular and interstitial Collagen III mRNA remained stable. This was unsurprising as the profibrotic influence of CyA had been reduced although clearly other profibrotic stimuli were still present. Tenascin is a glycoprotein constituent of the ECM that is produced when matrix remodelling is active and may reflect the development of new fibrosis rather than old scarring[206]. There were no significant changes in its expression following

CyA dose reduction alone supporting the findings with Collagen III. However in patients receiving Rapa, Collagen III mRNA increased in both compartments over the study. Glomerular TGFβ1 mRNA expression had a significant positive correlation with the levels of Collagen III mRNA. Since mean TGFβ1 expression remained stable in patients taking Rapa this implied that other profibrotic mediators e.g. PDGF, IGF etc (not assessed in this work) could be responsible for the increases in collagen III expression observed although further studies will be required to confirm this. Somewhat surprisingly tenascin expression remained unchanged in the Rapa group, but the significance that should be attached to this is unclear.

The MMPs and their tissue inhibitors (TIMPs) are one of several enzyme systems involved in breakdown of ECM. There is enormous functional heterogeneity in this system, the full complexities of which are not completely understood. MMP and TIMP expression was identified in both glomeruli and interstitium but significant changes in expression over the study were only noted in glomeruli. Both groups demonstrated upregulation of glomerular TIMP 1 and MMP-2 mRNA with patients taking Rapa also demonstrating an increase in TIMP 2 expression over time. As both antidegradative (TIMPs) and prodegradative (MMPs) genes were upregulated simultaneously the balance of these opposing forces will determine the fate of the ECM. However one could speculate that in patients taking Rapa increased TIMP-2 expression may tip the scales more against ECM degradation than in patients receiving a CyA dose reduction alone. Interestingly significant positive correlations were noted between TGFβ1 and TIMP-1, TIMP-2 and MMP-2. in both glomeruli and interstitium, confirming the findings from a previous study[235] and suggesting that TGFβ-1 may be influence the expression of MMPs and TIMPs although the mechanism underlying this is not known.

The role of iNOS and its product the vasodilator NO in both native and transplanted kidneys is incompletely understood. In vitro CyA inhibits macrophage, vascular smooth muscle cell and renal parenchymal cell NO production[414-416]. In vivo, CyA caused a reduction in urinary NO metabolites and renal iNOS mRNA and protein[166]. In a model of chronic CyA nephrotoxicity, reductions in NO synthesis were associated with upregulations of TGFβ, collagen I, biglycan, fibronectin and PAI-1 and increased renal fibrosis, whereas increases in NO had the opposite effect suggesting that NO mediated pathways may be important contributors to chronic CyA nephrotoxicity and thus CAN[167]. Glomeruli contain a high density of iNOS producing cells and in this work glomerular iNOS expression increased after CyA dose reduction as one might expect. Unlike CyA, Rapa has been shown to preserve iNOS expression in rat aortic allografts and it has been suggested, although not proven, that this may have a beneficial effect with respect to the development of arteriosclerosis[381]. iNOS expression increased in both glomeruli and the more sparsely populated interstitium, supporting a permissive role for Rapa in this respect. However although increases in NO were thought to be beneficial it has also been proposed that upregulations of iNOS play a role in the CyA activated apoptosis of renal cells that in turn pre-empts the acellular fibrosis of late CyA nephrotoxicity[168]. Thus it is currently unclear whether increased iNOS expression has a positive or negative influence on the progression of CAN and further work is necessary to look at this further.

The technique of RT-PCR-ELISA has been described in previous work from the Leicester unit[407,412]. A number of methodological modifications were made in order to minimise intra-batch and correct for inter-batch PCR variation (see methods). The major limitation of this technique is that semi-quantitative assessment of mRNA

levels only gives an indication of current gene transcription. The assumption usually made is that these transcripts reflect the amount protein produced and that this in turn produces a biological effect. However TGFβ is secreted as an inactive precursor in a complex bound with a latency-associated peptide[222]. Various factors including changes in pH, several proteolytic enzymes and thrombospondin can release the active form from this complex[223]. Furthermore the function of active TGFβ can also be controlled by the binding of a number of 'local' proteins such as decorin that inhibit its activity[224]. Thus the significance of changes in TGFβ1 mRNA expression is difficult to interpret. as it is difficult to distinguish between biologically active and inactive cytokine. Post-translational modifications are also important in determining MMP activity and thus a similar problem applies in this respect. Therefore in order to put these molecular changes into context it is necessary to assess the actual changes that have occurred in the extracellular matrix itself (Chapter 5).

Molecular analysis was performed on 2 major compartments in the allograft. Glomerular haemodynamic factors are known to be important determinants of progressive renal disease in both native and transplanted kidneys, ultimately causing glomerulosclerosis. This can be a patchy process and thus 3 glomeruli were assessed for each patient at study entry and after 6 months follow-up. However glomeruli only account for a small fraction of the allograft interstitium and in view of this whole interstitium was also analysed. Although glomeruli and interstitium are intimately anatomically related, changes in gene expression seen in glomeruli were not always accompanied by similar changes in the interstitium. Previous studies from our laboratory have noted that RT-PCR ELISA (as described above) tends to identify changes in gene expression in glomeruli more than in interstitium (unpublished data) although the reasons for this are not clear. Glomeruli and interstitium are composed of

different cell types and have unique functional roles and this may account for the differences observed. When the relationship between gene expression in glomeruli and interstitium was assessed there were no significant correlations noted between the 2 compartments, a finding consistent with the functional integrity suggested above. However it is also worth considering a methodological explanation. Glomeruli are relatively fragile and have a high density of cells thus requiring little manipulation in order to facilitate successful mRNA extraction. However allograft interstitium, particularly in CAN, contains significant amounts of extracellular matrix with a correspondingly lower cell density that resists manual disaggregation. Despite enthusiastic manipulation it is quite possible that mRNA extraction is less efficient in this setting potentially explaining the different findings in glomeruli and interstitium.

A number of glomerular mRNA species have been noted to have statistically significant relationships with estimated GFR. TGF-β1 expression was negatively correlated with GFR. This supported the results of an immunohistochemical study in 40 patients with chronic allograft fibrosis where the 72% of patients who expressed high levels of TGFβ had a much steeper decline in renal function than those with minimal or low expression of TGFβ[236]. Both glomerular TIMP-1 and TIMP-2 also demonstrated significant negative correlations with GFR suggesting that inhibition of ECM degradation plays an important role in determining the functional outcome of allografts with CAN. Furthermore it lends additional weight to arguments supporting the use of these molecular markers as surrogate markers of long term graft outcome.

Chapter 5

Does the addition of Rapamycin after Cyclosporin dose reduction influence histomorphometric evidence of renal allograft fibrosis?

5.1 Introduction and principles of Sirius Red staining

The progression of CAN is caused by expansion of the ECM and the development of fibrosis. This process is dominated by the accumulation of collagens I, III and IV in the allograft[206]. Measurement of the hydroxyproline content per mg protein tissue has been described to quantitatively assess the proportion of collagen in tissues[417]. However it has the disadvantage that it does not visualise what it is measuring and is thus susceptible to misleading results as a result of tissue necrosis or excessive collagen deposition around large blood vessels. Thus techniques utilising sectioned material have been proposed as this allows a direct correlation of collagen content with the histological image. Immunohistochemistry is one such method and the degree of staining using this technique for collagen III in renal allograft biopsy sections at 6 months has been found to predict renal function at 2 years[26]. This technique can be quite technically difficult and in addition does not identify all collagen subtypes accumulating in the allograft. Therefore alternative approaches such as Sirius Red staining have been advocated.

Sirius Red F3BA is an azo dye that stains the major collagen subtypes. Observed under polarized light, collagen bundles reveal a strong birefringency with either yellow-red or green colours[418]. It had been suggested that these colours could be used to distinguish the collagen subtypes I, II and III or between mature and foetal collagen[419,420]. However more recently it has been pointed out that this may be misleading, as the colour of individual fibres also appeared to vary according to the orientation of the slide on the microscope[421]. There are numerous papers describing Sirius Red as a method for visualizing collagen in tissues and it has been used to estimate cardiac, liver and lung and pancreatic fibrosis[422-425] as well as for the

quantitation of the reduction of renal fibrosis by pharmacological therapy in the rat 5/6th nephrectomy model of renal disease[426]. In both a rat model of liver fibrosis and in humans with liver fibrosis in non-A non-B hepatitis, Sirius red staining correlated strongly with total liver collagen as determined by collagen hydroxyproline assay[427,428]. In human renal biopsies Sirius red staining has been quantitated using automated image analysis and has been shown to correlate with the interstitial volume fraction of the cortex as measured by a manual point counting method[429].

5.2 Materials and methods

Renal allograft biopsies were obtained from each patient before the study and after 6 months as previously discussed. PCR experiments only require small samples from each individual and the remaining tissue was embedded in paraffin and 3μm sections mounted on microscope slides. Staining with Sirius red (*Appendix F*) involved deparaffinizing and hydrating each section before removal of the fixative and exposure to 0.1% Sirius red dye for 12 hours. Excess dye was removed and then sections were dehydrated prior to re-mounting. Sections were stained in batches of 20.

5.3 Computerised histomorphometry

Computerised histomorphometry is a well-established technique for the quantification of histological changes. The system used here has been validated in previous work by Nicholson et al[26]. Sirius red stained slides were viewed under polarised light using a Zeiss photomicroscope III (x10 objective) with an attached JVC TK 120E video camera. This was linked to an Apple Macintosh 7100/80 computer that was able to directly import the image into the image analysis program, NIH-Image (Fig 5.3(i)). Sequential grey-scale images of renal cortex (Fig 5.3(ii)) were analysed by moving

along the central line of each biopsy specimen from one end of the available cortex to the other, without overlapping. Glomeruli were ignored but if renal medulla was present this was not analysed. In order to calculate the area fraction of cortex stained with Sirius red, a threshold was applied to each image at a constant level that distinguished between the stained component (rendered black) and the unstained background (rendered white). The proportion of black to white pixels in the image was then calculated as a percentage, representing the percentage volume fraction of each image stained with Sirius red. Five or more images were obtained for each section of cortex and the mean value expressed as the Interstitial Volume Fraction (IVF). All measurements were performed by one person (RNS) and sections were analysed in a blinded manner on the same day.

The reproducibility of the histomorphometric system for the assessment immunostained collagen III has been confirmed in a previous study[26]. However in order to validate the Sirius red staining technique this was repeated. Ten serial sections were obtained from one biopsy specimen and either stained together and analysed on the same day or stained on separate days but analysed on the same day. As it was possible to perform the analysis on one day without changing microscope settings analyses of the same section on different days was unnecessary.

5.4 Statistical analysis

Patients included in the study were analysed on an intention to treat basis using the SPSS v 8.0 software package. Coefficients of variation were calculated in order to ascertain the reproducibility of this technique. The IVF stained with Sirius red was normally distributed and thus results were expressed as means (±sd). Statistical

analysis between groups and within each group over the study was performed using unpaired student's t-tests and p-values < 0.05 considered significant with p-values $\geq 0.05 - 0.10$ considered marginally significant.

5.5 Results

5.5(i) Reproducibility

The results of analysing serial sections from one biopsy are shown in Fig 5.5(i). The coefficient of variation was 16.2% for Group A (10 sections stained together and analysed on the same day) and 13.6% for Group B (10 sections stained on separate days but analysed on the same day). There was no significant difference noted between mean IVFs in groups A and B.

5.5(ii) Sirius Red staining

Fig 5.5(ii) shows Sirius red stained images from patients with mild and severe interstitial fibrosis respectively. The IVF for both groups before and after the study is shown in Fig 5.5(iii). There was a trend suggesting a reduction in IVF stained with Sirius red following CyA dose reduction alone over time and also compared to Rapa patients after 6 months. However in Rapa patients Sirius red IVF remained unchanged over follow-up. Somewhat surprisingly no statistically significant correlations were noted between IVF and GFR or IVF and mRNA expression for the genes studied.

Fig 5.3(i) Renal cortex visualised using polarised light

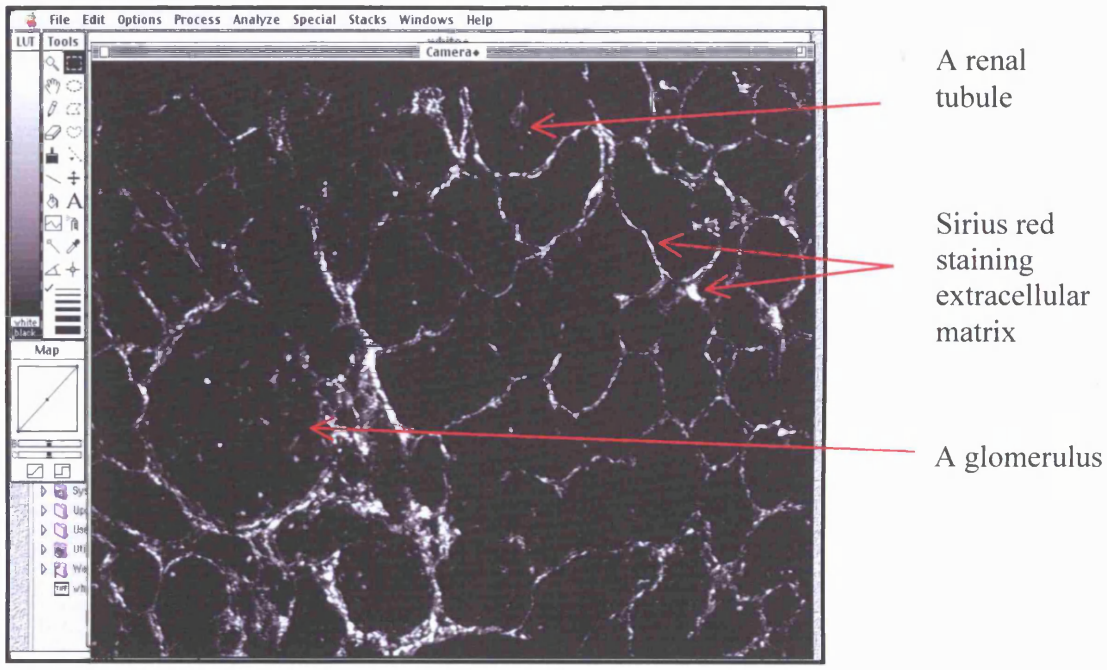


Fig 5.3(ii) Threshold grey-scale image used to assess interstitial volume fraction

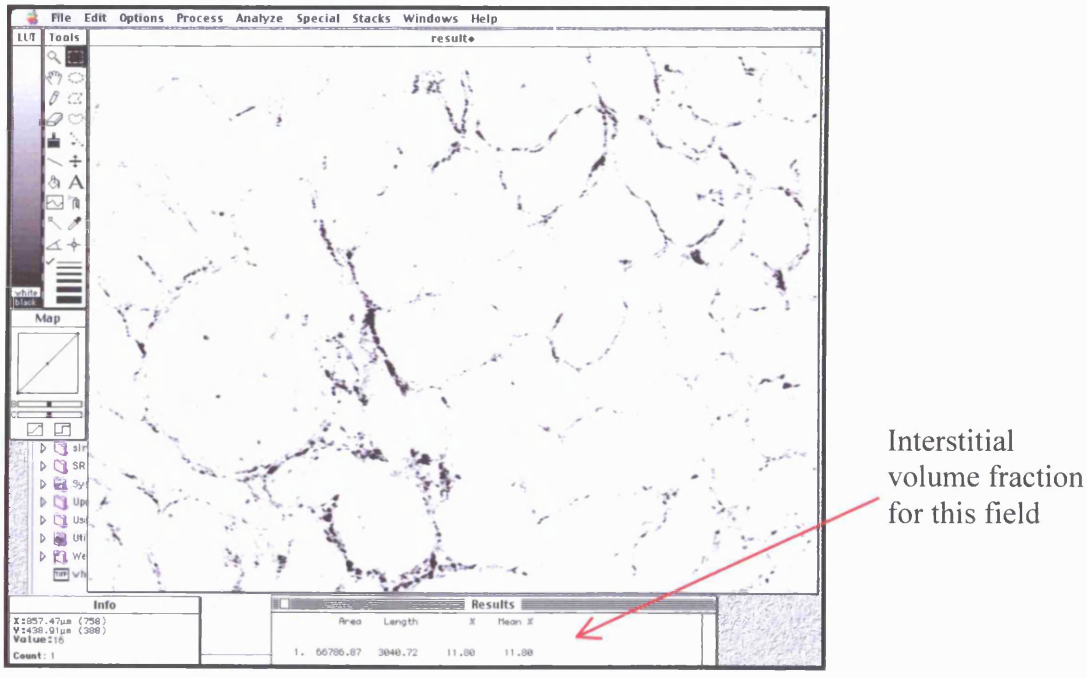
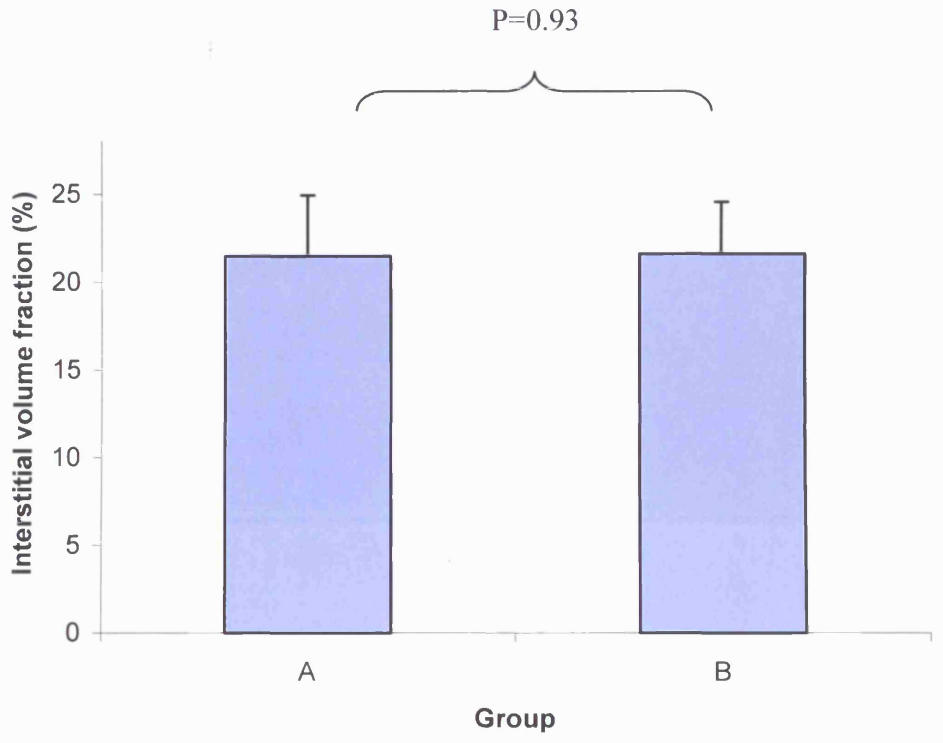


Fig 5.5(i) Sirius red staining reproducibility studies

Values expressed as mean ± sd (error bars). Statistical analysis was performed using unpaired student's t-tests. There was no significant difference between Groups A and B.

