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Transforming growth factor beta 1: Role in the progression of chronic renal failure

Mahmoud Salah Khalil

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Abstract

TGF-β1 plays an important role in the pathogenesis of experimental and clinical glomerulosclerosis and tubulointerstitial fibrosis. Associations have been described between polymorphisms of cytokine and growth factor genes and susceptibility to, or progression of, an increasing number of diseases. In this study, single nucleotide polymorphisms (SNPs) in the TGFB1 gene were investigated as possible markers for the progression of chronic renal failure (CRF). One hundred and forty two Caucasian patients with CRF were screened for four TGFB1 SNPs: T-509C in the promoter region; Arg25Pro and Leu10Pro in exon 1 and Thr263Ile in exon 5. There were significant differences between CRF patients and controls in allele frequencies of two of the SNPs (Leu10Pro and C-509T), indicating an association with susceptibility to CRF. We also observed a significant association between rate of progression of CRF (the slope of the reciprocal of serum creatinine v time) and genotype, both at codon 25 (odds ratio 3.77, 95% confidence interval, 2.2 - 6, p <0.001) and at the -509 promoter site (odds ratio 1.67, 95% confidence interval 1.1-2.5), p <0.005) in patients with primary nephropathy (excluding PKD). Genotype at codon 25 was also associated with severity of proteinuria (p= 0.038), plasma TGF-β1 protein levels (p = 0.01), and the severity of glomerulosclerosis (p<0.05). Genotype at C-509T was associated with the level of renal tubular TGF-β1 immunostaining (p = 0.0006) and with renal interstitial inflammatory cellular infiltration (p=0.015). There was a highly significant correlation between the degree of cellular infiltration in renal tissues and tubular TGF-β1 immunostaining.

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ABBREVIATIONS

AAV: ANCA-associated vasculitis

ABC: avidin-biotin-peroxidase

ACE: angiotensin I converting enzyme

ActR: activin receptor type

ADPKD: Autosomal dominant polycystic kidney disease

AEC: 3' amino- 9' ethyl-carbimazole

AGT: angiotensinogen

ALK: activin receptor-like kinase

AMHR: anti-mullerian hormone receptor

Ang II: angiotensin II

Apo: apolipoprotein

Arg: arginine

ARMS-PCR: amplification refractory mutation-screening polymerase chain reaction

AT1R: angiotensin type 1 receptor

bFGF: basic fibroblast growth factor

BMPs: bone morphogenic proteins

CAPD: continuous ambulatory peritoneal dialysis

CGN: crescentic glomerulonephritis

CIN: chronic interstitial nephritis

CrCl: creatinine clearance

CRD: chronic renal diseases

CREB: cAMP response element binding protein

CRF: chronic renal failure

CTGF: connective tissue growth factor

D': coefficient of linkage disequilibrium

DBP: diastolic blood pressure

DBPd: diastolic blood pressure at diagnosis

DBPf: diastolic blood pressure during follow up

DM: diabetes mellitus

DN: diabetic nephropathy

DNA: deoxyribonucleic Acid

dNTPs: deoxynucleotide triphosphates

EcNOS: endothelial constitutive nitric oxide synthase

ECM: extracellular collagenous matrix

EGF: epidermal growth factor

ELISA: enzyme linked immunoassay

ERK: extracellular signal-regulated kinases

ESRD: end stage renal disease

ESRF: end stage renal failure

ET1: endothelin 1

FGF: fibroblast growth factor

FSGS: focal and segmental glomerulosclerosis

GDFs: growth and differentiation factors

GDNF: glial cell line-derived neurotrophic factor

GLUT: glucose transporter

GN: glomerulonephritis

GS: glycine serine domain

gTGF-β1: glomeruli TGF-β1

gTGF-β1+: glomeruli stained with TGF-β1

gTGF-β1-: glomeruli did not stained for TGF-β1

HD: haemodialysis

ICI: interstitial cellular infiltration

ICI-: renal tissue without inflammatory cellular infiltration

ICI+: renal tissue with inflammatory cellular infiltration.

IGF-1: insulin-like growth factor-1

IF: interstitial fibrosis

IL-1: interleukin 1

IL1ra: interleukin 1 receptor antagonist

IL1RN*2: interleukin 1 receptor antagonist allele 2

Ile: isoleucine

KLK: kallikrein

LAP: latency associated peptide

LDL: low-density lipoproteins

Leu: leucine

LLC: large latent complex

LTBP: latent precursor molecule binding protein

LTGF-β: latent precursor molecule

MAP: mean arterial blood pressure

MAPK: mitogen-activated protein kinase

MCGN: mesangiocapillary glomerulonephritis

MCP-1: monocyte chemo-attractant protein 1

MDRD: modification of diet in renal disease

MH: mad homology

MIF-2: macrophage inhibitory factor-2

MIS: mullerian inhibitory substance

MMP: metalloproteinase

MN: membranous nephropathy

MRFIT: multiple Risk Factor Intervention Trial

mRNA: messenger ribonucleic acid

MTHFR: methylenetetrahydrofolate reductase

NGF: nerve growth factor

NO: nitric oxide

NOS: nitric oxide synthase

NP: non-progressive chronic renal failure

OU: obstructive uropathy

P: progressors chronic renal failure

PAI-1: plasminogen activator inhibitor-1

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PKD: polycystic kidney disease

Pro: proline

RI: type 1 transforming growth factor beta receptor

RII: type 2 transforming growth factor beta receptor

RIII: type 3 transforming growth factor beta receptor

RANTES: regulated on activation, normal T expressed and secreted

RRT: renal replacement therapy

SARA: Smad anchor for receptor activation

SBPd: systolic blood pressure at diagnosis

SBPf: systolic blood pressure during follow up

$1/S_{Cr}$: reciprocal of serum creatinine

SLC: small latent complex

SLE: systemic lupus erythematosus

SMAD: signalling mother against decapentaplegic protein

SNPs: single nucleotide polymorphisms

TBE: Tris borate EDTA

TβR: transforming growth factor beta receptor

TGF-α: transforming growth factor alpha

TGF-β1: transforming growth factor beta-1

Thr: therionine

TIF: tubulointerstitial fibrosis

TIMP: tissue inhibitors of metalloproteinase

TNF-α: tumour necrosis factor alpha

tRNA: transferase ribonucliec acid

TSP-1: thrombospondin1

tTGF-β1: tubular TGF-β1

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

Contents

I- Abstracts and posters:

1- **Khalil, M.S.;** Blakemore, A.I.F. and El Nahas, A.M. (2001): Transforming growth factor beta-1 (TGF-β1) polymorphisms as a predictor of progressive renal insufficiency. Am Soc Nephrol: 12: 817A.

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II- Paper:

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1.1 Introduction

Transforming growth factor beta-1 (TGF-β1) is a multifunctional growth factor implicated in the pathogenesis of experimental and clinical chronic renal failure (CRF) (Okuda, 1990; Yamamoto, *et al.*, 1993). It has potent fibrogenic properties through the stimulation of synthesis of extracellular collagenous matrix (ECM) and inhibition of its breakdown (Border and Noble, 1997). Although there are three types of TGF-β (1,2 and 3), TGF-β1 is the growth factor that has been implicated in fibrogenesis (Roberts and Sporn, 1997). There is considerable experimental and clinical evidence pointing to an important role of TGF-β1 in renal fibrogenesis (section 1.6.2) (Border and Noble, 1994, 1997, Peters, *et al.*, 1999, Basile, 1999). However, a clear-cut association between TGF-β1 and the progression of clinical nephropathies is lacking. In this thesis, the potential relationship between TGF-β1 genetic polymorphisms and the progression of human nephropathies is studied.

1.2 Chronic renal failure (CRF)

Chronic renal failure (CRF) is defined as irreversible, long-standing loss of renal function. End stage renal failure (ESRF) refers to advanced renal insufficiency when the glomerular filtration rate is below 10 ml/min prior to the initiation of either dialysis or renal transplantation (El Nahas and Winearls, 1997). The patients complain of a wide range of symptoms including poor appetite, vomiting, bone pain, headache, insomnia, itching, dry skin, malaise, fatigue with light activity, muscle cramps, and change in mental alertness (El Nahas & Winearls, 1997). A survey of UK hospital biochemistry records revealed that the prevalence of CRF (plasma creatinine concentration >150 μmol/l) is 2058 adults per million population (pmp) (UK Registry, 2001). The data also revealed the incidence of ESRF to be around 78 pmp/year (UK Registry, 2001). Data from the UK registry suggests that the incidence of ESRF in the UK is currently around 80-110 pmp/year (UK Registry, 2001). Corresponding data from the United States (US) suggest an incidence of 315 pmp/year; US prevalence is currently around 1217 pmp (USRDS, 2001). In addition, there is little doubt that the incidence of ESRF is steadily

increasing by around 8-10% every year (USRDS, 2001). One of the major causes of ESRF in the US is diabetes mellitus (DM) (41.8 %). In Norway, type 1 DM progresses to ESRF in 40% of patients, while type 2 is thought to lead to renal involvement in 20% of patients (Type 2 DM is 10 times as common as Type 1) (Bergrem and Leivestad, 2001). In the US, the second most common cause of ESRF is hypertension (25.4%). Thirdly, chronic glomerulonephritis accounts for 21% of patients with end stage renal diseases (ESRD). Polycystic kidney disease and urologic disease account for approximately 15% and 20% of cases of ESRD have no known cause (USRDS, 2001). On the other hand, in the UK, the most common cause of ESRF remains chronic glomerulonephritis as the incidence and prevalence of diabetic nephropathy remains below 20% (UK Registry 2001).

1.2.1 Natural history of chronic renal failure

Progression of CRF occurs following renal injury regardless of the underlying cause of nephropathy (For review see Locatelli and Del Vecchio, 2000). The reciprocal of serum creatinine ($1/S_{Cr}$) regression slope value against time (described below) determines the rate of decline of renal function (progression of CRF). When the pattern of the regression is a straight line this means that the rate of the progression of CRF occurs at a constant rate (Mitch, *et al.*, 1976, Rutherford, *et al.*, 1977, Bleyer, 1999). Generally speaking the rate of decline of renal function is constant, implying that the process causing the decline of renal function is continuous. It was reported in the late seventies, that the progression of CRF from various diseases was occurring at a constant rate (linear pattern of $1/S_{Cr}$ value against time) in the majority of patients (Mitch, *et al.*, 1976 and Rutherford, *et al.*, 1977). A non-linear decline of renal function occurred in around 15% of patients (Shah and Levey, 1992). Further, some patients have a change in the regression line of the reciprocal of serum creatinine against time suggesting a spontaneous acceleration or slowing down of the rate of decline. These spontaneous changes are referred to as breakpoints in the slope which may be due to intercurrent events such as, infection, dehydration, poor blood pressure control, or changes in the activity level of the underlying pathological processes which initiated the renal disease (Shah and Levey, 1992).

1.2.2 Factors influencing the progression of chronic renal failure

1.2.2.1 Age

Ageing is associated with physiological changes in the kidneys, including a reduction in renal plasma flow (Fliser, *et al.*, 1993), increase in filtration fraction (Baylis, *et al.*, 1990, Flisher, *et al.*, 1993) and a decrease in renal size due to parenchymal reduction (Emamian, *et al.*, 1993). Also, primary involution can be detected in the renal cortex with relative sparing of renal medulla (Hollenberg, *et al.*, 1974). Furthermore, 10 – 30% of the total glomeruli are sclerosed between the fourth and eighth decades of life (Kaplan, *et al.*, 1975). It is often assumed that GFR is nearly 50% at the age of 80 compared to values after puberty (Kaysen and Myers, 1985). Lindeman and coworkers (1985) showed that creatinine clearance was not decreased in one third of healthy elderly people. Many studies have shown that the incidence of renal failure from various renal diseases increases with age (Feest, *et al.*, 1990, McGowen, 1990 and Jungers, *et al.*, 1996). Berthoux and colleagues (1998) reported that renovascular diseases and diabetes mellitus (type 2) are the most common causes of ESRD in the elderly. The same study also showed that primary glomerulonephritis is common in the elderly as it constitutes about 12% of ESRD in the elderly. The prognosis of renal diseases in the elderly is also more severe than in the younger age group as they have, in general, a higher rate of progression compared to younger patients with similar nephropathies (For review see Locatelli and Del Vecchio, 2000).

1.2.2.2 Gender

Regardless of the cause of CRF, ESRD is more common in males than females (For review, Locatelli and Del Vecchio, 2000). The USRDS study (2001) revealed that the incidence of ESRD in men was 348 pmp/year compared to 242 pmp/year in women (URSDS, 2001). Furthermore, the rate of decline in renal function is usually faster in males regardless of the underlying nephropathy (Hannedouche, *et al.*, 1993). Some postulated that the decline of renal function is faster in males due to a higher protein ingestion, larger muscle mass and increased creatinine generation (Levey, *et al.*, 1989).

This is unlikely. Others argued that hormonal factors, including oestrogens, may play an important protective role in females with CRF (Velasquez and Bhathena, 2001; For review, Neugarten, *et al.*, 2000). Because the faster decline in renal function occurs only after puberty, it was assumed that sex hormones might play a role in it (Reckelhoff, *et al.*, 1997, Neugarten, *et al.*, 2000). The slope of progression in males correlates with the mean arterial blood pressure, which is generally higher in males. In females, it correlates with the type of nephropathy (For review see Locatelli and Del Vecchio, 2000).

1.2.2.3 Race

In the US, African-Americans have a four-fold higher incidence of ESRD compared to Caucasians (USRDS, 2001). Freedman and colleagues (1997) reported that the greater risk of ESRF in African-Americans might be due to a genetic predisposition. This may also be explained by higher levels of circulating fibrogenic growth factors in African-Americans compared to others (Suthanthiran, *et al.*, 2000). Also, it was reported that incidence of increased blood pressure levels in black individuals is twice as high as that in the white population (Cornoni, *et al.*, 1989). The high blood pressure may contribute to the higher incidence of ESRF. Furthermore, in essential hypertension there is increased prevalence of microalbuminuria in black individuals (Summerson, *et al.*, 1995). The prevalence of DM is twice as high in African-Americans as in Caucasians and ESRD due to DM is 3-6 times more common in African-Americans (Carter, *et al.*, 1996). Also, ESRF was higher in Native Americans and Asian/Pacific Islanders than Caucasians (USRDS, 2001). The incidence of CRF is increased in the first and second-degree relatives of those with renal disorders (Ferguson, *et al.*, 1988). In Caucasians this relationship is weaker than in African-Americans. Pugh and colleagues (1988) found that Mexican-Americans were also at a higher risk of developing ESRD than other populations. In the UK, reports suggest a higher prevalence of renal disease in some Asian individuals although the rate of progression of the nephropathies was not found to be faster than Caucasians (For review, Wing and Jones, 2000, UK Registry, 2001).

1.2.2.4 Systemic hypertension

Systemic hypertension is one of the most important factors contributing to the deterioration of renal function and elevated blood pressure, can be a cause or a consequence of renal injury (For review, Adamczak, *et al.*, 2002). The higher the blood pressure the faster the rate of decline of renal function and the progression of CRF (Tiernay, *et al.*, 1989). Brazy and coworkers (1989), showed that increase in mean diastolic blood pressure (DBP) > 90 mmHg was associated with a greater rate of decline in the reciprocal of serum creatinine versus time compared to patients with CRF with a mean DBP < 90 mmHg). Furthermore, there is improvement in renal function with intensive antihypertensive therapy (For review, Locatelli and Del Vecchio, 2000 and Adamczak, *et al.*, 2002). It has even been suggested that a stabilization and even a regression of the progression of CRF can be achieved with aggressive antihypertensive therapy (For review, Dworkin and Weir, 2000). The Modification of Diet in Renal Disease (MDRD) Study Group showed that the lower the mean arterial blood pressure (MAP) value, the slower the decline in GFR (Klahr, *et al.*, 1994). This study implied that lower blood pressure targets should be sought for patients with progressive CRF and high levels of proteinuria (Klahr, *et al.*, 1994). In those with protein excretion rate in excess of 3 g/24h, it suggested that the MAP should be reduced to levels around 92 mmHg to obtain the same protective effect of renal function a MAP of 97 mmHg would provide those with less proteinuria (Klahr *et al.*, 1994, Peterson, *et al.*, 1995).

1.2.2.5 Proteinuria

Proteinuria is an important prognostic indicator of renal disease. Kincaid-Smith and Becker (1978) showed that the presence of proteinuria predicted the progression of CRF in patients with chronic pyelonephritis. The presence of proteinuria indicates poor prognosis in most cases of primary glomerulonephritis (Williams, *et al.*, 1988 and Cameron, 1989). El Nahas and colleagues (1984) were the first to demonstrate that a reduction of proteinuria by dietary protein restriction predicted the renal functional response to the diet. Bjorck (1986) Apperloo (1994) and their colleagues showed that pharmacological interventions that slow the progression of CRF are associated with a reduction of proteinuria. More recently, a large body of experimental evidence has

suggested that proteinuria is not only a marker of poor prognosis but may also be a mediator involved in the progressive scarring process (Bruzzi, *et al.*, 1997, Eddy, 2001, Jafar, *et al.*, 2001). Remuzzi and Bertani (1998) postulated that the excessive filtration of macromolecules, including protein, into the glomerulus could accelerate sclerosis. Burton and Harris (1996) suggested that proteinuria might also cause tubular dysfunction and damage. Renal tubular damage caused by proteinuria can initiate inflammatory and fibrotic changes within the renal interstitium. The activation of proximal tubular cells by excessive exposure to proteins leads to their release of pro-inflammatory cytokines and chemokines as well as their release of profibrotic growth factors such as platelet derived growth factor (PDGF) and TGF-β1 (For review, Harris, 2000; Eddy, 2001; Wardle, 2001, 2002). It was also hypothesised that mesangial accumulation of proteins may produce mesangial cell injury and proliferation and consequently increases production of mesagial matrix and glomerulosclerosis (For review, Harris, 2000). Also, activation of tubular cells by proteinuria can stimulate their release of extracellular matrix (ECM) components, accelerating interstitial fibrosis (For review see, Harris, 2000). Consequently, proteinuria is, along with systemic hypertension, thought to be one of the most significant risk factors in progressive CRF. Increasingly, attention is paid when hypertension is treated in patients with CRF to lower proteinuria as well (Jafar, *et al.*, 2001, Adamczak, *et al.*, 2002). Data from a European study suggested that the control of systemic hypertension without the concomitant reduction of proteinuria is ineffective (Locatelli, *et al.*, 1996).

1.2.2.6 Dyslipidaemia

It was postulated over twenty years ago by Moorhead and his colleagues (1982) that lipids could be toxic to both the glomeruli and the tubulointerstitium. Since, a growing body of experimental and clinical data has supported this hypothesis (Attman, *et al.*, 1999; Samuelsson, *et al.*, 1997). An association has been shown between hypercholesterolaemia and the progression of diabetic (Krowleski, *et al.*, 1994) and nondiabetic (Samuelsson, *et al.*, 1997) nephropathies. Hyperlipidaemia can induce glomerular toxicity through the accumulation of low-density lipoproteins (LDL) as well as oxidized LDL in the mesangium, leading to structural and functional changes in mesangial cells. Also, hyperlipidaemia increase glomerular capillary pressure, which may

contribute to glomerular hypertension and sclerosis (Keane, *at al.*, 1988, Keane, 2000). It was also concluded that hyperlipidemia activates mesangial cells and lead to mesangial matrix accumulation (Keane, *et al.*, 1988). Harris and colleagues (1990) showed that a diet low in essential fatty acids protects against tubulointerstitial inflammation. Kees-Folt and coinvestigators (1994) showed that free fatty acids lead to the generation of a lipid chemotactic factor, which attracts monocytes and initiates tubulointerstitial inflammation. So a high level of non-essential fatty acids may play a role in the inflammatory and fibrotic processes in the renal interstitium. Further, it was advanced by some that the nephrotoxicity of proteinuria/albuminuria may be linked to their lipid-carrying capacity (For review, Harris, 2000). Lipids rather than protein may be the culprit regarding tubulointerstitial inflammation and fibrosis in heavy proteinuric states (For review, Harris, 2000). In experimental animals, there is a large number of experiments showing that the reduction of blood lipids levels is associated with a protective effect on kidney scarring (Keane, 2000, Praga, 2002). However, similar data is still lacking in humans with CRF, although a recent review analysis suggested that lowering lipids in patients with CRF may be beneficial (Fried, *et al.*, 2001).

1.2.2.7 Smoking

Smoking has a deleterious effect on renal function, as evidenced by the development of microalbuminuria and its progression to overt albuminuria in diabetic nephropathy (Chase, *et al.*, 1991). Smoking of 15 packs/year in non-diabetic patients increases the risk of development of ESRD by 5.8 fold (Orth, *et al.*, 1998). Haemodialysed diabetic patients who smoked cigarettes had higher systolic blood pressure and fibrinogen levels and had a higher incidence of myocardial infarction, than non-smokers (Biesenbach and Zazgornik, 1996). A retrospective multicentre European case-control study showed that smoking is an independent risk factor for ESRF in patients with both inflammatory and non-inflammatory renal disease, i.e. IgA glomerulonephritis and polycystic kidney disease (Orth, *et al.*, 2000). Smoking increases the severity of glomerulonephritis, particularly in men older than 40 and/or hypertensive patients (Stengel, *et al.*, 2000, Regalado, *et al.*, 2000). Furthermore, it was reported in the Multiple Risk Factor Intervention Trial (MRFIT) study that smoking increases the renal risk especially in the male population

(Walker, *et al.*, 1992). The calculated relative risk for ESRF was found to be 1.69 for smokers as compared to non-smokers (Shoji, *et al.*, 2001). The deleterious effect of cigarette smoking may be through exacerbation of other risk factors such as high blood pressure, proteinuria or hyperlipidaemia. Moreover, progressive kidney failure is associated with decreased elimination of nicotine by both renal and non-renal mechanisms (Molander, *et al.*, 2000). Furthermore, it was postulated that smoking induces renal damage by increasing blood pressure, alteration of intrarenal hemodynamics and activation of the sympathetic nervous system. Discontinuation of smoking improved renal prognosis and is probably a very effective measure to retard progression of renal failure (Praga, 2002).

1.2.2.8 Alcohol

It was reported that cross-sectional data from the 1983 (National Health Interview Survey) showed that hypertensive women consumed less alcohol than non-hypertensive women (Laforge, *et al.*, 1990). On the other hand, the same authors reported that alcohol consumption was significantly associated with a greater risk of hypertension amongst men. Moreover, beer consumption and spirits consumption above three drinks/day were significant predictors of male hypertension (Laforge, *et al.*, 1990). The consumption of more than two alcoholic drinks per day is associated with an increased risk of ESRD and hypertension in the general population (Laforge, *et al.*, 1990). On the other hand, a lower intake of alcohol (< 2 drinks/day) did not appear to be harmful (Perneger, *et al.*, 1999, Parekh and Klag, 2001). The mechanisms by which alcohol consumption leads to hypertension and, perhaps, renal disease are unknown.

1.2.2.9 Recreational drugs

Use of heroin and/or cocaine increases the risk of ESRD (Perneger, *et al.*, 2001). After adjusting for patients' age, sex, race, socioeconomic status, and history of hypertension and diabetes, persons who had ever used heroin or other opiates (any amount) were at increased risk for ESRD (Perneger, *et al.*, 2001). There was also an increased risk for individuals who consumed crack cocaine, although it was difficult to dissociate it from

heroin consumption (Perneger, *et al.*, 2001). The same authors suggested that cocaine might cause accelerated hypertension, acute renal failure from rhabdomyolysis, and progression of pre-existing renal disease.

1.3 Mechanisms of progression of chronic renal failure

1.3.1 Glomerulosclerosis

1.3.1.1 Systemic and glomerular hypertension

As previously discussed, systemic hypertension not only induces CRF but also induces a faster loss of renal function in acquired renal diseases and loss of renal function associated with normal ageing (Adamczak, *et al.*, 2002). In the subtotal nephrectomy model of CRF, the loss of function of some nephrons leads to hypertrophy of the remaining nephrons and decrease of vascular resistance (especially the afferent renal arterioles) due to impaired autoregulation (For review see Dworkin and Weir, 2000). This process leads to an increase in the glomerular capillary pressure of the remaining nephrons (glomerular hypertension) (Anderson, *et al.*, 1986). It was postulated that there was a significant association between systemic hypertension and the increment of glomerular capillary pressure (For review see Dworkin and Weir, 2000). The harmful effects of renal parenchymal disease, e.g. diabetes mellitus and systemic hypertension, are a result of production or accentuation of glomerular capillary hypertension, rather than induction of extraglomerular vascular injury or glomerular ischaemia. Histological examination reveals an increase of glomerular volume and glomerular sclerosis in hypertensive patients (For review see Dworkin and Weir, 2000). In the presence of an underlying nephropathy, the glomerular response to systemic hypertension is impaired (For review see Dworkin and Weir, 2000). This leads to a rise in glomerular pressure and progression of glomerulosclerosis. Studies suggest that the control of glomerular capillary pressure protects against kidney deterioration with or without control of systemic hypertension (Anderson and Brenner, 1987, For review, Anderson 2000, Dworkin and Weir 2000).

Lowenstein and coworkers (1970) also argued that systemic hypertension leads to ischaemia due to renal vascular disease which, in turn, leads to a decrease in glomerular perfusion/ischaemia. Glomerulosclerosis induces efferent arteriolar hypoperfusion and hypoxia (decreased oxygen delivery) of the tubular cells and their release of cytokines such as TGF-β1, platelet-derived growth factor (PDGF), endothelin and vascular endothelial growth factor (VEGF) (Orphanides, *et al.*, 1997; Fine, *et al.*, 1998).

Both systemic hypertension and non-hypertensive injury that cause loss of single nephron units results in hypertension in the remaining glomeruli (glomerular hypertension). Glomerular hypertension can lead to injury to the glomerular capillary wall causing it to leak plasma proteins into the urine (Anderson and Brenner, 1987; Anderson, 2000).

1.3.1.2 Role of proteinuria

Systemic and glomerular hypertension are both associated with proteinuria. Olson and colleagues (1985) demonstrated that glomerular hypertension leads to transudation of plasma proteins into the endothelial and subendothelial spaces which can promote glomerular hyalinosis, thereby narrowing and occluding glomerular capillaries. Increased trafficking and accumulation of proteinacious molecules in the glomerular mesangium may also contribute to the pathogenesis of glomerulosclerosis (Bertani and Remuzzi, 1998). Rennke and Klein (1989) showed that the increment of glomerular permeability to protein affected not only the glomerular mesangium but also the glomerular epithelial cells leading to structural and functional changes and this further increased the passage of protein molecules across the glomerular capillaries (For review, Harris 2000, Anderson 2000).

1.3.1.3 Renal hypertrophy

Loss of function of some of the nephrons leads to an increase in the size and cellular number of the remaining glomeruli (Wesson, 1989; Anderson and Meyer, 1997). Several growth factors such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and TGF-β1 are responsible for the

hypertrophy and hyperplasia of the remaining glomeruli (Fine, *et al.*, 1992). Glomerular hypertrophy has been put forward as a likely pathway to glomerulosclerosis (Ichikawa, *et al.*, 1986). These authors argued that it was glomerular hypertrophy rather than glomerular hypertension that initiated and perpetuated glomerulosclerosis (Ichikawa, *et al.*, 1986). Moreover, growth factors associated with hypertrophy might be also instrumental in the pathogenesis of glomerulosclerosis (Fogo and Ichikawa, 1989). Prominent amongst these growth factors, TGF-β1 is known to be a potent hypertrophic growth factor (Fine, *et al.*, 1992). Increase in glomerular size (hypertrophy) along with an increased glomerular capillary pressure (hypertension) would lead to a significant increase in the glomerular capillaries' wall tension. Such an increased wall tension with the associated shear stress would initiate a cascade of events culminating in glomerulosclerosis (For review, Dworkin and Weir, 2000). Initially, glomerular endothelium would be most affected by the haemodynamic strain. It would initiate a local inflammatory response. Glomerular inflammation with the accumulation of leukocytes and monocytes has been shown in immune and non-immune-mediated renal diseases (Erwig, *et al.*, 2000). Infiltration of the glomerular tuft by monocytes leads to their interaction with glomerular cells especially mesangial cells (Mene, *et al.*, 2002). Mesangial cells are activated and proliferate in response to the release by monocytes/foam cells of mitogenic growth factors such as PDGF (Johnson, 1994). The activation of mesangial cells has been linked to the trans-differentiation of these cells into myofibroblasts (Johnson, *et al.*, 1991). These cells have been shown to release ECM components including interstitial collagen III. Stretching of the glomeruli by hypertrophy and hypertension is also likely to lead to the stretching of glomerular epithelial cells (Rennke, 1994). This would lead to the denudement of areas of the basement membrane not covered by the stretched podocytes (Kriz, *et al.*, 2001). This would facilitate the leakage of proteins and other macromolecules. Furthermore, the activation of epithelial cells within the glomerulus has been shown to be associated with their release of fibrogenic growth factors and ECM components. This would also exacerbate glomerulosclerosis (Kriz, 1996). Fibrogenic growth factors such as TGF-β1 are likely to be involved in all the stages of glomerulosclerosis including hypertrophy, inflammation and fibrosis (Basile, 1999).

In summary, the hypotheses for induction of glomerulosclerosis have implicated glomerular hyperfiltration, hyperperfusion, glomerular hypertension, glomerular hypertrophy, nephrotoxicity of proteinuria and the role of growth factors such as PDGF and TGF-β1.

1.3.2 Tubulointerstitial fibrosis (TIF)

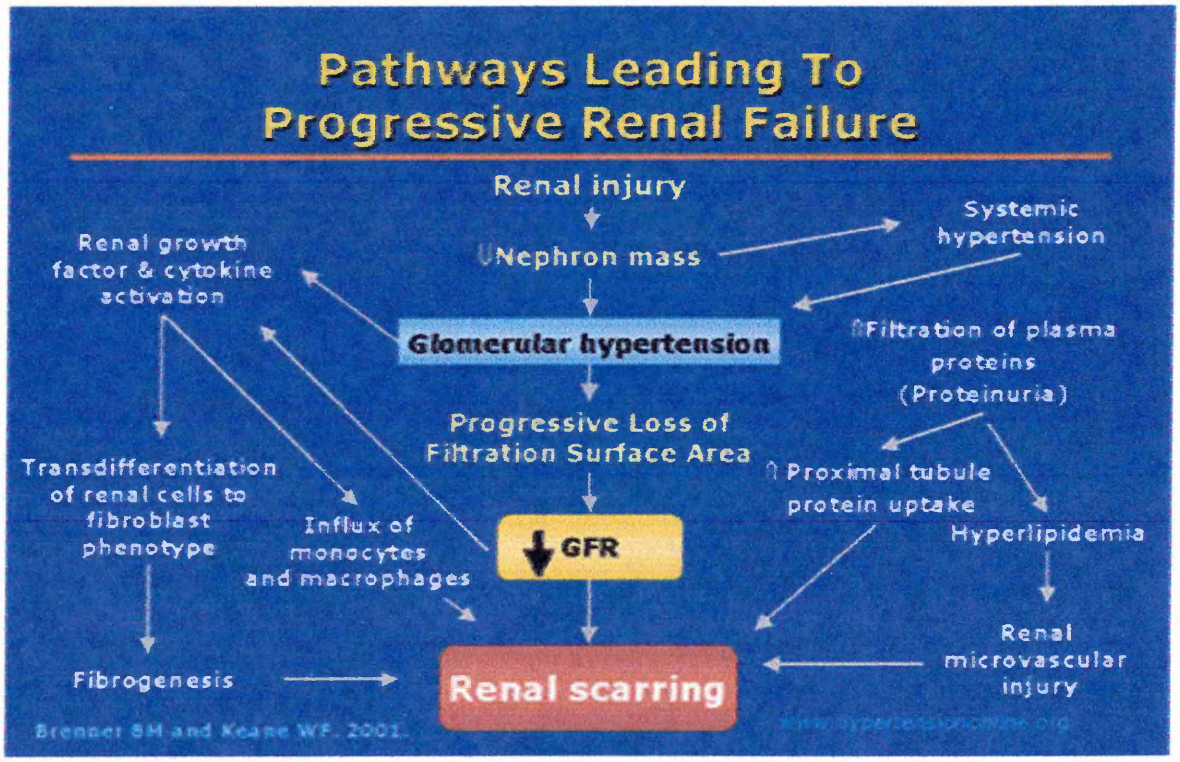
Local and systemic cytokines play a role in the induction of TIF. There are several cells responsible for the production of ECM (scar) in the kidneys. These cells are infiltrating macrophages (attracted by activated tubules as discussed above), tubular epithelial cells, peritubular capillary endothelial cells (after vasoconstriction or ischaemia/hypoxia) and fibroblasts/pericytes. These cells are resident kidney cells, migrating and transformed cells (For review, Jernigan and Eddy, 2000). The cells release fibrogenic factors such as TGF-β1, angiotensin II, endothelin 1, TGF-α, PDGF, and FGF (For review, Jernigan and Eddy, 2000). Accumulation of ECM leads to obliteration of peritubular capillaries and the death of tubular cells due to ischaemia (Bohle, *et al.*, 1981; Seron, *et al.*, 2001) and consequently, progressive renal insufficiency (Jernigan and Eddy, 2000; Eddy, 2001). There is a good correlation between the severity of tubulointerstitial fibrosis and renal function (Bohle, *et al.*, 1994).