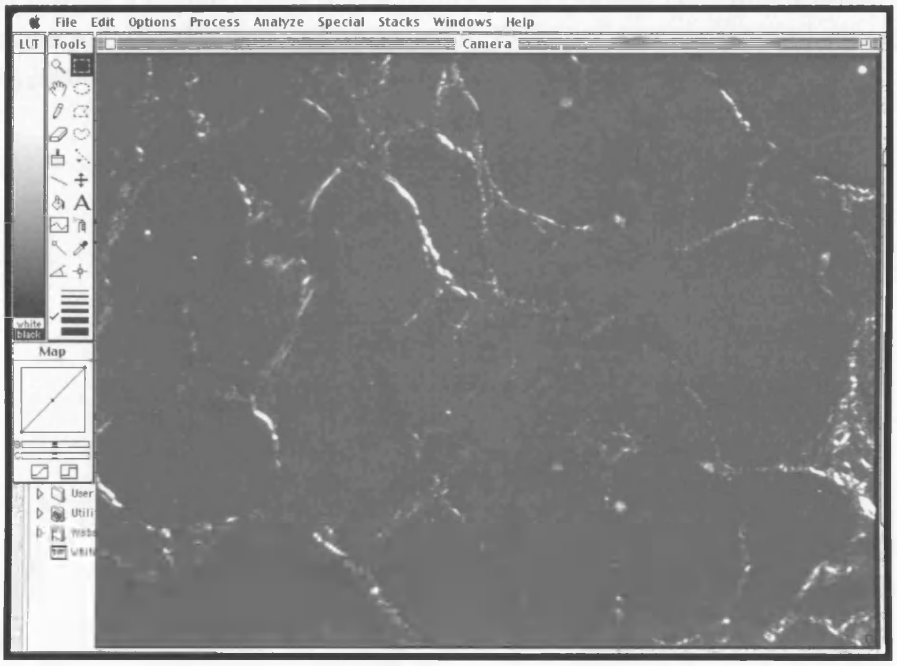


Column A - 10 sections stained together and analysed on the same day

Column B – 10 sections stained on separate days but analysed on the same day

Fig 5.5(ii) Sirius red stained allograft biopsies

Mild tubulo-interstitial fibrosis with delicate lacy collagen staining



Severe CAN with coarse, thickened and multi-laminated intertubular collagen

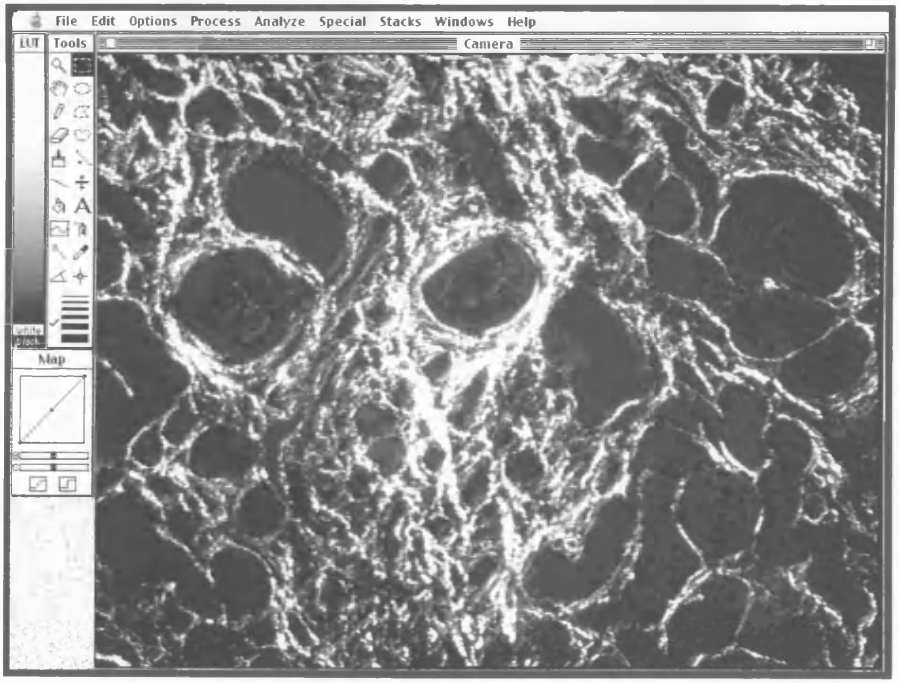
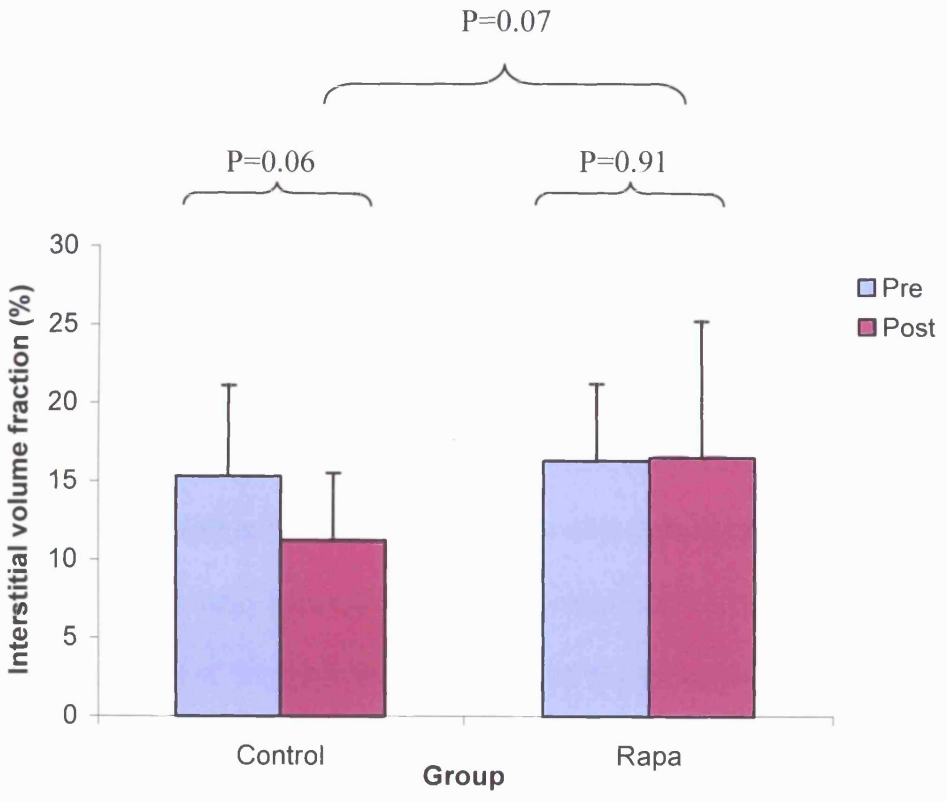


Fig 5.5(iii) Interstitial volume fraction stained with Sirius Red

Values expressed as mean ± sd (error bars). Statistical analysis was performed using unpaired student's t-tests. There was a reduction in Control IVF of marginal significance(p=0.06) that was not observed in Rapa patients. At 6 months there was a trend suggesting that control IVF was lower than Rapa IVF (p=0.07).



5.6 Discussion

Experiments assessing mRNA expression using RT-PCR-ELISA are limited because they only describe transcriptional changes in tissues. Such changes may not always represent changes in extracellular matrix protein and cytokine activity. The translation of mRNA to a protein product can be interrupted. Furthermore post-translational modifications can occur that either activate or inhibit proteins. Therefore in order to put the changes in gene expression (chapter 4) into context it was important to assess the effect of CyA dose reduction with or without the addition of Rapa on ECM deposition. Thus Sirius red staining was undertaken.

CyA dose reduction alone caused a marginally significant reduction in Sirius red stained cortex. CyA is a profibrotic agent in both native and transplanted kidneys and promotes the accumulation of ECM[233]. These findings suggest that reducing the CyA dose by 40% decreased the profibrotic stimulus associated with CyA and allowed the turnover of ECM to shift in favour of degradation rather than accumulation. Changes in the expression of a many genes are likely to be responsible for this shift. However it is worth noting that of the genes studied in chapter 4, expression of profibrotic genes fell (TGFβ) or were stabilised (Collagen III and TIMP-2) whilst expression of the degradative gene (MMP-2) was increased.

In patients taking reduced dose CyA and Rapa there was little change in Sirius red staining suggesting that the favourable changes in ECM turnover after CyA dose reduction alone had been attenuated. The molecular mechanisms underpinning either the reduction in degradation or the increase in synthesis of ECM responsible for this effect are highly complex and not fully characterised. However increases in both

TIMP-2 and Collagen III gene expression in Rapa patients, not observed in Controls (Chapter 4), imply that both mechanisms may play a role in this respect.

The failure of Sirius red IVF to correlate with gene expression was surprising, particularly as collagen III represents one of the most important of the collagen subtypes in renal interstitium[206]. A possible explanation may be the different time frames over which such changes occur. Gene expression is a highly sensitive parameter that may change significantly over relatively short periods of time in response to numerous stimuli. However although the turnover of ECM is a dynamic process, changes occur over much longer periods. Thus gene expression at 6 months follow-up reflects that occurring at that point in time whereas the Sirius red IVF reflects the changes that have occurred over the whole of the preceding 6-month period. Furthermore the relatively small number of genes studied represents only a fraction of those responsible for the changes observed. It is the complex interactions between these and the many other enzymes and cytokines present, rather than the level of individual genes expression that determines the Sirius red IVF potentially explaining why no direct correlation could be obtained.

The relationship between renal function and graft histology is one that has always been difficult to assess in CAN. Like gene expression renal function can change rapidly over short time periods. Furthermore it can be preserved despite abnormal histology until sudden deteriorations occur[14]. A number of studies have attempted to study this relationship using scoring systems for graft histology e.g. CADI (see Chapter 1) or immunohistochemistry to in an attempt to determine if these can be correlated with renal function at that time or alternatively predict renal function at some point in the future[26,27]. A similar study has documented a significant correlation

between Sirius red staining of human allografts and GFR[430]. However this and other studies were all performed on relatively new allograft recipients (between 6month and 2 years post-transplantation). No such work has been performed on older allografts, as in this study, where allograft damage is often extensive and renal function significantly impaired. It is therefore unclear whether the correlation between GFR and IVF persists in this setting. The results from this study suggest that it may not but clearly a definitive study involving large numbers of patients is necessary to confirm this.

Chapter 6

Conclusions and future developments

CAN remains the most common reason for late allograft loss in renal transplantation. However its multifactorial aetiology and complicated pathogenesis have made it a difficult problem to treat. Despite its potent immunosuppressive properties the introduction of Cyclosporin in the 1980s has had little impact on this as any potential benefit appeared to be counter-balanced in the longer term by nephrotoxicity and the development of allograft fibrosis. A number of small studies have advocated CyA dose reduction with the addition of alternative immunosuppressants in patients with CAN. Improvements in renal function and blood pressure were reported and such regimens are currently acknowledged as having the potential to retard the progression of CAN, although no large studies have been performed to confirm this[273-277].

Patients in the control arm of the current study received a 40% Cyclosporin dose reduction alone. In common with other work in the field, the number of patients studied was relatively small. However the major differences between this and other studies is that this was a prospective randomised study and that the molecular and histological changes underpinning changes in renal function were characterised. CyA dose reduction stabilised declining renal function in the majority of patients and was associated with a reduction in glomerular TGFβ expression, stabilisation of collagen III expression and increases in the expression of genes involved in the turnover of ECM. A reduction in ECM stained with Sirius red supported these findings and suggests that CyA dose reduction was associated with increased ECM degradation. Thus CyA dose reduction appears to have significant functional, molecular and histological advantages in established CAN (mean 5 years + post-transplant). The implication being that prior to dose reduction the profibrotic / nephrotoxic properties of CyA tended to outweigh the immunosuppressive benefits of this agent and that 40% CyA dose reduction had gone some way to redress this balance.

Despite the longevity of allografts studied, concerns exist that immune mediated injury may be precipitated by regimens that reduce levels of immunosuppression. However these were not substantiated as there were no episodes of AR and chronic immune damage appeared minimal on the basis of the molecular and histological results obtained. Thus CyA dose reduction appeared both safe and efficacious and the work of this and other studies should now encourage the design of a large RCT to confirm these findings. CyA dose reduction early in the life of the allograft is likely to prove more effective at limiting the speed and intensity with which CAN progresses, rather than later dose reductions when fibrosis secondary to CAN is well established. However it is in the early stages that allografts are at their most immunogenic and thus the risks of CyA dose reduction initiating episodes of AR or accelerating chronic immune injury are greatest. Clearly the timing of such intervention is important to maximise the potential benefits whilst minimising the associated risks and this needs to be addressed in future work.

Rapa is a new immunosuppressant that appears as effective as CyA at preventing AR in humans without the nephrotoxic properties of this agent[338,339]. Furthermore there is experimental evidence both from in vitro and in vivo work that Rapa may reduce the development and progression of histological changes associated with CAN (Chapter 2). However there have been few studies investigating the effect of Rapa on human CR / CAN. At the inception of this current work it was hoped that the addition of Rapa following CyA dose reduction would produce additional benefits for patients but the results do not support this. Renal function continued to deteriorate in this group. The reduction in TGFβ following CyA dose reduction alone was not seen and collagen III expression increased in both glomeruli and interstitium. Like CyA dose reduction alone expression of genes involved in ECM degradation was also enhanced

although in contrast ECM Sirius red staining in Rapa patients remained constant rather than being reduced. The combination of reduced dose CyA and Rapa appears to have attenuated the functional, molecular and histological benefits seen after CyA dose reduction alone. It has transpired in the last 12 months that the higher doses of Rapa and CyA used in denovo allograft recipients in the US multicentre trial was associated with worse renal function than controls[328]. There is good in vivo and human data to suggest that Rapa alone is not nephrotoxic at treatment doses and thus it has been suggested that the combination of Rapa and CyA may enhance the bioavailability and hence nephrotoxic effects of CyA, possibly at tissue level[328]. There is only limited experimental evidence to support this theory and work is underway to characterise the interaction between CyA and Rapa further. Although the doses of CyA and Rapa are lower in this study, allografts with impaired renal function secondary to CAN are particularly susceptible to nephrotoxicity and a similar explanation may account for the current findings.