1.3.2.1 Composition and formation of interstitial fibrosis

Interstitial fibrosis is the result of excessive accumulation of ECM in the renal interstitium. Matrix is composed of both normal interstitial proteins such as collagen (I, III, V, VII, XV) and proteins which are normally restricted to the basement membrane such as collagen IV and laminin (For review, Jernigan and Eddy, 2000). Two enzymatic pathways regulate matrix protein turnover. The first due to the activation of matrix metalloproteinases (MMP) and the second initiated by the generation of plasmin from plasminogen (For review, Branton and Kopp, 1999). Plasmin can degrade fibrin, fibronectin, and laminin (Liotta, *et al.*, 1981), and also activate the gelatinase class of MMPs (Wong, *et al.*, 1992). Furthermore, plasmin activates latent collagenases and TGF-β1 (Pollanen, *et al.*, 1991). Tissue and urokinase activators as well as plasminogen activator activate plasminogen. Interstitial collagenases such as MMPs, degrade the

interstitial matrix collagens (collagen I, II, III), stromelysin family members degrade the basement membrane proteins (collagen IV, V) and other proteins such as fibronectin, gelatinases degrade basement membrane proteins and elastin (Norman and Lewis, 1996). Furthermore, membrane type MMPs (MT-MMP) which degrade collagen III, fibronectin, laminin, have been described (Norman and Lewis, 1996). Tissue MMPs are activated by membrane-bound MMP proteins and inactivated by tissue inhibitors of metalloproteinases (TIMP-1-3). Plasminogen activator inhibitor-1 (PAI-1) inhibits the other collagenolytic pathway by inhibiting plasmin activation. During the course of renal scarring and interstitial fibrosis, there is a down-regulation of MMPs and upregulation of their inhibitors, TIMPs and PAI-1 (For review, Jernigan and Eddy, 2000). These processes lead to decreased breakdown of deposited ECM and, consequently, increase in its accumulation in the scarred kidney. Transforming growth factor-β1 has the capacity to directly inhibit MMPs and activate their inhibitors TIMPs and PAI-1. Therefore, TGF-β1 stimulation of ECM synthesis as well as inhibition of its breakdown would lead to irreversible renal fibrosis (Border and Noble, 1994, 1997 and Basile 1999).

Figure 6. Mechanisms leading to progressive renal failure.



(Brenner and Keane, 2001, www.hypertensiononline.org)

Pathways Leading To
Progressive Renal Failure
Renal injury
Nephron mass
Systemic hypertension
Glomerular hypertension
Filtration of plasma proteins (Proteinuria)
Progressive Loss of Filtration Surface Area
Proximal tubule protein uptake
Hyperlipidemia
↓ GFR
Renal growth factor & cytokine activation
Influx of monocytes and macrophages
Transdifferentiation of renal cells to fibroblast phenotype
Renal microvascular injury
Renal scarring
Fibrogenesis

1.4. Transforming growth factor-β

1.4.1 Transforming growth factor-β superfamily

The transforming growth factor β superfamily (Table 1) is a protein group that includes bone morphogenic proteins (BMPs), activin/inhibins, that inhibit pituitary secretion of follicle stimulating hormone, Mullerian inhibitory substance (MIS), which is produced by the testis and is responsible for the regression of the Mullerian ducts (to induce development of the female reproductive system), and DPP (decapentaplegic protein is part of the TGF-β superfamily that regulates *Drosophila* morphogenesis) (Leslie, 1999). The superfamily also includes glial cell line-derived neurotrophic factor (GDNF) and growth and differentiation factors (GDFs) (Leslie, 1999). Furthermore, this family includes the MAD system (the mother against decapentaplegic protein, which is part of the TGF-β superfamily that regulates *Drosophila* morphogenesis) and is also called Smad, which are the downstream effectors of TGF-β1 signalling. Smads molecular weights range from 42 to 60 kDa. The Smads have two domains, MH (Mad Homology) 1 in the amino terminal regions and MH2 in the C-terminal region (Massague, *et al.*, 1997).

The Smad system includes, Smad1, 2,3,5 and 8 which are activated by R1 (TGF-β type 1 receptor) ser/thr kinase receptor of TGF-β family including BMPs, DPP, and activins (Graff, *et al.*, 1996, Lui, *et al.*, 1996 and Kretzchmar, *et al.*, 1997, Roberts 2002). Smad4 is a common factor required for TGF-β1, activin and BMP signaling. Smad6 and Smad7 are inhibitory towards TGF-β1 signalling (Massague, 1990) (Table 1). The immediate TGF-β family includes five members (TGFβ1-5), however the mammalian isoforms of TGF-β1 are three (1-3). The structure of these isoforms is nearly identical, as they contain nine cysteine residues and share 76 to 80% of the amino acid sequence (Figure 1 and 2). It was reported that the mature active form of TGF-β1 structure conforms to a cysteine knot motif similar to nerve growth factor (NGF) and platelet-derived growth factor (PDGF) which do not belong to the TGF-β family and only share 10% of the amino acid sequence (Daopin, *et al.*, 1992; Schlunegger and Grutter, 1992). This knot is

held together by six cysteines joined together by three intra-chain disulfide bonds, which stabilize the beta sheet strands (Figure, 1).

Table 1. Members of TGF-β1 superfamily

Ligand	*Type II Receptor*	*Type I Receptor*	*Receptor regulating Smad*	*Common Smad*	*Inhibitory Smad*
TGF-β	TβRII	ALK5(TβR1)	Smad2 Smad3	Smad4	Smad6 Smad7
Activin	ActRII ActRIIB	ALK4(ActR1B			
BMPs	ActRII ActRIIB BMPRII	ALK2(ActRI) ALK3(BMPR1A) ALK(BMPRIB)	Smad1 Smad5 Smad8		
?	?	ALK1(TSR1)	Smad1		
MIS	AMHR	?	?		
?	?	ALK7	?		

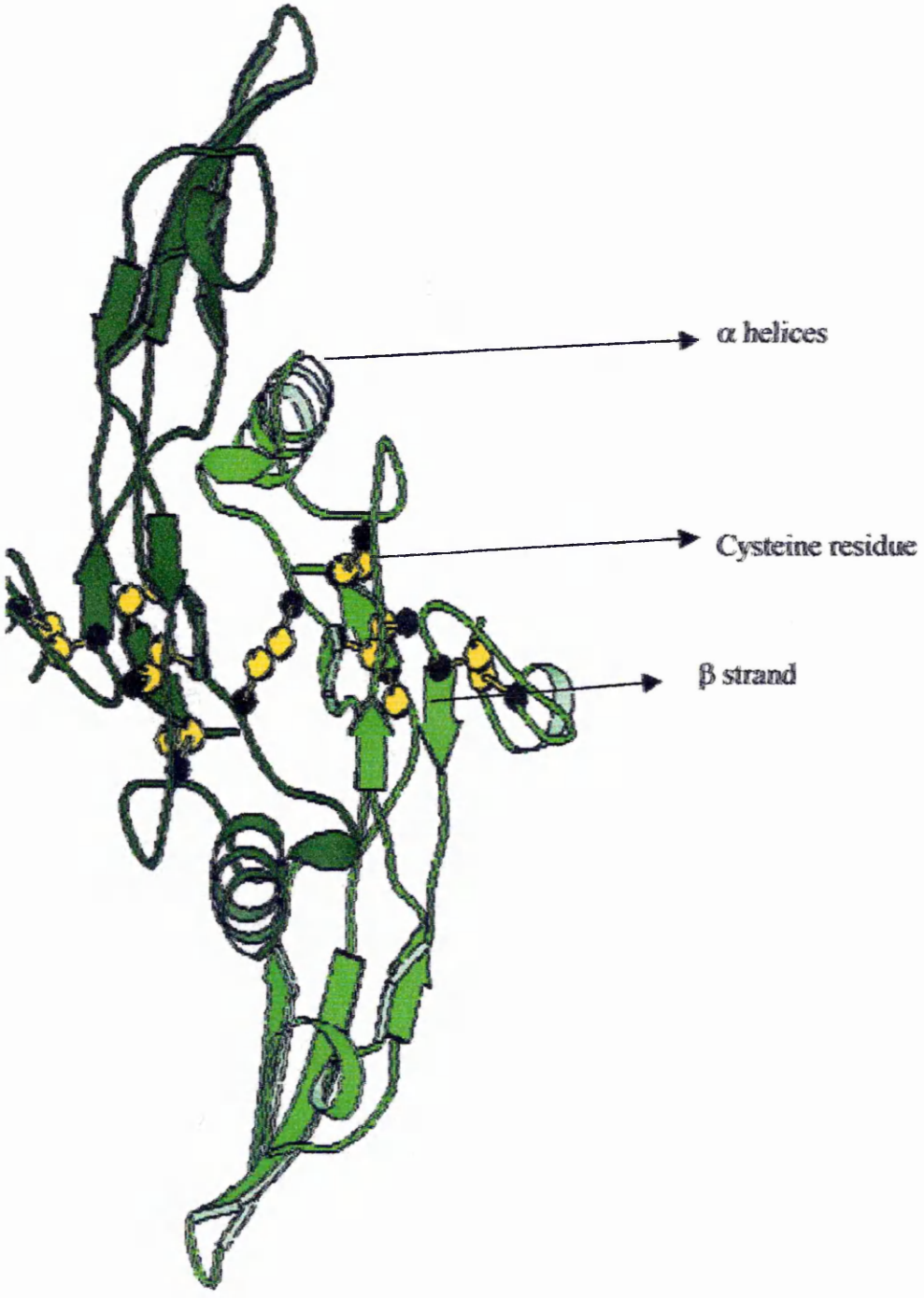
(Kluppel, et al., 1999, http://ana30.med.utoronto.ca/paths.htm)

Abrreviations: TGF-β: transforming growth factor beta; BMPs: bone morphogenic proteins; MIS: mullerian inhibitory substance; ALK: activin receptor-like kinase; Smads: signalling mother against decapentaplegic protein; AMHR: anti-Mullerian hormone receptor; ActR: Activin receptor type; TβR: transforming growth factor beta receptor.

1.4.2 Structure of TGF-β1

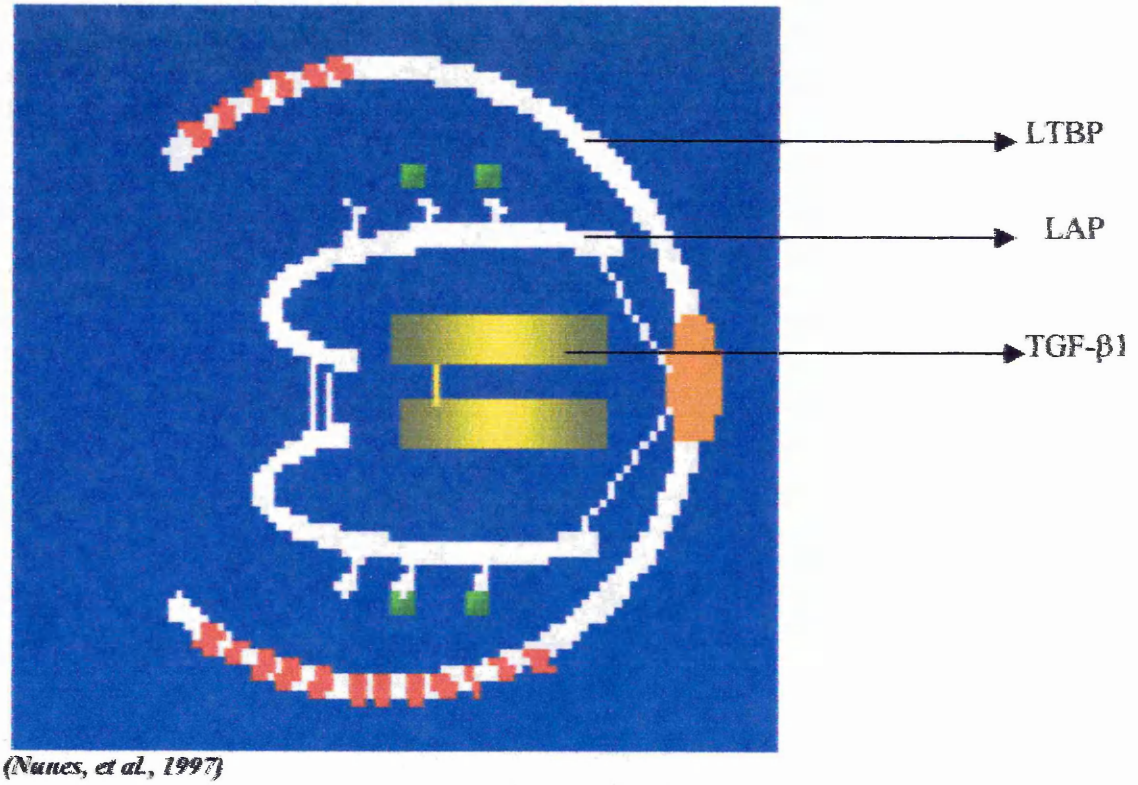
Transforming growth factor-β1 is a dimeric protein of 25kDa (Figure 1). It is secreted as inactive form called latent precursor molecule (LTGF-β1). The latent precursor molecule consists of 390-414 amino acids and contains an amino-terminal hydrophobic signal peptide region called latency associated peptide (LAP), consisting of 249 residues (Hinck, *et al.*, 1996). The latent precursor molecule (LTGF-β1) contains 112 amino acids at the C terminal, which is the potentially bioactive mature region (Figure 2). The latent precursor molecule LTGF-β1 is secreted as a large latent complex (LLC), bound via the LAP region to another protein called LTGF-β1 binding protein (LTBP) (Miyazono, *et al.*, 1991; Tailpale, *et al.*, 1995). The latent precursor molecule binding protein (LTBP) has an important role in the assembly and secretion of LTGF-β1 and serves to bind LTGF-β1 to extracellular matrices to enable proteolytic activation (Nunes, *et al.*, 1997). Latent precursor molecule secreted without LTBP, is called the small latent complex (SLC) (Munger, *et al.*, 1997). The mature (active) form is derived from the latent form, which consists of two large polypeptide chains linked by a disulfide bond to the latent TGF-β1 binding protein (LTBP). The molecular mass of this complex is 210 kDa. The latent precursor molecule is secreted and precleaved intracellularly at dibasic residues located between the LAP and the mature region. The LAP portion blocks the activation of the bioactive domains by keeping its folding. The folding of the bioactive domain assists by glycosylation of LAP, which undergoes mannose-6-phosphate addition at N-terminal (Schultz, *et al.*, 1995).

Figure 1. TGF-βl protein model



(http://cytokine.medic.kumamoto-u.ac.jp)

Figure 2. Cartoon of latent TGF-β1



(Nunes, et al., 1997)

Abbreviations: LTBP: latent precursor molecule binding protein; LAP: latency associated peptide; TGF-β: transforming growth factor beta.

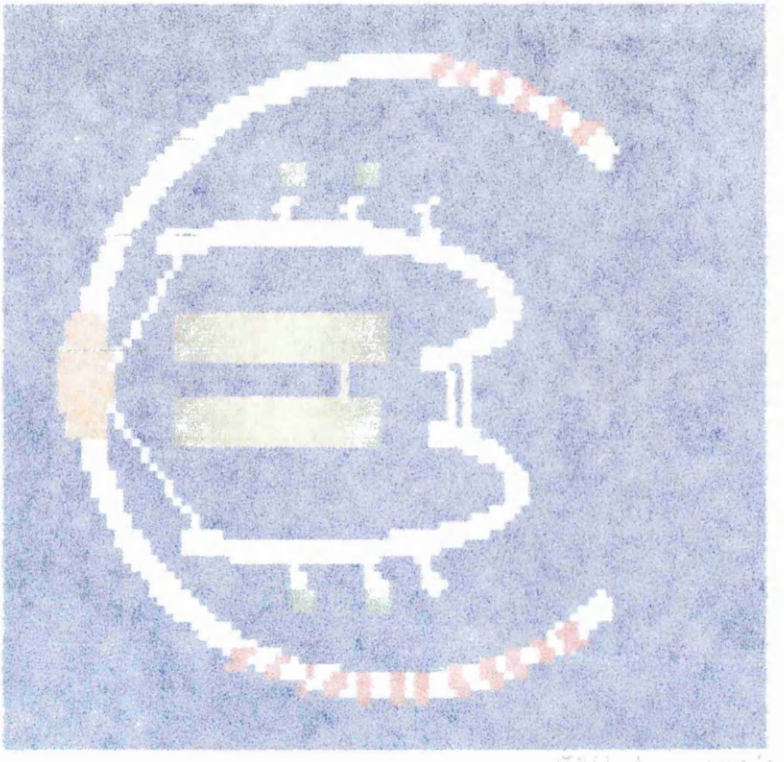


Table 2. TGF-β1 protein sequence

Key	From To	Length	Description
SIGNAL	1 23	23	*POTENTIAL.*
PROPEP	24 278	255	*BY SIMILARITY.*
CHAIN	279 390	112	*TGF-β1.*
DISULFID	285 294		*BY SIMILARITY.*
DISULFID	293 356		*BY SIMILARITY.*
DISULFID	322 387		*BY SIMILARITY.*
DISULFID	326 389		*BY SIMILARITY.*
DISULFID	355 355		INTERCHAIN.
CARBOHYD	82 82		N-LINKED (*POTENTIAL*).
CARBOHYD	136 136		N-LINKED (*POTENTIAL*).
CARBOHYD	176 176		N-LINKED (*POTENTIAL*).
SITE	244 246	3	CELL ATTACHMENT SITE (*POTENTIAL*).

```
        10         20         30         40         50         60
         |          |          |          |          |          |
MPPSGLRLLP LLLPLLRLLV LTPGRPAAGL STCKTIDMEL VKRKRIEAIR GQILSKLRLS

        70         80         90        100        110        120
         |          |          |          |          |          |
SPPSQGEVPP VPLPEAVLAL YNSTRDRVAG ESAEPEPEPE ADYYAKEVTR VLMVENTNKI

       130        140        150        160        170        180
         |          |          |          |          |          |
YEKVKKSPHS IYMLFNTSEL REAVPEPVLL SRAELRLLRL KLKAEQHVEL YQKYSNDSWR

       190        200        210        220        230        240
         |          |          |          |          |          |
YLSNRLLAPS DTPEWLSFDV TGVVRQWLSH GGEVEGFRLS AHCSCDSKDN TLQVDINGFS

       250        260        270        280        290        300
         |          |          |          |          |          |
SSRRGDLATI HGMNRPFLLL MATPLERAQH LHSSRQRRAL DTNYCFSSTE KNCCVRQLYI

       310        320        330        340        350        360
         |          |          |          |          |          |
DFRKDLGWKW IHEPKGYHAN FCLGPCPYIW SLDTQYSKVL ALYNQHNPGA SAAPCCVPQA

       370        380        390
         |          |          |
LEPLPIVYYV GRKPKVEQLS NMIVRSCKCS
```

(Derynck et al., 1985)

Note: *blue sequence shows the potentially bioactive mature region*

1.5 TGF-β1 activation

The activation of TGF-β1 requires plasmin, thrombin, tissue transglutaminase, endoglycosylases and retinoic acid (Sato, *et al*, 1990; Flaumenhaft, *et al.*, 1993; Nunes, *et al*, 1996; Rifkin, *et al.*, 1999). These compounds, in addition to thrombospondin1 (TSP-1), activate TGF-β1 through a specific binding interaction that alters the conformation of LAP (Ribeiro, *et al.*, 1999). It has been suggested that the presence of TSP-1 may prevent reformation of the inactive latent TGF-β1 complexes because when TSP-1 is bound to LAP it cannot rebind active TGF-β1 to confer latency (Ribeiro, *et al.*, 1999). There are two matrix proteoglycans, decorin and biglycan, which bind TGFβs for retention in extracellular matrices and may play a role in the regulation of TGF-β1 bioavailability (Yamaguchi, *et al.*, 1990; Border and Noble, 1994). Transforming growth factor-β1 is then activated by proteolytic cleavage to 112 amino acids. The structure contains nine strands, which form four fingers and three helices. It was thought that the function of LTBP is just a carrier for TGF-β, but Ruoslahti and Pierschbacher (1987) showed that the LTBP contains an endothelial growth factor like domain (Arg-Gly-Asp sequence) which mediates the interaction with connective tissue substances such as integrin.

1.5.1 TGF-β receptors

Three TGF-β receptors bind TGF-βs. TGF-β receptors distinguish between the TGF-β isoforms and bind these ligands with different affinities (Cheifetz, *et al.*, 1990; MacKay and Danielpour, 1991). These receptors are termed type I (RI) (50-60 kD) ranging from 503-532 amino acids, type II (RII) (75-85 kD) consisting of 567 amino acids and type III (RIII/betaglycan) consisting of 849 amino acids (280kD) (Lin, *et al.*, 1992). The TGF-β receptors exist as surface binding proteins (Lin, *et al.*, 1992; Massague, 1992; Kingsley, 1994). Both RI and RII are transmembrane signal-transducing receptors which contain serine/threonine kinase cytoplasmic domains (Kingsley, 1994; Newfeld, *et al.*, 1999). RI and RII kinase domains share 40% amino acid homology (Kingsley, 1994; Newfeld, *et al.*, 1999). RII contains active kinase and cooperates functionally with RI. Glycine-Serine sequence referred to as the GS domain (GS) is a highly conserved juxtamembrane region,

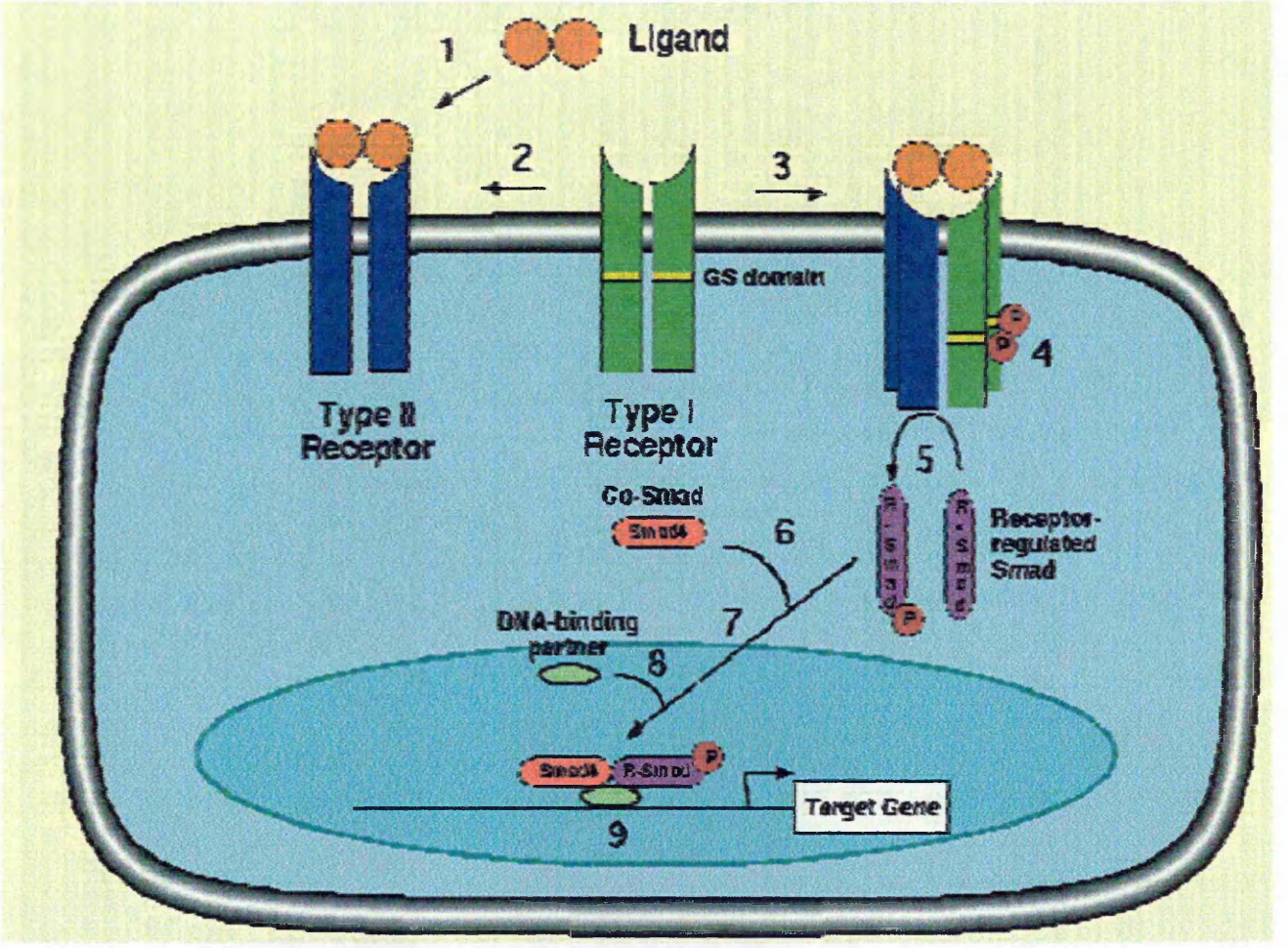
which plays an important role in the signal transduction pathway (Visser and Themmen, 1998). RII binds to the ligand consequently, phosphorylates and activates RI which initiates the signal-transduction (Attisano, *et al.*, 1993). RIII is a non-signalling receptor and it plays a role in concentrating TGF-β ligand on the surface of cells and in presenting TGF-β1-3 to the signaling receptors (Lopez-Casillas, *et al.*, 1994). Type III receptor consists of a core protein (110-139 kD) bound by side chains to chondroitin sulphate and heparin. Receptor type III also can be released by cells and acts as a soluble inhibitor of TGF-βs regulating their activity (Lopez-Casillas, *et al.*, 1994). Endoglin is another non-signaling receptor, which binds TGF-β to RIII/betaglycan (Letamendia, *et al.*, 1998). Endoglin binds TGF-β III, and it is found on endothelial cells, macrophages, and stromal cells (St. Jacques, *et al.*, 1994; Lastres, *et al.*, 1996). Endoglin (a receptor with significant homology to the type III receptor) is a homodimeric cell surface glycoprotein that complexes with RI and RII (Zhang, *et al.*, 1996).

1.5.2 TGF-β1 signal transduction pathway

Signal transduction is the mechanism by which TGF-β1 induces its function (Figure 3). Transforming growth factor-β receptors and Smads are required for signal transduction (for review, Roberts 2002). Transforming growth factor-β1 binds to RII. This leads to a phosphorylation and subsequently activation of RI to initiate its downstream signal transduction. Consequently, R1 directly interacts with phosphorylated Smad2 at C terminal serines (Macias-Silva, *et al.*, 1996). Smad2 phosphorylation occurs at C terminal MH2 domain on ser465 and ser467 of the SSXS motif (the site of phosphorylation that occurs on the last two serines of a conserved SSXS motif at the carboxy terminus of the Smad protein). This leads to phosphorylation of Smad3. Smad1 and 5 are activated directly by RI, Smad2 and 3, activin, and form a hetero-oligomeric complex with Smad4 (Chen, *et al.*, 1996). The complex (Smad2, Smad3 and Smad4) enters the nucleus and acts as a transcripitional activator of genes involving TGF-β1 function. The Smad MH1 domain binds to DNA through the DNA binding motif which is composed of an 11-residue hairpin turn that forms a loop that fits into the major groove of specific DNA

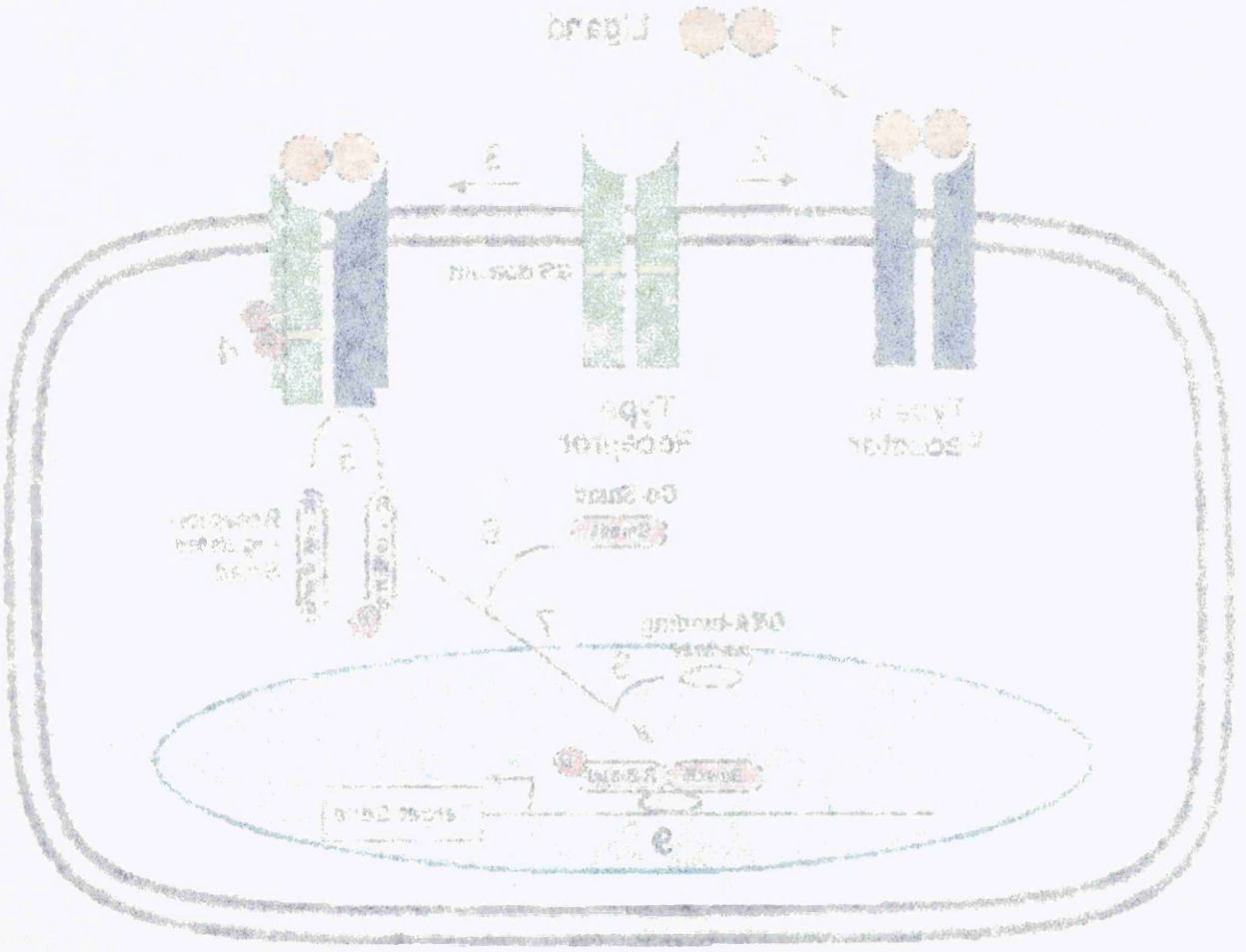
sequences (Shi, *et al.*, 1998). Smad 4 hetero-oligomerisation is absolutely essential for the C terminal domain of Smad 4 to perform its transcriptional transactivating function. This process needs a nuclear protein (orphan) that interacts with Smad 4 to mediate transcription (Shioda, *et al.*, 1998). Smad 4 plays an important role in promoting the binding of the Smad2/Smad4 (DNA binding partner) complex to DNA through its N terminus and in promoting transcriptional activation by Smad 1 and 2 through the Smad 4 C terminal region. The Smad 3/Smad4 hetero-oligomeric complex has been shown to cooperate with c-jun/c-fos to mediate TGF-β1 transcriptional responses (Zhang, *et al.*, 1998). Also, there is an intracellular protein called the Smad anchor for receptor activation (SARA) (Zhang, *et al.*, 1998). This protein plays a role in recruiting Smad2 and Smad3 to the TGFβ receptor complex (Tsukazaki, *et al.*, 1998). TGF-β1 is not bound to RII in the absence of RIII (i.e. TGF-β2). Moreover, RIII acts as a soluble inhibitor of TGF-β isoforms and consequently prevents their activity. On the other hand Smad6 and Smad7 bind to RI (without phosphorylation) and consequently inhibit RI binding to Smad2 or Smad3 and signaling (Massague, 1990) (Figure 3).

Figure 3. Transforming growth factor-beta (TGF-β1) signalling pathway



(http://www.grt.kyushu-u.ac.jp/spad/pathway/tgf-beta.htm)

Abbreviations: GS: Glycine-Serine sequence; P: phosphorylated head; Smad: signalling mother against decapentaplegic protein.



1.6 The role of transforming growth factor-β1

1.6.1 Transforming growth factor-β1 in health

Transforming growth factor-β1 expression was observed in normal glomeruli (Ando, *et al.*, 1995; Yamamoto, *et al.*, 1996), renal tubules, and the interstitium (Ando, *et al.*, 1998).

Transforming growth factor-β isoforms are expressed firstly in embryogenesis (Pelton, *et al.*, 1991) and cranial development (Roth, *et al.*, 1997). Transforming growth factor-β1 plays a major role not only in vasculogenesis, but also in maintenance of blood vessel wall integrity during embryogenic development (Kulkarni, *et al.*, 1993, Oshima, *et al.*, 1996). Transforming growth factor-β1 was found also to arrest the growth of most epithelial cells, neuroectodermal cells, hepatocytes, lymphocytes, myeloid cells (Moses, *et al.*, 1991, Massague, *et al.*, 1992 and Alexandrow and Moses, 1997) by blocking cell cycle transit late in the G1 phase of the cell cycle (Han, *et al.*, 1993).

Transforming growth factor-β1 plays a role in inflammation and tissue repair (Sporn and Robert, 1992; Roberts, 1998). The physiological function of this observation is unclear but TGF-β 1 levels could contribute to the maintenance of the normal glomerular microenvironment (Massague, 1990). Under normal conditions, remodeling of the tissue (synthesis and degradation) is under the control of several enzymes (Branton and Kopp, 1999). Plasminogen, which is produced by liver and kidneys, is delivered by circulation to other tissues. Plasmin, which is the active form of plasminogen, degrades fibrin, fibronectin and laminin (Liotta, *et al.*, 1981). Moreover, it activates MMP, latent collagenase and TGF-β1 (Pollanen, *et al.*, 1991 and Wong, *et al.*, 1992). Transforming growth factor β1 also inhibits the plasmin system (Tomooka, *et al.*, 1992). In response to a wound, TGF-β1 is activated in two stages. The initial activation of latent TGF-β1 occurs within an hour, followed by the second activation within several days (Yang, *et al.*, 1999). Furthermore, Pelton and colleagues (1991) found that TGF-β1 is increased in

keloid and Ghahary and colleagues (1993) reported a similar increase associated in hypertrophic scars from burns.

Experimentally, injection of TGF-β1 into wounded rabbits induces a faster rate of wound epithelialization and wound contraction than controls (Pandit, *et al.*, 1999). The role of TGF-β1 was found to be enhancement of fibroblast activity to contract (Montesano and Orci, 1988). Not only the systemic administration of TGF-β1 but also its local application improves the tensile strength of the wound healing scar (Becks, *et al.*, 1993). Interestingly, and surprisingly increase of circulating TGF-β1 in mice induced a decrease of scarring of the wound, a finding reflecting that the circulating TGF-β1 may not substitute the local (tissue) TGF-β1 (Shah, *et al.*, 1999). This finding shows the dissociation between circulating and tissues levels of TGF-β1.

1.6.2 Transforming growth factor-β1 and fibrosis

Transforming growth factor β1 stimulates the production of fibronectin, collagen, and proteoglycan in fibroblasts (Border, *et al.*, 1990). Border and Ruoslahti (1992) showed that fibrosis is a form of inappropriate injury repair and its development leads to tissue dysfunction and organ failure. Fibrosis represents an excess of normal repair process that follows tissue injury. Sporn and Roberts (1992) showed that TGF-β1 has a role in tissue repair, and has a fibrogenic effect.

This is because TGF-β1 stimulates the deposition of extracellular matrix by:

1 Stimulation of the synthesis of ECM e.g. fibronectin, collagens and proteoglycans;
2 Inhibition of proteases, tissue and urokinase activators as well as stimulation of plasminogen activator inhibitor (see composition and formation of interstitial scar) and consequent blocking of the degradation of the ECM;
3 Modulation of the expression of integrin receptors on the cells and, consequently, matrix deposition by stimulation of TIMPs and plasminogen activator inhibitor.

Furthermore, TGF-β1 induces proliferation of human renal fibroblasts: this process is mediated largely by fibroblast growth factor-2 (Strutz, *et al.*, 2001). The induction of

proliferation by TGF-β1 via induction of FGF-2 may play an important role in the autonomy of renal fibroblast growth and, thus, in the pathogenesis of human fibrogenesis (Strutz, *et al.*, 2001). It has also been postulated that TGF-β1 exerts its fibrogenic effect through the induction of connective tissue growth factor (CTGF) (Kothapalli, *et al.*, 1997).

One model for pulmonary fibrosis was induced by administration of bleomycin in rats. The total lung TGF-β1 content in these rats was several times higher than that in normal rats, which in turn increased the synthesis of collagens, fibronectin, and proteoglycans (Westergren-Thorsson, *et al.*, 1993). Alveolar macrophages produce TGF-β1, and this production could not be suppressed by high-dose corticosteroid treatment. This finding might be the explanation for the ineffectiveness of this treatment in patients with idiopathic pulmonary fibrosis (Khalil, *et al.*, 1993).

In humans, it was found that the TGF-β1 protein levels increased in alveolar walls at the sites at which ECM have accumulated in idiopathic pulmonary fibrosis (Broekelmann, *et al.*, 1991). Compared to normal subjects or patients with asthma, the bronchoalveolar cells obtained by lavage from patients with autoimmune diseases and lung fibrosis contained 10 times more TGF-β1 mRNA (Deguchi, 1992).

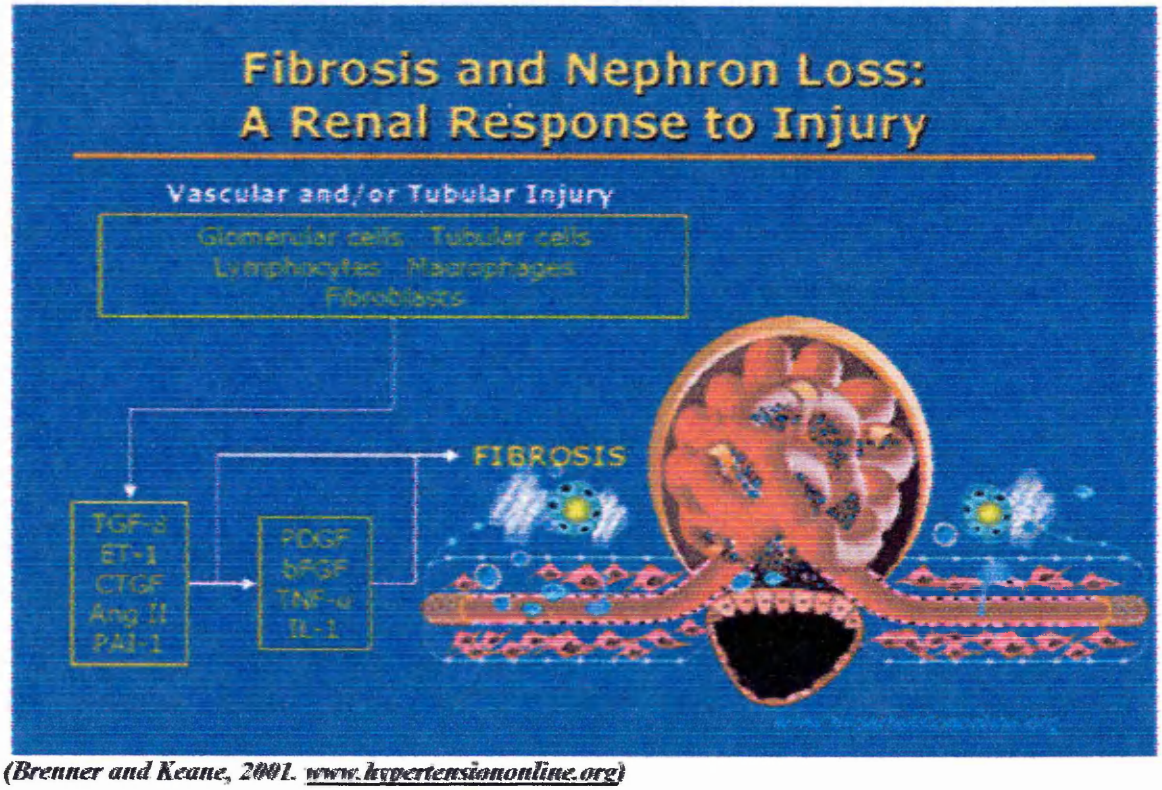
Czaja and coworkers (1989) found that liver mRNA for type I collagen (the predominant matrix component in injured liver) was increased in cultured rat hepatocytes incubated with TGF-β1. Also, the amount of TGF-β1 mRNA closely correlated with type I collagen mRNA in liver specimens from patients with chronic liver disease (Castilla, *et al.*, 1991). Chen and Colleagues (2002) also reported that there is a positive correlation between TGF-β1 mRNA concentrations in the liver and the histological activity of the liver disease. In chronic liver disease, TGF-β1 immunostaining was detected in fibrotic areas rather than areas with inactive disease (Nagy, *et al.*, 1991). Plasma level of TGF-β1 was found to be highly predictive of the development of hepatic fibrosis (veno-occlusive disease) in the recipients of bone marrow transplants (Anscher, *et al* 1993). The level of

expression of TGF-β1 is closely correlated with liver fibrosis associated with viral hepatitis (Murawaki, *et al.*, 1998).

Transforming growth factor-β1 and collagens are increased in tissue sections from patients with systemic sclerosis (Kulozik, *et al.*, 1990). Transforming growth factor-β1 was also found to be increased in stenosed arteries of both rats and humans (Nikol, *et al.*, 1992, Wolf, *et al.*, 1994). On the other hand, TGF-β1 has been known to have an anti-inflammatory action (Park, *et al.*, 2000). It down-regulates the inflammatory cytokine-induced expression of VCAM-1 in human glomerular endothelial cells, which could be the mechanism of its anti-inflammatory action in human glomerular diseases (Park, *et al.*, 2000). Furthermore, TGF-β1 inhibits proliferation of most cell types, including epithelial, endothelial, and haematopoietic cells (Roberts, 1998). Inhibition of renal tubular epithelial cell proliferation by TGF-β1 may promote the tubular cell hypertrophy that is characteristic of tubulointerstitial fibrosis (Roberts, 1998). Moreover, TGF-β and angiotensin II produced locally or delivered in the circulation, appear to play a central role in renal fibrosis (Figure 4). Transforming growth factor-β1 plays an important role in regulating the immune response as it was postulated that the knockout mice die from cardiopulmonary inflammation within weeks of weaning (Kulkarni, *et al.*, 1996).

In TGF-β1 transgenic mice (TGF-β1 gene under promoter control of murine albumin), the highest level of the TGF-β1 was found in the liver and also had high (> 10-fold over control) plasma levels of TGF-β1. Fibrosis and apoptotic death also developed in the liver (Kanzler, *et al.*, 1999). The fibrotic process was characterized by deposition of collagen around individual hepatocytes and within the space of Disse in a radiating linear pattern. Several extrahepatic lesions developed, including glomerulonephritis and renal failure, arteritis and myocarditis, as well as atrophic changes in pancreas and testis (Sanderson, *et al.*, 1995). Furthermore, TGF-β1 mediates pancreatic fibrosis through activation of pancreatic stellate cells and deposition of collagen type I and III at early time points (Vogelmann, *et al.*, 2001). The results from this transgenic model strongly support the proposed aetiological role for TGF-β1 in a variety of fibrotic and inflammatory disorders.

Figure 4. The role of different cytokines in induction of fibrosis



(Brenner and Keane, 2001. www.hypertensiononline.org)

Abbreviations: TGF-β: Transforming growth factor beta, ET1: endothelin 1, CTGF: connective tissue growth factor, Ang II: angiotensin II, PAI-1: plasminogen activator inhibitor 1, PDGF: platelet derived growth factor, bFGF: basic fibroblast growth factor, TNF-α: tumour necrotising factor alpha, IL-1: interleukin 1.

1.6.3 Transforming growth factor-β1 and renal diseases

1.6.3.1 Glomerulosclerosis

Transforming growth factor-β1 is the single most important fibrogenic growth factor in the pathogenesis of glomerulosclerosis (Border and Noble 1994, 1998, Peters, *et al.*, 1997, Basile 1999).

Transforming growth factor-β1 exerts its various effects within the glomeruli through interactions with its two receptors (type I and II). These receptors are expressed on glomerular endothelial and mesangial cells. Two intracellular signal pathways have been

implicated to mediate the effects of TGF-β1 on renal cells. These consist of the Smad family of proteins and the mitogen-activated protein kinase (MAPK) family (Chin, *et al.*, 1999). Recent data has shown Smad2 and Smad3 activation by TGF-β1 in human mesangial cells with resultant increased collagen transcription (Chin, *et al.*, 2001). Mitogen-activated protein kinase proteins include three groups including extracellular signal-regulated kinases (ERK), c-jun N-terminal kinases and p38 MAPK. In mesangial cells, the activation by TGF-β1 of ERK and p38 leads to increased collagen I transcription (Chin, *et al.*, 2001).

1.6.3.1 Experimental evidence

The first evidence for the role of TGF-β1 in increasing the production of ECM was increased production of ECM proteins, inhibition of protease activity, and increased integrin expression. These were obtained by incubation of normal glomeruli, mesangial cells and nonrenal cells with TGF-β1 (Okuda, *et al.*, 1990, Tomooka, *et al.*, 1992, for review Border and Noble 1994, Basile, 1999).

Transforming growth factor-β1 induces phenotypic changes in rat mesangial cells in vivo, with the expression of the α-smooth muscle actin (Imai, *et al.* 1994). This may lead to a switch in the type of collagen synthesized by these cells. Changes in mesangial (α-smooth muscle actin) and epithelial (desmin) cells can also be induced by the infusion of angiotensin II into rats (Johnson, *et al.* 1992). Of note angiotensin II is known to stimulate the synthesis and release by mesangial cells of TGF-β1 (Johnson, *et al.* 1992).

In a model of acute glomerulonephritis in rats obtained by a single injection of an antithymocyte antibody, extracellular matrix accumulation reached maximum levels after 2 weeks. The glomeruli returned to normal after 3 weeks (Okuda, *et al.*, 1990). Transforming growth factor-β1 is thought to inhibit the release of matrix metalloproteinases and stimulate the synthesis of their inhibitors (TIMPs) (Mozes, *et al.*, 1999). This would favour irreversible ECM deposition. Mice transgenic for TGF-β1 have high circulating levels of TGF-β1 and develop proteinuria and progressive glomerulosclerosis leading to death from uraemia (Mozes, *et al.* 1999). This is associated with increased matrix (collagen I and III) expression within the mesangium along with a

decreased collagenolytic activity due to increased synthesis of TIMP1 (18 fold increase) (Mozes, *et al.*, 1999).

Transforming growth factor-β1 is also a potent pro-apoptotic growth factor contributing to glomerular cells deletion. TGF-β1 transgenic mice model (under promoter control of murine albumin) had severe apoptosis of podocytes (glomerular epithelial cells). Apoptosis induced by phosphorylation of p38 and activation of caspase-3, leading to adhesion of glomerular capillaries to the Bowman's capsule and consequently glomerulosclerosis (Schiffer, *et al.*, 2001).

The second evidence was noticed by neutralizing the effects of TGF-β1 by adding anti-TGF-β1 antibody. This led to prevention of the accumulation of ECM (Border, *et al.*, 1994, Ziyadeh, *et al.*, 2000). Also, the natural antagonists, decorin, for TGF-β1 may play a modulating role. A fall in the glomerular content of decorin, a natural proteoglycan antagonist of transforming growth factor-β1 appears to precede the development of experimental glomerulosclerosis (For review, Basile, 1999).

The third evidence was through the transfection of the TGF-β1 gene into normal rat kidneys which led to increased production of TGF-β1 in glomeruli and, consequently, a rapid development of glomerulosclerosis (Isaka, *et al.*, 1993; Imai, *et al.*, 1994). The constant production of TGF-β1 leads to glomerulosclerosis fibrosis within weeks. This very closely resembles the histological findings in humans with chronic glomerulonephritis (For review, Border and Noble, 1994, Basile, 1999).

The fibrogenic effect of TGF-β1 has also been attributed to the stimulation by this growth factor of another fibrogenic growth factor, connective tissue growth factor (CTGF). The up-regulation of this growth factor in mesangial as well as tubular cells has been shown to follow TGF-β1-dependent and independent pathways (Yokoi, *et al.*, 2002). In

mesangial cells and fibroblasts CTGF mediates some of TGF-β1 induced increased ECM production by autocrine modes of action (Blom, *et al.*, 2001).

Transforming growth factor-β1 stimulates binding of bFGF to ECM, increase ECM heparan sulfate proteoglycan, and potentiate the mitogenic activity of bFGF (Nugent and Edelman, 1992). Glomerular cells express FGF-2 receptors (FGFR-1 and FGFR-3). The infusion of high does of FGF-2 causes mesangial proliferation as well as glomerulosclerosis (Floege, *et al* 1993; Kriz, *et al* 1995).

1.6.3.2 Clinical evidence

In mesangial proliferative glomerulonephritis, TGF-β1 intensity estimated by immunohistochemistry was closely correlated with the amount of ECM mesangial matrix (Yoshioka, *et al.*, 1993).

Glomeruli of patients with diabetic nephropathy had increased TGF-β1 protein and matrix proteins (Yamamoto, *et al.*, 1993).

The levels of renal TGF-β1 protein are increased in diabetic (Iwano, *et al.*, 1996) and non-diabetic kidney disease (Sutaria, *et al.*, 1998). In human immunodeficiency virus-associated nephropathy, chronic allograft rejection and radiation nephropathy, increased TGF-β1 has also reported (Datta, *et al.*, 1999).

Urinary TGF-β1 levels are increased in focal glomerulonephritis (GN), membranous GN and interstitial fibrosis but the results are not sufficiently consistent to establish a precise and predictive correlation (Honkanen, *et al.*, 1997; Murakami, *et al.*, 1997).

Tubulointerstitial fibrosis

Increased expression of TGF-β1 has been detected within most forms of experimental and clinical nephropathies associated with interstitial fibrosis (For review, Basile, 1999). Transforming growth factor-β1 can be derived from tubular cells, infiltrating macrophages as well as renal fibroblasts. Transforming growth factor-β1 has a high affinity to ECM which acts as storage areas for this growth factor. For its activation TGF-β1 has to be released from the associated latent TGF-β1 binding protein (LTBP) and the smaller latency associated protein (LAP). Once activated, TGF-β1 is fibrogenic through the stimulation of renal tubular as well as fibroblasts synthesis of ECM as well as the capacity of this growth factor to inhibit ECM breakdown through the stimulation of TIMPs. Many of the fibrogenic effects of TGF-β1 appear to be mediated through connective tissue growth factor (CTGF) (For review, Basile, 1999).

Transgenic mice for active form of TGF-β1 under promoter control of murine albumin and enhancer DNA sequence showed highest expression of TGF-β1 in the liver. This mice model also had elevated plasma TGF-β1 levels and consequently glomerulosclerosis and interstitial fibrosis (Kopp, *et al.*, 1996). Interventions aimed at neutralising TGF-β1 through the administration of neutralising antibodies or small molecular weight proteoglycan antagonsists such as decorin have been effective in attenuating interstitial fibrosis in rats with cresentic glomerulonephritis (Stokes, *et al.*, 2001).

Basic fibroblast growth factor (bFGF) is also thought to be a fibrogenic growth factor. It can act directly or indirectly through the stimulation of tubular TGF-β1 release. Basic FGF accumulates, like TGF-β1, in areas of interstitial fibrosis. Recently it was shown that TGF-β1 works through induction of FGF-2, which leads to hypertrophy of fibroblasts (Strutz, *et al.*, 2001).

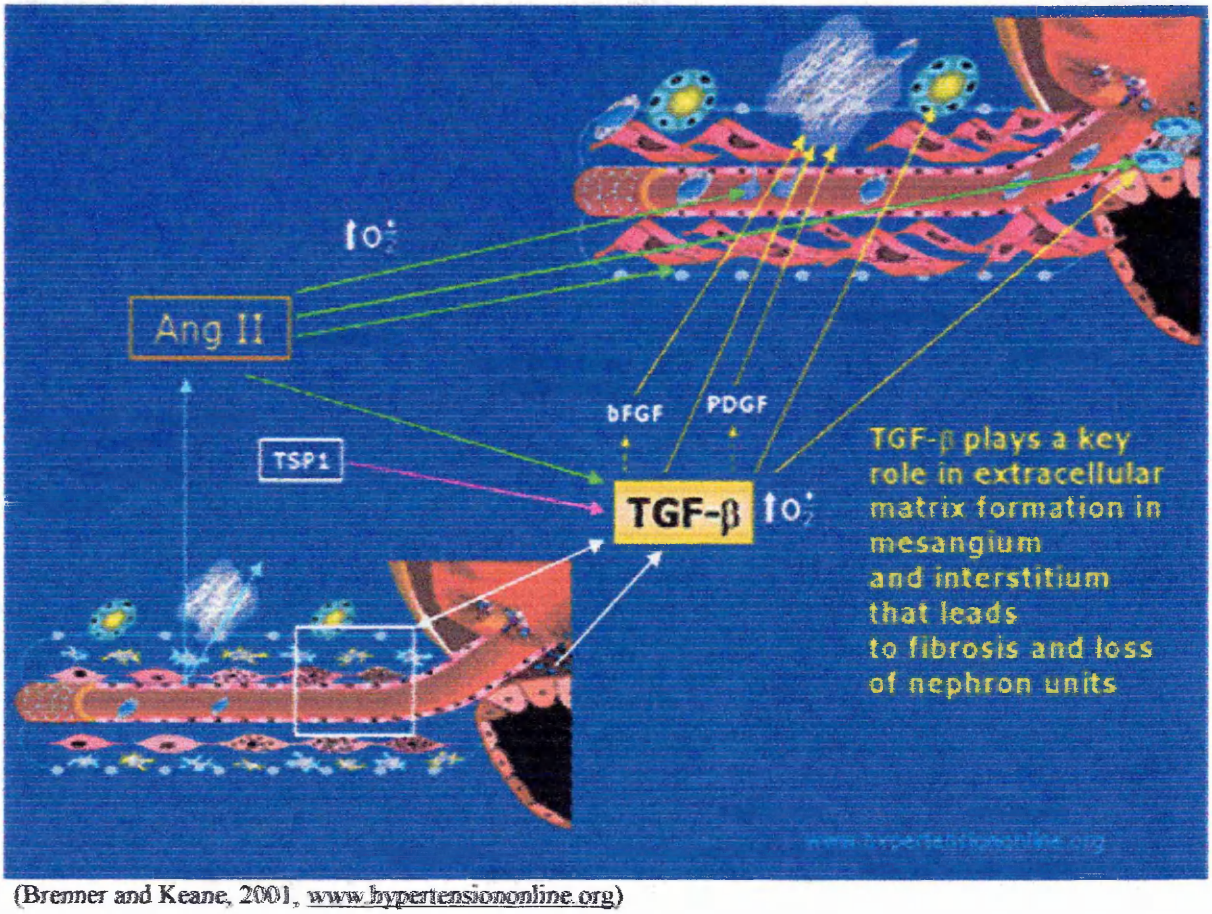
Bone morphogenic protein-7 (BMP-7) is a member of the TGF-β superfamily and has been localised to the collecting ducts (Lund, *et al.*, 2002). Proximal tubular and collecting duct cells express the type II BMP-7 receptor (Kopp, 2002). The administration of BMP-7 to rats with unilateral ureteral obstruction preserved tubular epithelial integrity, inhibited tubular cell apoptosis and prevented interstitial fibrosis (Piscione, *et al.*, 2001).

Circulating TGF-β1 is higher in African-Americans than Caucasians (Tell, *et al.*, 1996; Suthanthiran, *et al.*, 1998). The increased circulating TGF-β1 is associated with hypertension, tubulointerstitial fibrosis and glomerulosclerosis (Bottinger and Kopp, 1998). Circulating TGF-β1 is higher in the hypertensive than those with normal blood pressure in Afro-Caribbean (Suthanthiran, *et al.*, 1998).

It can be concluded that TGF-β1 increases deposition of extracellular matrix proteins, by several mechanisms:

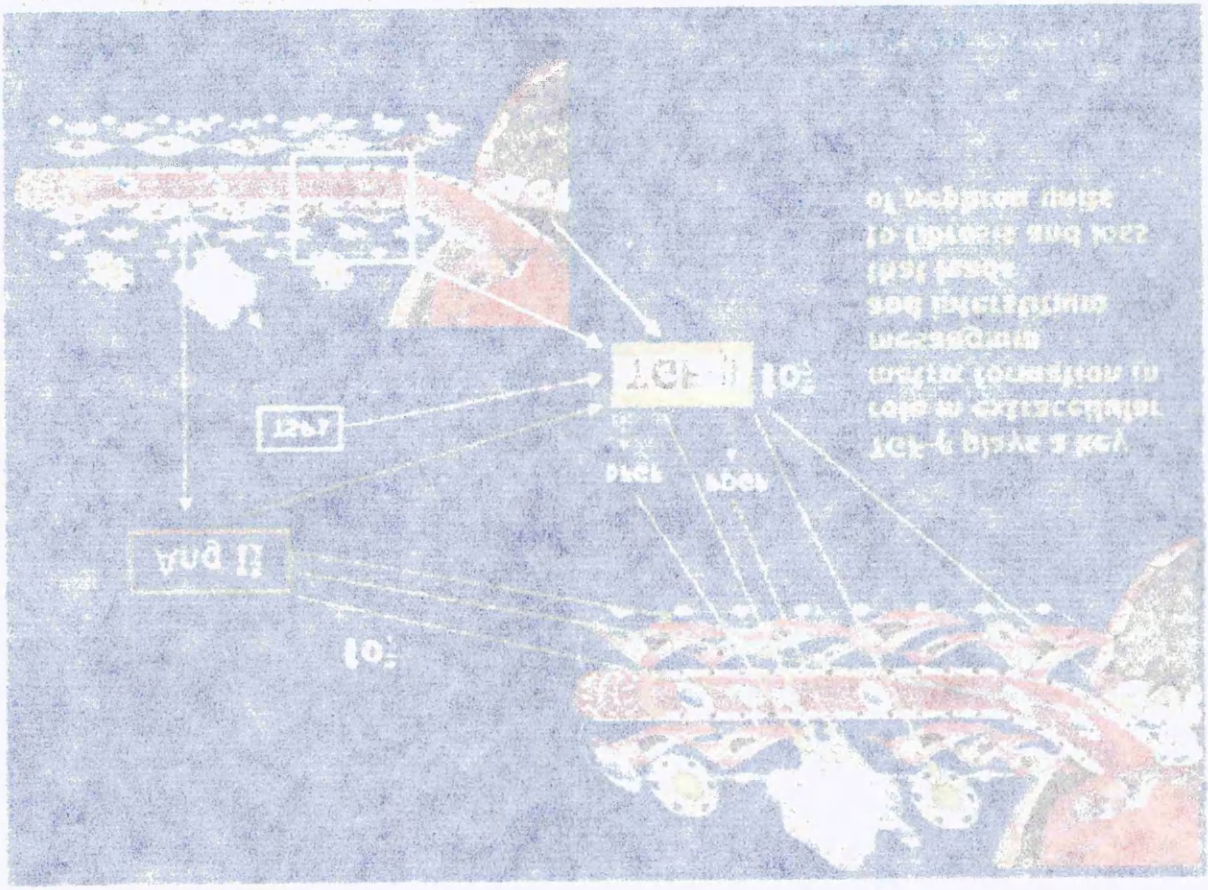
1) TGF-β1 stimulates production of several growth factors including basic fibroblast growth factor-2 (FGF-2), and connective tissue growth factor (CTGF) that stimulate the formation of extracellular matrix (ECM) proteins (Strutz, *et al.*, 2001).
2) TGF-β1 also has a direct effect on ECM protein expression (Tabibzadeh, 2002).
3) TGF-β1 stimulates the formation of the receptors (integrin) to which ECM proteins adhere (Yasuda, *et al.*, 1996).
4) TGF-β1 increases the expression of tissue inhibitors of metalloproteases (TIMP) that, in turn, prevents proteases from degrading ECM proteins (Ichikawa, *et al.*, 1996).

Figure 5. The role of TGF-β1 in induction of fibrosis



(Brenner and Keane, 2001, www.hypertensiononline.org)

Abbreviations: TSP-1: Thrombospondin 1, Ang II: angiotensin II, bFGF: basic fibroblast growth factor, PDGF: platelet derived growth factor.



1.7 Genetic aspects of chronic renal failure

New molecular genetics techniques have proved extremely powerful tools to investigate the links between genetic susceptibility and the progression of chronic renal diseases. Two research strategies have been applied to detect associations. The first is based on the *candidate gene approach aiming to detect an association between a particular* polymorphic DNA marker related to a gene coding for a factor thought to be involved in the progression of chronic renal disease. The second approach is based on the genome-wide search of unknown genes potentially associated with progressive CRF. In this approach, several hundred highly polymorphic microsatellite markers are tested for co-inheritance with the disease (Schork, 1997).

Several candidate genes have been investigated to seek a relationship between genotype and the initiation or the progression of CRF (Table 3).

Table 3. Candidate genes in ESRD *(For review, Buraczynska and Ksiazek, 2001)*

Gene	***Mechanism***
Vasoactive mediators: ***Renin-angiotensin system*** angiotensin I converting enzyme (ACE) angiotensinogen (AGT) angiotensin type 1 receptor (AT1R)	Effect on tissue hemodynamics and glomerular cell function
nitric oxide synthase (NOS) endothelin (ET) methylenetetrahydrofolate reductase (MTHFR)	Regulation of vascular tone Homocysteine metabolism
Kallikrein-kinin system	Inflammatory processes
Growth factors and cytokines: interleukin 1 (IL1) interleukin 1 receptor antagonist (IL1ra) tumour necrosis factor (TNF-(α) transforming growth factor (TGF-β)	Kidney mesangial cell prolife-ration,extracellular matrix production, Role in renal hypertrophy

Molecular genetics studies might give a clue for the causative or initiative factor of ESRF (Table 4).