Although the results from this thesis do not encourage the addition of Rapa in humans taking reduced dose CyA, several small studies in patients with chronic allograft damage have reported encouraging results with CyA elimination and the addition of Rapa[398,406]. This takes advantage of the immunosuppressive and antiproliferative properties of Rapa as well as avoiding the nephrotoxicity of CyA and is thus potentially beneficial with respect to CAN on two accounts. Such regimens may well provide the best hope for the attenuation of CAN over the next decade but will require a large RCT to prove their efficacy and safety before use becomes widespread.

Appendices

Appendix A Dynabead extraction of mRNA

Appendix B Reverse transcription

Appendix C Polymerase chain reaction

Appendix D Agarose gel electrophoresis

Appendix E Enzyme linked immunosorbent assay

Appendix F Sirius red staining

Appendix A: Dynabead extraction of mRNA

Sample Preparation

1. Glomerular and interstitial samples were stored at 4°C in individual eppendorf tubes containing 100µl of Lysis binding buffer until required.
2. All equipment used was made mRNA 'free' by immersion in H_2O_2 for 20 minutes before rinsing with DEPC water (1L DEPC water: 1 ml DEPC, 1 L sterile distilled water).
3. Samples were homogenised for 2 minutes using a "Pellet Pestle." and pressed at least 3 times through a 21-gauge needle attached to a 1 ml syringe.
4. 5 µl of 1 mg/ml proteinase K (1 mg proteinase K in 1 ml 0.05 M Tris pH 7.65) was added to the biopsy lysate and this was then incubated for 1 hour at 37°C.
5. The lysate was then centrifuged for 1 minute at 10,000 *g* to pellet any debris and this was then aspirated and discarded.

Dynabead Preparation

6. 10 µl Dynabeads were dispensed into a sterile Eppendorf and pelleted by exposure to a magnetic field (Dynal MPC) for 10 seconds until the supernatant was clear.
7. The supernatant was removed and the dynabead pellet was resuspended in 20 µl of Lysis/Binding Buffer (50 ml Lysis/Binding Buffer: 5 ml 1 M Tris pH 8.0, 1 ml of 0.5 M EDTA pH 8.0, 1.06 g LiCl, 5 ml of 10% LiDS/ SDS, 250 µl of 1 M DTT, 38.75 ml DEPC water).
8. Steps 6 and 7 were repeated once.
9. The Dynabead pellet was subsequently resuspended in 10 µl of Lysis/Binding Buffer.

Extraction of mRNA

9. The biopsy lysate was added to the Dynabeads taking great care avoid transfer of any small particles of debris not previously identified. The mixture was allowed to anneal for 10 minutes at room temperature.
10. The Dynabeads were then pelleted using the Dynal MPC for 10 seconds and the supernatant discarded
11. The Dynabeads were then mixed thoroughly with 50 µl Washing Buffer + LiDS (50 ml Washing Buffer + LiDS: 0.5 ml of 10% SDS, 49.5 ml Dynal

Washing buffer) before they were pelleted until the solution was clear and the supernatant discarded. This step was repeated once.

12. The Dynabeads were subsequently mixed with 50 µl Washing Buffer (50ml Washing buffer: 0.5 ml 1 M Tris pH 8.0, 0.1 ml of 0.5 M EDTA pH 8.0, 0.318 g of LiCl, 49.4 ml DEPC water) before they were pelleted once again and the supernatant discarded. This step was repeated twice.

13. The remaining Dynabead pellet was resuspended in 10µl of DEPC labelled water prior to reverse transcription.

Appendix B: Reverse transcription

1. The dynabeads suspended in 10µl DEPC water were known as the RT+ sample. 6.5 µl DEPC-treated water was pipetted into a second 0.5 ml Eppendorf and 2 µl of Dynabeads from the RT+ sample added to this. This was known as the RT- sample.

2. An RT mastermix (5 µl AMV RT 5X buffer, 2.5 µl 10 mM DEPC-treated dNTPs (2.5 ml DEPC treated dNTPs: 250 µl dATP, 250 µl dTTP, 250 µl dCTP, 250 µl dGTP, 1500 µl DEPC water), 0.6 µl 40 U/µl Rnasin (25 U), 8.4 µl DEPC-treated water per reaction) was made and 16.5 µl added to each RT- sample.

3. Into the remainder of the mastermix, 0.5 µl of 10 U/µl AMV RT (5 U) was added for every RT+ sample and 17 µl added to each RT+ sample.

4. Samples were mixed well and incubated at 42°C for 1 hour using the thermal cycler. In order to confirm the efficiency of mRNA extraction and reverse transcription, a PCR was performed and the products identified by agarose gel electrophoresis.

5. Samples were subsequently stored in 24 µl 1x TE buffer (100 ml 1xTE buffer: 0.1211g Tris, 0.0372g EDTA, 0,5ml DEPC water and adjust with 1M HCL to ph 8.0, 99.5 ml DEPC water) at 4°C.

Appendix C: Polymerase chain reaction

1. AJ 10X buffer (2ml AJ 10 X buffer: 900 µl of 1 M Tris pH 8.8, 220 µl of 1 M $(NH_4)_2SO_4$, 90 µl of 1 M $MgCl_2$, 40 µl *each* of 100 mM ultrapure dATP/dGTP/dCTP/dTTP, 110 µl of 20 mg/ml ultrapure BSA, 8.8 µl of 1 mM EDTA pH 8, 501.8 µl of sterile distilled water, 9.4 µl of neat β-mercaptoethanol) was prepared. This was mixed thoroughly and then centrifuged at 3000-4000 *g* for 1 minute to pellet precipitated BSA (an inhibitor of PCR) which was removed.

2. A PCR mastermix was prepared (5 µl AJ 10X buffer, 2 µl of 5 pmol/µl forward + reverse primer mix, 0.1µl Jumpstart Taq (10U/µl) and 41.9 µl sterile distilled water per sample).

3. The Dynabeads were pelleted using the Dynal MPC, the supernatant discarded, and then resuspend in 1 X TE buffer to the correct volume (depending on the volume of dynabeads already used).

4. 1 or 2 µl (depending on efficiency of mRNA reaction and reverse transcription) from each RT+ sample was pipetted into an eppendorf. If 2 µl was used the dynabeads were pelleted using a Dynal MPC and 1µl of supernatant removed thus doubling the concentration of dynabeads in the remaining 1µl.

5. 49 µl PCR mastermix was added to each sample.

6. PCRs to check the quality of an mRNA extraction utilised both RT- and RT+ samples. Once this was confirmed experimental PCRs used only RT+ samples.

7. A water blank containing a complete reaction mixture, but without the Dynabeads, to check for contamination of AJ buffer, primers, water and Taq was run for each experiment.

8. A positive control containing 1 µl 1:100000 pure DNA (specific for the primers used) with 49µl PCR mastermix was also run for each experiment.

9. A drop of mineral oil was placed over each sample at this stage in order to prevent evaporation. Samples were then transferred to the thermal cycler and PCR process initiated using the appropriate program (See optimal cycle number and temperature settings).

Appendix D – Agarose gel electrophoresis

1. Casting trays were prepared

2. 100 ml of 1x TAE (1 litre 50x TAE: 242 g Tris, 842.9 ml sterile distilled water, 100ml of 0.5 M EDTA, 57.1 ml glacial acetic acid. To make 1x TAE dilute 50x with sterile distilled water) was mixed with 3g of agarose in a conical flask to make a 3% solution.

3. This was microwaved at full power for 3 minutes to form a gel. 15 µl of ethidium bromide solution (20 ml ethidium bromide solution: 0.2 g ethidium bromide, 20 ml sterile distilled water) was added and the gel was poured into the casting tray to set.

4. The gel was then submerged in an "electrophoresis" tank of 1 X TAE and 15-20 µl of ethidium bromide added at the "positive electrode" end of the tank.

5. A series of Eppendorf tubes were arranged and 15µl of DNA from each PCR sample was mixed with 2 µl of 5x loading buffer (20 ml 5x loading buffer: 20 mg bromophenol blue, 8 ml sterile distilled water, 2 ml of 50 x TAE, 10 ml glycerol) in each of these.

6. 15µl of each sample + loading buffer was pipetted into individual wells in the agarose gel and a further well was filled with 15µl of 100 bp ladder.

7. A DC current of 150V was applied across the gel for 45 minutes.

8. The gel was subsequently photographed under ultraviolet illumination to confirm the success of the PCR reaction and to rule out contamination

Appendix E – Enzyme linked immunosorbent assay

End Labelling of Oligonucleotides with Digoxygenin

1. 10 µl of the 10 pmol/µl oligonucleotide probe of interest was pipetted into a 0.5 ml Eppendorf.

2. A labelling mastermix was made up by mixing 4 µl Boehringer TdT 5X buffer, 2 µl of Boehringer 25 mM $CoCl_2$, 2 µl of 1 mM Digoxigenin-11-dUTP, 1 µl of 10 mM dATP and 3 µl of 15U/µl Boehringer rTdT.

3. This was added to the probe and incubated at 37°C for 15 minutes.

4. The labelling reaction was halted with 1 µl of 0.2 M EDTA and 77 µl of ultrapure water was added to give a final labelled oligonucleotide probe concentration of 1 pmol/µl oligo.

5. This was stored in 25µl aliquots at –20°C until required.

Controls

A number of controls were used for each ELISA plate in order to ensure that the results were accurate:

a. A substrate only blank, (SOB) *i.e.* an untreated pre-streptavidinylated well
b. A PBS/BSA blank, (PBS) *i.e.* no DNA added
c. A non-specific binding control for each probe used, (NSB) *i.e.* an equivalent dilution of unrelated test DNA instead of the target DNA (*e.g.*β- actin instead of GAPDH)
d. A positive DNA control for each target DNA tested, (POS) *i.e.* an equivalent dilution of the same DNA species, previously amplified and then frozen in aliquots
e. A positive plate/enzyme control, (PLATE) *i.e.* a 1:10 dilution of digoxigenin-labelled biotinylated PCR primer

DNA Binding

1. A 1% solution of PBS/BSA was made up (0.3g BSA added to 30mls PBS).

2. 100 µl PBS/BSA was added to each well, except SOB, and left for 15 minutes at room temperature before aspiration of each plate using the Labsystems Wellwash Ascent leaving each well dry.

3. 25 µl PBS/BSA was then added to each well of the plate, except SOB and 2 µl of PCR product or DNA positive, or unrelated DNA, or diluted digoxigenin-

labelled biotinylated primer, added to the appropriate wells. A further 75 µl PBS/BSA was pipetted into each well except SOB and left for 30 minutes at room temperature.

4. 100 µl of 0.25 M NaOH was then added to each well except SOB and left. for 10 minutes at room temperature.

5. The ELISA plate was subsequently washed 3 times with Amersham wash buffer (1L Amersham wash buffer: 200 µl Tween 20, 1L PBS), using the Labsystems Wellwash Ascent, leaving each well dry.

6. The appropriate probe labelled with digoxigenin (see above) was further diluted to 0.2 pmol per 100 µl of Rapid Hyb buffer and 100µl of this solution added to each well except SOB and PLATE. 100 µl neat Rapid Hyb buffer was added to PLATE. The ELISA plate was then covered with labfilm and incubated for 2 hours at 42°C.

Immunosorption

1. The ELISA plate was washed 3 times with Amersham wash buffer, using the Labsystems Wellwash Ascent, leaving the wells dry.

2. Anti-digoxigenin-antibody was diluted 1:500 in PBS/BSA and 100 µl added to each well except SOB and BSA.

3. After 30 minutes at room temperature the ELISA plate was washed 3 times with Amersham wash buffer, using the Labsystems Wellwash Ascent, leaving the wells dry.

4. DEA (1L DEA: 105 ml (99%) diethanolamine, 800 ml distilled water adjusted to pH 9.8 using concentrated HCl and then made up to 1 litre with purified water) was prewarmed to 37°C and 5mg PNPP were dissolved per 5 ml DEA used.100 µl of this solution was added to each well and the ELISA plate incubated for 1-5 hours at 37°C.

5. The colour of each well on the ELISA plate was subsequently measured at 405 nm with a 630 nm differential using the Labsystems Multiskan EX.

Appendix F – Sirius red staining

1. Paraffin sections baked at 37°C overnight were deparaffinized and hydrated by immersing them in the following solutions:-.
    a. 2x xylene for 5 minutes
    b. 2x 100% ethanol for 2 minutes
    c. 1x 95% ethanol for 2 minutes
    d. 1x 80% ethanol for 2 minutes
    e. 1x 60% ethanol for 2 minutes

2. These sections were bathed in 0.1% Picrosirius red solution (100ml 0.1% Picrosirius red solution: 10 ml 1% Sirius red (0.1g Sirius red, 10ml sterile distilled water), 90 ml saturated picric acid (3g picric acid, 90 ml sterile distilled water), picric acid crystals added to saturate solution (pH 2.0)) for 12 hours.

3. Subsequently they were immersed in 0.01 M HCl for 2 minutes before dehydration by in 1x 70% ethanol for 45 seconds, 1x 80% ethanol for 2 minutes, 1x 95% ethanol for 2 minutes, 2x 100% ethanol for 2 minutes each and 2x xylene for 2 minutes each.

4. Sections were then mounted with DPX mountant and coverslips.

References

1. Cecka J M. The UNOS scientific renal transplant registry – Ten years of kidney transplants. Clin Transpl 1-14, 1997

2. Hariharan S, Johnson C P, Bresnahan B A, Taranto S E, McIntosh M J et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Eng J Med 342:605-12, 2000

3. Fellstrom B, Akuyrek L M, Backman U, Jonsson C, Larsson E et al. The relative influence of antigen dependent and independent factors for the development and progression of chronic rejection. Trans Proc 31: 2717-2718, 1999

4. Wolfe R A, Ashby V B, Milford E L, Ojo A O, Ettenger R E et al. Comparison of mortality in all patients on dialysis awaiting transplantation and recipient of a first cadaveric transplant. N Eng J Med 341: 1725-1730, 1999

5. Hume D, Merrill J P, Miller B F, Thorn G W. Experiences with renal homo-transplantation in the human: Report of nine cases. J Clin Invest 34: 327-382, 1955

6. Kuss R, Legrain M, Mathe G, Nedey R, Camey M. Homo-transplantation renale chez l'homme hors d'out lien de parente: Survie jusqu'au dix-septieme mois. Rev Franc Etudes Clin et Biol 7: 1048-1066, 1962

7. Goodwin W E, Kaufman J J, Mims M M, Turner R D, Glassock R et al. Human renal transplantation I. Clinical experience with six cases of renal homotransplantations. J Urol 89: 13-24, 1963

8. Porter K A, Thompson W B, Owen K, Kenyon J R, Mowbray J F et al. Obliterative vascular changes in four human kidney transplants. BMJ 2: 639-45, 1963

9. Hamburger J, Crosnier J, Dormant J. Observations in patients with a well tolerated homo-transplanted kidney: Possibility of a new secondary disease. Ann N Y Acad Sci 120: 558-77, 1964

10. Najarian J S, Foker J E. Interpretations of transplanted kidney morphology. Trans Proc 1: 1022-31, 1969

11. Paul L C. Chronic allograft nephropathy: An update. Kid Int 56: 783-793, 1999

12. Solez K M, Axelsen R A, Benediktsson H, Burdick J F, Cohen A H et al. International standardisation of criteria for the diagnosis of renal allograft rejection: The Banff working classification of kidney transplant pathology. Kid Int 44: 411-22, 1993

13. Seron D, Moreso F, Ramon J M, Hueso M, Condom E et al. Protocol renal allograft biopsies and the design of clinical trials to prevent or treat chronic allograft nephropathy. Transplantation 69: 1849-55, 2000

14. Isoniemi H M, Krogerus L, Von Willebrand E, Taskinen E, Ahonen J et al. Histopathological findings in well functioning long-term renal allografts. Kidney Int 41:151-160, 1992

15. Gouldsebrough D R, Axelsen R A. Arterial endothelialitis in chronic renal allograft rejection. A histopathological and immunohistochemical study. Neph Dial Transplant 9: 35-40, 1994

16. Hayry P, Mennander A, Raisanen-Sokolowski A, Ustinov J, Lemstrom K et al. Pathophysiology of vascular wall changes in chronic allograft rejection. Transplant Rev 7: 1-20, 1993

17. Monga G, Mazzucco G, Novara R, Real L. Intertubular capillary changes in kidney allografts. An ultra-structural study in patients with transplant glomerulopathy. Ultrastruct Pathol 14: 201-209, 1990

18. Monga G, Mazzucco G, Messina M, Motta M, Quarenta S et al. Intertubular capillary changes in kidney allografts. A morphological investigation on 61 renal specimens. Modern Path 5: 125-130, 1992

19. Morozumi K, Oikawa T, Fukuda M, Sugito K, Takeuchi O et al. Diagnosis of chronic rejection using peritubular and glomerular capillary lesions. Trans Proc 28: 508-511, 1996