Table 4. Genetics polymorphisms of ESRF

Gene family	*Associations reported*	*References*
Renin-angiotensin system		
ACE gene(I/D) insertion/deletion		
DD	• Progression of diabetic nephropathy in type I and type II diabetes mellitus	Mclaughlin, *et al.*, 1996; Velming, *et al.*, 1999
	• Progression of ESRF in non-diabetics	Samuelsson, *et al.*, 2000; Gumprecht, *et al.*, 2000, Lovati, *et al.*, 2001
	• Progression of IgA nephropathy	Tanaka, *et al.*, 1998
	• Initiation of IgA nephropathy	Yorioka *et al.*, 1995
	• Progression of parenchymal damage in uropathy	al-Eisa, *et al.*, 2000
	• Initiation of the development of focal segmental glomerulosclerosis	Hori, *et al.*, 2001
	• Worsening of polycystic kidney disease	Baboolal, *et al.*, 1997, Perez-Oller, *et al.*, 1999
angiotensinogen (AGT)		
M235T (TT)	• Initiation of the development of diabetic nephropathy in type I diabetes mellitus	Rogus, *et al.*, 1998
	• Progression of CRF in non-diabetics	Lovati, *et al.*, 2001
		Tomino, *et al.*, 1999;
AT1R (A1166C) (AC/CC)	• Susceptibility of CRF	Buraczynska, *et al.*,2001; Filler, *et al.*, 2001
Endothelial NO synthetase		
Glu298Asp	Progression of type I and type II diabetic nephropathy	Fogarty, *et al.*, 1996; Freedman, *et al.*, 1997; Loughry, *et al.*, 1998

Gene family	*Associations reported*	*References*
MTHFR		
C677T (TT)	Predisposition to and progression of diabetic nephropathy	Kimura, *et al.*, 2000
A1298C (CC)	Lower incidence of diabetic nephropathy	Shpichinetsky, *et al.*, 2000; Szabo, *et al.*, 2001
Kallikrein-kinin system		
C699A (CC) *C181T (TT)*	Less common in CRF and protects from progression to ESRF	Bachvarov, *et al.*, 1998
Growth factors and cytokines		
IL1RN *IL1B*	• Development of diabetic nephropathy • Progression to ESRF in non-diabetics	Hughes, *et al.*, 1996 Freedman, *et al.*, 1997; Loughrey, *et al.*, 1998
TGF-β1		
Leu10Pro	• development of renal failure in patients undergoing heart transplantation who are prescribed cyclosporin and steroids	Bean, *et al.*, (2000)
Thr263Ile	• development of nephropathy in diabetic patients	Picot, *et al.*, (1998)
Arg25Pro	• increased both systolic and diastolic blood pressure in ESRF patients	Li, *et al.*, (1999)
Mitochondrial gene		
A3243G mutation	Progression of CRF	Iwasaki, *et al.*, 2001
Glucose transporter gene		
XbaI (+/+)	Development of albuminuria in type II diabetes mellitus	Crzeszczak, *et al.*, 2001
Apolipoprotein		
ApoE2 and apoE4	Development of proteinuria in glomerulonephritis	Oda, *et al.*, 1999
ApoE2	Development of ESRF	Oda, *et al.*, 1999

1.7.2 Vasoactive genes

1.7.2.1 Angiotensin 1 converting enzyme (ACE) gene

Angiotensin 1 converting enzyme gene at 287 bp has three genotypes at intron 16 (I/D) insertion/deletion sites. The patient may be homozygous for I allele (II), heterozygous (ID) or homozygous for D allele (DD) (Filler, *et al.*, 2001).

Cambien and colleagues (1992) showed that increased incidence of cardiovascular disease and increased salt sensitivity and consequent hypertension is associated with the insertion/deletion polymorphism. Both II and ID genotype had a lower activity of the circulating ACE enzyme compared to DD genotype (Filler, *et al.*, 2001). Several studies showed an association with both initiation and progression of CRF (Table 4). On the other hand, Grzeszczak and colleagues (1998) found that there was no association between the ACE gene I/D polymorphism and the development of nephropathy in type 2 DM. There was also no significant association of this polymorphism and progression of CRF (Nakai, *et al.*, 1997, Gumprecht, *et al.*, 2000, Aucella, *et al.*, 2000, Schena, *et al.*, 2001). Many studies supported that there was no deviation in terms of allele frequencies (I/D) in CRF patients (non-diabetics) from normal controls (Hashimoto, *et al.*, 1998, Gumprecht, *et al.*, 2000, Schena, *et al.*, 2001, Lovati, *et al.*, 2001).

For some forms of glomerulonephritis, ACE gene polymorphism may not influence the extent of acute mesangial proliferation and crescents formation. However, the ID/DD genotypes are associated with chronic lesions, such as capsular adhesions or glomerulosclerosis (Tanaka, *et al.*, 1998).

Homozygosity for the D allele implies a worse renal prognosis in adult polycystic kidney disease (ADPKD), based on both the significantly lower median renal survival time and significantly greater percentage of patients who reach ESRD before the age of 50 years, without implying a greater prevalence of hypertension (Baboolal, *et al.*, 1997, Perez-

Oller, *et al.*, 1999). The data from ACE gene polymorphisms studies in polycystic kidney disease (PKD) remain controversial (Hashimoto, *et al.*, 1998; Lee, *et al.*, 2000).

1.7.2.2. Angiotensinogen gene and angiotensin type 1 receptor

Angiotensinogen gene (AGT) polymorphisms are involved in the regulation of blood pressure and, consequently, have been investigated for association with induction of nephropathy (Rogus, *et al.*, 1998). A point mutation in the angiotensinogen gene resulting in substitution of the amino acid threonine for methionine at position 235 (M235T) is associated with essential hypertension (Jeunemaitre, *et al.*, 1992). The frequency of the M allele was significantly higher in patients with diabetic nephropathy than in diabetic patients without renal involvement. The AGT-TT genotype was associated with a faster progression to ESRD in patients with glomerulonephritis (Rogus, *et al.*, 1998, Lovati, *et al.*, 2001). On the other hand, there were no significant associations of AGT polymorphisms with regard to the slope of 1/creatinine before or after renal transplantation (Yoshida, *et al.*, 1996, Filler, *et al.*, 2001).

The C allele in the angiotensin type 1 receptor (AT 1 R) gene polymorphism increases the susceptibility of chronic renal disease (Table 4) (For review, Buraczynska and Ksiazek, 2001).

1.7.2.3 Nitric oxide synthase and endothelin genes

Nitric oxide (NO) and endothelin (ET) are mediators that are released in inflammation and regulate vascular tone and blood pressure (For review, Freedman, *et al.*, 2000). Abnormalities in NO and ET-1 metabolism or synthesis were revealed to have a role in initiation and progression of CRF, regardless of the cause (Freedman, *et al.*, 2000).

The allele A (A/B) at intron 4 of endothelial constitutive nitric oxide synthase (ecNOS) occurs significantly more frequently in cases with ESRF caused by diabetic and non-diabetic nephropathy (Asakimori, *et al.*, 2001; Yokoyama, *et al.*, 1998).

Moreover, the Glu298Asp mutation (G-->T) in exon 7 of NOS3 was common in ESRD patients regardless of the nephropathy cause (Suzuki, *et al.*, 2000).

1.7.2.4 Methylenetetrahydrofolate gene

Hyperhomocysteinemia is frequent among patients with renal failure. Furthermore, it can accelerate the progression of CRF (Gupta and Robinson, 1997) The C to T mutation at C677T in the gene encoding methylenetetrahydrofolate (MTHFR), tends to decrease MTHFR activity, and consequently elevate circulating homocysteine levels in patients homozygous for the T allele (TT) (Kimura, *et al.*, 2000). The same authors also revealed that the homozygous (TT) patients were more likely to develop diabetic nephropathy than patients with other genotypes. Furthermore, Shpichinetsky and colleagues (2000) reported that C677T and A1298C markers were associated with diabetic nephropathy in type II diabetic patients.

1.7.2.5 Kallikrein-kinin system

Several studies have demonstrated the role in regulating of kallikrein-kinin system in inflammatory processes in the kidney and its protective effect against the development of renal pathology (Bachvarov, *et al.*, 1998). The kallikrein (KLK3) b allele frequency is associated with ESRD (Yu *et al.*, 1998). Kinins effects are mediated through B1 and B2 kinin receptors. The C allele (G-->C) at position -699 of the promoter region of the bradykinin-1 receptor gene (B1R) gene was found to be less frequent among patients with ESRD compared with healthy control subjects (Bachvarov, *et al.*, 1998, Zychma, *et al.*, 1999). On the other hand, there was a significant increase in the frequency of the C-allele at B1R gene polymorphisms in diabetic and non-diabetic nephropathy compared to controls (Knigge, *et al.*, 2000).

1.7.2.6 Cytokine genes

The carriers patients for the IL-1beta allele 2 (IL1beta2) or interleukin 1 receptor (IL-1Ra) interleukin 1 receptor antagonist allele 2 (IL1RN*2) in intron 2, was associated with a higher risk of IgA nephropathy (Liu, *et al.*, 2001). These alleles were tightly linked. On the other hand, carriage of the TNF-alpha allele 2 (TNF2) was associated with a decreased risk of IgA nephropathy (Syrjanen, *et al.*, 2002). Carriage of the IL1RN*2 allele was associated with a significantly poorer long-term outcome with a median survival time of 72 months as compared with those without IL1RN*2 in IgA nephropathy (Shu, *et al.*, 2000).

Blakemore and coinvestigators (1996), showed that IL1RN*2 could be a marker for the severity of inflammatory processes and complications of type 2 DM including nephropathy. Freedman and colleagues (1997) showed an association between the same allele and ESRD.

1.8 Other genes

The A-->G transition at nucleotide 3243 of the mitochondrial tRNA was found to be associated with faster progress to ESRF in type II DM (Iwasaki, *et al.*, 2001, Watson, *et al.*, 2001).

Grzeszczak and colleagues (2001) reviewed that the XbaI allele in the glucose transporter (GLUT1) gene protects against the development of diabetic nephropathy in Caucasian patients with type 2 diabetes.

In glomerulonephritis, patients with proteinuria had higher apoE2 and apoE4 allele frequency and lower apoE3 allele frequency than the controls (Oda, *et al.*, 1999). Furthermore, ESRD patients had higher apoE2 allele frequency and lower apoE4 allele frequency than the controls. Higher prevalence of nephrotic syndrome was found in proteinuric glomerulonephritis patients with apoE2 (Oda, *et al.*, 1999).

1.9 TGF-β1 and TGF-B1 gene

1.9.1 TGF-B1 gene structure

The transforming growth factor-B1 (TGF-B1) gene has been located to 19q13.1-13.3 (Fujii, *et al.*, 1986). The gene is more than 100kb and includes seven exons. The active part of TGF-β1 is encoded by part of exons 5, 6, and 7 (Derynck, *et al.*, 1987). It was reported that there are seven polymorphic regions in the upstream and coding regions (Lympany, *et al.*, 1998). Three biallelic polymorphisms [-988 (C/A), -800 (G/A) and –509 (C/T)] untranslated region are located in the upstream regions (promoters). A C insertion site was found to be located at position 72 of the untranslated region, and three biallelic polymorphisms located at codon 10 (Leu/Pro) and 25 (Arg/Pro)] of exon 1 and codon 263 (Thr/Ile) of exon 5 (Cambien, *et al.*, 1996).

1.8.2 TGF-B receptor gene

Separate genes is responsible for the production of each of the three TGFB receptors. TGFBRI has been located at 9q33-p34, TGFBRII at 3p22 and TGFBRIII at 1p32-p33 [http://bioinfo.weizmann.ac.il/card-bin/cardship (accessed on 11/12/1999)]. The transforming growth factor-β type II receptor [which is the most important in signal transduction (see section 1.5.3)] gene consists of seven exons and six introns. The sizes of the exons range from 128 to 800 bp. Four polymorphic regions were found in TGFBRII. One of these polymorphisms (A/G) has been located at the seventh base of intron 2, another (C/T) (a silent mutation) was found to be located at codon 389, the third, (C/T) at codon 439 and the fourth (A/C) at nucleotide 1710 of the 3' (non-translated region) (Chidambaram, *et al.*, 1996).

1.8.3 TGF-B1 polymorphisms

Transforming growth factor-B1 polymorphism can influence the pathogenesis of fibrotic diseases (For review, Border and Noble, 1994; Basile, 1999). Patients homozygous for the G allele at codon 25 have higher systolic blood pressure than those who are C-homozygous or heterozygous (Li, *et al.*, 1999). In type I diabetes mellitus, there is a

significant association between the presence of the T allele (Thr263Ile) and the development of diabetic nephropathy (Pociot, *et al.*, 1998). Also there is a significant association between the presence of the T allele and high levels of serum TGF-β1 (active + acid-activatable latent) protein levels (Grainger, *et al.*, 1999).

1.8.3.1 TGF-B1 polymorphisms and diseases

Because of the implication of TGF-β1 in the pathogenesis of several diseases, researchers have recently given great attention to the association between TGF-B1 polymorphisms and different diseases. TGF-B1 polymorphisms are associated with a wide range of different diseases (Table 5).

Although there was an association between polymorphism at (C-509T) and circulating TGF-β1, there was no significant associations with the other studied genotypes (G-800A) (Grainger, *et al.*, 1999).

Table 5. TGF-β1 polymorphisms and different forms of diseases

TGF-β1 gene polymorphisms	***Associations***	***References***
C-509T (T allele)	• Associated with decreased bone mineral density and osteoporosis • Increased both active and acid-activated latent TGF-β1 in normal people	Yamada, 2001 Grainger, *et al.*, 1999
Leu10Pro (T allele)	• Increased risk of osteoporosis • Associated with higher SBP in whites • Increased risk of coronary artery disease • Increased susceptibility to myocardial infarction in Japanese • Development of end stage heart failure caused by cardiomypathy • Acute rejection of the heart within one year after its transplantation • deterioration the pulmonary function in cystic fibrosis	Langdahl, *et al.*, 1997, Bertoldo, *et al.*, 2000 Rivera, *et al.*, 2001 Syrris, et al., 1998 Yokota, *et al.*, 2000 Holweg, *et al.*, 2001 Holweg, *et al.*, 2001 Arkwright, *et al.*, 2000
Arg25Pro (G allele) (C allele)	• Graft vascular disease diagnosed by angiography in one year after heart transplantation • Increased risk of coronary artery disease • Development of fibrosis in lung graft • Deterioration of the pulmonary function in cystic fibrosis • Increased risk of myocardial infarction and reduced risk for systolic hypertension	Yokota, *et al.*, 2000 Syrris, *et al.*, 1998 El-Gamel, *et al.*, 1997. Arkwright, *et al.*, 2000 Cambien, *et al.*, 1996

1.8.4 TGF-B1 polymorphism and renal diseases

Bean and colleagues (2000) found that there is a significant association between the presence of C allele at Leu10Pro and the development of renal failure in patients undergoing heart transplantation who were prescribed cyclosporin and steroids. Picot and co-workers (1998) found that there is a weak, but significant association between the presence of the T allele at Thr263Ile and the development of nephropathy in diabetic patients. On the other hand, the Leu10Pro polymorphism showed no significant association with progression of diabetic nephropathy (Akai, *et al.*, 2001). Li and co-workers (1999) found a significant association between homozygotes for the arginine allele (G) at codon 25 and increased systolic and diastolic blood pressure in ESRF patients.

It can be concluded that TGF-β1 is a strong candidate for association with renal failure because of its known biological effects and because of reported associations with both fibrosis and increased blood pressure. Also, it was observed that some patients with CRF progress quickly to ESRF compared with others who have stable renal function for a long time. This encouraged me to investigate whether TGF-β1 polymorphisms can answer why some patients with CRF develop ESRF faster than those who may have a constant but impaired renal function for a long time although both groups have the same management.

Aim of this thesis

This thesis investigates TGF-B1 polymorphisms that may be associated with CRF. In order to determine if there is an association between these polymorphisms and:

1- Susceptibility to CRF

2- Progression of CRF

3- Clinical features of CRF

4- TGF-β1 levels in the serum, plasma and tissue of patients with CRF

Overview of study design

Deoxyribonucleic Acid (DNA) samples for genotyping were prepared from a cohort of patients with CRF recruited from the Sheffield Kidney Institute, Northern General Hospital, Sheffield, UK. Serum and plasma for TGF-β1 measurement were also stored and clinical parameters (including blood pressure, proteinuria, and creatinine levels) were recorded. Furthermore, TGF-β1 was estimated in the available patients histological sections.

1) Association with susceptibility to CRF was investigated by comparison of allele and genotypes frequency in-patients with those in normal controls.
2) Patients were designated as having progressive or non-progressive disease on the basis of the reciprocal of creatinine slope over 2-9 years. Allele and genotype frequencies were compared between these two groups to establish whether associations were present with progression of CRF.
3) Associations with clinical features and with circulating and renal tissue TGF-β1 levels were also investigated.

It was hoped, by this approach, to determine whether sequence variation in the gene encoding TGF-β1 is an important factor in susceptibility to, and/or progression of, CRF. If this is the case, they might be useful as prognostic indicators and, possibly, in the future might inform clinical management.

Chapter 2

Materials and Methods

2.1 Collection of patients blood samples

One hundred and forty six samples have been collected from patients with chronic renal failure (CRF) (appendix forms). Ethical approval for the study was obtained from the North Sheffield Ethics Committee, Northern General Hospital Trust. Informed consent was obtained from all patients and controls included in the study.

2.1.1 Inclusion/exclusion criteria

The study included patients with creatinine concentration >150 μmol/l, who were white Caucasians, of any gender and age group, with various degrees of renal insufficiency including some on dialysis replacement therapy (ESRF). Patients with all types of underlying renal diseases including diabetic nephropathy, hypertensive glomerulosclerosis, obstructive uropathy and polycystic kidney disease were evaluated.

The study excluded patients with creatinine concentration <150 μmol/l, non-Caucasians as well as the relatives of patients included in the study.

Blood samples were collected for analysis from CRF/ESRF patients:

Firstly, blood samples were collected from patients ESRF on renal replacement therapy:

a) Forty-one patients on continuous ambulatory peritoneal dialysis (CAPD): From the kidney outpatient clinic at the Northern General Hospital, Sheffield.
b) Twenty-nine patients on haemodialysis: These samples have been collected from patients at the Sheffield Kidney Institute at the Northern General Hospital, Sheffield.

Secondly, blood samples were collected from patients with CRF not on renal replacement therapy:

c) Seventy-six samples were collected from the outpatient clinic at the Northern General Hospital, Sheffield.

The control DNA samples (80 samples) were obtained from a white Caucasian population from Sheffield (UK) and they were stored in a -20°C freezer at Sheffield Hallam University.

2.1.4 Clinical data

The following parameters were recorded: Patients' age, gender, renal diagnosis, renal histology (when a renal biopsy was available), blood pressure, serum creatinine, proteinuria, serum cholesterol and triglycerides levels. Parameters such as systemic blood pressure and proteinuria were recorded at the time of diagnosis as well as during follow-up (mean of the follow-up values). Treatment was documented.

The slope of the regression analysis of the reciprocal of serum creatinine value (1/serum creatinine) against time was taken as an indicator of the rate of progression of CRF. At least 5 serum creatinine measurements were included for each patient.

Patients with chronic renal failure (CRF) were classified into: -

(i) Progressors (P) – this group included both patients who had a 1/serum creatinine against time regression slope significantly inferior to zero and patients with ESRF who were on renal replacement therapy. End stage renal failure patients were considered as progressors because these patients already progressed to ESRF.

(ii) Non-progressors (NP) – these patients have a 1/serum creatinine against time regression slope, which was not significantly different from zero.

Analyses were done for all patients first. Then individual diseased categories where excluded and the analyses were reported. This also involved analysis with and without conditions such as adult dominant polycystic kidney disease (ADPKD) and obstructive uropathy.

2.1.5 Blood samples collection and processing

Twenty mls of venous blood was collected from each patient. Ten mls was anticoagulated with EDTA. The other 10mls were not treated with anticoagulant. The anticoagulated blood samples were used for DNA extraction and the coagulated blood samples were used for serum preparation. These samples were separated by centrifugation (1000 g) for 10 minutes to separate the cells from the plasma from the coagulated blood and the serum from the anticoagulated samples. Samples were aliquoted and stored at –20°C until required for analysis.

2.2 Genomic DNA extraction techniques

2.2.1 Buffers for DNA extraction

Buffers A, B and Sodium perchlorate, see appendix.

2.2.2 Protocol for genomic DNA extraction

After defrosting, each sample (10mls) was poured into a 50 mls universal tube (Bioline) and 40 mls of buffer A was added. This led to the hypotonic lysis of the cells and consequently allowed removal of haemoglobin and other cellular proteins. The samples were mixed end-over-end for 4 minutes at room temperature (RT) and were centrifuged (Sorvall RT 6000D) at RT, 3000 g for 15 minutes. The supernatants were discarded and the pellets (containing the nuclei of white cells) were resuspended in Buffer A to 20 ml. Samples were centrifuged again, at RT, 3000 g for 15 minutes. The supernants were removed and discarded. One ml of Buffer B was added to each tube to resuspend the pellets. The contents were transferred to 1.5ml Eppendorf tubes and 300 µl of sodium perchlorate (5M) was added to each tube, to precipitate the proteins. The samples were mixed end-over-end for 10 minutes followed by microcentrifugation (12.000 g) for 10 minutes. From each supernant, 600µl were taken in a clean Eppendorf then 700µl of ice cold chloroform (Sigma) was added. The samples were mixed end-over-end for 3 min and were microcentrifuged (10.000 g) for 10 minutes. The top layer was transferred to clean Eppendorf. Twice the volume of ice-cold ethanol was added to each Eppendorf.

The samples were gently mixed end-over-end, then microcentrifuged (Sorvall Super T21) at 10.000 g, for 15 minutes at 4°C. Supernatants were discarded and the DNA was exposed to air for 10 minutes to dry. The pellet was resuspended in 200-500 µl of sterile distilled water. This was mixed by end-over-end for 1 hour at 4°C.

2.2.3 Assessment of DNA purity and concentration

Genomic DNA concentration was estimated by measuring the absorbance at 260 nm (using GeneQuant spectrophotometer, Pharmacia Biotechnology). The DNA samples were diluted (5 µl of stock DNA was diluted in 995µl of sterile distilled water). One cm-path-length quartz cuvette was used.

One unit of absorbance at 260nm corresponds to 50µg/ml of DNA. The DNA concentration was calculated using the equation (absorbance at 260nm/0.02 (constant factor x dilution factor). The stock DNA was diluted in sterile distilled water to a final concentration of 50-100ng/µl. Then samples were stored at –20°C. The ratio of absorbance at 260nm to 280nm in a 1cm-path-length-quartz cuvette was used to assess DNA purity. The sample was considered pure when the ratio was 1.8. Generally, a ratio of 1.6 to 2 was obtained. The quality of the DNA was examined by using 0.8% agarose gel electrophoresis.

2.3 Polymorphism analysis

2.3.1 *Polymerase chain reaction (PCR) screening for TGF-β1 gene polymorphisms*

The polymerase chain reaction was performed on the purified DNA extracted from patients with CRF/ESRF and the controls. Four polymorphisms in the TGF-B1 gene were investigated C-509T, Leu10Pro, Arg 25Pro and Thr263Ile.

The primers for amplification of the different polymorphisms are shown in table 2.1.

Table 2.1. The different primers were used for detection of polymorphisms

Polymorphic site	*primer*	*Primer sequences*	*Reference*
C-509T	F	5' CAG ACT CTA GAG ACT GTC AG-3'	Grainger, *et al.*, 1999
	R	5' GTC ACC AGA GAA AGA GGA C-3'	
Leu10Pro	F1	5'CTCCGGGCTGCGGCTGCTGCT-3'	
	F2	5'CTCCGGGCTGCGGCTGCTGCC-3'	Yoshiji, *et al.*, 1998
	R	5'GTTGTGGGTTTCCACCATTAG-3'	
Arg25Pro	F1	5' ACT GGT GCT GAC GCC TGG CCC-3'	Li, *et al.*, 1999
	F2	5' ACT GGT GCT GAC GCC TGG CCG-3'	
	R	5' TGC TGT TGT ACA GGG CGG CGA GCA-3'	
Thr263Ile	F1	5' ACC GGC CTT TCC TGC TTC TCA TGG CCC T-3'	Pociot, *et al.*, 1998
	F2	5' TGG CCA CCA TTC ATG GCA TGA GTC GGC CTT TCC TGC TTC TCA TGG ACA C-3'	
	R	5' AAG GCC TCC ATC CAG GCT ACA AGG CTC AC-3'	

2.3.2 Preparation of primers

Primers were obtained from the Medical Genetics Department of the Royal Hallamshire Hospital, Sheffield. Primer pellets were resuspended in 100µl of nanopure water. Five µl of the aliquot was added to 995µl of sterile distilled water. Concentrations were estimated by measuring the absorbance at 260nm (by using a GeneQuant spectrophotometer) using 1cm-pathlength quartz cuvette. Concentration was adjusted to 40pmol/µl. The aliquoted primers were kept at –20°C until required for PCR.

2.3.3 Genotyping for Leu10Pro and Arg25Pro

Amplification Refractory Mutation-Screening Polymerase Chain Reaction (ARMS-PCR) was used for this purpose. The system is simple, reliable and non-isotopic. Furthermore, it clearly distinguishes between heterozygotes at a locus from homozygotes for either allele. The system requires neither restriction enzyme digest nor sequencing of PCR products (Newton *et al.*, 1989). For Amplification Refractory Mutation-Screening Polymerase Chain Reaction two sense (forward) primers were used. The sense primers had identical sequences except at 3' nucleotide. If the primer sequence is identical to the allele, it will bind to the complementary strand of the allele sequence, permitting amplification. On the other hand, if the primer is not complementary to the allele, amplification will be prevented. A common antisense (reverse) primer was used. Two reactions were performed for each patient; each one contained one of the two forward primers and the common primer in addition to the other reagents. Polymerase Chain Reaction reactions were carried out in a final volume of 50µl. Each reaction contained the following final concentrations: PCR buffer 20mM Tris-HCl, 50mM potassium chloride (KCl) (Gibco), 1.5mM magnesium chloride ($MgCl_2$) (Gibco), 20 pmol of each primer, 0.2 mM of deoxynucleoside triphosphates (dNTPs) (Gibco), 2.5 units of Taq DNA polymerase (recombinant from *Thermus aquaticus*) (Perkin Elmer for Leu10Pro) (Gibco for Arg25Pro) and 100ng of DNA template. Negative controls for each reaction were used (the DNA template was replaced by 2µl of water) to detect any possible contamination. The PCR mix was overlaid with 40µl of mineral oil (Sigma). The overlying oil prevents evaporation and keeps the temperature of the PCR mix during the reaction in the thermocycler. The tubes were put in the thermocycler (Biometra). The temperature program was, 1 cycle: 95°C for 10 minutes, 1 cycle: 94°C for 5 minutes; 5 cycles: 94°C for 60 seconds, 60°C for 60 seconds, 72°C for 60 seconds; 30 cycles: 94°C for 30 seconds, 56 °C for 30 seconds, 72°C for 30 seconds; followed by 1 cycle: 72°C for 5 minutes. PCR products were stored at 4°C until electrophoresis was carried out. Furthermore, dimethylsulfoxide (DMSO) (7%) was also used to increase the specificity of the reaction for the Leu10Pro genotype.

2.3.4 Genotyping for Thr263Ile

Amplification Refractory Mutation-Screening Polymerase Chain Reaction (ARMS-PCR) was also used for the Thr263Ile genotyping. Two sense (forward) primers that differed in size and one antisense (reverse) primer were used for amplification of C/T polymorphism for each DNA sample. These three primers were used in a single reaction mix with the final volume of 50µl. Polymerase chain reaction buffer was used in a concentration of 20mM Tris-HCl, 50mM KCl, 1.5mM of $MgCl_2$, 20pmol of each primer, 0.2 mM of deoxynucleoside triphosphates (dNTPs), 2.5 units of Taq DNA polymerase (Bioline) (recombinant from *Thermus aquaticus*) and 100ng of DNA template. Negative controls were used (the DNA template was replaced by 2µl of water) to detect any possible contamination. The PCR reaction mix was overlaid with 40µl of mineral oil.

The tubes were put in a thermocycler. Standard PCR conditions were, 1 cycle: 95°C for 10 minutes; 30 cycles: 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds; followed by 1 cycle: 72°C for 5 minutes. Polymerase chain reaction products were stored at 4°C until electrophoresis was carried out.

Following amplification, agarose gel electrophoresis was used to visualize the reaction products.

2.3.4 Genotyping C-509T by restriction digest with Bsu 36I

One sense (forward) primers and one antisense (reverse) primer were used for amplification of C/T polymorphism for each DNA sample. Reactions were carried out in a final volume of 50 µl. Polymerase Chain Reaction buffer was used in a concentration of 20mM Tris-HCl, 50mM KCl, 1.5mM of $MgCl_2$, 20pmol of primer, 0.2 mM of deoxynucleoside triphosphates (dNTPs), 2.5 units of Taq DNA polymerase (Bioline) (recombinant from *Thermus aquaticus*) and 100ng of DNA template. Negative control was used (the DNA template was replaced by 2µl of water) to detect any possible contamination. The PCR reaction mix was overlaid with 40µl of mineral oil. The tubes were put in the thermocycler. Standard PCR conditions were, 1 cycle: 95°C for 5

minutes; 34 cycles: 95°C for 1 minute, 60°C for 1minute, 72°C for 1 minute; followed by 1 cycle: 72°C for 5 minutes. The PCR products were precipitated with three volumes of 96% ethanol and 1/10 volume of 3M sodium acetate (pH 5.2) and incubated at -20°C for an hour. Then centrifuged at 12000 g. Then DNA was resuspended in 20µl of *Bsu36I* (Promega) digestion mix containing 10 units of enzyme, followed by incubation at 37°C overnight. The following morning, the digestion products were analysed by agarose gel electrophoresis.

2.4 Electrophoretic Techniques

Genomic DNA, PCR products and digests were analysed by agarose gel electrophoresis. To analyse genomic DNA, 0.8% agarose gels were prepared by adding 0.4 g of agarose to 50 ml of 1x of Tris borate EDTA (TBE) buffer. To detect PCR products and digests 2% agarose gels were prepared by adding 1 g of agarose to 50 ml of 1x TBE buffer. 10x TBE buffer was made (0.89M Tris, 0.89M boric acid, and 0.025M EDTA water, pH 8.0). This was kept at room temperature until diluted (to 1x TBE) and used. Heating for 1 minute in the microwave oven agarose suspension was dissolved. After the gel was cooled, 2 µl of ethidium bromide (Sigma) (stock solution 10mg/ml in water) was added. Then the dissolved agarose was set in the gel apparatus (Biometra, Luton). To check the quality of genomic DNA, 5µl of loading dye (1% bromophenol blue in 40% sucrose) was added to 15 µl of DNA sample and subjected to electrophoresis in the agarose gel. For PCR samples, 5µl of loading dye was added to 10 µl of the PCR products. DNA marker (ϕX174DNA/HaeIII) (Promega) was electrophoresed in each gel to size the product. For marker (ϕX174DNA/HaeIII) preparation, 0.5 µl of the marker stock (1 µg/ µl) was added to 15µl of water and 5 µl of loading dye. Electrophoresis was carried out at 80 volts for one hour at room temperature. Photographs of the gels were taken by Polaroid camera or by digital camera (Kodak electrophoresis documentation and analysis system, Rochester).

2.4 *Measurement of circulating (plasma and serum) TGF-β1*

Plasma total acid-activated TGFβ1 protein levels were measured by using an ELISA [Quantikine Human TGF-β1 Immunoassay kit (R&D Systems, Abingdon, Oxford, UK)] (Danielpour et al., 1989, 1993; Tsang et al., 1990). The assay was carried out according to the manufacturer instructions. The sensitivity of the assay was 7 pg/ml. The cross reactivities were 12.4% for latent TGF-β1, 0.08 for TGF-β2, and 0.12% for TGF-β3 which are all non-significant. The samples were diluted (1:5 in a calibrator diluent) and assayed in duplicates. An automatic plate reader (Wallace) read the ELISA plates at 450nm. Control plasma was collected from healthy 20 volunteers. Furthermore, the precision of the measurement was studied (see chapter 5 section 5.3.1.1)

2.5 Renal histology evaluation

Four-micron sections from paraffin-embedded renal biopsy material were stained with haematoxylin and eosin, periodic acid Schiff and Masson's trichrome. The severity of the histological changes (glomerulosclerosis and tubulointerstitial fibrosis) were scored according to an arbitrary scale (from 1-4) where 1 = normal (no pathology), 2 = mild changes (affecting < 25% of the area), 3 = moderate changes (25-50%) and 4 = severe (>50%) as previously described (Muchaneta-Kubara, *et al.*, 1995). In addition, we counted the inflammatory cellular infiltrate (ICI) of the renal interstitium by counting the number of inflammatory cells per low power field (x200 magnification). The individual score was based on the mean of the number of cells in the fields evaluated. Each measurement was carried out on 6 – 10 fields per slide.

Histology reports were reviewed and attributed an arbitrary score from 1 to 4 depending on the severity of scoring parameters (no changes = 1, mild changes = 2, moderate changes = 3, severe changes = 4).