20. Olsen T S. Pathology of allograft rejection. In Burdick J F, Racusen L C, Solez K (eds): Kidney Transplant Rejection, ed2, New York, Dekker, 1992, pp 333-357

21. Solez K. International standardisation for criteria for the histological diagnosis of chronic rejection in renal allografts. Clin Transplant 8: 345-350, 1994

22. Habib R, Zurowska A, Hinglais N, Gubler M C, Antignac C et al. A specific glomerular lesion of the allograft: Allograft glomerulopathy. Kid Int 44 (Supp 42): S104-S111, 1993

23. Habib R, Broyer M. Clinical significance of allograft glomerulopathy. Kid Int 44 (Supp 43): S95-98, 1993

24. Strutz F. Novel aspects of renal fibrogenesis. Neph Dial & Transplant 10: 1526-1532, 1997

25. Racusen L C, Solez K, Colvin R B, Bonsib S M, Castro M C. et al. The Banff 97 working classification of renal allograft pathology. Kid Int 55: 713-723, 1999

26. Nicholson M L, Bailey E, Williams S, Harris K P G, Furness P N: Computerized histomorphometric analysis of protocol renal transplant biopsy specimens for surrogate markers of chronic rejection. Transplantation. 68: 236-41, 1999

27. Isoniemi H. Taskinen E. Hayry P. Histological chronic allograft damage index accurately predicts chronic renal allograft rejection. Transplantation 58:1195-8, 1994

28. Kasiske B L, Heim-Duthoy K L, Tortorice K L, Rao K V. The variable nature of chronic declines in renal allograft function. Transplantation 51: 330-334, 1991

29. Massy Z A, Guijarro C, Wiederkehr M R, Ma J Z, Kasiske B L. Chronic renal allograft rejection: Immunological and non-immunological risk factors. Kid Int 49:518-524, 1996

30. First M R, Vaidya P N, Maryniak R K, Weiss M A, Munda R et al. Proteinuria following transplantation. Transplantation 38: 607-612, 1984

31. Raine A E G. Does antihypertensive failure modify chronic allograft failure? Kid Int 48: S107-S111, 1995

32. Modena F M, Hostetter T H, Salahudeen A K, Najarian J S, Matas A J et al. Progression of kidney disease in chronic renal allograft rejection. Transplantation 52: 239-244, 1991

33. Kasiske B L, Kalil R S N, Lee H S, Rao K V. Histopathological findings associated with a chronic progressive decline in renal allograft function. Kid Int 40: 514-524, 1991

34. Foster M C, Wenham P W, Rowe P A, Burden R P, Morgan A G et al. The late results of renal transplantation and the importance of chronic rejection as a cause of chronic allograft loss. Ann R C S Eng 1: 44-47, 1989

35. Paul L C, Hayry P, Foegh M, Dennis M J, Mihatsch M J et al. Diagnostic criteria for the chronic rejection /accelerated graft atherosclerosis in heart and kidney transplants. Joint proposal from the Fourth Alexis Carrel conference on chronic rejection and accelerated arteriosclerosis in transplanted organs. Trans Proc 25: 2022-2023, 1993

36. Cecka J M. Outcome statistics of renal transplants with an emphasis on long term survival. Clin Transpl 8: 324-327, 1994

37. Burke B A, Chavers B M, Gillingham K J, Kashtan C E, Manivel C E et al. Chronic renal allograft rejection in the first 6 months post-transplant. Transplantation 60: 1413-1417, 1995

38. Kon S, Templar J, Dodd S M, Rudge C J, Raftery. Diagnostic contribution of renal allograft biopsies at various time intervals after transplantation. Transplantation 63: 547-550, 1997

39. Valente J F, Hariharan S, Peddi V R, Schroeder T J, Ogle C K et al. Causes of renal allograft loss in black versus white transplant recipients in the cyclosporin era. Clin Transplant 11: 231-6, 1997

40. Kim S I, Kim M S, Kim Y S, Jeong H J, Park K. Biopsy proven chronic rejection in living donor kidney transplantation: Risk factors and graft survival. Trans Proc 29: 100, 1997

41. Matas A J, Humar A, Payne W D, Gillingham K J, Dunn D L et al. Decreased acute rejection in kidney transplant recipients is associated with decreased chronic rejection. Ann Surg 230: 493-500, 1999

42. Pallardo L M, Sanchez J, Puig N, Sanchez P, Beneyto I et al. Chronic rejection in 500 kidney transplant patients treated with Cyclosporin: Incidence and risk factors. Trans Proc 27: 2215-2216, 1995

43. Vanrenterghem Y, Roels L, Lerut T, Waer M, Gruwez J, M et al. Long term prognosis after cadaveric kidney transplantation. Trans Proc 19: 3702-3704, 1987

44. Rao K V, Anderson R C, Lacombe M J. Experience of renal transplantation in the second and third decades at the Hennepin County medical centre. Clinical Transplants: Eds Terasaki P I, UCLA tissue typing laboratory, Los Angeles, USA. pp233-239

45. Legendre C, Thervet E, Skhiri H, Mamzer-Bruneel M F, Cantarovich F et al.. Histological features of chronic allograft nephropathy revealed by protocol biopsies in kidney transplant recipients. Transplantation 65: 1506-9, 1998

46. Solez K, Vincenti F, Filo R. Histopathological findings from 2 year protocol biopsies from a US multicentre kidney transplant trial comparing tacrolimus versus cyclosporin. Transplantation 66: 1736-1740, 1998

47. Opelz G. for the Collaborative Transplant study. Strength of HLA-A, HLA-B, and HLA-DR mismatches in relation to short and long-term kidney graft survival. Transpl Int 5(Supp 1): S621-S624, 1992

48. Opelz G, for the Collaborative Transplant study. Factors influencing long term graft loss. Trans Proc 32: 647-649, 2000

49. Matas A J, Gillingham K, Humar A, Payne W, Kandaswamy R et al. The impact (?) of HLA-ABDR match on chronic rejection. Abstract 670: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

50. Matas A J. Chronic rejection--definition and correlates. Clinical Transplantation. 8: 162-7, 1994

51. Citterio F, Torricelli P, Serino F, Foco M, Pozzetto U et al. Low exposure to Cyclosporin is a risk factor for the occurrence of chronic rejection after kidney transplantation. Trans Proc 30: 1688-1690, 1998

52. Guyot C, Nguyen J, Cochat P, Foulard M. Bouissou F et al. Risk factors for chronic rejection in paediatric renal allograft recipients. Paed Nephrol 10: 723-7, 1996

53. Sumethkul V, Jirasiritham, Chiewsilp P. Optimum maintenance dosage of Cyclosporin and the impact on the occurrence of chronic rejection: An extensive multivariate analysis. Trans Proc 27: 844-845, 1995

54. Kahan B D, Welsh M, Schoenberg L, Rutzky L P, Katz S M et al. Variable oral absorption of Cyclosporin: A biopharmaceutical risk factor for chronic renal allograft rejection. Transplantation 62: 599-606, 1996

55. Feucht H E, Opelz G. The humeral immune response towards HLA class II determinants in renal transplantation. Kid Int 50: 1464-1475, 1996

56. Davenport A, Youmie M E, Parson J E M, Klouda P T. Development of cytotoxic antibodies following renal allograft transplantation is associated with reduced graft survival due to chronic vascular rejection. Neph Dial Transplant 9: 1315-1319, 1994

57. Cecka J. Early rejection - determining the fate of renal transplants. Trans Proc 23: 1263, 1991

58. Gulinaker A, Macdonald A, Sungurtekin U, Belitsky P. The incidence and impact of early rejection episodes on graft outcome in recipients of first cadaver kidney transplants. Transplantation 53: 323-328, 1992

59. Lindholm A, Ohlman S, Albrechtsen D, Tufveson G, Persson H et al. The impact of acute rejection episodes on long term graft function and outcome in 1347 primary renal transplants. Transplantation 56: 307-315, 1993

60. Matas A J, Gillingham K J, Payne W D, Najarian N S. The impact of acute rejection episodes on long-term renal allograft survival (t1/2). Transplantation 57: 857-859, 1994

61. Van Saase J, Van der Woude F, Thoroggod J, Hollander A A, van Es LA et al. The relationship between acute vascular and interstitial renal allograft rejection and subsequent chronic rejection. Transplantation 59: 1280-1285, 1995

62. Chavers B, Mauer M, Gillingham K, Matas A. Histology of acute rejection impacts renal allograft survival in patients with a single rejection episode. J Am Soc Nephrol 6: 1076, 1995

63. Gulanikar A C, MacDonald A S, Sungurtekin U, Belitsky P. The incidence and impact of early rejection episodes on graft outcome in recipients of first cadaver kidney transplants. Transplantation. 53: 323-8, 1992

64. Alexopoulos E, Leontsini M, Papadimitriou M. Macrophages and HLA-DR (+) cells in acutely rejecting kidney transplants predict subsequent graft survival, even after reversal of the acute rejection episode. Nephrologie 4: 113-117, 1998

65. Basadonna G P, Matas A J, Gillingham K J, Payne W D, Dunn D L et al. Early versus late acute renal allograft rejection: Impact on chronic rejection. Transplantation 55: 993-995, 1993

66. Isoneimi H, Kyllonen L, Eklund B, Hockerstedt K, Salmela K et al. Acute rejection under triple immunosuppressive therapy does not increase the risk of late first cadaveric allograft loss. Trans Proc 27: 875-877, 1995

67. Massy Z A, Guijarro C, Ma J Z, Wiederkehr M, Kasiske B L. Not all acute rejection leads to graft loss from chronic rejection after renal transplantation. Abstract: 13th International Congress of Nephrology, Madrid, 1995

68. Vereerstraeten P. Abramowicz D. de Pauw L. Kinnaert P. Absence of deleterious effect on long-term kidney graft survival of rejection episodes with complete functional recovery. Transplantation 63: 1739-43, 1997

69. Cosio F G. Pelletier R P. Falkenhain M E. Henry M L. Elkhammas E A et al. Impact of acute rejection and early allograft function on renal allograft survival. Transplantation 63: 1611-5, 1997

70. Opelz G. Critical evaluation of the association of acute with chronic graft rejection in kidney and heart transplant recipients. The Collaborative Transplant Study. Trans Proc 29: 73-6, 1997

71. Flechner SM. Modlin CS. Serrano DP. Goldfarb DA. Papajcik D et al. Determinants of chronic renal allograft rejection in cyclosporin treated recipients. Transplantation. 62: 1235-41, 1996

72. Mazzucchi E, Lucon A M, Nahas W C, Neto E D, Saldanha L B et al. Histological outcome of acute cellular rejection in kidney transplant recipients after treatment with methyl-prednisolone. Transplantation 67: 430-4, 1999

73. Rush D N, Henry S F, Jeffery J R, Schroeder T J, Gough J. Histological findings in early routine biopsies of stable renal transplant recipients. Transplantation 57: 208-11, 1994

74. Rush D N, Karpinski M E, Nickerson P, Dancea S, Birk P et al. Does subclinical rejection contribute to chronic rejection in renal transplant patients? Clin Transplantation 13: 441-6, 1999

75. Rush D N, Nickerson P J, Gough J, McKenna R, Grimm P et al. Beneficial effects of treatment of early sub-clinical rejection: A randomised study. J Am Soc Nephrol 9: 2129-34, 1998

76. Hayry P. Pathophysiology of chronic rejection. Trans Proc 28 (Supp1): S7-S10, 1996

77. Olsen T S. Pathology of allograft rejection. In Burdick J F, Racusen L C, Solez K (eds): Kidney Transplant Rejection, ed2, New York, Dekker, pp 333-357, 1992

78. Nadasdy T, Krenacs T, Kalmar K N, Csajbok E, Bouda K et al. Importance of plasma cells in the infiltrate of renal allografts. An immunohistochemical study. Pathol Res Pract 187: 178, 1991

79. Hayry P, Isoniemi H, Yilmaz S, Mennander A, Lemstrom K et al. Chronic allograft rejection. Immun Rev 134: 33-81, 1993

80. Brenner B M, Cohen R A, Milford E L. In renal transplantation one size may not fit all. J Am Soc Nephrol 3: 162-169, 1992

81. Dunnill M S, Halley W. Some observations on the quantitative anatomy of the kidney. J Pathol 69: 467-83, 1973

82. Nyengaard J R, Bendtsen T F. Glomerular number and size in relation to age, kidney weight and body surface in normal man. Anat Rec 232: 194-201, 1992

83. Rao K V, Kasiske B L, Odlund M D, Ney A L, Anderson R C. Influence of cadaveric donor age on post-transplant renal function and graft outcome. Transplantation 49: 91-95, 1990

84. Kuo P C, Johnson L B, Schweitzer E J, Alfrey E J, Waskerwitz J et al. Utilization of the older donor for renal transplantation. Am J Surg 172: 551-5, 1996

85. Neugarten J, Silbiger S. The impact of gender on renal transplantation. Transplantation 58: 1145-1152, 1994

86. Mackenzie H S, Azuma H, Helmut G R, Tilney N L, Brenner B M. Renal mass as a determinant of late allograft outcome: Insights from experimental studies in rats. Kid Int 48 (Supp 52): S38-S42, 1995

87. Land W, Messmer K. The impact of ischaemia-reperfusion injury on specific and non-specific early and late chronic events after transplantation. Transplant Rev 10: 108-127, 1996

88. Opelz G. Cadaver kidney graft outcome in relation to ischaemia time and HLA match. Trans Proc 30: 4294-4296, 1998

89. Cecka J M, Terasaki P I. The UNOS scientific renal transplant registry, in Clinical transplants 1995, edited by Cecka J M and Terasaki P I, Los Angeles, UCLA Tissue typing laboratory, 1996, pp1-18

90. Connolly J K, Dyer P A, Martin S, Parrott N R, Pearson R C et al. Importance of minimising HLA-DR mismatch and cold preservation time in cadaveric renal transplantation. Transplantation 61: 709-14, 1996

91. Lehtonen S R, Isoniemi H , Salmela K T, Taskinen E I, von Willebrand E O. et al. Long-term graft outcome is not necessarily affected by delayed onset of graft function and early acute rejection. Transplantation 64: 103-7, 1997

92. Troppmann C, Gillingham K, Gruessner R, Dunn D L, Payne W D et al. Delayed graft function in the absence of rejection has no long-term impact. A study of cadaver kidney recipients with good graft function at 1 year after transplantation. Transplantation 61: 1331-7, 1996

93. Montagnino G, Tarantino A, Cesana B, Rossini G, DaMilano I et al. Prognostic factors of long term allograft survival in 632 CyA treated recipients of a primary renal transplant. Transplant Int 10: 268-275, 1997

94. Shoskes D, Cecka M. Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. Transplantation 55: 1697-1701, 1998

95. Yokoyama I, Uchia K, Kobayashi T, Tominaga Y, Orihara A et al.. Effect of prolonged delayed graft function on long-term graft outcome in cadaveric kidney transplantation. Clin Transplant 8: 101, 1994

96. Cacciarelli T, Sumrani N, Delaney V, Hong J H, DiBenedetto A et al. The influence of delayed graft function on long-term graft outcome in the Cyclosporin era. Clin Nephrol 39: 335-9, 1993

97. Goes N, Urmson J, Ramassar V, Halloran P F. Ischaemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-γ, TGFβ1, GM-CSF, IL-2 and IL-10. Transplantation 59: 565, 1995

98. Land W, Schneeberger H, Schleibner S, Illner W D, Abendroth D et al. The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants. Transplantation 57: 211-7, 1994

99. Troppmann G, Gillingham K, Benedetti E, Almond P S, Gruessner R W et al. Delayed graft function, acute rejection, and outcome after cadaver renal transplantation - A multivariate analysis. Transplantation 59: 962-968, 1995

100. Ojo A O, Wolfe R A, Held P J, Port F K, Shmouder R L. Delayed graft function: Risk factors and implications for renal allograft survival. Transplantation 63: 968-974, 1997

101. Terasaki P I, Cecka J M, Gjertson D W, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. N Eng J Med 333: 333-336, 1995

102. Mertes P M. Transplantation Biology: Cellular and molecular aspects, ed Tilney N L, Strom T B, Paul L C. Philadelphia, Penn, pp275, 1996

103. Takada M, Nadeau K C, Hancock W W, Mackenzie H S, Shaw G D et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation 65: 1533-42, 1998

104. Pratschke J, Wilheim M J, Kusaka M, Beato F, Milford E L et al. Accelerated rejection of renal allografts from brain dead donors. Ann Surg 232: 263-271, 2000

105. Cecka J M, Gjertson D W, Cho Y, Terasaki P I, for the UNOS scientific renal transplant registry. HLA polymorphisms, ethnicity and graft survival: United Network for Organ Sharing. Trans Proc 25: 2446-2447, 1993

106. Georgi B, Sumrani N, Maursky V, Hassoun A, Hong J H et al. Racial differences in long-term renal allograft outcome. Trans Proc 28: 1623-5, 1996