2.6 Estimation of TGF-β1 in renal tissue

Immunohistochemistry was done to localise and estimate TGF-β1 protein at tissue level. A specific human anti-TGF-β1 antibody was used for this purpose (Promega). Renal biopsies were fixed in 10% neutral-buffered formalin, paraffin-embedded, sectioned at 3 μm and stained using a standard immunoperoxidase staining technique. Briefly, sections were deparaffinized and rehydrated in descending concentrations of alcohol. Following blocking of endogenous peroxidase activity with 3% H_2O_2 in methanol and nonspecific binding sites with a protein blocker, the primary antibody was added with overnight incubation at 40°C. *On day 2*, the biotinylated secondary antibody (Vector) was added at a concentration of 2% for 30 minutes (37°C) followed by addition of the avidin-biotin-peroxidase (ABC) complex (Vector). Visualization of the reaction was performed using 3' amino- 9' ethyl-carbimazole (AEC) (Vector) as the chromogen. All steps were performed at room temperature in a humidified chamber unless otherwise specified. Controls included sections incubated in the absence of the primary antibody as well as some incubated with a non-immune appropriate immunoglobulin. Control renal tissues were obtained from cadaveric donor healthy kidneys.

2.6.1 Morphometric analysis

For the evaluation of TGF-β1 staining, sections were analysed using a drawing tube and a 25 point squared lattice relying on a standard morphometric analysis based on point counting (Williams, *et al.*, 1987, Muchaneta-Kubara, *et al.*, 1995). Data was collected from a series of adjacent fields (10-20) extending perpendicularly from the cortex to the medulla. The percentage points falling on stained structures (cell or interstitium) were estimated, and the percentage of glomeruli stained was calculated. Slides were scanned independently and subsequently, I reviewed my scoring with my supervisor who finalized the scoring with me.

2.8 Statistical Analysis

Analysis of allele frequencies and carriage rates was carried out using the Chi-squared test. Comparison of the means (blood pressure and proteinuria and estimated TGF-β1

protein levels either in blood or in renal tissues) was carried out using F-tests followed by the t-tests (unpaired, two-tailed, for equal or unequal variance, as appropriate) (see below). Correlation and regression between the different measurements was carried out using ANOVA test on Microsoft Excel and SPSS. The Hardy-Weinberg equation (O'Neil. 1997, http://anthro.palomar.edu) was applied to compare the expected versus observed distribution of genotypes (see below). A p value < 0.05 was considered significant.

2.8.1 Hardy-Weinberg equation

The Hardy-Weinberg equation was used to predict the expected distribution of genotypes at given allele frequencies.

$p^2 + 2pq + q^2 = 1$

(p = frequency of the common allele, q = frequency of the rare allele).

Using the above formula, the expected and observed frequencies of genotyping were compared. If the differences between the observed and the expected frequencies are not statistically significant, the balance of genotypes does not differ from there expected in a population in Hardy-Weinberg equilibrium.

2.8.2 Chi-squared analysis (χ^2)

Chi-squared analysis tests the null hypothesis (no difference between the observed and expected values).

$$\chi^2 = \Sigma \frac{(Observed - Expected)^2}{Expected}$$

Degree of freedom equal number of genotype – 1 for testing the Hardy-Weinberg expectations Comparison of genotypes and carriage rates between different groups (i.e. control Vs patients, or progressors Vs non-progressors) was carried out using contingency tables. For these, the degree of freedom are calculated by (number of raws – 1) χ (number of columns – 1) An example is given below.

If Yates' correction for continuity is to be applied, due to cell counts below 5, the calculation is the same except for each cell, subtract an additional 0.5 from the difference of O - E, prior to squaring and then dividing by E.

Example of 2x2 contingency table was used to calculate χ^2

		Variable 1		*Total*
		Progressors	*Non-progressors*	
Variable 2	*Carriage of allele 2*	**a**	**b**	**a + b**
	Non-carriage of allele 2	**c**	**d**	**c + d**
		a + c	**b + d**	**a + b + c + d**

$\chi^2 = (ad / cb)^2 / (a + b)(c + d)(a + c)(b + d) \times (a + b + c + d)$

The null hypothesis was rejected and the statistic was considered significant if the p value < 0.05.

2.8.3 Odds ratio (OR) and confidence interval

Odds ratio analysis was done manually by calculating ab/cd. Confidence interval (CI) was calculated by finding standard error (SE) of log e (antilog) of OR.

SE (log e OR) = square roots of (1/a + 1/b + 1/c + 1/d)

Y= In OR – (1.96 x SE (In OR)

X= In OR + (1.96 x SE (In OR)

CI= is the anti log for the Y and X values.

A p value < 0.05 was considered significant.

2.8.4 F-test

Using F test (EXCEL) tested the significant of differences in tested variance between two values. The null hypothesis was rejected if the p (t) < 0.05 (statistically significant).

2.8.5 t-test

The significance of any difference between two means was tested by the appropriate unpaired t test (for samples of equal or unequal variance as assessed by the F-test) by using EXCEL. The null hypothesis was rejected if the p (t) < 0.05 (statistically significant).

2.8.6 Regression and correlation

Regression analysis was used to assess the significance of the slope of the reciprocal of the 1/serum creatinine against time. If the p value for the regression < 0.05, the patient was considered to have progressive CRF. On the other hand if the p value for the regression > 0.05 the patient was considered to be a non-progressor. Furthermore, for comparing the variances regression and correlation were used. The null hypothesis was rejected if the p(r) < 0.05 (statistically significant).

2.8.7 Coefficient of variation (CV):

The following formula was used to calculate CV to study the precision of the measurement of the circulating TGF-β1 protein levels in the plasma.

$$\frac{\text{Standard deviation X 100}}{\text{Mean of assay}}$$

2.8.7 Coefficient of linkage disequilibrium (D')

The following formula was used to calculate the D' value (departure from linkage equilibrium).

$$D' = (P_{ab} \cdot P_{AB} - P_{aB} \cdot P_{Ab}) / (P_{ab} \cdot P_{AB} + P_{aB} \cdot P_{Ab})$$

Abbreviations:

P_{ab}: proportion of the carrier patients for allele a and b

P_{AB}: proportion of the carrier patients for allele A and B

P_{aB} : proportion of the carrier patients for allele a and B

P_{Ab}: proportion of the carrier patients for allele A and b

Chapter 3

Clinical data

3.1 Introduction

The relative frequency of different causes of CRF varies from country to country. In the UK, chronic glomerulonephritis remains the main cause of ESRF (Wing and Jones, 2000). An increasing number of patients suffer from ESRF due to diabetic nephropathy. In the US, diabetic nephropathy is currently the most common cause of ESRF along with hypertensive nephrosclerosis (USRDS, 2001). Diabetic nephropathy accounts for up to 40% of all patients on renal replacement therapy in the US (USRDS, 2001). Other causes of CRF include hypertensive nephrosclerosis, chronic interstitial nephropathies, obstructive uropathy as well as polycystic kidney disease (Wing and Jones, 2000).

The progression of chronic renal failure (CRF) characterised clinically by a heterogeneous rate of decline of renal function (El Nahas and Winearls, 1997). Histologically, this reflects the various degrees of underlying renal scarring and the associated progressive deletion of intrinsic renal cells and their replacement by collagenous extracellular matrix (ECM).

There is a wide range of factors known to influence the rate of progression of CRF (Silver, *et al.*, 1989; Sedor, 1992). These include genetic, racial and environmental factors (USRDS, 2001). In addition, some modifiable factors such as systemic hypertension, proteinuria, dyslipidaemia and smoking have been implicated (For review, Locatelli and Del Vecchio, 2000).

Of all the modifiable risk factors, systemic hypertension is the most important (Adamczak, *et al.*, 2002). An elevation of systolic as well as diastolic blood pressure is associated with a faster rate of progression of CRF in patients with diabetic and non-diabetic nephropathies (Brazy, *et al.*, 1989; Ruggenenti, *et al.*, 1998). It is believed that the transmission of systemic hypertension to poorly autoregulated glomeruli lead to their scarring (For review, Dworkin and Weir, 2000). Therefore, the control of systemic as well as glomerular hypertension is crucial for delaying the progression of CRF in experimental animals (For review, Dworkin and Weir, 2000). In humans, a growing number of references suggest that the control of hypertension is also protective (Maschio,

et al., 1996; Ruggenenti, *et al.*, 2001; Adamczak, *et al.*, 2002). In addition, the use of angiotensin converting enzyme (ACE) inhibitors has been shown to provide additional protection (For review, Dworkin and Weir, 2000).

Proteinuria is also an important prognostic marker for the progression of CRF (For review, Harris, 2000). Both diabetic and non-diabetic nephropathies associated with heavy proteinuria have a poor prognosis. Recent research suggests that proteinuria may also contribute to scarring of the kidney (Harris, 2000). It has been postulated that excessive filtration of proteins and their accumulation in the glomerular mesangium leads to mesangial overload and sclerosis (Bertani and Remuzzi, 1997). Also, excessive filtration of proteins and their reabsorption by proximal tubular cells activates these cells and leading to release of chemokines, cytokines and growth factors (Harris, 2000). These factors can in turn attract inflammatory cells into the renal interstitium, which activate renal fibroblasts. The proliferation of activated renal fibroblasts and their synthesis of extra-cellular matrix (ECM) lead to renal fibrosis (Jernigan and Eddy, 2000). It is, therefore, important to reduce proteinuria when we treat patients with progressive CRF (Jafar, *et al.*, 2002). This may be one of the advantages associated with control of blood pressure and the use of ACE inhibitors.

Moorhead and his colleagues (1982) postulated that dyslipidaemia can accelerate the progression of glomerular and tubular scarring. In animals, hyperlipidaemia is associated with accelerated renal fibrosis and its control is protective (Keane, 2000). In humans an association has been reported between hypercholesterolemia progression of diabetic and non-diabetic CRF (Krowleski, *et al.*, 1994; Samuelsson, *et al.*, 1997). It is, therefore, important to monitor and treat hypercholesterolemia in patients with progressive CRF (Keane, 2000; Fried, *et al.*, 2001).

Lifestyle factors, such as smoking, alcohol consumption and the abuse of recreational drugs have all been associated with a higher risk of ESRF (Perneger, *et al.*, 2001 and Bakir and Dunea, 2001).

3.2 Methodology

One hundred and forty six patients with CRF were studied. The study included patients with creatinine concentration >150 μmol/l, white Caucasians, of any gender and age group, with various degrees of renal insufficiency including some on dialysis replacement therapy (86 end-stage renal failure [ESRF] patients: 46 on continuous ambulatory peritoneal dialysis [CAPD] and 40 on haemodialysis [HD]. The inclusion and exclusion criteria were discussed previously (see materials and methods, chapter 2, section 1.3).

Patients with CRF who were not on renal replacement therapy (RRT) were followed up retrospectively for 58.8±4.8 (mean ±SEM) months. Furthermore, patients with chronic renal failure (CRF) were classified into: -

(i) Progressors (P) – this group includes both patients who had a 1/serum creatinine against time regression slope significantly inferior to zero and patients who were on renal replacement therapy (ESRF).

(ii) Non-progressors (NP) – these patients have a 1/serum creatinine against time regression slope, which was not significantly different from zero.

3.2. 1 Parameters

The following parameters were studied: Patients' age, gender, weight, height, smoking habits, renal diagnosis, renal histology (when a renal biopsy had been undertaken), blood pressure, serum creatinine, proteinuria, haematuria, serum cholesterol and triglycerides levels. Parameters such as systemic blood pressure and proteinuria were recorded at the time of diagnosis as well as during follow-up (mean of the follow-up values). The slope of the regression analysis of the reciprocal of serum creatinine value (1/serum creatinine) against time was taken as an indicator of the rate of progression of CRF.

3.2.2 Renal histology analysis

Renal biopsies were available in 25 patients. Their biopsy material was available in the archives of the histopathology Department at the Northern General Hospital Trust. Paraffin-embedded section (4 μ) were stained with conventional dyes as detailed in chapter 2, section 6.2.

I analysed, histomorphometrically the severity of renal scarring parameters [glomerulosclerosis (GS) and tubulointerstitial fibrosis (IF)] according to an arbitrary score (from 1= normal to 4= severe) as detailed in the Material and Methods chapter (chapter 2 section 6.2). Furthermore, the pathological processes were divided into two categories. Glomerulosclerosis (GS) were divided into, a) normal + mild scores and b) moderate + severe. For interstitial fibrosis (IF) the available scores were only score 3 which was considered moderate and score 4 which was considered severe IF. I also evaluated the severity of the interstitial cellular infiltration (ICI) by counting individual cells within a microscopic field (x 200). An average of ten consecutive and random fields were counted. The mean of the cellular count for each patient was the given ICI score.

3.2.3 Statistical Analysis

The results are given as mean ± standard error of the mean (M ± SEM). Analysis of the difference between the means of the studied parameters was carried out using F tests followed by t tests (*unpaired, two tailed, for equal or unequal variance, as appropriate*). Chi square analysis tests the *null hypothesis*. Yates' correction was applied for the 2x2 contingency table if any of its cell less than 5. Correlation and regression between the different measurements was carried out relying on regression analyses. A p value < 0.05 was considered significant.

3.3 Results

3.3.1 Clinical Observations

One hundred and twelve patients (39 females and 73 males) were classified as progressors, 34 patients (8 females and 26 males) were non-progressors. The characteristics of the progressors and non progessors are shown in table 3.1

Table 3.1

The clinical characteristics of the CRF patient groups (mean ±SEM)

Parameters	*Whole group (n = 146)*	*P = 112*	*NP =34*	*P(f)*	*P (t)*
Age (years)	57.48 ± 1.4	57 ± 1.5	58.9 ± 2.3	0.2	0.4
Serum creatinine (µmol/l)	345.5 ± 18.7	514 ± 32	274 ± 23	0.4	**0.0005***
1/creatinine slope	-0.00003 ± 0.00000903	-0.00009 ± 0.00002	0.000009 ± 0.000004	0.002	**0.000007***
SBPd (mmHg)	152.41 ± 2.26	155.1 ± 2.7	146.6 ± 4.9	0.03	0.13
SBPf (mmHg)	148.52 ± 1.8	153.1± 2.3	140.5 ± 3.8	0.5	**0.008***
DBPd (mmHg)	87.31 ± 1.22	88.1 ± 1.4	86.6 ± 3.3	0.007	0.5
DBPf (mmHg)	84.65 ± 1	85.8 ± 1.2	83.7 ± 2.9	0.003	0.5
Proteinuria at diagnosis g/24h	2.49 ± 0.187	2.8 ± 0.3	1.3 ±0.3	0.00006	**0.0004***
Proteinuria during follow up g/24h	2.32 ± 0.174	2.7 ± 0.3	1.1 ± 0.2	0.000033	**0.00007***
S.Cholesterol (mmol/l)	5.6 ± 0.14	5.4 ± 0.15	6.2 ± 0.4	0.1	**0.04***
S.Triglycerides (mmol/l)	2.8 ± 0.18	2.6 ± 0.2	3.6 ± 0.6	0.08	**0.044***

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up. DBPf: diastolic blood pressure during follow up. S.Cholesterol: serum cholesterol, S.Triglycerides: serum triglycerides. P: progressors, NP: non-progressors, p (f): p value for f test. p (t): p value for t test.

Of note the clinical data relating to progression (1/Cr slope) was calculated for patients with CRF who were not on RRT.

The distribution of different disease entities is listed in table 3.2.

Table 3.2.

Relative frequency of different causes of CRF

Diagnoses	***Total***	***P***	***NP***	***% of total patients***
Hypertensive nephrosclerosis	28	20	8	19.7
CIN	23	12	11	16.2
Obstructive uropathy	15	10	5	10.6
Glomerulonephritis	15	10	5	10.6
DN	12	10	2	9.2
Systemic vasculitis	11			8.1
i) Wegeners granulomatosis	6	4	2	4.2
ii) SLE	3	1	2	2.1
iii) Rheumatoid arthritis	2	1	1	1.4
PKD	7	3	4	4.9
Amyloidosis	2	1	1	1.4
Unknown	29	14	15	20.4

Abbreviations: CIN: chronic interstitial nephritis including analgesic nephropathy and chronic pyelonephritis, DN: diabetic nephropathy, SLE: systemic lupus erythematosus, PKD: polycystic kidney disease.

3.3.2 Pathological data

Most of the patients did not have a renal biopsy. Pathological study was done on the available sections from 24 patients who underwent a renal biopsy. Seventeen patients were diagnosed as having glomerulonephritis, 5 patients had chronic interstitial nephritis and 2 of them were diagnosed as having amyloidosis (Table 3.3). According to the pathological processes, the patients with glomerulosclerosis were classified into mild (11

patients), moderate (4 patients) and severe (9 patients) (the assessment of the severity was discussed in section 3.2). The patients with tubulointerstitial fibrosis (IF) also were classified into mild (1 patient), moderate (19 patients) and severe (4 patients). Glomerulosclerosis were more severe in crescentic glomerulonephritis (CGN) than the other pathological forms (Table 3.3). Patients with chronic interstitial nephritis (CIN) had less severe interstitial fibrosis compared to the other pathological forms (Table 3.3).

Table 3.3

The distribution of different pathological diagnoses amongst CRF patients and the severity (mean ±SEM) of the renal scarring parameters

Pathology	*n=*	*GS*	*IF*	*ICI (cell/field)*
MCGN	6	3.1 ± 0.4	3.2 ± 0.15	20 ± 12.6
FSGS	5	2 ± 0.5	3 ± 0	20 ± 14
MN	2	1.5 ± 0.5	3 ± 0	7.5 ± 7.5
DN	2	2.5 ± 1.5	3.5 ± 0.5	57.5 ± 2.5
CGN	2	4 ± 0	3.5 ± 0.5	60 ± 0
CIN	5	2.4 ± 0.7	2.8 ± 0.5	42.6 ± 9
Amyloidosis	2	2.5 ± 0.5	3 ± 0	60 ± 0

Abbreviations: GS: glomerulosclerosis, IF: interstitial fibrosis, ICI: interstitial cellular infiltration, MCGN: mesangiocapillary glomerulonephritis, FSGS: focal and segmental glomerulosclerosis, MN: membranous nephropathy, DN: diabetic nephropathy, CGN: crescentic glomerulonephritis, CIN: chronic interstitial nephritis

There was a significantly higher number of patients with severe IF in the progressors compared to the NP. On the other hand, severe glomerulosclerosis was found in a similar number of P and NP (table 3.4).

Table 3.4

The distribution of pathological processes among the studied CRF patients

Pathology	***P***	***NP***	***Total***	***χ^2 (at one degree of freedom)***	***P for χ^2***
GS Mild	10	1	12	2.1	0.15
Severe	9	4	13		
Total	*19*	*5*	*24*		
IF Moderate	9	10	20	10.2	**0.002***
Severe	5	0	2		
Total	14	10	24		

Abbreviations: GS: glomerulosclerosis, IF; interstitial fibrosis, P: progressors, NP: non-progressors.

*= <0.05 (significant).

It was noted that there were no significant relationship between the different pathological processes and any of the studied parameters (table 3.5).

Table 3.5. The relationship between the pathological processes and the different studied parameters (mean ± SEM)

	ICI		***GS***		***IF***	
	With ICI n=14	***Without ICI n=10***	***Mild n=11***	***Severe n=13***	***Moderate n=19***	***Severe n=5***
I) Proteinuria (g/24hrs) 1- At diagnosis P(f) P(t)	4.1±1.4 **0.02** 0.97	4.1±1.5	3.0±09 0.12 0.5	4.0±1.2	3±0.7 **0.01** 0.4	6.3±2.8
2- During follow up P(f) P(t)	4.7±1.4 0.3 0.6	3.5±1.3	2.9±0.8 0.07 0.6	3.7±1.3	2.9±0.7 **0.009** 0.5	5.5±2.7
II) Blood pressure (mmHg) 1- SBPd P(f) P(t)	149±3 **0.02** 0.9	150±8	144±7 0.2 0.4	153±9	146±6 0.4 0.4	160±10
2- DBPd P(f) P(t)	93±3 **0.05** 0.2	84±6	86±6 0.3 0.6	91±7	90±5 0.1 0.6	84±4
3- SBPf P(f) P(t)	145±6 0.2 0.54	151±6	154.5±6 0.5 0.2	150±5	148±5 0.3 0.5	156±7
4- DBPf P(f) P(t)	82±4 0.2 0.3	87.3±3.5	84±3 0.03 0.4	90±5	87±3.5 0.3 0.5	87±5
III) 1/Cr slope P(f) P(t)	0.00001± 0.00002 0.06 0.2	-0.00005± 0.00002	0.000008± 0.00003 0.2 0.9	-0.00002± 0.00003	-0.000009 ±0.000003 0.4 0.6	-0.000008 ±0.000001
IV) Serum lipids (mmol/l) 1- Serum Cholesterol P(f) P(t)	4±0.5 0.06 0.2	6.1.4	5±1.3 0.2 0.9	5±1.5	5.95±0.8 0.3 0.1	3.3±0.4
2- Serum Triglycerides P(f) P(t)	2.5±0.5 0.1 0.8	2.8±1.6	5±0.8 0.07 0.07	1.9±0.5	2.7±0.6 0.1 0.1	1.4±0.3

Abbreviations: see tables 3.1 and 3.4

3.4 Factors affecting the progression of CRF

Table 3.1 summarizes the data collected (see appendix) for the patients studied.

3.4.1 Gender

The number of males was higher than females. It was noted also that 72 males and 35 females were classified as progressors, and 23 males and 12 females were classified as non-progressors. The 1/creatinine slopes were significantly lower in males (Table 3.6). This means that progression is faster in males than in females. Also, proteinuria at follow-up was significantly higher in males than females (Tables 3.6) (Figure 3.1). There was no difference between other parameters studied between males and females (Table 3.6).

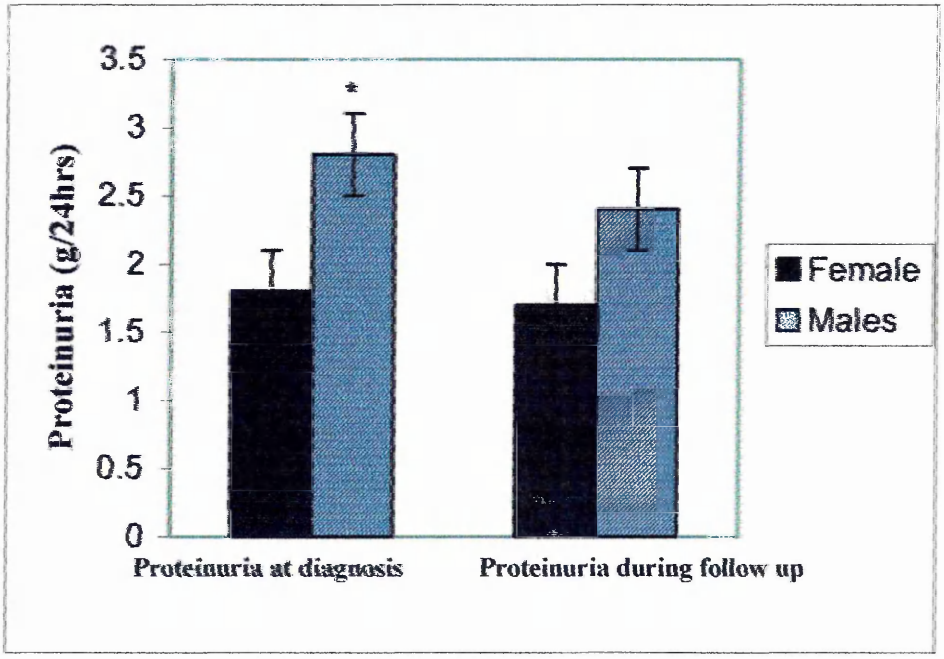
Table 3.6

The clinical characteristics (mean ± SEM) of the studied patients according to gender

CRF parameters	*Females (N=47)*	*Males (N=99)*	*p(f)*	*p(t)*
Proteinuria at diagnosis (g/24h)	1.7±0.3	2.5±0.3	0.013	0.06
Proteinuria during follow up (g/24h)	1.8±0.3	2.9±0.3	0.14	0.047*
SBPd (mmHg)	155±4	153±4	0.14	0.6
DBPd (mmHg)	89±2	87±2	0.3	0.3
SBPf (mmHg)	152±3	149±2	0.14	0.2
DBPf (mmHg)	84.3±4	87.2±1.3	0.14	0.2
S.Cholesterol (mmol/l)	5.9±0.3	5.6±0.3	0.09	0.3
S.Triglycerides (mmol/l)	2.5±0.3	3±0.3	0.1	0.14
S.Creatinine (µmol/l)	340±50	524±57	0.006	0.02*
1/Creatinine slope	-0.000009± 0.0000000019	-0.00004± 0.000000008	0.0003	0.055
CrCl (ml/min)	19±4	23±2	0.003	0.5

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up. DBPf: diastolic blood pressure during follow up. S.cholesterol: serum cholesterol, S.triglycerides: serum triglycerides. CrCl: creatinine clearance, p(f): p value for f test. p(t): p value for t test.

Figure 3.1. Proteinuria (mean ± SEM) at diagnosis and during follow up in CRF patients according to gender



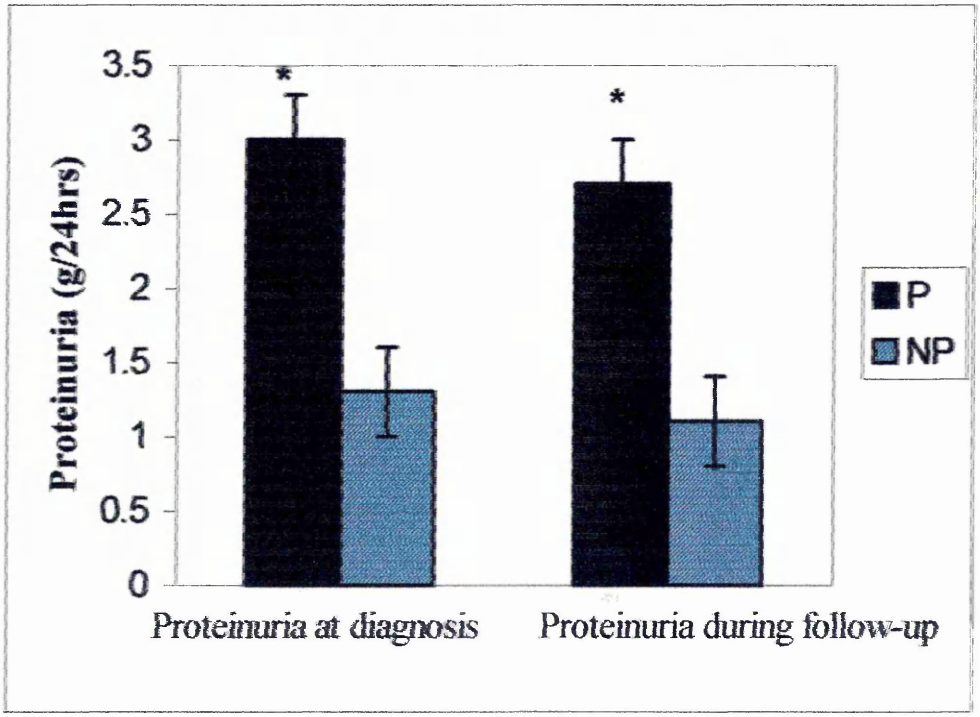
* = $p<0.05$.

3.4.2 Proteinuria

It was found that overall means of proteinuria at diagnosis was 2.5±0.24 g/24hrs and 2.2±0.2 g/24hrs during follow-up. We noted that 41 patients had proteinuria at onset and 31 patients had proteinuria at follow up (> 300 mg/24hr). Proteinuria, both at onset and during follow up, was significantly higher in progressors compared to non-progressors ($p < 0.0005$) (figure 3.2).

Figure 3.2.

Proteinuria (mean±SEM) at diagnosis and during follow up in progressors (P) and non-progressors (NP) with CRF



*= P <0.05.

Further, I compared the proteinuria in diabetic nephropathy to the remaining patient group. In diabetic nephropathy, proteinuria levels both at diagnosis and during follow up were not different from that of the remaining patients (at diagnosis 2.3±0.2g/24h, during the follow up 2.1±0.2g/24h) (Table 3.7).

Table 3.7

Proteinuria and 1/Cr slope (mean ± SEM) among the different causes of CRF

	n=	***Proteinuria at diagnosis (g/24hrs)***	***Proteinuria during follow up (g/24hrs)***	***1/Cr slope***
Hypertensive nephrosclerosis	14	2.2 ± 0.8	2.4 ± 0.9	-0.00003±0.00001
Systemic vasculitis	10	2.6±0.6	2.5±0.7	-0.0000014±0.000013
GN	9	1.7±0.3	2±0.27	-0.000007±0.00002
Diabetic nephropathy	7	3.1±0.8	2.8±0.7	-0.00003±0.000014
CIN	7	1.2±0.4	1.5±0.3	-0.00002±0.00001
Obstructive uropathy	6	1.9±0.6	1.7±0.5	-0.000003±0.00001
PKD	1	1.7±0.5	1.6±0.4	-0.00002±0
Unknown	13	2.5±0.5	2.5±0.5	-0.00001±0.000015

Abbreviations: CIN: chronic interstitial nephritis, PKD: polycystic kidney disease GN: glomerulonephritis.

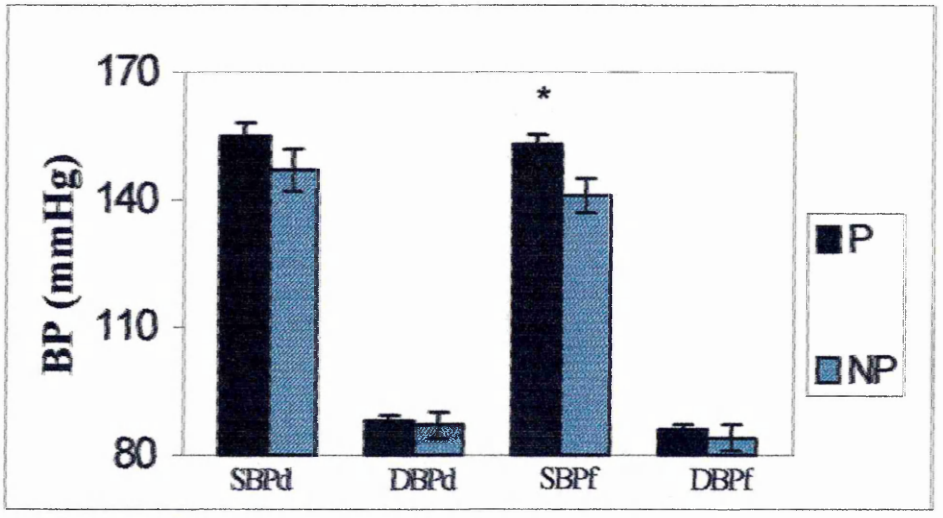
Of note the clinical data relating to progression (1/Cr slope) was calculated for patients with CRF who were not on renal replacement therapy (RRT).

3.4.3 Blood pressure

In the overall population, blood pressure values at diagnosis did not differ significantly from those during follow-up (Figure 3.3). However, systolic blood pressure during follow up was significantly higher in the progressors than in the non-progressors (Figure 3.3). On the other hand, there was no significant difference in diastolic blood pressure values between progressors and non-progressors (Figure 3.3).

Figure 3.3.

Blood pressure values (mean ± SEM) in CRF progressors (P) and non-progressors (NP)



* : p value = 0.01.

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: mean systolic blood pressure during follow-up, DBPf: mean diastolic blood pressure value during the follow-up period.

3.4.4 Serum Lipids

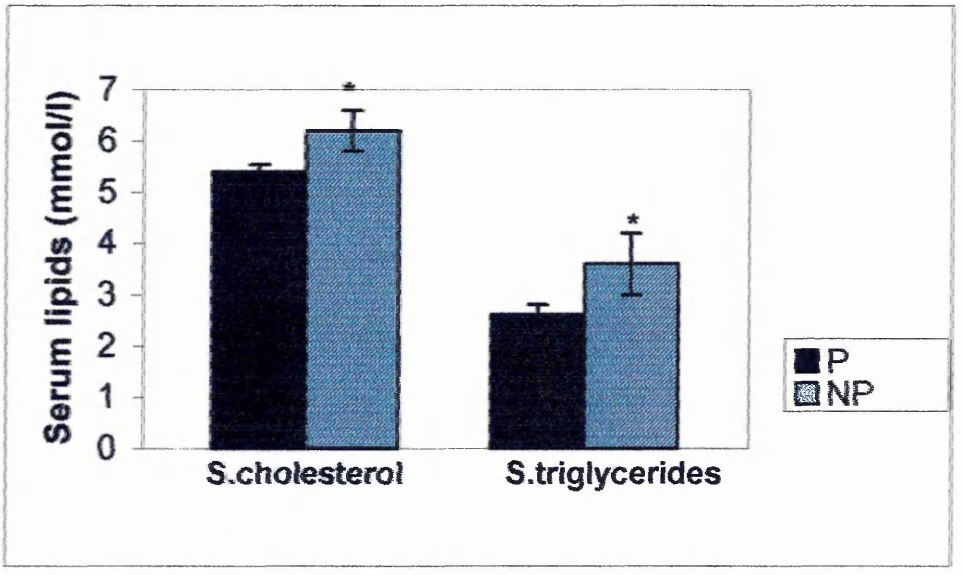
The mean serum cholesterol level was 5.7±0.16 mmol/l and the serum triglycerides level was 2.8±0.19 mmol/l in the overall patient group.

Both serum cholesterol and triglycerides were significantly higher in non-progressors compared to progressors (Figure 3.4).

There was no significant difference in circulating lipids levels in the patients with diabetic nephropathy (S.cholesterol = 5.5±0.37 mmol/l, S.triglycerides = 3.3±0.8 mmol/l) compared to the non-diabetic CRF (S.cholesterol= 5.8±0.17 mmol/l, S.triglycerides= 2.8±0.19mmol/l).

Figure 3.4.

Serum lipids (mean ±SEM) in CRF progressors (P) and non-progressors (NP)



Abbreviations: S.cholesterol: serum cholesterol, S.triglycerides: serum triglycerides.

*= $p < 0.05$

3.4.5 Correlations between the different parameters of CRF

There was a significant correlation between the age of the patients and proteinuria, both at onset and during follow up. Furthermore, the age was significantly correlated with diastolic blood pressure both at onset and during the follow-up (Table 3.8).

There was also a significant correlation between diastolic blood pressure at diagnosis and both proteinuria and serum triglyceride levels at diagnosis. Moreover, there were significant correlations between serum triglyceride levels and both systolic and diastolic blood pressure during the follow-up. There were also significant correlation between the 1/creatinine slope and both proteinuria at diagnosis and serum cholesterol levels. Furthermore, the mean of proteinuria during the follow up was significantly correlated with systolic blood pressure at onset (Table 3.8).

Table 3.8. Correlation between the different CRF parameters

CRF parameters	*n=*	*r =*	*R^2 =*	*P (r)*
I) Proteinuria at onset (g/24hr) with				
1- age of the patients (year)	123	0.164	0.028	**0.048***
2- SBPd (mmHg)	119	0.144	0.024	0.087
3- DBPd (mmHg)	119	0.174	0.03	0.053
4- SBPf (mmHg)	119	0.128	0.016	0.15
5-DBPf (mmHg)	119	0.25	0.06	**0.005***
6- S.Cholesterol (mmol/l)	67	0.026	0.001	0.83
7- S.Triglycerides (mmol/l)	60	0.152	0.023	0.2
8- 1/creatinine slope	76	0.36	0.13	**0.001***
II) Proteinuria during follow up (g/24hr) with				
1- age of the patients (year)	119	0.29	0.084	**0.001***
2- SBPd (mmHg)	113	0.173	0.03	0.056
3- DBPd (mmHg)	113	0.163	0.27	0.07
4- SBPf (mmHg)	113	0.185	0.34	0.36
5-DBPf (mmHg)	113	0.21	0.05	**0.017***
6- S.Cholesterol (mmol/l)	56	0.231	0.054	0.07
7- S.Triglycerides (mmol/l)	56	0.144	0.21	0.3
8- 1/creatinine slope	67	0.122	0.15	0.3

CRF parameters	*n=*	*r =*	*R^2 =*	*P (r)*
III) SBPd (mmHg) with				
1- age (year)	130	0.005	0.000	0.9
2- S.Cholesterol (mmol/l)	60	0.1	0.012	0.4
3- S.Triglycerides (mmol/l)	57	0.2	0.04	0.3
4-1/creatinine slope	34	0.128	0.016	0.3
IV) DBPd (mmHg) with	130	0.215	0.046	**0.016***
1- age of the patients (year)				
2- S.Cholesterol (mmol/l)	60	0.128	0.016	0.34
3- S.Triglycerides (mmol/l)	57	0.04	0.002	0.06
4- 1/creatinine slope	34	0.1	0.01	0.4
V) SBPf (mmHg) with				
1- age (year)	130	0.015	0.0002	0.9
2- S.Cholesterol (mmol/l)	60	0.005	0.007	0.9
3- S.Triglycerides (mmol/l)	57	0.245	0.06	0.06
4- 1/creatinine slope	34	0.13	0.016	0.3
VI) DBPf (mmHg) with				
1-age (year)	130	0.33	0.102	**0.0003***
2- S.Cholesterol (mmol/l)	60	0.116	0.013	0.4
3- S.Triglycerides (mmol/l)	57	0.08	0.007	**0.028***
4- 1/creatinine slope (mmol/l)	34	0.08	0.005	0.6
VII) S.Cholesterol (mmol/l) with				
1- age of the patients (year)	72	0.09	0.004	0.45
2- 1/creatinine slope	33	0.126	0.01	0.48
VIII) S.Triglycerides (mmol/l) with				
1- age of the patients (year)	68	0.07	0.005	0.6
2-1/creatinine slope	32	0.344	0.118	**0.05***

Abbreviations: see table 3.5

*= p value <0.05

3.5 Discussion

In this analysis, the clinical and histological parameters and their relationship to the rate of progression of CRF were examined.

Initial analysis centered on investigating differences relating to gender and parameters influencing the progression of CRF. The 1/creatinine slope was significantly lower in males than females, reflecting the faster progression of CRF in males. Moreover, the severity of proteinuria both at diagnosis and during follow-up was higher in men. These findings were expected, as it is known that males with CRF have a faster rate of progression than females (Berthoux, *et al.*, 1998). This may be attributed to the differences in proteinuria that I have reported. It was also reported that regardless of the cause of chronic renal failure, ESRD is more common in males than females (Locatelli and Del Vecchio, 2000 and Hannedouche, *et al.*, 1993).

Age was the second factor studied. There was no significant difference between the progressors and non-progressors. This finding was contrary to observations made by Fliser and colleagues (1993) who showed that the progression of CRF was faster with increased age. This difference might be due to the small number of patients that I have studied. On the other hand, there was a statistically significant correlation between age and the severity of proteinuria, both at diagnosis and during follow-up. This may be relevant, as proteinuria is a known factor affecting the progression of CRF. Age itself may not be a strong prognostic factor on its own but it may be the associated risk factors such as the increased age-related proteinuria or systemic hypertension which increase the risk of progression of CRF (For review, Locatelli and Del Vecchio, 2000). In support of that, age of the studied patients was significantly correlated with diastolic blood pressure at diagnosis and systolic blood pressure during follow up. A large number of studies have shown that systemic blood pressure increase with age (Malberti, *et al.*, 1997). However, very few have made the association of age-blood pressure level and progression of CRF.

Proteinuria was the third factor studied. It has been reported that proteinuria is a marker for the progression of CRF (Jafar, *et al.*, 2001; Locatelli and Del Vecchio, 2000). There were significant associations between both proteinuria at onset and mean of proteinuria during follow-up as well as the rate of progression of CRF. Many studies also found that the level of proteinuria was correlated with the rate of decline of renal function (Williams, *et al.*, 1988 and Wight, *et al.*, 1992). It has been argued by that the proteinuria itself may be nephrotoxic accelerating the progression of both glomerulosclerosis (Bertani and Remuzzi, 1993) and that of tubulointerstitial scarring (For review, Harris, 2000). This may be mediated through the transudation of circulating proteins into the mesangium of damaged glomeruli causing the activation of mesangial cells (For review, Harris, 2000). In the tubules, exposure to high amounts of filtered proteins may stimulate and activate tubular epithelial cells to synthesise and release cytokines, chemokines as well as fibrogenic growth factors (For review, Jernigan and Eddy, 2000; Eddy, 2001).

Hypertension was the fourth risk factor studied. There was no significant difference in terms of blood pressure and progression of CRF. This contrasts with data reported by Locatelli *et al.*, (2000). This might be due, firstly to the small size of the studied cohort; secondly it might be due to the fact that patients were receiving anti-hypertensive therapy thus controlling and normalizing some of the blood pressure readings. There was statistically significant correlations between systolic blood pressure, diastolic blood pressure, at diagnosis and during follow-up with proteinuria both at the time of diagnosis and follow-up. Further, this is a well-known fact that with increasing blood pressure proteinuria is raised. There were also positive correlations between blood pressure, proteinuria and the levels of serum triglycerides.

Surprisingly, there were higher circulating lipid levels (both cholesterol and triglycerides) in non-progressors compared to the progressors. This finding was contrary to those discussed by Harris (2000). Lipids might be the culprits regarding tubulointerstitial inflammation and fibrosis in heavy proteinuric states (Attman, *et al.*, 1999; Samuelsson, *et al.*, 2000). The discrepancy between my findings and the literature may be explained

by the fact that some of the hyperlipidemic patients may be under treatment thus lowering their blood levels.

Pathological processes were studied. There was no significant association between the severity of progression of CRF and the severity of glomerulosclerosis. It was assumed that this might due to the scanty specimen available for pathological examination and the small number of glomeruli available for analysis. On the other hand, it was noted that there was a higher severity of tubulointerstitial fibrosis in the progressors compared to the non-progressors. This was also expected, as other workers have reported that there is a significant association between the progression of CRF and the severity of interstitial fibrosis (Risdon, *et al.*, 1966 and Bohle, *et al.*, 1981; For review, Jernigan and Eddy, 2000). The prognostic superiority of the tubulointerstitial scarring over glomerulosclerosis may be a simple reflection that the area covered by the former within a renal biopsy is considerably larger than that representing glomeruli. On the other hand, it may be a genuine difference reflecting the importance of tubulointerstitial lesions in relation to decline in renal function.

3.6 Conclusion

- Men were found to have a higher frequency of progressive CRF/ESRF than women.
- The age was significantly correlated with diastolic blood pressure at diagnosis and systolic blood pressure during the follow-up.
- There was a highly significant association between the severity of progression of CRF and the severity of proteinuria both at diagnosis and during the follow-up.
- The progression of CRF was associated with tubulointerstitial fibrosis rather than glomerulosclerosis.

Chapter 4

Investigation of four single nucleotide polymorphisms in the TGF-β1 gene

4.1 Introduction

Genetic definitions

Each human genome has approximately three billion bases, which are arranged in a unique order (the sequence of bases) and is estimated to contain approximately 30.000-40.000 genes. Bases that vary within DNA sequences from one individual to another, are known as single nucleotide polymorphisms (SNPs). The term "haplotype" refers to a combination of SNPs on a chromosome, usually within the context of a particular gene.

Polymorphism is a word derived from the Greek for 'many forms' variations in the DNA sequence. It either occurs at one nucleotide or more than one nucleotide within a certain area, which is called polymorphic region of a gene.

An allele is a variant form of a gene. Allele frequency is a measurement of how common an allele is in the studied population. Carriage rate is the proportion of individuals carrying one copy of the allele. If the co-inheritance of one allele with another at a nearby locus is significantly higher than expected within the given population, it is said that these alleles are in "*linkage disequilibrium*" (Sved, 1971). Genetic association is an increased frequency of a paticular allele or genotype in a sample of individuals with a particular disease trait.

Genotyping is the determination of the specific composition of an individual's genome (the genetic information present which present in a particular organism) with respect to a specific set of markers (a marker is specific polymorphic location on a chromosome, the inheritance of which can be tested).

Genotyping facilitates analytic studies by allowing determination of whether the alleles are associated with, or causing, a particular disease. There are numerous diseases in which a particular polymorphism is associated with diseases. This makes research in the field stimulating and rewarding. Also, this type of studies may indicate markers of disease susceptibility and/or predictors for disease activity. Furthermore, the markers may

lead to the early diagnosis of the disease or early prediction of the severity of that condition. Consequently, early medical intervention, may be applied to control disease activity.

TGF-β1 polymorphisms and diseases

Alleles of transforming growth factor-β1 have been implicated in the pathogenesis of a variety of fibrotic diseases.

One of single nucleotide polymorphism at Leu10Pro (C→T) confer a significantly increased risk of osteoporosis, as shown by measurements of bone mineral density (Langdahl, *et al.*, 1997, Bertoldo, *et al.*, 2000). The same allele was associated with the development of end-stage heart failure in patients with cardiomyopathy (Holweg, *et al.*, 2001).

Another marker at Arg25Pro (G→C) is associated with increased risk of myocardial infarction although, surprisingly, it seems to be associated with a reduced risk of systolic hypertension (Cambien, *et al.*, 1996). The G allele was associated with the development of fibrotic lung pathology in cystic fibrosis prior to lung transplantation and fibrosis in the graft (El-Gamel, *et al.*, 1997). Furthermore, carriage of the G and T allele at Arg25Pro and Leu10Pro respectively is associated with a deterioration of pulmonary function in-patients suffering from cystic fibrosis (Arkwright, *et al.*, 2000).

A single base change in the TGF-β1 promoter region, at C-509T (C→T) was associated with higher active and acid-activated latent circulating TGF-β1 levels (Grainger, *et al.*, 1999). Furthermore, the same allele was associated with decreased bone mineral density and osteoporosis (Yamada, 2001).

TGF-β1 and the progression of chronic renal failure

Transforming growth factor-β1 has been implicated in the pathogenesis of renal fibrosis and the associated progression of CRF (Yoshioka, *et al.*, 1993; Border and Noble, 1994). It is believed that this growth factor is capable of stimulating the synthesis and decreasing the breakdown of collagenous extracellular matrix (ECM) (Yoshioka, *et al.*, 1993). Circulating TGF-β1 levels have been found to be higher in African-American patients with progressive CRF (Suthanthiran, *et al.*, 2000). Similarly, tissue levels of TGF-β1 have been found by immunohistochemistry, to be up-regulated within the kidneys of patients with diabetic (Yamamoto, *et al.*, 1993) and non-diabetic (Datta, *et al.*, 1999) nephropathies. Therefore, the study of TGF-β1 polymorphism has been undertaken recently in patients with renal diseases.

Transforming growth factor-β1 polymorphism at Leu10Pro (T→C) is significantly associated with the development of renal failure in patients undergoing heart transplantation and treated with cyclosporin A (Baan, *et al.*, 2000). Also, TGF-β1 polymorphism at Thr263Ile in exon 5 (C→T) shows a weak association with the development of nephropathy in diabetic patients (Pociot, *et al.*, 1998). Polymorphism at Arg25Pro (C→G) was associated with elevation of both systolic and diastolic blood pressure in ESRF patients (Li, *et al.*, 1999). Furthermore, in the same study, it was noted that carriers of the same allele also had higher circulating TGF-B1 levels (Li, *et al.*, 1999). On the other hand, a study in African-Americans with ESRF failed to show any significant association between TGF-B1 or PDGF genetic polymorphisms and susceptibility to progression of CRF to ESRD (Freedman, *et al.*, 1997).

Aim of the study

With the above studies in mind, I have undertaken to investigate associations between TGF-B1 polymorphisms and susceptibility to, and/or, progression of CRF.

I have studied a variety of polymorphic regions as shown on table 4.1.

Table 4.1. Transforming growth factor-B1 polymorphic regions studied

Site	*Bases*	*Amino acid*
I- C-509T		
Common allele T	5'CTT'3	Non-coding
Rare allele C	5'CCT'3	Non-coding
II- Leu10Pro		
Common allele T	5'CTG'3	Leucine
Rare allele C	5'CCG'3	Proline
III- Arg25Pro		
Common allele G	5'CGG'3	Arginine
Rare allele C	5'CCG'3	Proline
IV- Thr263Ile		
Common allele C	5'ACC'3	Threonine
Rare allele T	5'ATC'3	Isoleucine

These alleles are common in Caucasians, other ethnic groups may be different.

Material and Methods

Patients

A hundred and forty four patients with CRF/ESRF and 73 white Caucasian controls (for details see Chapter 2 Section 2.1.4) were included in this study. Patients with CRF were divided into progressors (P) and non-progressors (NP) depending on their 1/serum creatinine against time slope. We therefore studied 112 progressors and 32 non-progressors. As previously described, all patients with ESRF were considered progressors. Patients who were not on renal replacement therapy (RRT) were followed-up retrospectively for 58.8±4.8 months (mean ± SEM). The patients were also classified into total CRF patient group (n = 143) and CRF patients without polycystic kidney disease (PKD) or obstructive uropathy (OU) (n = 126)

Polycystic kidney disease (PKD) was excluded sometimes from the analysis because it is inherited by particular genes: PKD1 on 16p and PKD2 on 4q13-23. Furthermore, OU was sometimes excluded from the analysis because the progression of CRF might be due to mechanical causes rather than the effect of TGF-B1 polymorphisms.

Blood pressure (systolic and diastolic) at diagnosis and during follow-up, serum creatinine levels during the follow-up period from which the 1/serum creatinine against time slope was derived and proteinuria at diagnosis and during follow-up. For details see Chapter 2 Section 2.1.4.

Twenty four renal biopsies were available for histological analysis. Emphasis was on glomerulosclerosis and tubulointerstitial fibrosis scores as well as level of inflammatory cellular infiltration. An arbitrary severity score from 0-4 was applied to all these parameters. For details see Chapter 2 Section 2.5.

Methodology

Deoxyribonucleic acid (DNA) was extracted from leukocytes from peripheral blood samples using the protocol given in the Material and Methods section (chapter 2 section 2.2.2).

Genomic DNA concentration was estimated by measuring the absorbance at 260 nm. The ratio of absorbance at 260nm to 280nm (see chapter 2, section 2.2.3), allowed assessment of the purity of DNA.

Polymerase chain reaction (PCR) was performed on the purified DNA extracted from patients with CRF/ESRF and controls.

Four polymorphisms in the TGF-B1 gene were investigated: Leu10Pro; Arg25Pro; Thr263Ile and C-509T (see Chapter 2, Section 2.3.1). Genomic DNA, PCR products and digestion products were visualised by agarose gel electrophoresis (see Chapter 2, Section 2.4).

Statistical analysis

The Hardy-Weinberg equation was used to predict the expected distribution of genotypes at given allele frequencies. Chi squared analysis was used to test the null hypothesis (see Chapter 2, Section 2.8) that there was no difference in genotype distributions between two groups. Comparisons between groups in relation to a range of clinical parameters relied on the student t test, which was carried out using Microsoft EXCEL. A p value of less than 0.05 was considered significant. P-value were not subjected to Benferoni correction (ie. Each p-value multiplied by the number of independent tests carried out), since tests were not independent.

The coeffiecient of the linkage disequilibrium (D') was calculated by using the following formula.

$$D' = (P_{ab} \cdot P_{AB} - P_{aB} \cdot P_{Ab}) / (P_{ab} \cdot P_{AB} + P_{aB} \cdot P_{Ab})$$

Alleles A and a are segregating at one locus, and B and b are segregating at the second. There are, therefore, four possible gametes, ab, aB, Ab and AB. The results should be between -1 and +1. If D'=1, this means that there is complete association.

4.3 Results

4.3.1 Investigation of DNA quality

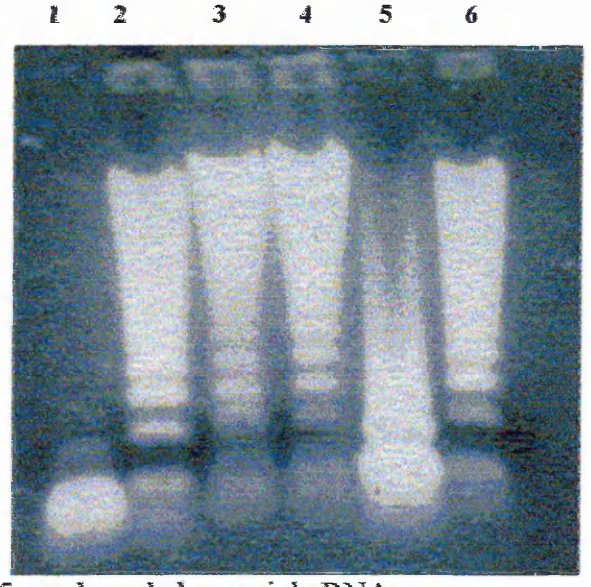
4.3.1.1 Yield

Deoxyribonucleic acid was extracted from 5 – 10 ml of venous blood. Samples were resuspended in 200 - 400µl of distilled water. Optical density at 260nm ranged from 0.016 to 0.295 (concentration ranging from 800µg/ml to 14.75µg/ml). Differences in DNA concentrations were due to differences in the initial volume of the blood amount from which DNA was extracted. White blood cell count also affects the DNA concentration. The yield ranged from 20 – 30 µg/ml of blood. The ratio between optical density at 260nm and 280nm was used to assess the purity of DNA. In the studied samples, the ratio ranged from 1.6 to 2. Most of samples gave a ratio of around 1.8, indicating that most of the extracted DNA samples were pure.

4.3.1.2 Analysis by agarose gel electrophoresis

Agarose (0.8%) gel electrophoresis was used to investigate the quality of the extracted DNA from the white blood cells of the CRF patients. Although there was considerable degradation of the DNA (Figure 4.1), its ability to act as a template for PCR was not affected.

Figure 4.1. Agarose gel electrophoresis (0.8%) of extracted DNA from the blood samples of the patients with CRF

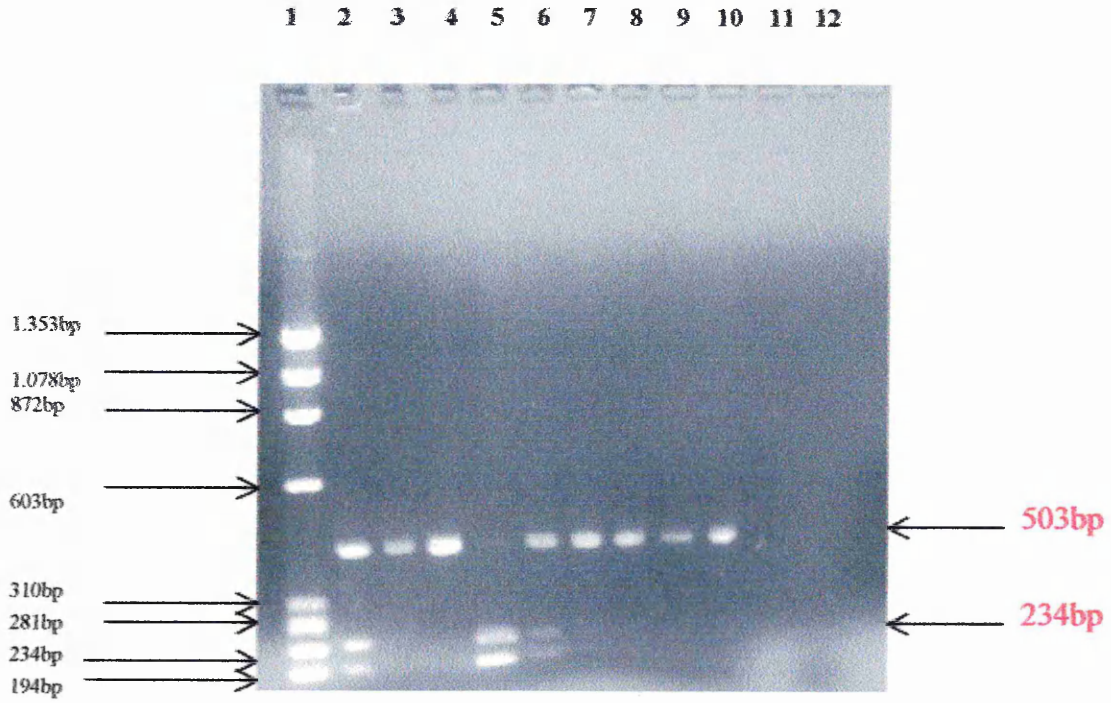


Legend: samples 1 and 5 are degraded or mainly RNA.

4.4 C-509T

Polymerase chain reaction (PCR) products of 56 control and 142 patients were digested with *Bsu*36I for 12 hours. The PCR product sizes were 503bp before digestion and 234bp if digestion happened. The results of the genotyping were checked by the Hardy-Weinberg equation (Figure 4.2, Table 4.2).

Figure 4.2. The genotyping at C-509T



Lane 1	Marker (ϕX174 DNA/HaeIII)
Lane 2	Heterozygote (TC)
Lane 3 and 4	Homozygote (TT)
Lane 5	Homozygote (CC)
Lane 6	Heterozygote (TC)
Lane 7-10	Homozygote (TT)
Lane 11	Negative control

Table 4.2. C-509T genotyping in control and CRF groups

Genotyping	*TT*	*TC*	*CC*	*Total*	*Allele frequencies*
1- Control group	22	28	5	55	(T = 0.66, C = 0.34)
2-CRF group	83	52	7	142	(T = 0.77, C = 0.23)

The genotype results were checked by the Hardy-Weinberg equation. The genotyping was within equilibrium (Table 4.3). The distribution of genotypes observed did not differ from that expected from a population in Hardy-Weinberg equilibrium.

Table 4.3. Hardy-Weinberg analysis of C-509T

	Observed genotypes	*%*	*Expecting genotypes*
1- Control group			
Heterozygous	28	46	25.3
Homozygous T	22	42	23.1
Homozygous C	5	12	6.6
Total	55	100	55
2-CRF group			
Heterozygous	52	35	49.7
Homozygous T	83	60	85.2
Homozygous C	7	5	7.1
Total	142	100	142

Hardy-Weinberg equation in the control group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(22 - 23.1)^2}{23.1} + \frac{(28 - 25.3)^2}{25.3} + \frac{(5 - 6.6)^2}{6.6} = 0.728$$

p value for χ^2 at two degree of freedom = 0.7

Hardy-Weinberg equation in the CRF group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(83 - 85.2)^2}{85.2} + \frac{(52 - 49.7)^2}{49.7} + \frac{(7 - 7.1)^2}{7.1} = 0.164$$

p value for χ^2 at two degree of freedom = 0.9

The balance of genotypes observed did not differ from that of expected from Hardy-Weinberg equation in either control or CRF groups (p value > 0.05).

4.4.1 Susceptibility to CRF

The carriage rate of the T allele was significantly higher in both the whole CRF patients group and CRF patients without PKD and obstructive uropathy groups compared to the control group (Tables 4.4 and 4.5). According to the significance of the regression of the 1/serum creatinine slope, CRF patients were classified into progressors and non-progressors (chapter 2). The carriage rate of the T allele was significantly higher in the progressors compared to the control group in total CRF patients (Table 4.4) and in CRF patients without PKD and OU (Table 4.5).

Table 4.4. Testing for association of C-509T genotype with susceptibility to CRF

	With C (CC+TC)	*Without C (TT)*	*Total*	*χ^2 at one degree of freedom*	*P value*
CRF patients	59	83	142	**5.25**	**0.022***
Control group	33	22	55		
Total	92	105	197		
CRF progressors	41	69	110	**7.66**	**0.0056***
Control group	33	22	55		
Total	74	91	165		

Table 4.5. Testing of the susceptibility of genotype at C-509T to CRF in patients without PKD or obstructive uropathy

	With C (CC+TC)	*Without C (TT)*	*Total*	*χ^2 (at two degree of freedom)*	*P value*
CRF patients	55	71	126	**4.1**	**0.043***
Control group	33	22	55		
Total	88	93	181		
CRF progressors	38	60	98	**6.38**	**0.012***
Control group	33	22	55		
Total	71	82	153		

4.4.2 Progression of CRF

The distribution of progressors and non-progressors according to genotype at this locus is shown in table 4.6. The distribution of genotypes in the CRF group following removal of the patients with OU and PKD is shown in table 4.7.

The number of progressive CRF patients was significantly higher in patients homozygous for the T allele (TT) compared to others (Tables 4.6 and 4.7) both in the total CRF patients (odds ratio 1.67, 95% confidence interval 1.1-2.5) and after removal PKD and obstructive uropathy (odds ratio 2.44, 95% confidence interval 1.5-3.7).

Table 4.6. Testing for association of genotype at C-509T with progression of CRF

	With C (CC+TC)	***Without C (TT)***	***Total***	***χ^2 at one degree of freedom***	***P value***
CRF progressors	41	69	110	**3.68**	**P=0.055***
CRF Non-progressors	18	14	32		
Total	59	83	142		

Table 4.7. Testing for association of genotype at C-509T with progression of CRF in patients without PKD or OU

	With C (CC+TC)	***Without C (TT)***	***Total***	***χ^2 (at two degree of freedom)***	***P value***
CRF progressors	**38**	60	98	**4.26**	**0.039***
CRF Non-pogressors	17	11	28		
Total	55	71	126		

Figure 4.3. a. The distribution of progressors and non-progressors according to C-509T genotype in CRF patients without PKD or OU

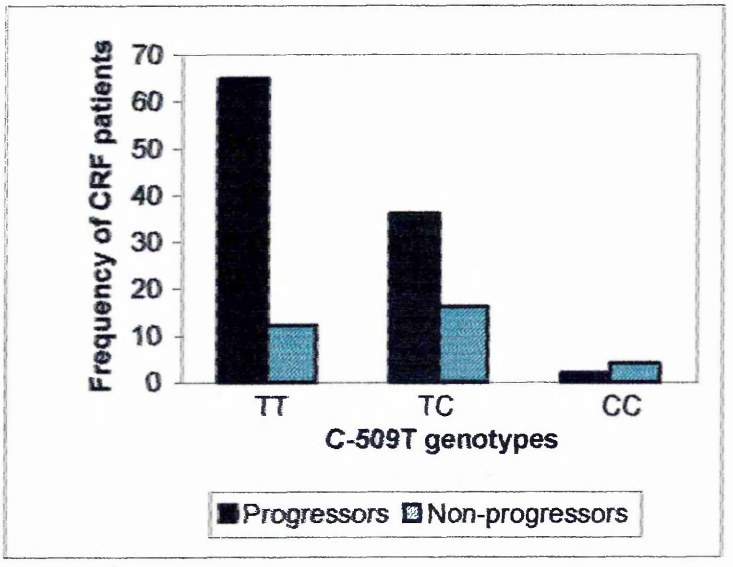
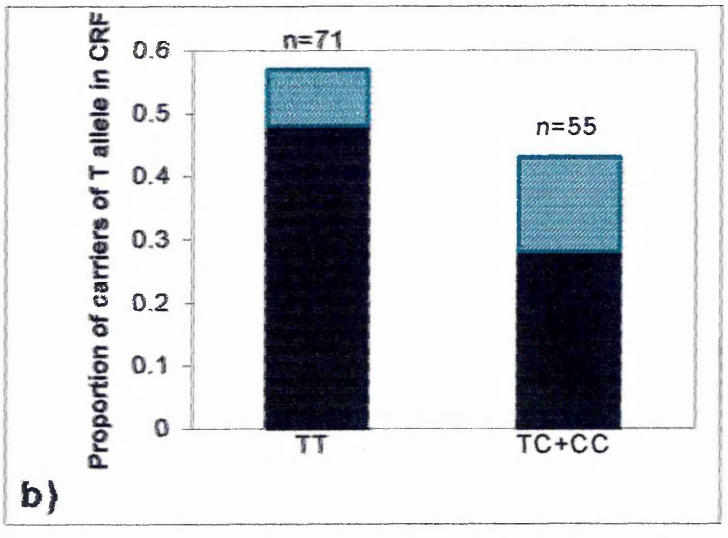


Figure 4.3. b. The proportion of progressors and non-progressors according to C-509T genotype



The number of carriers of the T allele was significantly higher in the progressors compared to the non-progressors in all the CRF patients and after exclusion of PKD and OU.

It was noted earlier (Chapter 3, section 3.4.1) that some CRF clinical parameters (proteinuria at diagnosis and serum creatinine levels) were significantly higher in males than females. The allele frequencies were then tested according to the gender (Figure 4.4). It was found that there was no significant difference in allele frequencies between males and females (Table 4.6).