107. Katznelson S, Gjertson D W, Cecka J M. The effect of race and ethnicity on kidney allograft outcome. Clin Transpl 379-394, 1995

108. Mackenzie H S, Brenner B M. Current strategies for retarding the progression of renal disease. Am J Kid Dis 31: 161-170, 1998

109. Opelz G, Wujciak T, Ritz E. Association of chronic kidney allograft failure with recipient blood pressure. Collaborative transplant study. Kid Int 53: 217-222, 1998

110. Mange K C, Cizman B, Joffe M, Feldman H I. Arterial hypertension and renal allograft survival. JAMA 283: 633-638, 2000

111. Kasiske B L, Kalil R S N, Lee H S, Rao K V. Histopathological findings associated with a chronic progressive decline in renal allograft function. Kid Int 40: 514-524, 1991

112. Cosio F G, Dillon J J, Falkenhain M E, Tesi R J, Henry M L et al. Racial differences in renal allograft survival: The role of systemic hypertension. Kid Int 47: 1136-1141, 1995

113. Benediktsson H, Chea R, Davidoff A, Paul L C. Antihypertensive drug treatment in chronic renal allograft rejection in the rat. Effect on structure and function. Transplantation 62: 1634-1642, 1996

114. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. N Eng J Med 339: 1448-1456, 1998

115. Mackenzie H S, Brenner B M. Current strategies for retarding the progression of renal disease. Am J Kid Dis 31: 161-170, 1998

116. Feehally J, Harris K P, Bennett S E, Walls J. Is chronic renal transplant rejection a non-immune phenomenon? Lancet 2(8505): 486-488, 1986

117. Jindal R M. Post-transplant hyperlipidaemia. Postgrad Med J 73: 785-793, 1997

118. Zhao B, Ehringer W D, Dierichs R, Miller F N. Oxidized low-density lipoprotein increases endothelial intracellular calcium and alters cytoskeletal f-actin distribution. Europ J Clin Invest 27: 48-54, 1997

119. Chana R S, Wheeler D C, Thomas G J, Williams J D, Davies M. Low-density lipoprotein stimulates mesangial cell proteoglycan and hyaluronan synthesis. Neph Dial Transplant 15: 167-72, 2000

120. Lee H S, Kim B C, Hong H K, Kim Y S. LDL stimulates collagen mRNA synthesis in mesangial cells through induction of PKC and TGF-beta expression. Am J Physiol 277: F369-76, 1999

121. Adams D, Karnovsky M. Hypercholesterolaemia does not exacerbate arterial intimal thickening in chronically rejecting rat cardiac allografts. Trans Proc 21: 437-439, 1989

122. Mennander A, Tikkanen M, Raisanen-Sokolowski A, Paavonen T, Ustinov J et al. Chronic rejection in rat aortic allografts. Effect of hypercholesterolaemia on allograft arteriosclerosis. J Heart Lung Transplant 12: 123-31, 1993

123. Raisanen-Sokolowski A, Tilly-Kreis M, Ustinov J, Mennander A, Paavonen T et al. Hyperlipidaemia accelerates allograft arteriosclerosis (chronic rejection) in the rat. Arterioscl Thromb 14: 2032-2042, 1994

124. Dimeny E, Fellstrom B, Larsson E, Tufveson G, Lithell H. Chronic vascular rejection and hyperlipoproteinaemia in renal transplant patients. Clin Transplant 7: 482-490, 1993

125. Isonniemi H, Nurminen M, Tikkanen M, Von Willebrand E, Krogerus L et al. Risk factors predicting chronic rejection of the renal allograft. Transplantation 57: 68-72, 1994

126. Hillebrand G F, Schlosser S, Schneeberger H, Lorenz B, Zanker B et al. No clinical evidence of hyperlipidaemia as a risk factor for chronic renal allograft failure. Trans Proc 31: 1391-1392, 1999

127. Dimeny E, Wahlberg J, Lithell H, Fellstrom B. Hyperlipidaemia in renal transplantation - Risk factor for long-term graft outcome. Eur J Clin Invest 25: 574-583, 1995

128. Vesco L, Busson M, Bedrossian J, Bitker M O, Hiesse C et al. Diabetes mellitus after renal transplantation: Characteristics, outcome and risk factors. Transplantation 61: 1475-8, 1996

129. Shaffer D, Simpson M A, Madras P N, Sahyoun A I, Conway P A et al. Kidney transplantation in diabetic patients using Cyclosporin. Five year follow-up. Arch Surg 130: 283-8, 1995

130. Koskinen P, Lemstrom K, Bruggeman C, Lautenschlager I, Hayry P. Acute CMV infection induces a subendothelial inflammation in the allograft vascular wall. A possible linkage with enhanced allograft arteriosclerosis. Am J Pathol 144: 41-50, 1994

131. Lemstrom K, Sihvola R, Bruggeman C, Hayry P, Koskinen P et al. Cytomegalovirus infection enhanced cardiac allograft vasculopathy is abolished in DHPG prophylaxis in the rat. Circulation 95: 2614-2616, 1997

132. Inkinen K, Soots A, Krogerus L, Bruggeman C, Ahonen J et al. CMV increases collagen synthesis in chronic rejection in rat renal allografts. Trans Proc 31: 1361, 1999

133. Koskinen P K, Yilmaz S, Kallio E, Bruggeman C A, Hayry P J et al. Rat cytomegalovirus infection and chronic kidney allograft rejection. Transpl Int 9 (Suppl): S3-S4, 1996

134. Van Dam J G, Li F, Yin M, You X M, Grauls G et al. Effects of cytomegalovirus infection and prolonged cold ischaemia on chronic rejection of rat renal allografts. Transp Int 13: 54-63, 2000

135. Fujinami R S, Nelson J A, Walker L, Oldstone M B. Sequence homology and immunological cross reactivity of human cytomegalovirus with HLA-DRβ chain: A means for graft rejection and immunosuppression. J Virol 62: 100-5, 1988

136. Beck S, Barrell B G. Human cytomegalovirus encodes a protein homologous to MHC class I antigens. Nature 331: 269-72, 1988

137. Koskinen P. The association of the induction of vascular cell adhesion molecule-1 with cytomegalovirus antigenaemia in human heart allografts. Transplantation 56: 1103-8, 1993

138. Ustinov J A, Lahtinen T T, Bruggeman C A, Hayry P J, Lautenschlager I T. Direct induction of class II molecules by cytomegalovirus in rat heart microvascular endothelial cells is inhibited by ganciclovir. Transplantation 58: 1027-31, 1994

139. Lautenschlager I, Soots A, Krogerus L, Inkinen K, Kloover J et al. Time-related effects of Cytomegalovirus infection on the development of chronic renal allograft rejection in the rat. Intervirology 42: 279-284, 1999

140. Everett J P, Hershberger R E, Norman D J, Chou S, Ratkovec R M et al. Prolonged cytomegalovirus infection with viraemia is associated with the development cardiac allograft vasculopathy. J Heart Lung Transplant 11: S133-7, 1992

141. Hruban R H, Wu T C, Beschorner W E, Cameron D E, Ambinder R F et al. Cytomegalovirus nucleic acids in allografted hearts. Hum Pathol 21: 981-982, 1990

142. Rubin R H, Tolkoff-Rubin N E, Oliver D, Rota T R, Hamilton J et al.. Multicenter seroepidemiologic study of the impact of cytomegalovirus infection on renal transplantation. Transplantation. 40: 243-9, 1985

143. Bock G H, Sullivan E K, Miller D, Gimon D, Alexander S et al. Cytomegalovirus infections following renal transplantation - Effects of antiviral prophylaxis: A report of the North American Paediatric renal transplant co-operative study. Paediatr Nephrol 11: 665-71, 1997

144. Nadasdy T, Smith J, Laszik Z, Waner J L, Johnson L D et al.. Absence of association between Cytomegalovirus infection and obliterative transplant arteriopathy in renal allograft rejection. Mod Pathol 7: 289-94, 1994

145. Pouteil-Noble C, Ecochard R, Landrivon G, Donia-Maged A, Tardy J C et al. Cytomegalovirus infection – an aetiological factor for rejection? A prospective study of 242 patients. Transplantation 55: 851-7, 1993

146. Jamil B, Nicholls K M, Becker G J, Walker R G. Influence of anti-rejection therapy on the timing of CMV disease and other infections in renal transplant recipients. Clin Transpl 14: 14-18, 2000

147. Humar A, Gillingham K J, Payne W D, Dunn D L, Sutherland D E R et al. Association between cytomegalovirus disease and chronic rejection in kidney transplant recipients. Transplantation 68: 1879-1883, 1999

148. Bennett W M. Insights into chronic cyclosporin nephrotoxicity. Int J Clin Pharmacol Ther 34: 515-519, 1996

149. Vercateren S B, Bosmans J L, Elseviers M M, Verpooten G A, DeBroe M E. A meta-analysis and morphological review of cyclosporin-induced nephrotoxicity in auto-immune disease. Kid Int 54: 536-45, 1998

150. Zietse R, Balk A H, vd Dorpel M A, Meeter K, Bos E et al. Time course of the decline in renal function in cyclosporin treated heart transplant recipients. Am J Nephrol 14: 1-5, 1994

151. Tinawi M, Miller L, Bastani B. Renal function in cardiac transplant recipients: Retrospective analysis of 133 consecutive patients in a single centre. Clin Transplant 11: 1-8, 1997

152. Dieterle A, Gratwohl A, Nizze H, Huser B, Mihatsch M J et al. Chronic cyclosporin associated nephrotoxicity in bone marrow transplant patients. Transplantation 49: 1093-1100, 1990

153. Andoh T F, Burdmann E A, Bennett W M. Nephrotoxicity of immunosuppressive drugs: Experimental and clinical observations. Semin Nephrol 17: 34-35, 1997

154. Davies D R, Bittmann I, Pardo J. Histopathology of calcineurin inhibitor induced nephrotoxicity. Transplantation 69 (12 Suppl): S11-13, 2000

155. Mihatsch M J, Ryffel B, Gudat F. The differential diagnosis between rejection and cyclosporin toxicity. Kid Int-Suppl 52: S63-9, 1995

156. Antonovych T T, Sabnis S G, Austin S G, Palestine A G, Balow J E et al. Cyclosporin A induced arteriolopathy. Trans Proc 20 (3 Suppl 3): 951-958, 1988

157. Moruzumi K, Gudat F, Thiel G. Is cyclosporin associated glomerulopathy a new glomerular lesion in renal allografts using CSA? Abstract at 14th International Congress of the Transplantation Society, Aug 1992

158. Mihatsch M J, Thiel G, Ryffel B. Histopathology of cyclosporin nephrotoxicity. Trans Proc 20: 759-771, 1988

159. Kagami S, Border W A, Miller D E, Noble N A. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor beta expression in rat glomerular mesangial cells. J Clin Invest 93: 2431-2437, 1994

160. Johnson D W, Saunders H J, Johnson F J, Huq S O, Field M J et al. Fibrogenic effects of cyclosporin A on the tubulo-interstitium: Role of cytokines and growth factors. Exp Nephrol 7: 470-78, 1999

161. Edwards B D, Chalmers R J, O'Driscoll J B, Mitchell D M, Smith R J et al. Angiotensin II as a risk factor for Cyclosporin nephrotoxicity in patients with psoriasis. Clin Neph 41: 350-6, 1994

162. Parra T, de Arriba G, Arribas I, Perez de Lema G, Rodriguez-Puyol D et al. Cyclosporin A nephrotoxicity: Role of thromboxane and reactive oxygen species. J Lab & Clin Med 131: 63-70, 1998

163. Gladue R P, Newborg M F. The protective effects of the thromboxane synthetase inhibitor Dazmegrel on nephrotoxicity in cyclosporin treated rats. Transplantation 52: 837-841, 1991

164. Kohan D E. Endothelins in the normal and diseased kidney. Am J Kid Dis 29: 2-26, 1997

165. Hutchinson I V. An endothelin-transforming growth factor beta pathway in the nephrotoxicity of immunosuppressive drugs. Curr Opin Nephrol Hypertens 7: 665-671, 1998

166. Vaziri N D, Ni Z, Zhang Y P, Ruzics E P, Maleki P et al. Depressed renal and vascular nitric oxide synthase expression in cyclosporin induced hypertension. Kid Int 54: 482-91, 1998

167. Shihab F S, Yi H, Bennett W M, Andoh T F. Effect of nitric oxide production on TGFβ-1 and matrix proteins in chronic cyclosporin nephrotoxicity. Kid Int 58: 1174-1185, 2000

168. Amore A, Emancipator S N, Cirina P, Conti G, Ricotti E et al. Nitric oxide mediates cyclosporin-induced apoptosis in cultured renal cells. Kid Int 57: 1549-59, 2000

169. Vieira J M Jr, Noronha I L, Malheiros D M, Burdmann E A. Cyclosporin induced interstitial fibrosis and arteriolar TGFβ expression with preserved renal blood flow. Transplantation 68: 1746-53, 1999

170. Lally C, Healy E, Ryan M P. Cyclosporin A-induced cell cycle arrest in renal epithelial cells. Kid Int 56: 1254-1257, 1999

171. Thomas S E, Andoh T F, Pichler R H, Shankland S J, Couser W G et al. Accelerated apoptosis characterizes cyclosporin associated interstitial fibrosis. Kid Int 53: 897-908, 1998

172. Esposito C, Fornoni A, Cornacchia F, Belloti N, Fasoli G et al. Cyclosporin induces different responses in human epithelial, endothelial and fibroblast cell cultures. Kid Int 58: 123-130, 2000

173. Shihab F S, Andoh T F, Tanner A M, Yi H, Bennett W M. Expression of apoptosis regulatory genes in chronic cyclosporin nephrotoxicity favours apoptosis. Kid Int 56: 2147-59, 1999

174. Johnson D W, Saunders H J, Johnson F J, Huq S O, Field M J et al. Cyclosporin exerts a direct fibrogenic effect on human tubulointerstitial cells: Roles of insulin-like growth factor-1, transforming growth factor β1, and platelet derived growth factor. J Pharmacol & Exp Therapeut 289: 535-42, 1999

175. Wolf G, Thaiss F, Stahl R A. Cyclosporin stimulates expression of transforming growth factor β1 in renal cells. Possible mechanism of cyclosporin's antiproliferative effects. Transplantation 60: 237-241, 1995

176. Fornoni A, Lenz O, Tack I, Potier M, Elliot S J et al. Matrix accumulation in mesangial cells exposed to cyclosporin A requires a permissive genetic background. Transplantation 70: 587-93, 2000

177. Ahuja S S, Shrivastav S, Danielpour D, Balow J E, Boumpas D T. Regulation of transforming growth factor β1 and its receptor in human T lymphocytes. Transplantation 60: 718-23, 1995

178. Wolf G, Zahner G, Ziyadeh F N, Stahl R A. Cyclosporin A induces transforming growth factor β in a cultured murine proximal tubular cell line. Exp Nephrol 4: 304-8, 1996

179. Khanna A, Kapur S, Sharma V, Li B, Suthanthiran M. In vivo hyperexpression of transforming growth factor β1 in mice. Stimulation by cyclosporin. Transplantation 63: 1037-9, 1997

180. Khanna A K, Cairns V R, Becker C G, Hosenpud J D. Transforming growth factor beta (TGFβ) mimics and anti-TGFβ antibody abrogates the in vivo effects of cyclosporin: Demonstration of a direct role of TGFβ in immunosuppression and nephrotoxicity of cyclosporin. Transplantation 67: 882-9, 1999

181. Shin G T, Khanna A K, Ding R, Sharma V K, Lagman M et al. In vivo expression of transforming growth factor β1 in humans. Stimulation by cyclosporin. Transplantation 65: 313-18, 1998

182. Hueso M, Bover J, Seron G, Gil-Vernet S, Sabate I et al. Low-dose cyclosporin and MMF in renal allograft recipients with sub-optimal renal function. Transplantation 66: 1727-31, 1998

183. Van der Mast B J, Van Besouw N M, De Kuiper P, Vaessen L M, Ijzermans J N et al. A longitudinal study of TGFβ1 protein levels in renal allograft recipients converted from CsA to MMF or Aza. Clin Transplant 14: 66-9, 2000

184. Pankewycz O G, Miao L, Isaacs R, Guan J, Pruett T et al. Increased renal tubular expression of transforming growth factorβ in human renal allografts correlates with cyclosporin toxicity. Kid Int 50: 1634-40, 1996

185. Mohaned M A, Robertson H, Booth T A, Balupuri S, Kirby J A et al. TGFβ expression in renal transplant biopsies: A comparative study between Cyclosporin and Tacrolimus. Transplantation 69: 1002-5, 2000