Figure 4.4. The distribution of C-509T genotype according to gender

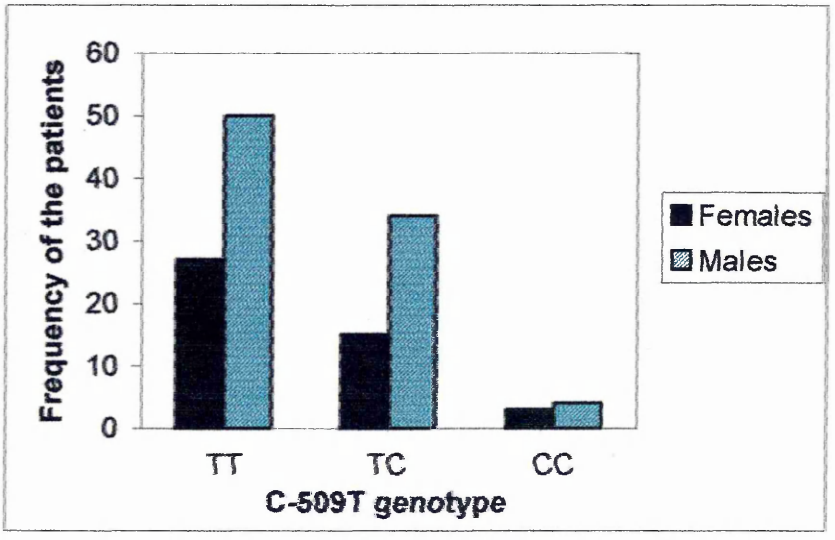


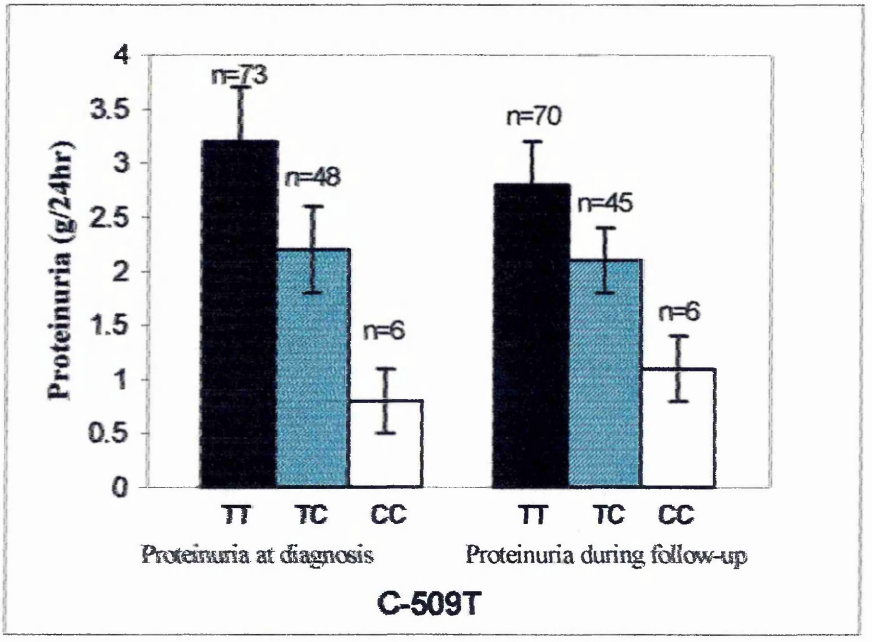
Table 4.6. The allele frequency of C-509T genotype according to gender

	Females	*Males*	*Total*	*Chi square (at one degree of freedom)*	*P value*
CC+TC frequency	17	42	59	0.103	0.75
TT frequency	26	57	83		
Total	43	99	142		

4.4.3 Proteinuria

There was no difference in proteinuria at diagnosis or during follow-up between patients with CRF of different C-509T genotypes (Figure 4.5).

Figure 4.5. The distribution of proteinuria (mean±SEM) according to C-509T genotype in the CRF patients



On the other hand, proteinuria during-follow up was higher in T-compared to C-homozygous in the total CRF patients (Table 4.7). Furthermore, proteinuria at diagnosis was higher in T-homozygotes compared to C-homozygotes in CRF patients without PKD or OU. Furthermore, the F-test was significant (Table 4.7) indicating a significant difference in variance between groups.

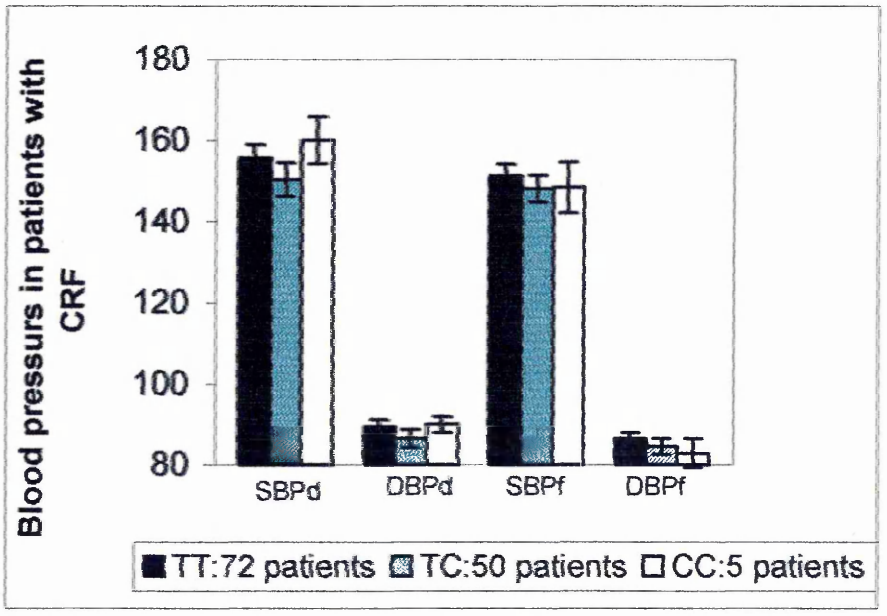
Table 4.7. Proteinuria in patients homozygous for the T and C alleles according to genotype at C-509T

	Means±SEM	*Observations*	*P(f)*	*P(t)*
CRF total patient group				
i) Proteinuria at diagnosis				
CC	1.5±0.5	6	**0.03***	0.09
TT	2.7±0.4	73		
ii) Proteinuria during follow up			**0.003***	**0.028***
CC	1.4±0.3	6		
TT	2.4±0.3	70		
CRF patient without PKD or OU				
i) Proteinuria at diagnosis				
CC	0.83±0.3	4	**0.01***	**0.0017***
TT	2.8±0.4	64		
ii) Proteinuria during follow up	1.1±0.3	4	**0.01***	0.36
CC				
TT	2.5±0.4	59		

4.4.4 Blood pressure

There was no significant difference between blood pressure of CRF patients according to C-509T polymorphism (Figure 4.6).

Figure 4.6. C-509T genotype according to blood pressure (mean±SEM) in the CRF without PKD or OU



Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow-up, DBPf: diastolic blood pressure during follow-up.

4.4.5 Pathological processes

Twenty-three patients' renal sections were analysed. The pathological processes were classified into two main categories; glomerulosclerosis (GS) and tubulointerstitial fibrosis (IF).

4.4.5.1 Glomerulosclerosis (GS)

The distribution of genotypes in relation to the severity of the GS is shown in figure 4.7. There was no difference in distribution of the different allele at C-509T genotype according to the severity of GS (Chi square at one degree of freedom = 0.38, p= 0.5).

Figure 4.7. a. The distribution of the severity of glomerulosclerosis (GS) according to C-509T genotype

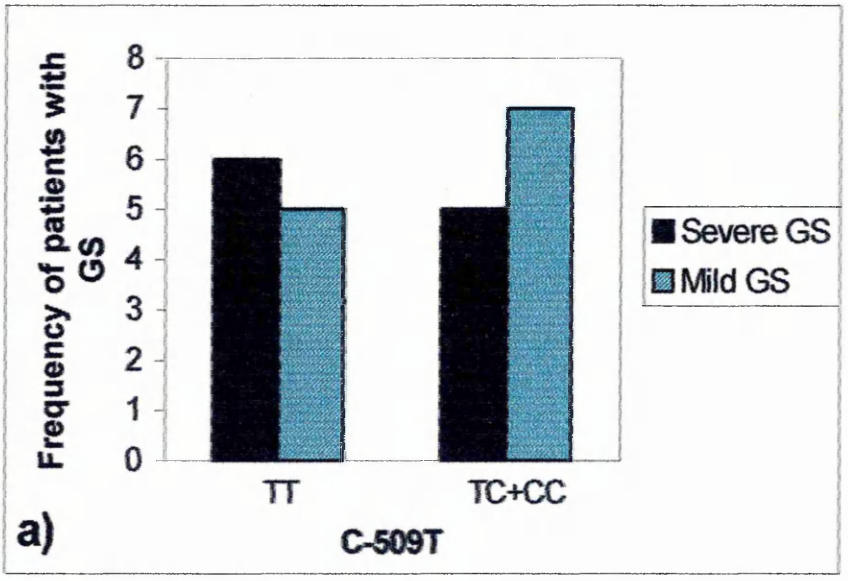
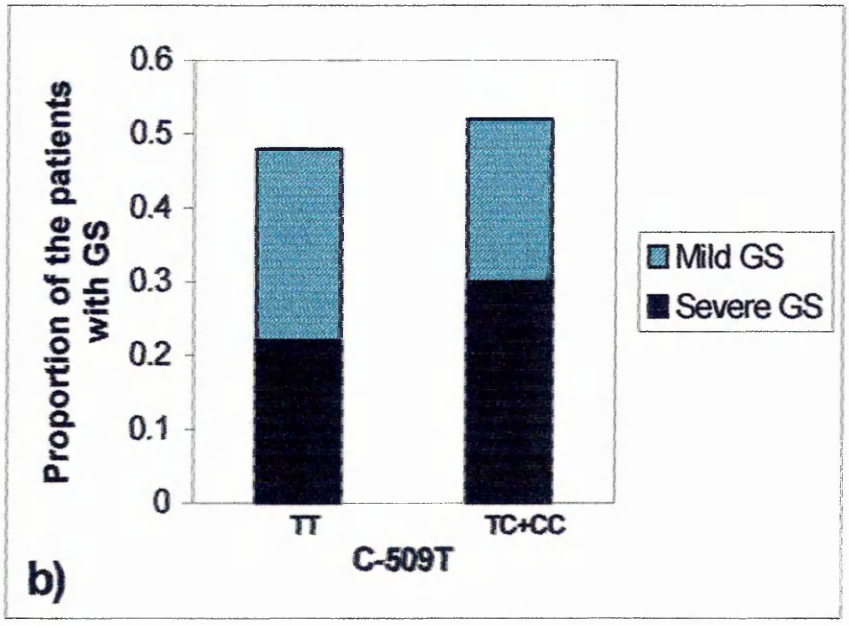


Figure 4.7. b. The proportion of mild and severe GS according to C-509T genotype



4.4.5.2 Tubulointerstitial fibrosis (IF)

The distribution of genotyping in relation to the severity of GS is shown in table 4.8. There was no difference in distribution of the different allele at C-509T genotype according to the severity of IF (p value = 0.1).

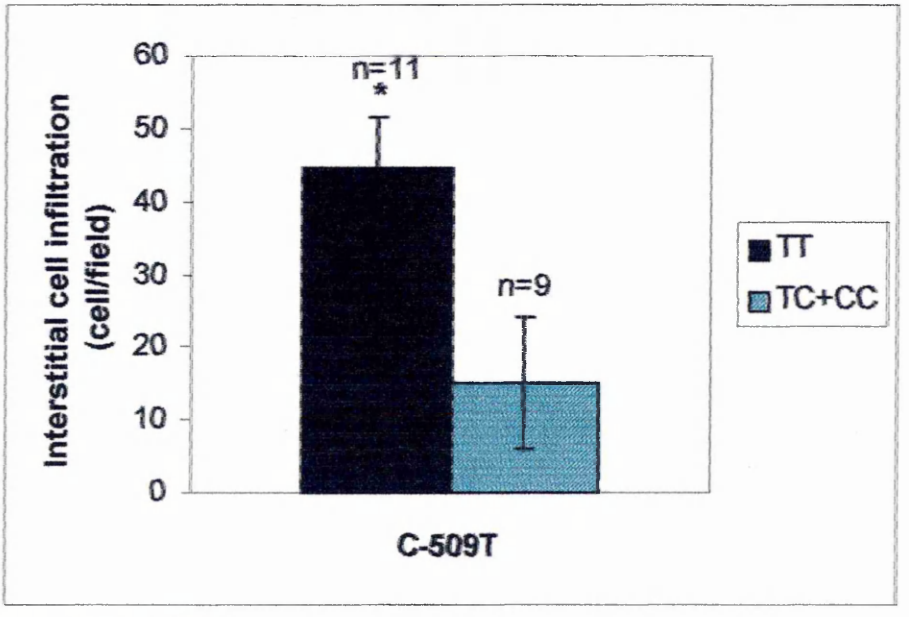
Table 4.8. The distribution of the severity of IF according to C-509T genotype

	TT	*TC/CC*	*Total*	*χ^2 (Yates' correction)*
Mild IF	6	10	16	2.7
Severe IF	5	2	7	
Total	11	12	23	

4.4.5.3 Renal interstitial cell infiltration (ICI)

Genotyping was performed for 21 patients who had pathology sections available. There was a significant difference in the severity of ICI in T-homozygotes compared to C-homozygotes and heterozygotes (p= 0.0098) (Figure 4.8).

Figure 4.8. The distribution of the severity of ICI (cell/field) (mean±SEM) according to C-509T genotype

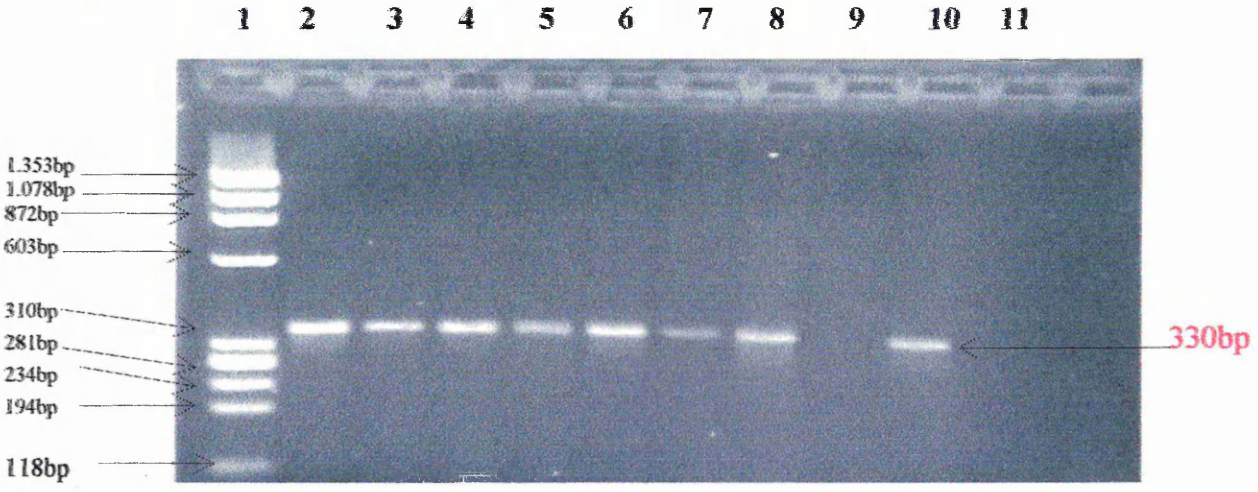


: p value = 0.0098

4.5 Leu10Pro

One hundred and twenty-four CRF patients and 71 white Caucasian controls were genotyped. Eighteen samples were excluded because they showed very faint bands (they might contain only small amounts of DNA). Figure 4.2 shows an example of a typical genotyping experiment. For each patient, 2 lanes are used, each represents a different allele of the polymorphism. Lanes 2, 4, 6, 8 and 10 represent the T allele and lanes 3, 5, 7, 9 and 11 represent the C allele.

Figure 4.9. ARMS PCR for genotyping at Leu10Pro



Lane 1	Marker (ϕX174 DNA/HaeIII)
Lane 2 & 3	Heterozygote (TC)
Lane 4&5	Heterozygote (TC)
Lane 6&7	Heterozygote (TC)
Lane 8&9	Homozygote (TT)
Lane 10 & 11	Homozygote (TT).

It is important to establish that an ARMS-PCR assay is adequately distinguishing between alleles. One way to assess this is to determine whether the balance of genotypes observed in the control sample differs from that predicted by the Hardy-Weinberg equation from the observed allele frequencies (p and q).

Table 4.9. Leu10Pro genotypes in the control and CRF patients group

Genotyping	*CC*	*TC*	*TT*	*Total*	*Allele frequencies*
1- Control group	11	35	27	73	(T = 0.6, C = 0.4)
2-CRF group	25	71	29	125	(T = 0.52, C = 0.48)

Table 4.10. Genotyping results for Leu10Pro in the control cohort and in CRF patients

	observed genotypes	*% of studied population*	*expected genotypes*
1- Control group			
Heterozygous	35	48	35.04
Homozygous T	27	37	27.01
Homozygous C	11	15	10.95
Total	73	100	73
2- CRF patients group			
Heterozygous	71	50	62.5
Homozygous T	29	27	33.8
Homozygous C	25	23	28.8
Total	125	100	125

Hardy-Weinberg equation in the control group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(27 - 27.01)^2}{27.01} + \frac{(35 - 35.04)^2}{35.04} + \frac{(11 - 10.95)^2}{10.95} = 0.08$$

p value for χ^2 at two degree of freedom = 0.96

Hardy-Weinberg equation in the CRF group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(29 - 33.8)^2}{33.8} + \frac{(71 - 62.5)^2}{62.5} + \frac{(25 - 28.8)^2}{28.8} = 2.3$$

p value for χ^2 at two degree of freedom = 0.3

The balance of genotypes observed did not differ from Hardy-Weinberg expectations in either control or CRF groups (p value > 0.05).

4.5.1 Susceptibility to CRF

Carriers of the C allele were more common in the CRF group than in the controls (Table 4.11). Furthermore, it was observed that there was a higher number of carriers of the C allele amongst the patients with progressive CRF compared to the control group (Table 4.11).

Table 4.11. Testing of the susceptibility of genotype at Leu10Pro to CRF in patients group

	With C (CC+TC)	***Without C (TT)***	***Total***	***χ^2 at one degree of freedom***	***P value***
Total patient group	96	29	125	**4.3***	**0.038***
Control group	46	27	73		
Total	142	56	198		
CRF progressors	73	19	92	**5.4***	**0.02***
Control group	46	27	73		
Total	119	46	165		
CRF Non-progressors	23	10	33	0.47	0.49
Control group	46	27	73		
Total	69	37	106		

4.5.2 Progression of CRF

The distribution of Leu10Pro genotypes in progressors and non-progressors CRF patients is shown in Figures, 4.10 and 4.11.

Figure 4.10. The distribution of CRF progressors and non-progressors according to Leu10Pro genotypes

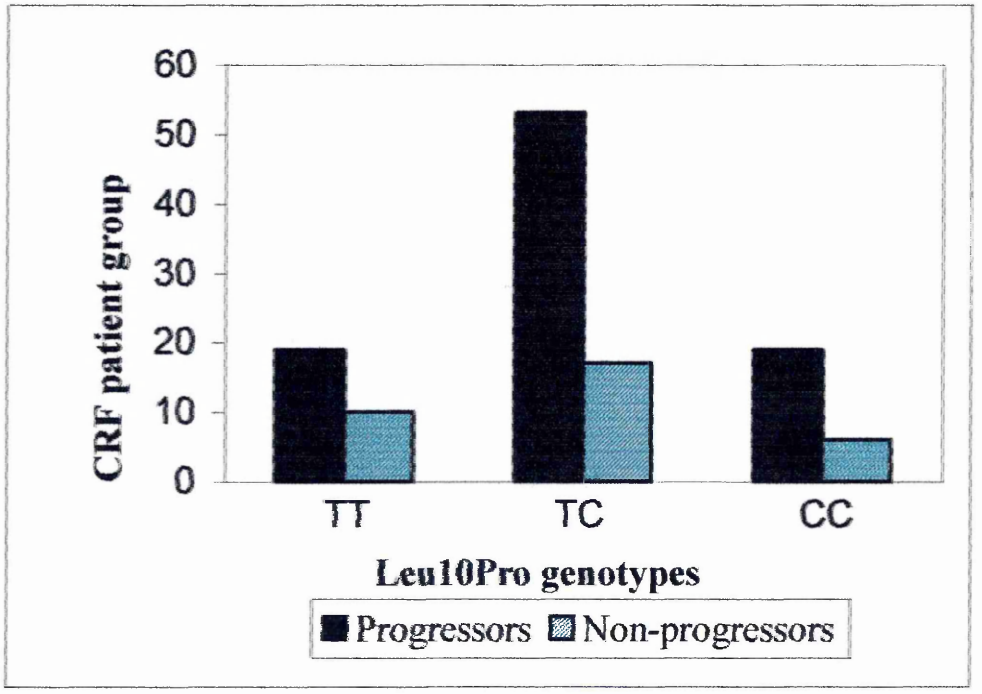


Figure 4.11. a. The distribution of progressors (P) and non-progressors (NP) among Leu10Pro genotypes

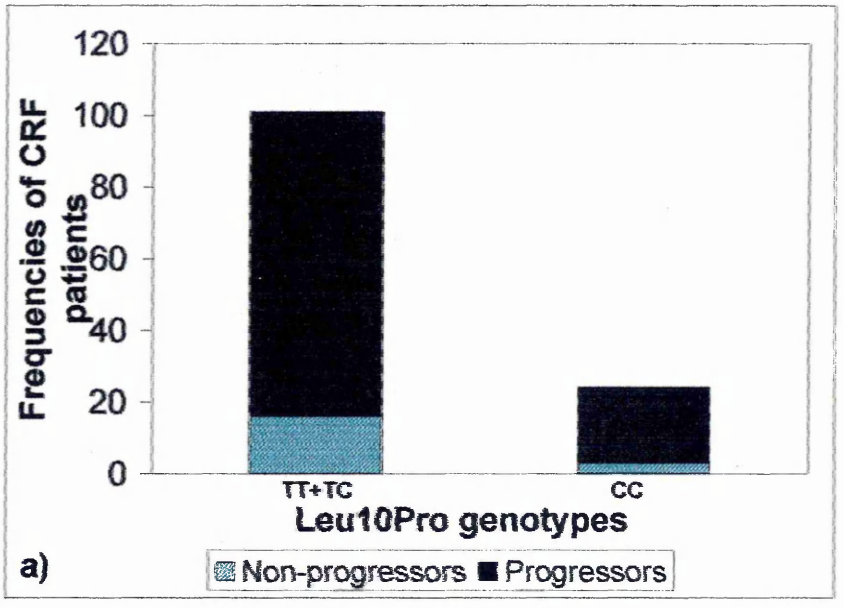
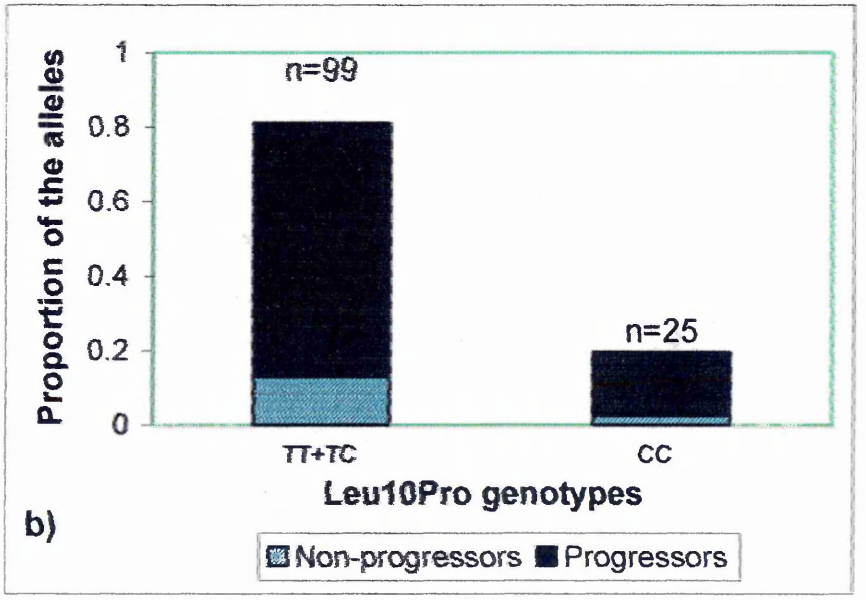


Figure 4.11. b. The proportion of progressors and non-progressors among Leu10Arg genotypes



There was no difference in the distribution of any of the alleles between the progressors compared to non-progressors (Table 4.11).

Table 4.11. Testing for association of genotype at Leu10Pro with progression of CRF

	With C (CC+TC)	*Without C (TT)*	*Total*	*χ^2 at one degree of freedom*	*P value*
CRF patient group CRF progressors	73	19	92	1.27	0.26
CRF Non-progressors	23	10	33		
Total	96	29	125		

It was noted earlier (Chapter 3, section 3.4.1) that the CRF parameters (proteinuria and serum creatinine) were higher significantly in males than females. The allele frequencies were tested according to gender (Figure 4.12). It was found that there was no significant difference in allele frequencies between males and females (Table 4.12).

Figure 4.12. The distribution of Leu10Pro genotype according to gender

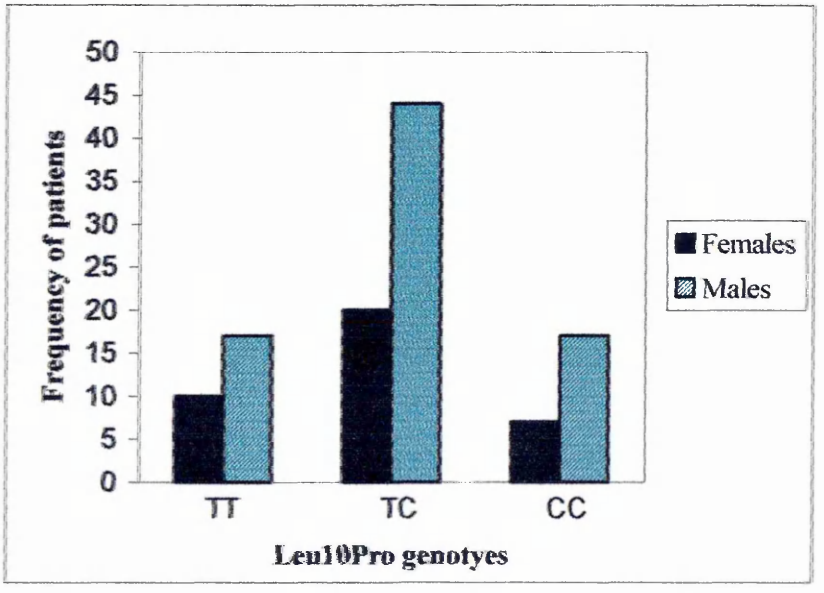


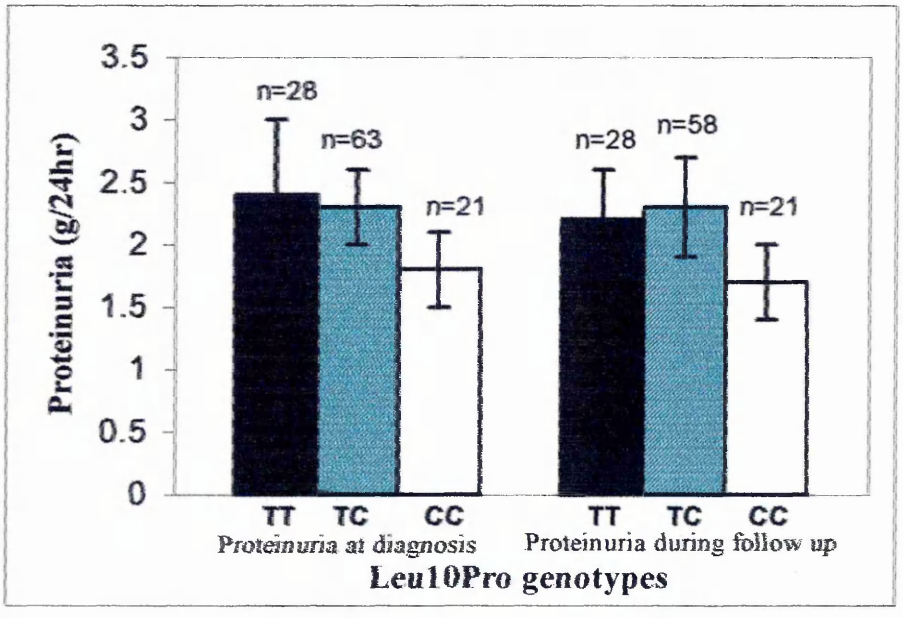
Table 4.12. The allele frequency of Leu10Pro genotype according to gender

	Females	*Males*	*Total*	*Chi square (at one degree of freedom)*	*P value*
CC+TC frequency	27	69	96	0.092	0.76
TT frequency	9	20	29		
Total	36	89	125		

4.5.3 Proteinuria

There was no difference in proteinuria at diagnosis or during the follow-up between patients with CRF of different Leu10Pro genotypes [p(F-test) = 0.03, p(t-test) = 0.46] or during follow-up [p(F-test) = 0.026, p(t-test = 0.8)] (Figure 4.13).

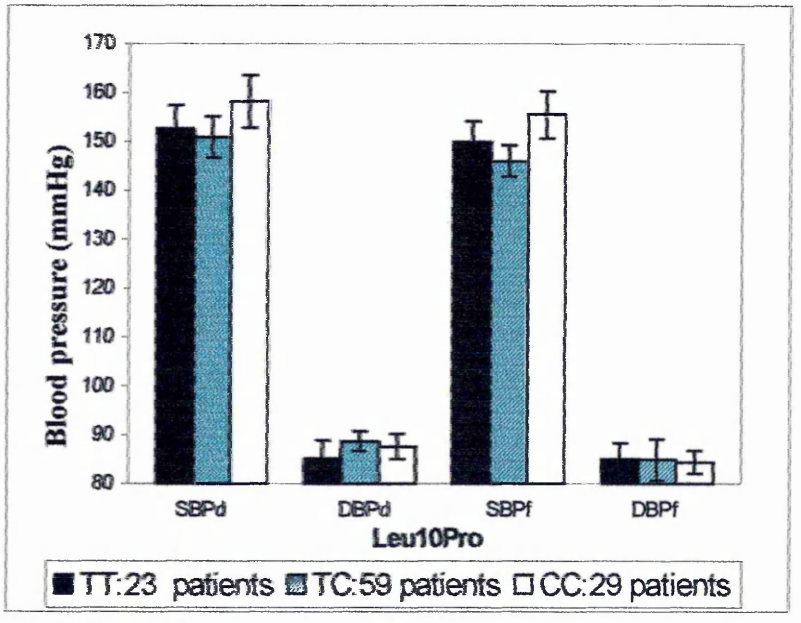
Figure 4.13. The distribution of proteinuria (mean±SEM) according to Leu10Pro genotype in the CRF patients



4.5.4 Blood pressure

There was no significant difference between blood pressure in CRF patients according to Leu10Pro polymorphism (Figure 4.14).

Figure 4.6. Leu10Pro genotype according to blood pressure (mean±SEM) in the CRF patients group



Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow-up, DBPf: diastolic blood pressure during follow-up.

4.5.5 Glomerulosclerosis (GS)

Twenty-two patients were analysed. Three of the 5 patients homozygous for the T allele (TT) had severe GS compared to 9 of 17 the heterozygotes and homozygotes for the C allele (Figure 4.15). This result was not significant (p value > 0.05 at one degree of freedom).