186. Bicknell G R, Williams S T, Shaw J A, Pringle J H, Furness P N et al. Differential effects of cyclosporin and tacrolimus on the expression of fibrosis associated genes in isolated glomeruli form renal transplants. Br J Surgery 87: 1569-1575, 2000

187. Ceol M, Anglani F, Vianello D, Murer L, Del Prete D et al. Direct effect of chronic cyclosporin treatment on collagen III mRNA expression and deposition in rat kidneys. Clin Nephrol 53: 8-9, 2000

188. Tullius S G, Hancock W W, Heemann U, Azuma H, Tilney N L. Reversibility of chronic renal allograft rejection. Transplantation 58: 93-99, 1994

189. Paul L C. Benediktsson H. Chronic transplant rejection: Magnitude of the problem and pathogenetic mechanisms. Transplant Rev 7: 96-113, 1993

190. Hayry P. Chronic Rejection. An update on mechanism. Trans Proc 30: 3993-3995, 1998

191. Newby A C, Southgate K M, Davies M. Extracellular matrix degrading metalloproteinases in the pathogenesis of arteriosclerosis. Basic Res Cardiol 89(Supp 1): 59-70, 1994

192. Kato Y, Periasamy M. Growth and differentiation of smooth muscle cells during vascular development. Trends Cardiovasc Med 6: 100-106, 1996

193. Matzinger P. Tolerance, danger, and the extended family. Ann Rev Immunol 12: 991-1045, 1994

194. Orosz C G, Pelletier R P. Chronic remodelling pathology in grafts. Curr Opin Imunol 9: 676-80, 1997

195. Healy E, Brady H R. Role of tubule epithelial cells in the pathogenesis of tubulointerstitial fibrosis induced by glomerular disease. Curr Opin Nephrol Hypertens 7: 525-30, 1998

196. Schelling J R, Nkemere N, Kopp J B, Cleveland R P. Fas-dependent fracticidal apoptosis is a mechanism of tubular epithelial cell deletion in chronic renal failure. Lab Invest 78: 813-824, 1998

197. Halloran P F, Melk A, Barth C. Rethinking chronic allograft nephropathy. The concept of accelerated senescence. J Am Soc Nephrol 10: 167-181, 1999

198. Diamond J R, Karnovsky M J. Focal and segmental glomerulosclerosis. analogies to atherosclerosis. Kid Int 33: 917-924, 1988

199. Hostetter T H, Olson J L, Rennke H G, Venkatachalam M A, Brenner B M. Hyperfiltration in remnant nephrons. A potentially adverse response to renal ablation. Am J Physiol 241: F85-F93, 1981

200. Remuzzi G, Bertani T. Is glomerulosclerosis a consequence of altered glomerular permeability to macromolecules? Kid Int 35: 384-394, 1989

201. Yoshida Y, Fogo A, Ichikawa I. Glomerular haemodynamic changes vs hypertrophy in experimental glomerular sclerosis. Kid Int 35: 654-664, 1989

202. Eddy A A. Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol 7: 2495-2508, 1996

203. Mackenzie H S, Tullius S G, Heemann U W, Azuma H, Rennke H G et al. Nephron supply is a major determinant of long term renal allograft outcome in rats. J Clin Invest 94: 2148-2152, 1994

204. Feldman H I, Fazio I, Roth D, Berlin J A, Brayman K et al. Recipient body size and cadaveric renal allograft survival. J Am Soc Nephrol 7: 151-157, 1996

205. Kingma I, Chea R, Davidoff A, Benediktsson H, Paul L C. Glomerular capillary pressures in long-surviving rat renal allografts. 56: 53-60, 1993

206. Furness P N. Extracellular matrix of the kidney. J Clin Path 49: 355-359, 1996

207. Nagase H, Woessner J F. Matrix Metalloproteinases. J Biol Chem 274: 491-4, 1999

208. Waller J R, Nicholson M L. Molecular mechanisms of renal allograft fibrosis. Br J Surg 99: 1429-1441, 2001

209. Katsura M, Ito A, Hirakawa S, Mori Y. Human recombinant interleukin-1 alpha increases biosynthesis of collagenase and hyaluronic acid in cultured human chorionic cells. FEBS Letters 244:315-8, 1989

210. Salo T, Turpeeniemi-Hujanen R, Tryggvason K. Tumour promoting esters and cell proliferation stimulate secretion of basement membrane type IV collagen degrading metalloproteinase by human fibroblasts. J Biol Chem 260: 8526-31, 1985

211. Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem 378: 151-60, 1997

212. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V et al. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nature Genetics 17: 439-44, 1997

213. Gomez DE. Alonso DF. Yoshiji H. Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Euro J Cell Biol 74: 111-22, 1997

214. Murphy G. Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymology 248: 496-510, 1995

215. Tang W W, Feng L, Xia Y, Wilson C B, Extracellular matrix accumulation in immune-mediated tubulointerstitial injury. Kid Int 45: 1077-84, 1994

216. Duymelinck C, Deng J T, Dauwe S E, De Broe M E, Verpooten G A. Inhibition of the matrix metalloproteinase system in a rat model of chronic cyclosporin nephropathy. Kid Int 54: 804-18, 1998

217. Stetler-Stevenson W G, Brown P D, Onisto M, Levy A T, Liotta L A. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumour cell lines and human tumour tissues. Journ Biol Chem 265: 13933-8, 1990

218. Wick M, Burger C, Brusselbach S, Lucibello F C, Muller R. A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation, and senescence. Journ Biol Chem 269: 18953-60, 1994

219. Frisch S M, Francis H. Disruption of epithelial cell matrix interactions induces apoptosis. J Cell Biol 124: 619-626, 1994

220. Strutz F, Okada H, Lo C W, Danoff T, Carone R L et al. Identification and characterisation of a fibroblast marker FSP1. J Cell Biol 130: 393-405, 1995

221. Paul L C, Van Es L A, Stuffers-Heiman M, De La Riviere G B, Kalff M W. Antibodies directed against tubular basement membranes in human renal allograft recipients. Clin Immunol Immunopath 14: 231-237, 1979

222. Waltenberger J, Miyazono K, Funa K, Wanders A, Fellstrom B et al. Transforming growth factor-beta and organ transplantation. Trans Proc 25: 2038-40, 1993

223. Riberio S M, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich J E. The activation sequence of thrombospondin-1 interacts with the latency associated peptide to regulate activation of latent transforming growth factor-beta. J Biol Chem 274: 13586-93, 1999

224. Border W A, Noble N A, Yamamoto T, Harper J R, Yamaguchi Y et al. Natural inhibitor of transforming growth factor beta protects against scarring in experimental kidney disease. Nature 360: 361-4, 1992

225. Ignotz R A, Endo T, Massague J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. J Biol Chem 158: 6443-6, 1987

226. Border W A, Okuda S, Languino L R, Ruoslahti E. Transforming growth factor-beta regulates production of proteoglycans by mesangial cells. Kid Int 37: 689-95, 1990

227. Nakamura T, Miller D, Ruoslahti E, Border W A. Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor-beta-1. Kid Int 41: 1213-21, 1992

228. Paul L C, Saito K, Davidoff A, Benediktsson H. Growth factor transcripts in renal transplants. Am J Kid Dis 28: 441-50, 1996

229. Shihab F S, Tanner A M, Shao Y, Weffer M I. Expression of TGF-β1 and matrix proteins is elevated in rats with chronic rejection. Kid Int 50: 1904-13, 1996

230. Mannon R B, Kopp J B, Ruiz P, Griffiths R, Bustos M et al. Chronic rejection of mouse kidney allografts. Kid Int 55: 1935-44, 1999

231. Lantz I, Dimeny E, Larsson E, Fellstrom B, Funa K. Increased immunoreactivity of transforming growth factor-beta in human kidney transplants. Transpl Immunol 4: 209-14, 1996

232. Shihab F S, Yamamoto T, Nast C C, Cohen A H, Noble N A et al. Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. J Am Soc Nephrol 6: 286-94, 1995

233. Sharma V K, Bologa R M, Xu G P, Li B, Mouradian J et al. Intragraft TGF-beta 1 mRNA: a correlate of interstitial fibrosis and chronic allograft nephropathy. Kid Int 49: 1297-1303, 1996

234. Suthanthiran M. Molecular analyses of human renal allografts: Differential intragraft gene expression during rejection. Kid Int - Suppl 58: S15-21, 1997

235. Nicholson M L, Bicknell G R, Barker G, Doughman T M, Williams S T et al. Intragraft expression of transforming growth factor-beta 1 gene in isolated glomeruli from human renal transplants. Br J Surg 86: 1144-1148, 1999

236. Cuhaci B, Kumar M S A, Bloom R D, Pratt B, Haussman G et al. Transforming growth factor-beta levels in human allograft fibrosis correlate with decline in renal function. Transplantation 68: 785-790, 1999

237. Border W A, Noble N A. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. Hypertension 31: 181-8, 1998

238. Lewis J B, Helderman J H. Is it time for ACE inhibitors in chronic allograft nephropathy? Am J Kid Dis 35: 154-156, 2000

239. Campistol J M, Inigo P, Jimenez W, Lario S, Clesca P H et al. Losartan decreases plasma levels of TGFβ-1 in transplant patients with chronic allograft nephropathy. Kid Int 56: 714-9, 1999

240. Szabo A, Lutz J, Schleimer K, Antus B, Hamar P et al. Effect of angiotensin-converting enzyme inhibition on growth factor mRNA in chronic renal allograft rejection in the rat. Kid Int. 57: 982-91, 2000

241. Paredes D, Sola R, Guirado L, Ibeas J, Agraz I et al. Treatment of kidney transplants with chronic rejection using angiotensin converting enzyme inhibitors. Trans Proc 29: 2587-8, 1997

242. Barnas U, Schmidt A, Haas M, Oberbauer R, Mayer G. The effects of prolonged angiotensin-converting enzyme inhibition on excretory kidney function and proteinuria in renal allograft recipients with chronic progressive transplant failure. Neph Dial Transpl 11: 1822-1824, 1996

243. Kobashigawa J A, Katznelson S, Laks H, Johnson J A, Yeatman L et al. Effect of pravastatin on outcomes after cardiac transplantation. N Eng J Med 333: 621-627, 1995

244. Wenke K, Meiser B, Thiery J, Nagel D, Von Scheidt W et al. Simvastatin reduces graft vessel disease and mortality after heart transplantation. Circulation 96: 1398-1402, 1997

245. Katznelson S, Wilkinson A H, Kobashigawa J A, Wang X M, Chia D et al. The effect of pravastatin on acute rejection after kidney transplantation – A pilot study. Transplantation 61: 1469-74, 1996

246. Knoll GA. Bell RC. Tacrolimus versus cyclosporin for immunosuppression in renal transplantation: meta-analysis of randomised trials. BMJ. 318: 1104-7, 1999

247. Mayer A D, Dmitrewski J, Squifflet J P, Besse T, Grabensee B et al. Multicentre randomised trial comparing tacrolimus (FK506) and cyclosporin in the prevention of renal allograft rejection: A report of the European Tacrolimus Multicentre Renal Study Group. Transplantation. 64: 436-43, 1997

248. Pirsch J D, Miller J, Deierhoi M H, Vincenti F, Filo R S. A comparison of tacrolimus (FK506) and cyclosporin for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Groups. Transplantation. 63: 977-83, 1997

249. Zhang J G, Walmsley M W, Moy J V, Cunningham A C, Talbot D et al. Differential effects of cyclosporin A and tacrolimus on the production of TGF-beta: implications for the development of bronchiolitis obliterans after lung transplantation. Transpl Int 11(Suppl 1): S325-7, 1998

250. Morris-Stiff G J, Baboolal K, Dunstan F, Jurewicz A W. Conversion from cyclosporin (Neoral) to tacrolimus (Prograf) in renal allograft recipients with chronic graft nephropathy: Results of an observational study. Transpl Int 12: 288-292, 1999

251. Bismuth H. Comparison of FK506 and Cyclosporin based immunosuppression: FK506 therapy significantly reduces the incidence of acute, steroid-resistant, refractory and chronic rejection whilst possessing a comparable safety profile. European FK506 Multicentre Liver Study Group. Trans Proc 27: 45-9, 1995

252. Gibbs J F, Husberg B S, Klintmalm G B, Backamn L, Levy M et al. Outcome analysis of FK506 therapy for acute and chronic rejection. Trans Proc 25: 622-23, 1993

253. Hauser I A, Renders L, Radeke H H, Sterzel R B, Goppelt-Struebe M. Mycophenolate mofetil inhibits rat and human mesangial cell proliferation by guanosine depletion. Neph Dial Transpl 14: 58-63, 1999

254. Raisanen-Sokolowski A, Aho P, Myllarniemi M, Kallio E, Hayry P. Inhibition of early chronic rejection in rat aortic allografts by mycophenolate mofetil (RS61443). Transplantation Proceedings. 27: 435, 1995

255. Azuma H, Binder J, Heemann U, Schmid C, Tullius S G et al. Effects of RS61443 on functional and morphological changes in chronically rejecting rat kidney allograft. Transplantation 59: 460-66, 1995

256. Halloran P, Mathew T, Tomlanovich S, Groth C, Hooftman L et al. Mycophenolate mofetil in renal allograft recipients: A pooled efficacy analysis of three randomised double-blind clinical studies in prevention of rejection. The International Mycophenolate Mofetil renal transplant study groups. Transplantation 63: 39-47, 1997

257. European Mycophenolate Mofetil Cooperative Study Group: Mycophenolate mofetil in renal; transplantation: 3 year results from the placebo-controlled trial. Transplantation 68: 391-6, 1999

258. Pascual J, Marcen R, Orofino L, Quereda C, Mampas F et al. Evidence that addition of azathioprine improves renal function in cyclosporin treated patients with allograft dysfunction. Transplantation 52: 276-279, 1991

259. Rocher L, Hodgson R, Merion R, Swartz R, Keavey S et al. Amelioration of chronic renal allograft dysfunction in cyclosporin treated patients by addition of Azathioprine. Transplantation 47: 249-254, 1989

260. Campistol J M, Mazuecos A, Segura J, Osuna A, Herrero J et al. Mycophenolate mofetil slows the decline of renal function in patients with biopsy proven chronic rejection: A collaborative pilot study. Trans Proc 31: 2267-2269, 1999

261. Fritsche L, Sperschneider H, Budde K, Giessing M, Morgera S et al. Conversion to mycophenolate mofetil for chronic progressive deterioration of renal allograft function: first clinical experiences in 44 patients. Trans Proc. 30: 1190-1, 1998

262. Glicklich D, Gupta B, Schurter-Frey G, Greenstein S M, Schechner R S et al. Chronic renal allograft rejection: No response to mycophenolate mofetil. Transplantation 66: 398-99, 1998

263. Senn B, Rochat P, Schwarzkopf A K, Weinreich T, Binswanger U. Mycophenolate Mofetil in ongoing chronic kidney allograft dysfunction. Trans Proc 30: 4075-4076, 1998

264. Hall B M, Tiller D J, Hardie I, Mahony J, Mathew T et al. Comparison of three immunosuppressive regimens in cadaver renal transplantation: Long term cyclosporin, short term cyclosporin followed by azathioprine and prednisolone and azathioprine and prednisolone without cyclosporin. N Eng J Med 318: 1499-1507, 1988

265. Spielberger M, Aigner F, Schmid T, Bosmuller C, Konigsrainer A et al. Long term results of cadaveric renal transplantation after conversion from cyclosporin to azathioprine: A controlled randomised trial. Trans Proc 20: 169-170, 1988

266. Sobh M A, Eldein A B, Moustafa F E, Ghoneim M A. Elective conversion from cyclosporin to azathioprine in living related donor kidney transplants. Neph Dial Transpl 2: 258-260, 1987

267. Helling T S, Wood B C, Nelson P W, Shelton L L, Eisenstein C L et al. Elective conversion from cyclosporin to azathioprine in sensitised patients following cadaveric renal transplantation. Am J Kid Dis 15: 137-140, 1990

268. Land W, Castro L A, Hillebrand G, Gunther K, Gokel J M. Conversion rejection consequences by changing the immunosuppressive therapy from Cyclosporin to Azathioprine after kidney transplantation. Trans Proc 15: 2857-2861, 1983

269. Vanrenterghem Y, Michielsen P. A controlled trial of one versus three months cyclosporin and conversion to azathioprine in renal transplantation. Trans Proc 17: 1162-1163, 1985

270. Kasiske B L, Heim-Duthoy K, Ma J Z. Elective cyclosporin withdrawal after renal transplantation. JAMA 269: 395-400, 1993

271. Smak Gregoor P J, van Gelder T, van Besouw N M, van der Mast B J, Ijzermans J N et al. Randomised study on the conversion of treatment with cyclosporin to azathioprine or mycophenolate mofetil followed by dose reduction. Transplantation 70: 143-8, 2000

272. Ducloux D, Fournier V, Bresson-Vautrin C, Rebibou J M, Billerey C et al. Mycophenolate mofetil in renal transplant recipients with cyclosporin associated nephrotoxicity: A preliminary report. Transplantation 65: 1504-6, 1998

273. Mourad G, Vela C, Ribstein J, Mimran A. Long term improvement in renal function after Cyclosporin dose reduction in renal transplant recipients with histologically proven chronic Cyclosporin nephrotoxicity. Transplantation 65: 661-7, 1998

274. Kleim V, Repp H, Frei U. Long term improvement of chronic renal graft dysfunction in cyclosporin treated patients by addition of azathioprine and reduction of cyclosporin. Trans Proc 26: 2553-2555, 1994

275. Weir M R, Anderson L, Fink J C, Gabregiorgish K, Schweitzer E J et al. A novel approach to the treatment of chronic allograft nephropathy. Transplantation 64: 1706-10, 1997

276. Islam MS. Francos GC. Dunn SR. Burke JF Jr. Mycophenolate mofetil and reduction in cyclosporin dosage for chronic renal allograft dysfunction. Trans Proc 30: 2230-1, 1998

277. Nicholson M L, Jain S J, Metcalfe M S, White S A, Furness P N. Abstract 437: Chronic allograft nephropathy - A prospective randomised controlled trial of cyclosporin dose reduction with or without Mycophenolate Mofetil. Transplantation 69: S227, 2000

278. Hunsicker L G, Bennett L E. Design of trials of methods to reduce late renal allograft loss: The price of success. Kid Int - Suppl 52: S120-123, 1995

279. Paul L C, Sijpkens Y W J. Surrogate end points in chronic kidney graft rejection studies. Trans Proc 31: 1293-1294, 1999

280. Dimeny E. Wahlberg J. Larsson E. Fellstrom B. Can histopathological findings in early renal allograft biopsies identify patients at risk for chronic vascular rejection? Clin Transpl 9: 79-84, 1995

281. Nicholson M L, McCulloch T A, Harper S J, Wheatley T J, Edwards C M et al. Early measurement of interstitial fibrosis predicts long-term renal function and graft survival in renal transplantation. Br J Surg 83: 1082-5, 1996

282. Vezina C, Kudelski A, Sehgal S N. Rapamycin , a new antifungal antibiotic. Taxonomy of the producing Streptomycete and isolation of the active principle. J Antibiot 28: 721-6, 1975

283. Sehgal S N, Baker H, Vezina C. Rapamycin, a new antifungal antibiotic. Fermentation , isolation and characterization. J Antibiot 28: 727-32, 1975

284. Ho S, Clipstone N, Timmermann L, Northrop J, Graef I et al. The mechanism of action of cyclosporin A and FK506. Clin Immunol & Immunopath 80(3 Pt 2):S40-5, 1996

285. Sehgal S N. Rapamune (Sirolimus, Rapamycin): An overview and mechanism of action. J Therap Drug Monitoring 17: 660-665, 1995

286. Morris R E. New small molecule immunosuppressants for transplantation: Review of essential concepts. J Heart Lung Transplant 12(6 Pt 2): S275-86, 1993

287. Cardenas M E, Lorenz M, Hemenway C, Heitmen J. Yeast as model T cells. Drug Disc Des 2:103-26, 1994

288. Chiu M I, Katz H, Berlin V. RAPT1, a mammalian homologue of yeast TOR interacts with the FKBP12-Rapamycin complex. Proc Nat Acad Sci USA. 91:12574-12578, 1994

289. Chen Y, Chen H, Rhoad A O, Warner L, Caggiano T L. A putative Sirolimus (Rapamycin) effector protein. Biochem Biophys Res Commun 203: 1-7, 1994

290. Brown E J, Albers M W, Shin T B, Ichikawa K, Keith C T et al. Purification and molecular cloning of FRAP a direct target of G1 arrest complex FKBP12-Rapamycin. Nature 369: 756-761, 1994

291. Sabers C J, Martin M M, Brunn G J, Williams J M, Dumont F J et al. Isolation of protein target of FKBP12-Rapamycin complex in mammalian cells. J Biol Chem. 270: 815-822, 1994

292. Sonenberg N, Gingras A C. The mRNA 5' cap-binding protein eIF-4E and control of cell growth. Curr Opin Cell Biol 10: 268-275, 1998

293. Beretta L, Gingras A C, Svitkin Y V, Hall M N, Sonenberg N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J 15: 658-664, 1996

294. Sabatini D M, Pierchala B A, Barrow R K, Schell M J, Snyder S H. The Rapamycin and FKBP12 target (RAFT) displays phospatidylinositol 4-kinase activity. J Biol Chem 270: 20875-8, 1995

295. Jeffries H B J, Fumagalli S, Dennis P B, Reinhard C, Pearson R B et al. Rapamycin suppresses 5'TOP mRNA translation through inhibition of $p70^{s6k}$. EMBO J 12: 3693-3704, 1997

296. Chung J, Kuo C J, Crabtree G R, Blenis J. Rapamycin-FKBP12 specifically blocks growth-dependent activation of and signalling by the 70kd S6 protein kinases. Cell 69: 1227-1236, 1992

297. Terada N, Lucas J J, Szepesi A, Franklin R A, Domenico J et al. Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cell cycle. J Cell Physiol 268: 22825-22829, 1993

298. Sehgal S N. Rapamune: Mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 31: 335-340, 1998

299. Bertagnolli M M, Yang L, Herrmann S H, Kirkman R L. Evidence that Rapamycin inhibits interleukin-12 induced proliferation of activated T lymphocytes. Transplantation 58: 1091-6, 1994

300. Sehgal S N, Bansbach C C. An in vitro immunological profile of Rapamycin. Ann. N. Y. Acad. Sci. 685: 58-67, 1993

301. Aaguaard-Tillery K M, Jelinek D. Inhibition of human B Lymphocyte cell cycle progression and differentiation by Rapamycin. Cell Immunol 152: 493-507, 1994

302. Kim H S, Raskova J, Degiannis D, Raska K J. Effects of Cyclosporin and Rapamycin on immunoglobulin production by preactivated human B cells. Clin Exp Immunol 96: 508-12, 1994

303. Dumont F J, Kastner C A. Transforming growth factor beta 1 inhibits interleukin-1 induced but enhances ionomycin induced interferon-γ production in a T

cell lymphoma: Comparison with the effects of Rapamycin. J Cell Physiol. 160:141-53, 1994

304. Zheng X X, Strom T B, Steele A W. Quantitative comparison of Rapamycin and cyclosporin, effects on cytokine gene expression studied by reverse transcriptase-competitive polymerase chain reaction. Transplantation 58: 87-92, 1994

305. Kahan B D, Gibbons S, Tejpal N, Stepkowski S M, Chou T C. Synergistic interactions of Cyclosporin and Rapamycin to inhibit immune performances of normal human peripheral blood leukocytes in vitro. Transplantation 51: 232-9, 1991

306. Calne R, St J Collier D, Lim S, Pollard S G, Samaan A et al. Rapamycin for immunosuppression in organ allografting. Lancet 2 (8656) : 227, 1989

307. Morris R E, Meiser B M. Identification of a new pharmacological action for a old compound. Med Sci Res 17: 609-610, 1989

308. Eng C P, Gullo-Brown J G, Chang J Y, Sehgal S N. Inhibition of skin graft rejection in mice by Rapamycin : A novel immunosuppressive macrolide. Trans Proc 23: 868, 1991

309. Ochiai T, Gunji Y, Nagata M, Komori A, Asano T et al. Effects of Rapamycin in experimental organ allografting. Transplantation 56: 15-19, 1993

310. Schmidbauer G, Hancock W W, Wasowska B, Binder J, Padberg W et al. Rapamycin abrogates accelerated rejection in sensitized rats by selectively suppressing intragraft cell activation, adhesion binding properties and modulating serum alloantibody responses. Trans Proc 27: 427-9, 1995

311. Stepkowski S M, Kahan B D. Rapamycin and cyclosporin synergistically prolong heart and kidney allograft survival. Trans Proc 23: 3262-4, 1991

312. Stepkowski S M, Chen H, Daloze P, Kahan B D. Rapamycin, a potent immunosuppressive drug for vascularised heart, kidney and small bowel transplantation in the rat. Transplantation 51: 22-6, 1991

313. Vu M D, Qi S, Xu D, Wu J, Peng J et al. Synergistic effects of Mycophenolate mofetil and Sirolimus in prevention of acute heart, pancreas and kidney allograft rejection and in reversal of ongoing heart allograft rejection in the rat. Transplantation 66: 1575-1580, 1998

314. Chen H, Wu J, Xu D, Aboujaoude M, Daloze P. The effect of Rapamycin on orthoptic small bowel transplantation in the rat. Trans Proc 24: 1157, 1992

315. Kahan B D, Chang J Y, Sehgal S N. Preclinical evaluation of a new potent immunosuppressive agent, Rapamycin. Transplantation 52: 185-191, 1991

316. Fryer J, Yatscoff R W, Pascoe E A, Thliveris J. The relationship of blood concentrations of Rapamycin and cyclosporin to suppression of allograft rejection in a rabbit heterotopic heart allograft model. Transplantation 55: 340-45, 1993

317. Knight R, Ferraresso M, Serino F, Katz S, Lewis R et al. Low dose Rapamycin potentiates the effects of subtherapeutic doses of Cyclosporin to prolong renal allograft survival in the mongrel canine model. Transplantation 55: 947-949, 1993

318. St J Collier D, Calne R, Thiru S, Lim S, Pollard S G et al. Rapamycin in experimental renal allografts in dogs and pigs. Trans Proc 22: 1674-1675, 1990

319. Almond P S, Moss A, Nakhleh R, Melin M, Chen S et al. Rapamycin: Immunosuppression, hypo-responsiveness and side effects in a porcine renal allograft model. Transplantation 56: 275-281, 1993

320. Granger D K, Cromwell J W, Chen S, Goswitz J, Morrow D T et al. Prolongation of renal allograft survival in a large animal model by oral Rapamycin monotherapy. Transplantation 59: 183-186, 1995

321. St J Collier D, Calne R Y, Pollard S G, Friend P J, Thiru S. Rapamycin in experimental renal allografts in primates. Trans Proc 23: 2246-2247, 1991

322. Chen H F, Luo H, Daloze P, Xu D, Shan X et al. Long term in vivo effects of Rapamycin on humoral and cellular immune responses in the rat. Immunobiol. 188: 303-315, 1993

323. Chen H F, Wu J P, Luo H Y, Daloze P M. Reversal of ongoing heart, kidney and pancreas rejection and suppression of accelerated heart allograft rejection in the rat by Rapamycin. Transplantation 56: 661-666, 1993

324. Wang M, Stepkowski S M, Ferraresso M, Kahan B D. Evidence that Rapamycin rescue therapy delays rejection of major and minor histocompatible heart allografts in rats. Transplantation 54: 704-709, 1992

325. Stepkowski S M, Tian L, Napoli K L, Ghobrial R, Wang M E et al. Synergistic mechanisms by which sirolimus and cyclosporin inhibit rat heart and kidney allograft rejection. Clin Exp Immunol 108: 63-68, 1997

326. Longoria J L, Roberts R F, Marboe C C, Stouch B C, Starnes V A et al. Sirolimus potentiates cyclosporin in prevention of acute lung rejection. J Thoracic & Cardiovasc Surg. 117: 715-718, 1999

327. Qi S, Xu D, Peng J, Liu D, Chen H. Synergistic effect of rapamycin and cyclosporin in prevention of acute kidney allograft rejection in the mouse. Microsurgery 19: 344-7, 1999

328. Kahan B D; For The Rapamune US Study Group. Efficacy of Sirolimus compared with Azathioprine for reduction of acute renal allograft rejection. A randomised multicentre study. Lancet 356: 194-202, 2000

329. MacDonald A S. The Rapamune Global Study Group. A worldwide phase III randomised controlled safety and efficacy study of a sirolimus/cyclosporin regimen

for prevention of acute rejection in recipients of primary mismatched renal allografts. Transplantation 2001; 71: 271-280

330. Kahan B D for the US and Global multicentre Sirolimus trial groups. Two year follow-up of the pivotal multi-centre trials of Sirolimus; Abstract 960: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

331. Kahan B D for the US and Global multicentre Sirolimus trial groups. Two year safety and efficacy of Sirolimus in renal transplantation; Abstract 144: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2001

332. Kahan B, Podbielski J, Kimberley L, Katz S M, Meier-Kriesche H et al. Immunosuppressive effects and safety of a Sirolimus / Cyclosporin combination regimen for renal transplantation. Transplantation 66: 1040-46, 1998

333. Kerman R H, Katz S M, Van Buren C T, Kahan B D. Addition of Rapamycin to Cyclosporin prednisolone based immunosuppression reduces the clinical risk of pre-transplant anti-HLA antibody allosensitisation; Abstract 155: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

334. Neylan J F for the Rapamune US study group. Racial effects on the safety and efficacy of Sirolimus in renal transplantation; Abstract 962: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

335. Kahan B D, Julian B A, Pescovitz M D, Vanrenterghem Y, Neylan J. Sirolimus reduces the incidence of acute rejection episodes despite lower cyclosporin doses in Caucasian recipients of mismatched primary renal allografts: A Phase II trial. Transplantation 68:1526-1532, 1999

336. Gonwa T, Alveranga D, Ancona G, Brinker K, Cambi V et al. Sirolimus permits early elimination of Cyclosporin in recipients of cadaveric renal allografts; Abstract 958: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

337. Hricik D E, for the Rapamune renal function study group. Improved renal function with Cyclosporin elimination in Sirolimus treated renal transplant recipients: One year results from a phase II trial. Abstract 54: AST/ASTS Chicago, USA 2001

338. Groth C G, Backman L, Morales J, Calne R, Kreis H et al. Sirolimus (Rapamycin) based therapy in human renal transplantation. Transplantation 67: 1036-1042, 1999

339. Kreis H, Cisterne J M, Land W, Wramner L, Squifflet J et al. Sirolimus in association with mycophenolate mofetil induction for the prevention of acute graft rejection in renal allograft recipients. Transplantation 69: 1252-1260, 2000

340. Slaton J W, Kahan B D. Case report- Sirolimus rescue therapy for refractory renal allograft rejection. Transplantation 61: 977-979, 1996

341. Hong J, Kahan B D. Sirolimus rescue therapy for refractory rejection in renal transplantation; Abstract 963: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

342. Ryffel B, Weber E, Mihatsch M J. Nephrotoxicity of immunosuppressants in rats: Comparison of macrolides with cyclosporin. Exp Nephrol 2: 324-333, 1994

343. Golbaekdal K, Nielson C B, Djurhuus J C, Pedersen E B. Effects of Rapamycin on renal haemodynamics, water and sodium excretion and plasma levels of angiotensin II, aldosterone, atrial natriuretic peptide, and vasopressin in pigs. Transplantation 58: 1153-57, 1994

344. Whiting P H, Woo J, Adam B J, Hasan N U, Davidson R J et al. Toxicity of Rapamycin - A comparative and combination study with cyclosporin at immunotherapeutic dosage in the rat. Transplantation 52: 203-208, 1991

345. DiJoseph J F, Sharma R N, Chang J Y. The effect of Rapamycin on kidney function in the Sprague-Dawley rat. Transplantation 53: 507-13, 1992

346. Andoh T F, Burbmann E A, Fransechini N, Houghton D, Bennett W M. Comparison of acute Rapamycin nephrotoxicity with cyclosporin and FK506. Kid Int 50: 1110-1117, 1996

347. Morales J M, Wramner L, Kreis H. Sirolimus does not exhibit the nephrotoxicity associated with Cyclosporin administration: Pooled data analysis of 2 randomised phase II trials in renal transplant recipients; Abstract 848: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

348. Murgia M G, Jordan S, Kahan B D. The side effect profile of Sirolimus: A phase I study in quiescent cyclosporin-prednisolone treated renal transplant patients. Kid Int 49: 209-216, 1996

349. Andoh T F, Lindsley J, Franceschini N, Bennett W M. Synergistic effects of cyclosporin and rapamycin in a chronic nephrotoxicity model. Transplantation 62: 311-6, 1996

350. Podder M, Stepkowski S M, Napoli K L, Kahan B D. Pharmacokinetic toxicities caused by Sirolimus or Cyclosporin alone or in combination.; Abstract PO503W: International Congress of the Transplantation Society, Rome 2000

351. Napoli K L, Wang M E, Stepkowski S M, Kahan B D. Relative tissue distributions of cyclosporin and sirolimus after concomitant per-oral administration in the rat: Evidence for pharmacokinetic interactions. Ther Drug Monit 20: 123-133, 1998

352. Mahalati K, Kahan B D. Is Cyclosporin-Sirolimus combination actually associated with renal impairment in kidney transplant recipients? Abstract 156: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

353. Quesniaux V F J, Wehrli S, Steiner C, Joergenson J, Schuumann H J et al. The immunosuppressant Rapamycin blocks in vitro responses to haemopoetic cytokines and inhibits recovering but not steady state haematopoiesis in vivo. Blood 84: 1543-52, 1994

354. Du X X, Williams D A. Interleukin-11: A multifunctional growth factor derived from the haemopoetic micro-environment. Blood 83: 2023-2030, 1994

355. Paul S R, Bennett F, Calvetti J A, Kelleher K, Wood C R et al. Molecular cloning of a cDNA encoding interleukin –11, a stromal cell derived haematopoietic and lymphopoietic cytokine. Proc Nat Acad Sci USA 87: 7512-7516, 1990

356. Satterthwaite R, Aswad S, Sunga V, Shidban H. Incidence of new onset hypercholesterolaemia in renal transplant patients treated with FK506 and Cyclosporin. Transplantation 65: 446-9, 1998

357. Cavaille M V, Elashoff M R. A comparison of Tacrolimus and Cyclosporin for immunosuppression after cadaveric renal transplantation. Transplantation 65:142-145, 1998

358. Copley J B, Staffield C, Lindberg J, Hansen A, Bailey C et al. Cyclosporin to Tacrolimus. Effect on hypertension and lipid profiles in renal allografts. Trans Proc 30: 1254-1256, 1998

359. Legendre C for the Sirolimus European Renal Transplant Study Group. Sirolimus vs Cyclosporin – A comparison of lipid parameters over two years; Abstract PO502W: International Congress of the Transplantation Society, Rome 2000

360. Blum C. Lipid changes with Sirolimus treatment in recipients of primary mismatched renal allografts; Abstract 955: American Society of Transplantation and Transplant Surgeons (AST/ASTS), Chicago, USA May 2000

361. Brattstrom C, Wilczek H, Tyden G, Bottiger Y, Sawe J et al. Hyperlipidaemia in renal transplant recipients treated with Sirolimus (Rapamycin). Transplantation 65: 1272-1274, 1998

362. Kahan B D. Rapamycin: Personal algorithms for use based on 250 treated renal allograft recipients. Trans Proc 30: 2185-2188, 1998

363. Bouberudin S, Hiesse C, Goupy C, Kriaa F, Marchand B, Charpentier B. Incidence and consequences of post transplant lymphoproliferative disease. J Nephrol 10: 136-45, 1997

364. Garber K. Rapamycin's resurrection: a new way to target the cancer cell cycle. J Nat Cancer Inst 93: 1517-9, 2001

365. Marx S O, Jayaraman T, Go L O, Marks A R. Rapamycin inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ Res 76: 412-417, 1995

366. Gregory CR, Pratt R E, Huie P, Shorthouse R, Dzau V J et al. Effect of treatment with Cyclosporin, FK506, Rapamycin, MMF or deoxyspergualin on vascular smooth muscle proliferation in vitro and in vivo. Trans Proc 25: 770-771, 1993

367. Obata T, Kashiwagi A, Maegawa H, Nishio Y, Ugi S et al. Insulin signalling and its regulation of system A amino acid uptake in cultured rat vascular smooth muscle cells. Circ Res 79: 1167-1176, 1996

368. Mohacsi P J, Tuller D, Hulliger B, Wijngaard P L. Different inhibitory effects of immunosuppressive drugs on human and rat aortic smooth muscle and endothelial cell

proliferation stimulated by platelet derived growth factor or endothelial cell growth factor. J Heart and Lung Transplant 16: 484-492, 1997

369. Akselband Y, Harding M W, Nelson P A. Rapamycin inhibits spontaneous and bFGF driven proliferation of endothelial cells and fibroblasts. Trans Proc 23: 2833-2836, 1991

370. Simm A, Nestler M, Hoppe V. PDGF-AA, a potent mitogen for cardiac fibroblasts from adult rats. J Mol Cell Cardiol 29: 357-368, 1997

371. Nair R, Huang X, Shorthouse R, Adams B, Brazelton T et al. Antiproliferative effect of Rapamycin on growth factor stimulated human adult lung fibroblasts in vitro may explain its superior efficacy for prevention and treatment of allograft obliterative airway disease in vivo. Trans Proc 29: 614-615, 1997

372. Cao W, Mohasci P, Shorthouse R, Pratt R, Morris R E. Effects of Rapamycin on growth factor stimulated vascular smooth muscle cell DNA synthesis. Transplantation 59: 390-95, 1995

373. Wang W, Chan J, Lee W, Chan L. Effect of Rapamycin and FK506 on renal mesangial cell proliferation; Abstract O660: International Congress of the Transplantation Society, Rome 2000

374. Hultsch T, Martin R, Hohman R J. The effect of the immunophilin ligands Rapamycin and FK506 on proliferation of mast cells and other haematopoetic cell lines. Mol Biol Cell 3: 981-987, 1992

375. Zeevi A, Woan G Z, Yao R, Venkataramanan S, Todo S et al. Comparative in vitro studies on the immunosuppressive activities of MMF, Bredinin, FK506, Cyclosporin and Rapamycin. Trans Proc 23: 2928-2930, 1991

376. Dodge I, Demirci G, Strom T B, Li X C. Rapamycin induces TGFβ production by lymphocytes. Transplantation 70: 1104-1106, 2000

377. Khanna A K. Mechanism of the combination immunosuppressive effects of Rapamycin with either Cyclosporin or Tacrolimus. Transplantation 70: 690-4, 2000

378. Gregory C, Huie P, Billingham M E, Morris R E. Rapamycin inhibits arterial intimal thickening caused by both allo-immune and mechanical injury. Transplantation 55: 1409-1418, 1993

379. Morris R E, Cao W, Huang X, Gregory C R, Billingham M E et al. Rapamycin inhibits vascular smooth muscle DNA synthesis in vitro and suppresses narrowing in arterial allografts and in balloon injured carotid arteries: Evidence that Rapamycin antagonizes growth factor action on immune and non-immune cells. Trans Proc 27: 430-431, 1995

380. Gregory C R, Huang X, Pratt R E, Dzau V J, Shorthouse R et al. Treatment with Rapamycin and MMF reduces arterial intimal thickening produced by mechanical injury and allows endothelial replacement. Transplantation 59: 655-661, 1995

381. Pham S M, Shears L L, Kawaharada N, Li S, Venkataramanan R, Sehghal S. High local production of nitric oxide as a possible mechanism by which Rapamycin prevents transplant arteriosclerosis. Trans Proc 30: 953-954, 1998

382. Shears L L, Kawaharada N, Tzeng E, Billiar T R, Watkins S C et al. Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. J Clin Invest 100: 2035-42, 1997

383. Tzeng E, Shears L L, Robbins P D, Pitt B R, Geller D A et al. Vascular gene transfer of human nitric oxide synthase. Characterisation of activity and effects of myointimal hyperplasia. Mol Med 2: 211-25, 1996

384. Geerling R.A, De Bruin R W F, Scheringa M, Bonthius F, Jeekel J et al. Suppression of acute rejection prevents graft arteriosclerosis after allogenic aorta transplantation in the rat. Transplantation 58: 1258-1263, 1994

385. Jain S, Bicknell G R, Whiting P H, Nicholson M L. Rapamycin reduces the expression of fibrosis associated genes in an experimental model of renal ischaemia re-perfusion injury; Abstract PO464W: International Congress of the Transplantation Society, Rome 2000

386. Morris R E, Huang X, Gregory C R, Billingham M E, Rowan R et al. Studies in experimental models of chronic rejection. Use of Rapamycin and Isosazole derivatives for the suppression of graft vascular disease and obliterative bronchiolitis. Trans Proc 27: 2086-2089, 1995

387. Zhu J, Wu J, Frizell E, Liu S L, Bashey R et al. Rapamycin inhibits hepatic stellate cell proliferation in vitro and limits fibrogenesis in an in vivo model of liver fibrosis. Gastroenterology 117: 1244-6, 1999

388. Meiser B M, Billingham M E, Morris R E. Effects of cyclosporin, FK506, and rapamycin on graft vessel disease. The Lancet 338: 1297-1298, 1991

389. Schmid C, Heemann U, Tilney N L. Factors contributing to the development of chronic rejection in heterotopic rat heart transplantation. Transplantation 64: 222-228, 1997

390. Hasegawa S, Mitchell R, Nagano H, Stinn J, Libby P. Rapamycin reduces graft coronary arteriosclerosis and attenuates expression of IFNγ, MHC II and VCAM-1 in murine cardiac allografts. J Heart Lung Transpl 16: A97, 1997

391. Wasowsksa B, Hancock WW, Onodera K, Korom S, Stadlbauer T H W et al. Rapamycin and Cyclosporin A treatment: A novel regimen to prevent chronic allograft rejection in sensitized hosts. Trans Proc 29: 333, 1997

392. Wasowska B, Wieder K J, Hancock W W, Zheng X X, Berse B et al. Cytokine and alloantibody networks in long-term cardiac allografts in rat recipients treated with Rapamycin. J Immunol 156: 395-404, 1996

393. Poston R S, Billingham M, Grant Hoyt E, Pollard J, Shorthouse R et al. Rapamycin reverses chronic graft vascular disease in a novel cardiac allograft model. Circulation 100: 67-74, 1999

394. Coupes B M, Williams S, Roberts I S D, Short S D, Johnson R W G. Plasma TGFβ-1 expression during a randomised study of therapy with Cyclosporin A and Sirolimus versus Sirolimus in renal allograft recipients; Abstract PO544: International Congress of the Transplantation Society, Rome 2000

395. Xavier P, Oliveira J G G, Henriques C, Mota A, Sampaio S et al. Cytokine changes with Rapamycin in renal transplants; Abstract PO500W: International Congress of the Transplantation Society, Rome 2000

396. Tang A W C, Walker R G, Nicholls K M, Becker G J. Sirolimus does not potentiate the indices of chronic Cyclosporin nephrotoxicity; Abstract PO532: International Congress of the Transplantation Society, Rome 2000

397. Vankawala R, Kode R K, Damask A M, Stabler S, Laftavi M R et al. Conversion from Mycophenolate to Rapamycin for acute and chronic rejection. Abstract 1069: AST/ASTS Chicago, USA 2001

398. Peraldi M N, Morelon E, Mamzer-Bruneel M F, Burke J, Kreis H. Renal function improvement after switch from calcineurin dependent drugs to Sirolimus in renal transplant recipients with chronic graft nephropathy; Abstract PO633: International Congress of the Transplantation Society, Rome 2000

399. Nishida S, Pinna A, Weppler D, Verzaro R, Levi D et al.. Rescue of chronic rejection after liver transplantation with Sirolimus; Abstract PO808: International Congress of the Transplantation Society, Rome 2000

400. Haddad H, MacNeil D M, Howlett J, O'Neill B. Sirolimus, a new potent immunosuppressant agent for refractory cardiac transplantation rejection: two case reports. Can J Cardiol 16: 221-4, 2000

401. Cockcroft D W, Gault M H. Prediction of creatinine clearance from serum creatinine. Nephron 16: 31-41, 1976

402. Kasiske B and Keane M. Laboratory assessment of renal disease. In Brenner and Rector's The Kidney 5th published by W B Saunders Co, 1143-1146, 1996

403. Butterworth PC. Harris KP. Nicholson ML. Comparison of glomerular filtration rate and serum creatinine in renal transplant patients. Trans Proc 29: 2776-7, 1997

404. Saunders R N, Metcalfe M S, Nicholson M L. Rapamycin in transplantation: A review of the evidence. Kid Int 59: 3-17, 2001

405. Johnson R W G, Kreis H, Oberbauer R, Brattstrom C, Claesson K et al. Sirolimus allows early Cyclosporin withdrawal in renal transplantation resulting in improved renal function and lower blood pressure. Transplantation 72: 777-786, 2001

406. Dominguez J, Mahalati K, Kiberd B, McAlister V C, MacDonald A S. Conversion to Rapamycin immunosuppression in renal transplant recipients: Report of an initial experience. Transplantation 70: 1244-7, 2000

407. Bicknell G R, Shaw J A, Pringle J H, Furness P N. Amplification of specific mRNA from a single human renal glomerulus, with an approach to the separation of epithelial cell mRNA. J Pathol 180: 188-93, 1996

408. Inis M Gelfand D. Optimisation of PCRs. In Innis M, Gelfand D, Sninsky J, White T eds. PCR protocols: A guide to methods and applications. San Diego: Academic Press Inc, 3-11, 1990

409. Saiki R. The design and optimisation of the PCR. In Erlich H ed. PCR technology. Principles and applications for DNA amplification New York: W H Freeman & Co, 7-16, 1992

410. Kawasaki E, Wang A. Detection of gene expression. In Erlich H ed. PCR technology. Principles and applications for DNA amplification New York: W H Freeman & Co, 89-97, 1992

411. Mullis K, Faloona F, Scharf S, Saiki R, Horn G et al. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp Quant Biol 51 Pt 1: 263-73, 1986

412. Hall L L, Bicknell G R, Primrose L, Pringle J H, Shaw J A et al.. Reproducibility in the quantification of m RNA levels by RT-PCR-ELISA and competitive RT-PCR-ELISA. Biotechniques 24: 652-8, 1998

413. Murphy G J, Bicknell G R, Saunders R N, Metcalfe M S, Nicholson M L. Combined Rapamycin / Neoral therapy effectively inhibited allograft vasculopathy in rat allografts but attenuated changes in profibrotic gene expression associated with Rapamycin alone. Abstract 467: AST/ASTS Chicago, USA 2001

414. Conde M, Andrate J, Bedoya F J, Maria Santa C, Sorino F. Inhibitory effect of cyclosporin A and FK506 on nitric oxide production by cultured macrophages: Evidence of a direct effect on nitric oxide synthase activity. Immunol 84: 476-481, 1995

415. Marumo T, Nakaki T, Hishikawa K, Suzuki H, Kato R et al. Cyclosporin A inhibits nitric oxide synthase induction in vascular smooth muscle cells. Hypertension 25: 764-768, 1995

416. Wu M, Yang C, Bens M, Yu H, Huang J et al. Cyclosporin inhibits nitric oxide production in medullary ascending limb cultured cells. Neph Dial Transplant 13: 2814-2820, 1998

417. Reddy G K, Enwemeka C S. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 29: 225-9, 1996

418. Weatherford T W. Staining of collagenous and non-collagenous structures with picrosirius red F3BA. J Med Sci 9: 383-388, 1972

419. Junqueira L C U, Cossermelli W, Brentani R. Differential staining of collagens type I, II and III by Sirius red and polarization microscopy. Arch Histol 41: 267-274, 1978

420. Bonafe J L. Pieraggi M T, Rochiccioli P. Etude du collagene dermique par la coloration du rouge Sirius au cours de maladies congenitales ou aquises du tissu conjonctif. Ann Dermatol Vener 108: 635-640, 1981

421. Pierard G E. Sirius Red polarization method is useful to visualise the organisation of connective tissues but not the molecular composition of their fibrous polymers. Matrix 9: 68-71, 1989

422. Campbell S E, Janicki J S, Matsubara B B, Weber K T. Myocardial fibrosis in the rat with mineralocorticoid excess. Prevention of scarring by amiloride. Am J Hypertens 6: 487-495, 1993

423. Rubio C A, Porwit A. Quantitation of fibrosis in liver biopsies. Anal Quant Cytol Histol 10: 107-108, 1988

424. Malkusch W, Rehn B, Bruch J. Advantages of Sirius Red staining for quantitative morphometric collagen measurements in lungs. Exp Lung Res 21: 67-77, 1995

425. Valderrama R, Navarro S, Campo E, Camps J, Gimenez A et al. Quantitative measurement of fibrosis in pancreatic tissue: Evaluation of a colorimetric method. Int J Pancreatol 10: 23-29, 1991

426. Flores O, Arevalo M, Gallego B, Hidalgo F, Vidal S et al. Beneficial effect of the long term treatment with the combination of an ACE inhibitor and a calcium channel blocker on renal injury in rats with $5/6^{th}$ nephrectomy. Exp Nephrol 6: 39-49,1998

427. James J, Bosch K S, Zuyderhoudt F M, Houtkooper J M, Van Gool J. Histophotometric estimation of volume density of collagen as an indication of fibrosis in rat liver. Histochem 85: 129-133, 1989

428. Menabe N, Chevallier M, Chossegros P et al. Interferon–alpha 2B therapy reduces liver fibrosis in chronic non-A non-B hepatitis. A quantitative histological evaluation. Hepatol 18: 1344-1349, 1993

429. Moreso F, Gratin C, Vitria J, Condom E, Poveda R et al. Automatic evaluation of renal interstitial fraction. Trans Proc 27: 1721-1727, 1995

430. Moreso F, Seron D, Vitria J, Grinyo J M, Colome-Serra F M et al. Quantification of interstitial chronic renal damage by means of texture analysis. Kid Int 46: 1721-1727, 1994