Figure 4.15. a. The distribution of severity of glomerulosclerosis (GS) according to Leu10Pro genotype

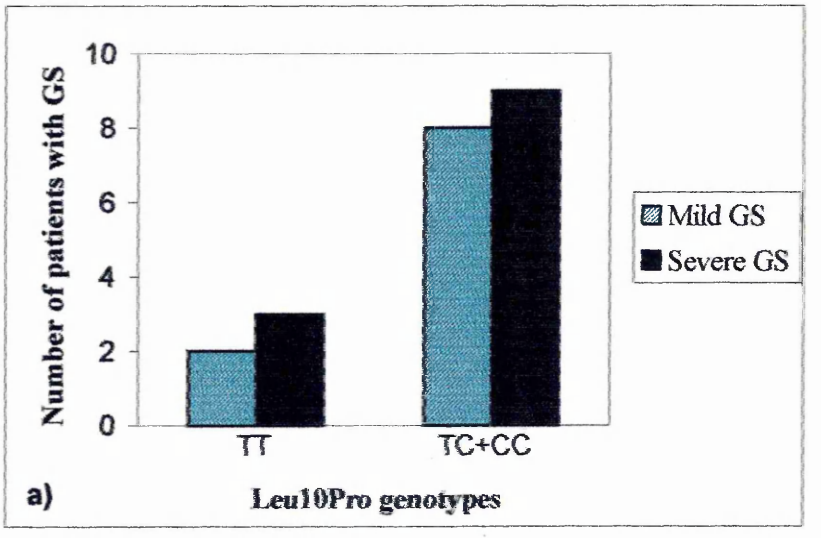
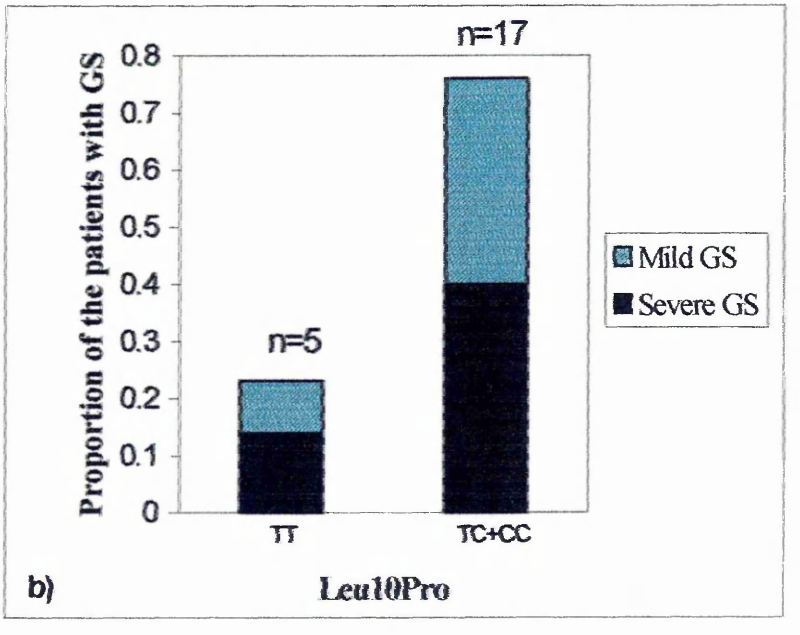


Figure 4.15. b. The proportion of mild and severe GS according to Leu10Pro genotype



4.5.6 Tubulointerstitial fibrosis

The distribution of patients with tubulointerstitial fibrosis according to Leu10Pro genotype is shown in (Table 4.13). The result was not significant (p value = 0.4 at one degree of freedom).

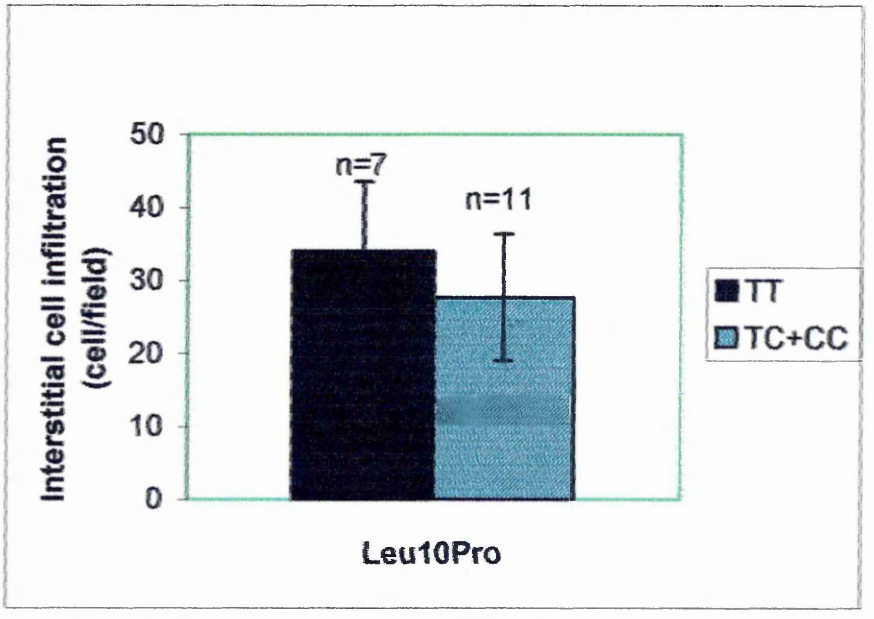
Table 4.13. Distribution of genotypes among patients with interstitial fibrosis (IF)

	CC	*TC/TT*	*Total*	*χ^2 (Yates' correction)*
Mild IF	5	12	17	0.7
Severe IF	1	4	5	
Total	6	16	22	

4.5.7 Renal interstitial cell infiltration (ICI)

Genotyping was done for 19 patients who had pathology sections available. There was no significant difference between the severity of ICI in T-homozygotes compared to other patients (Figure 4.16).

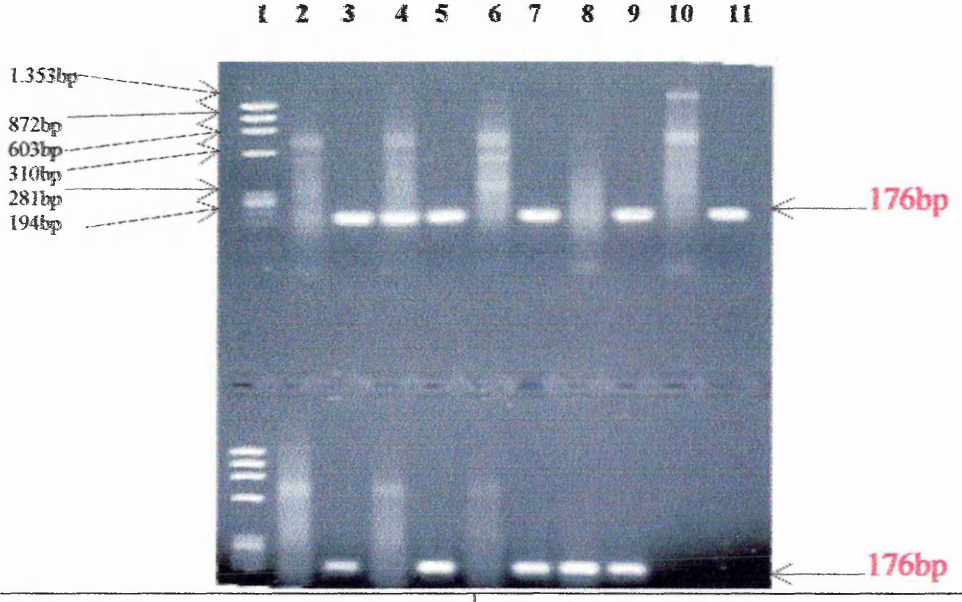
Figure 4.16. The distribution of the severity of IF according to Leu10Pro genotype



4.6 Arg25Pro

One hundred and forty three CRF patients and 78 white Caucasian individuals from Sheffield were genotyped (Figure 4.17). There are two lanes for each subject tested. Even numbered lanes represent the C-allele and odd numbered lanes (expect lane 1) represent the G-allele.

Figure 4.17. Figure 4.2. ARMS PCR for genotyping at Arg25Pro



1st row Lane 1	Marker (ϕX174 DNA/HaeIII)
Lane 2&3	Homozygote (GG)
Lane 4&5	Heterozygote (CG)
Lane 6&7	Homozygote (GG)
Lane 8&9	Homozygote (GG)
Lane 10&11	Homozygote (GG)
2nd row Lane 1	Marker (ϕX174 DNA/HaeIII)
Lane 2&3	Homozygote (GG)
Lane 4&5	Homozygote (GG)
Lane 6&7	Homozygote (GG)
Lane 8&9	Heterozygote (CG)
Lane 10&11	Negative control (no DNA template)

Allele frequencies were not significantly different in CRF patients compared to the control group (Table 4.14).

Table 4.14. Arg25Pro genotypes in the control group and CRF patients

Genotyping	*GG*	*GC*	*CC*	*Total*	*Allele frequencies*
1- Control group	59	19	0	78	(T = 0.87, C = 0.13)
2-CRF patients group	112	30	1	143	(G = 0.89, C = 0.11)

The balance of genotypes did not differe from that expected in a population in Hardy-Weinberg equilibrium in either the patients or control groups (Table 4.15).

Table 4.15. Hardy-Weinberg analysis for Arg25Pro in the control and CRF patients groups

	Observed genotypes	*%*	*Expected genotypes*
i) Control			
Heterozygous	19	21	16.5
Homozygous G	59	77	60.1
Homozygous C	0	2	0.02
Total	78	100	78
ii) CRF patients group			
Heterozygous	112	78	111
Homozygous G	30	21	30
Homozygous C	1	1	2
Total	143	100	143

Hardy-Weinberg equation in the control group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(59 - 60.1)^2}{60.1} + \frac{(19 - 16.5)^2}{16.5} + \frac{(0 - 0.02)^2}{0.02} = 0.42$$

p value for χ^2 at two degree of freedom = 0.77

Hardy-Weinberg equation in the CRF group:

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(30 - 30)^2}{30} + \frac{(112 - 111)^2}{111} + \frac{(1 - 2)^2}{2} = 0.5$$

p value for χ^2 at two degree of freedom = 0.8

The balance of genotypes observed did not differ from Hardy-Weinberg expectations in either control or CRF groups (p value > 0.05).

4.6.1 Susceptibility to CRF

There was no significant difference in the carriage of the G allele in the CRF patient group compared to the control group.

Table 4.16. Testing of the susceptibility of genotype at Arg25Pro to CRF in CRF patients group

	Without C (GG)	***With C (CG+CC)***	***Total***	χ^2	***P value (at one degree of freedom)***
CRF patients group	112	31	143	0.2	0.65
Control group	59	19	78		
Total	171	50	221		
CRF pogressors	94	18	112	2	0.15
Control group	59	19	78		
Total	153	37	190		

When patients with PKD or OU were excluded from the analysis, there was a trend for the G allele to be higher in patients with CRF compared to the control group (Table 4.17).

Table 4.17. Testing for association of genotype at Arg25Pro with susceptibility to CRF in CRF patients without PKD or obstructive uropathy

	Without C (GG)	*With C (CG+CC)*	*Total*	χ^2	*P value (at two degree of freedom)*
CRF progressors	88	14	102	3.35	0.06
Control	59	19	78		
Total	147	33	180		

4.6.2 Progression of CRF

The distribution of genotypes among progressors and non-progressors patients is shown in table 4.16, and figures 4.18.

Table 4.16. Testing for association of Arg25Pro genotype with progression of CRF

	Without C (GG)	*With C (CG+CC)*	*Total*	χ^2	*P value (at one degree of freedom)*
CRF progressors	94	18	112	**9.57**	**0.00198***
CRF non-progressors	18	13	31		
Total	112	31	143		

Figure 4.18. a. The distribution of progressors and non-progressors according to the Arg25Pro genotype

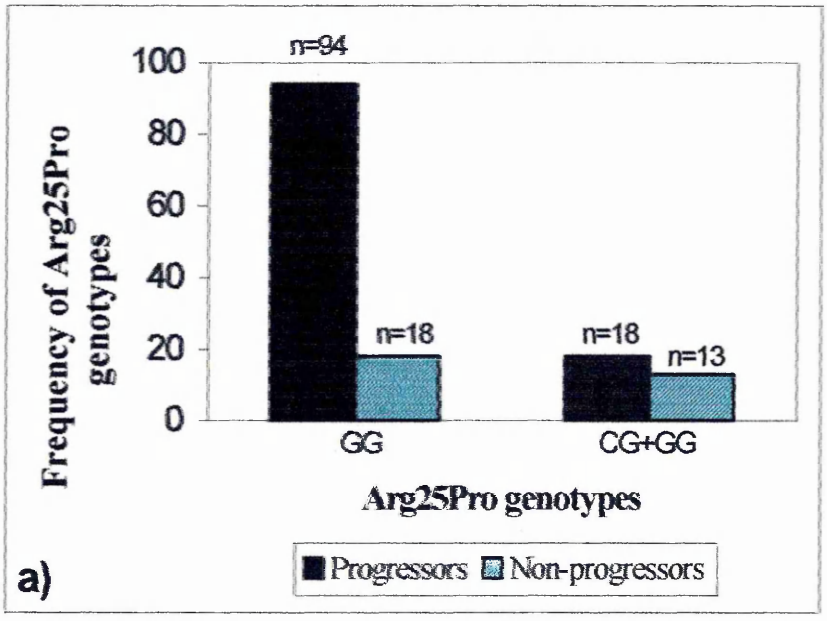
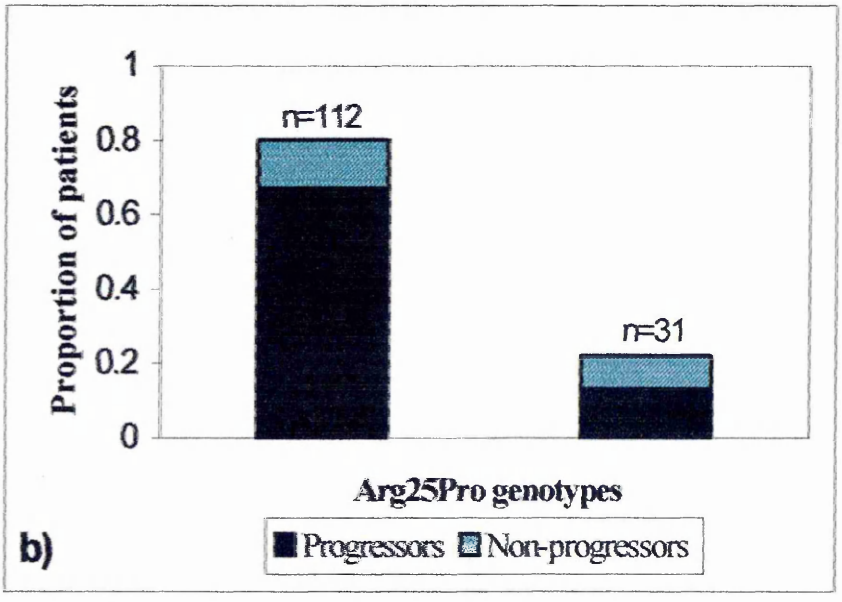


Figure 4.18. b. The proportion of progressors and non-progressors according to the Arg25Pro genotype



There was a higher risk of progressive CRF in the G-homozygotes (GG) compared to patients with other genotypes in both the total CRF group (Table 4.16) and after exclusion of patients with PKD and obstructive uropathy. The homozygous patients for the G allele had a significantly higher number of progressors compared to the other genotypes in the total CRF patients group (odds ratio 3.77, 95% confidence interval, 2.2 - 6) and after excluding PKD and obstructive uropathy (odds ratio=5.00, 95% confidence interval 2.7 - 9).

It was noted earlier (Chapter 3, section 3.4.1) that some CRF parameters (proteinuria and serum creatinine) were significantly higher in males. The allele frequencies were then tested according to gender (Figure 4.19). It was found that there was no significant difference in allele frequencies according to gender (Table 4.18).

Figure 4.19. The distribution of the Arg25Pro genotype according to gender

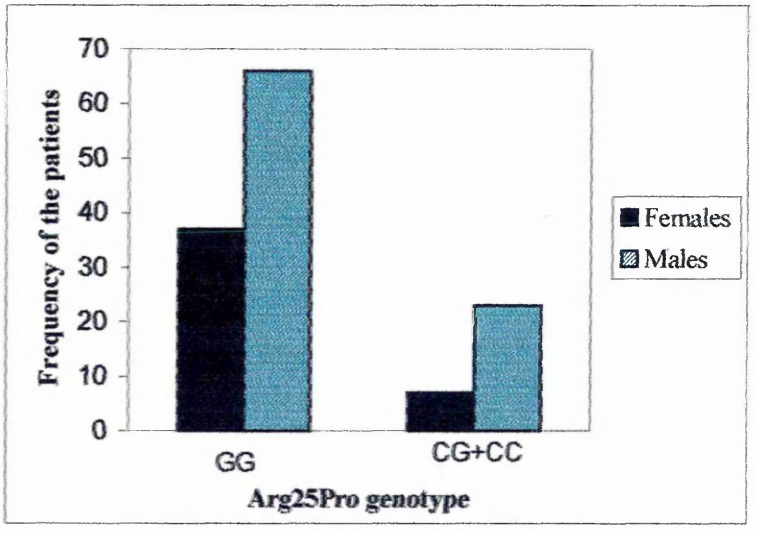


Table 4.18. The allele frequency of Arg25Pro genotype according to gender

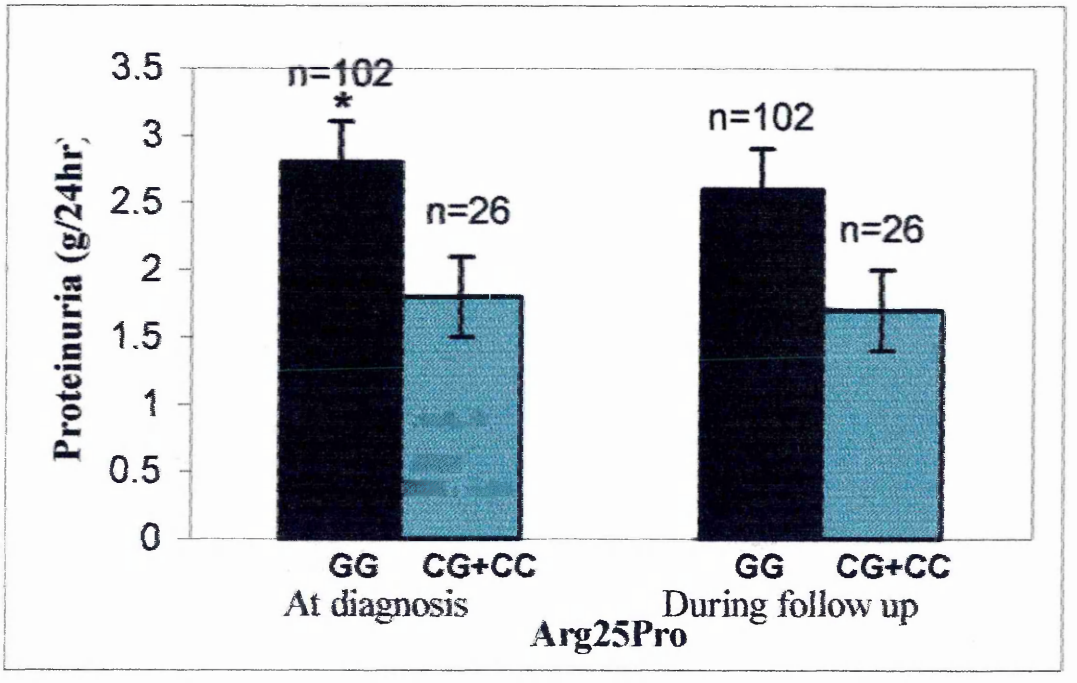
	Females	*Males*	*Total*	*Chi square (at one degree of freedom)*	*P value*
G-frequency	37	76	113	1	0.3
C-frequency	7	23	30		
Total	44	99	143		

4.6.3 Proteinuria

There was no difference in proteinuria at diagnosis or during the follow-up between patients different Arg25Pro genotypes.

On the other hand, it was noted that in CRF patient without PKD or obstructive uropathy, there was a higher level of proteinuria at diagnosis in G-homozygotes (P = 0.038) (Figure 4.20).

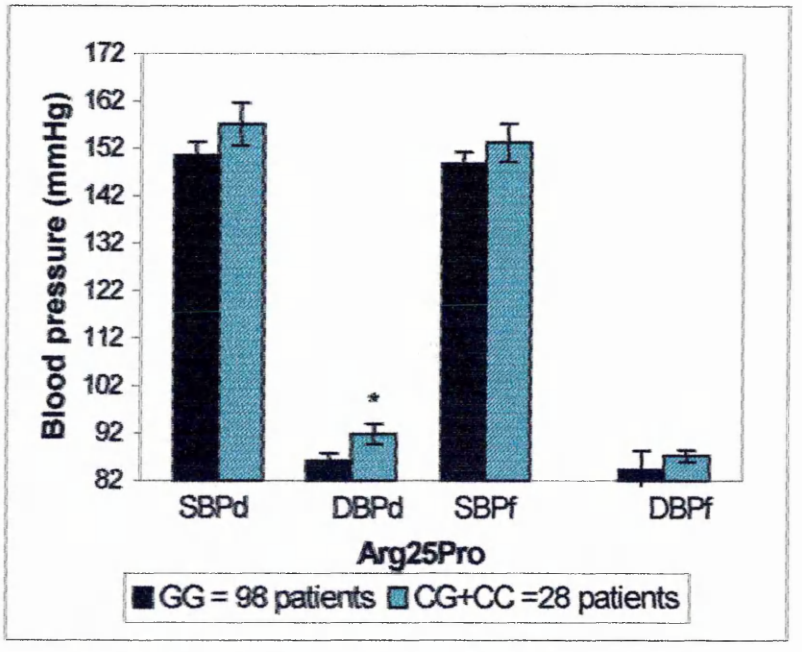
Figure 4.20. Proteinuria (mean±SEM) according to Leu10Pro genotype in the CRF patients



4..6.4. Blood pressure

Although there was no significant association between Arg25Pro genotype and increased systolic blood pressure at diagnosis, there was a significant association between the presence of the C allele and increased diastolic blood pressure at onset in the CRF group. There was no significant association between genotype and systolic or diastolic blood pressure during follow-up in the CRF group (Figure 4.21). On the other hand there was no association between the blood pressure and genotype in CRF patients without PKD or OU.

Figure 4.21. Arg25Pro genotype and blood pressure (mean±SEM) in the CRF patients group



Abbreviations: SBPd: Systolic blood pressure at diagnosis, DBPd: Diastolic blood pressure at diagnosis, SBPf: Systolic blood pressure during follow-up, DBPf: Diastolic blood pressure during follow-up.

*= statistically significant (p=0.045).

Twenty eight patients were diagnosed as having hypertensive glomerulosclerosis. There was a highly significant association of hypertensive nephrosclerosis with homozygosity for the G allele ($\chi^2 = 11$ at one degree of freedom, $p = 0.0009$) (Table 4.19).

Table 4.19. The distribution of Arg25Pro genotyping according to CRF progression in hypertensive nephrosclerosis

	GG	*CG/CC*	*Total*	*χ^2 (Yates' correction)*
CRF Progressors	19	1	20	11
CRF Non-progressors	3	5	8	
Total	22	6	28	

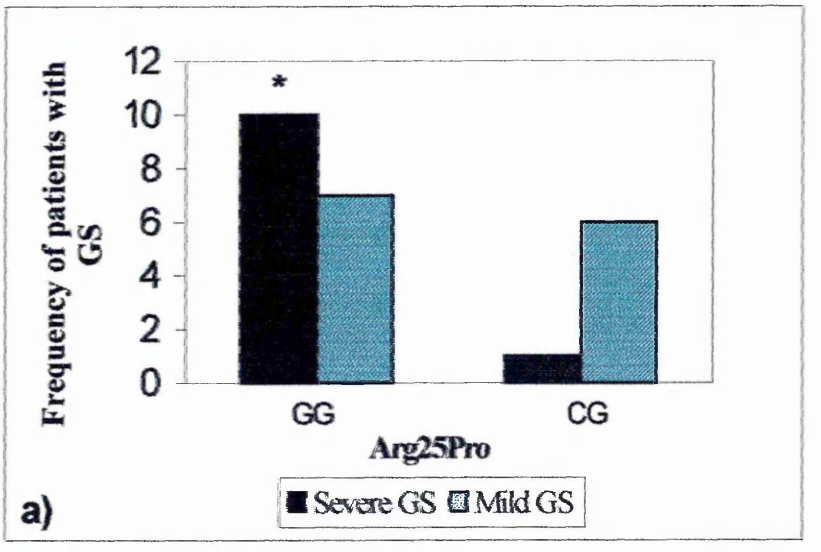
4.6.5 Pathological processes

Twenty-four renal sections were analysed. The pathological processes were classified into two main categories; glomerulosclerosis (GS) and tubulointerstitial fibrosis (IF).

4.6.5.1 Glomerulosclerosis (GS)

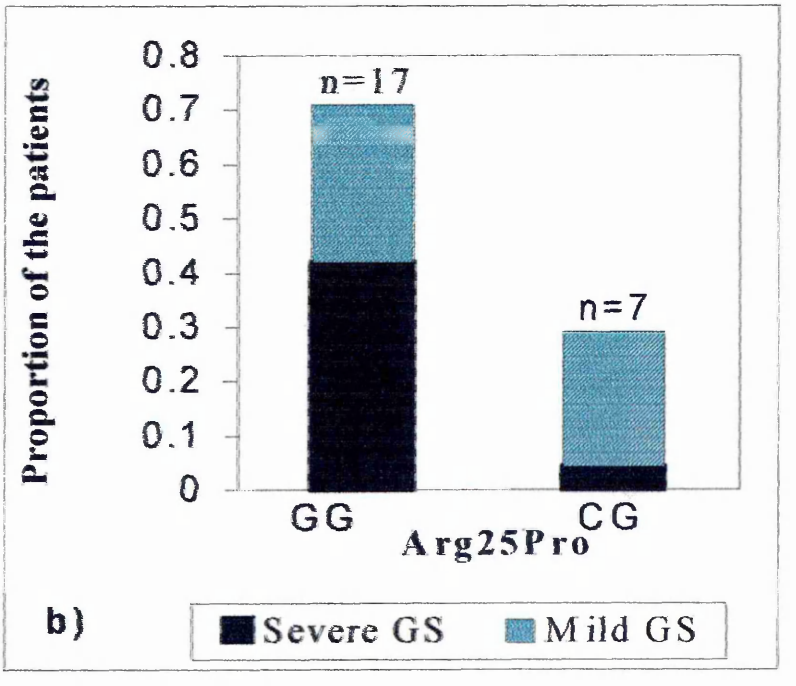
Twenty-four sections from the CRF patients were studied. It was noted that homozygosity for the G allele was much more common in severe GS compared to mild GS [χ^2 (Yates' correction = 4.2, p value = 0.04 at one degree of freedom] (Figure 4.22).

Figure 4.22. a. The distribution of severe and mild glomerulosclerosis (GS) according to Arg25Pro genotype



*: p value = 0.04

Figure 4.22.b The proportion of mild and severe GS according to Arg25Pro genotype



4.6.5.2 Tubulointerstitial fibrosis (IF)

The distribution of genotypes according to the severity of IF is shown in table 4.20. There was no statistically significant difference between the Arg25Pro genotype and the severity of IF (p= 0.65 at one degree of freedom).

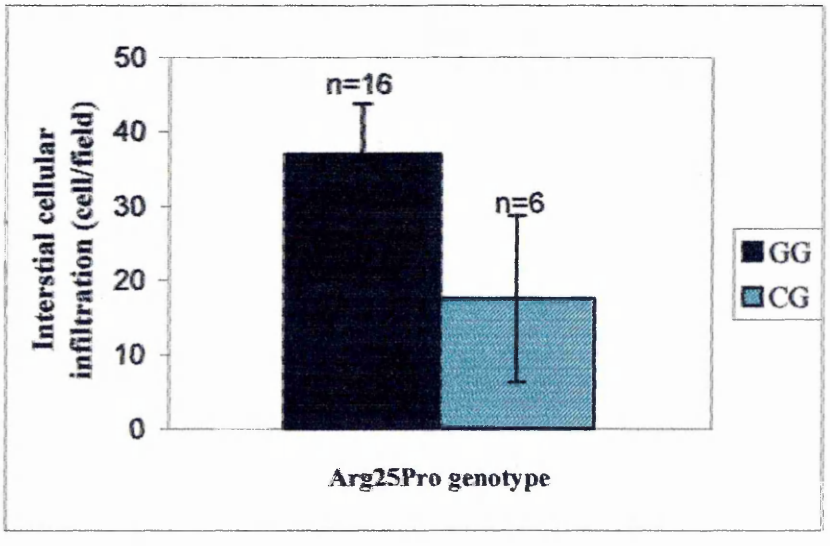
Table 4.20. The distribution of Arg25Pro genotype according to the severity of interstitial fibrosis

	GG	*GC/ CC*	*Total*	*χ^2 (Yates' correction)*
Moderate interstitial fibrosis	13	6	19	0.2
Severe interstitial fibrosis	3	2	5	
Total	16	8	24	

4.6.5.3 Renal interstitial cellular infiltration (ICI)

Genotyping was performed for 22 patients who had pathology sections available. There was no significant difference between the extent of ICI in patients homozygous for the G allele compared to others (p value = 0.3) (Figure 4.23).

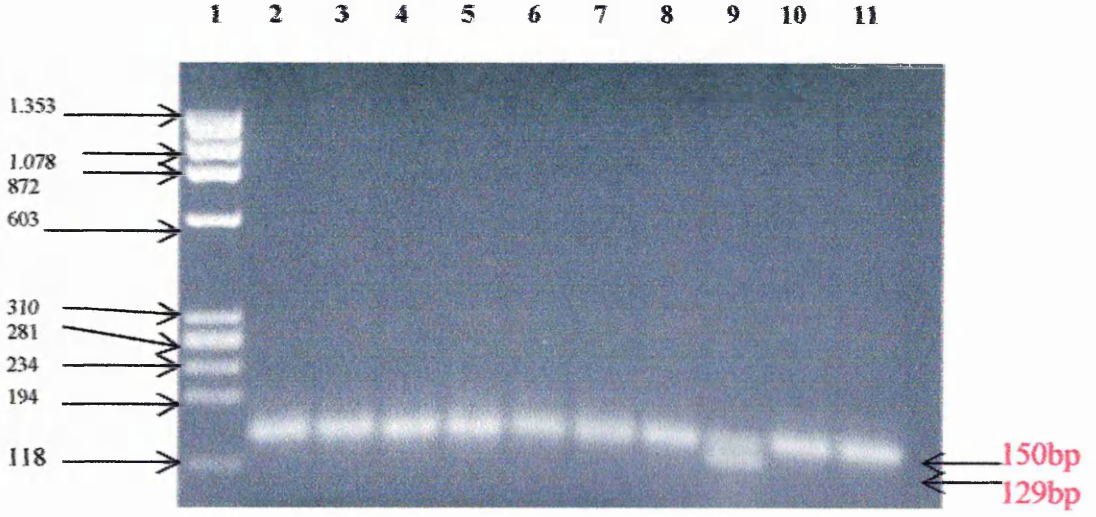
Figure 4.23. The distribution of Arg25Pro according to interstitial cellular infiltration (mean±SEM)



4.7 Thr263Ile

One hundred and forty two CRF patients were genotyped by using mutation screen PCR (figure 4.24).

Figure 4.24. Genotyping at Thr263Ile



Lane 1 is the marker (ϕX174 DNA/HaeIII), lanes samples 2 - 8 are homozygous for the C allele, sample 9 is heterozygous (CT)

Five patients were heterozygous (CT), the remaining patients were homozygous for the C (CC) allele. There was no significant association between the different alleles at Thr263Ile and the different parameters of CRF.

The genotyping results were checked by the Hardy-Weinberg analysis. The genotyping balance of genotypes did not differ significantly from that expected from a population in Hardy-Weinberg equilibrium (Table 4.21).

Table 4.21. Hardy-Weinberg analysis of Thr263Ile

	No of observed genotyping frequencies	*%*	*No of expecting genotyping frequencies*
CRF patients group			
Heterozygous	5	3.5	4.97
Homozygous C	137	96.43	136.9
Homozygous T	0	0.0031	0.043
Total	142	100	142

Hardy-Weinberg equation in CRF group

$$\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$\frac{(137-136.9)^2}{136.9} + \frac{(5-4.97)^2}{4.97} + \frac{(0-0.043)^2}{0.043} = 0.0.00042$$

p value for χ^2 at two degree of freedom = 0.9

The balance of genotypes observed did not differ from Hardy-Weinberg expectations (p value > 0.05).

4.7.1 Progression of CRF

There was no significant difference between the genotypes of the progessors and non-progressors (Table 4.22) (odds ratio 2.9, 95% confidence interval -1.1 - 9).

Table 4.22. The progression of CRF according to Thr263Ile genotype

	CC	*TC*	*Total*	*χ^2 at one degree of freedom (Yates' correction)*	*P value*
CRF patients				0.72	0.4
Progressors	108	3	142		
Non-progressors	29	2	55		
Total	92	105	197		

There was no significant association between Thr263Ile genotype and proteinuria at diagnosis or during the follow-up (Table 4.30). Further, there was no significant difference between Thr263Ile genotype and blood pressure at diagnosis or systolic blood pressure during the follow-up. There was a significantly higher diastolic blood pressure during follow-up in the C-homozygotes compared to heterozygotes (Table 4.23).

Table 4.23. Different parameters in CRF patients according to genotype at Thr263Ile

	Means±SEM	*Observations*	*P(f)*	*P(t)*
i) Proteinuria (g/24hr) at diagnosis				
CC	2.45±0.25	134	0.3	0.7
TC	2.96±0.4	5		
ii) Proteinuria (g/24hr) during follow-up				
CC	2.3±0.22	133	0.5	0.7
TC	2.8±0.1	5		
SBPd (mmHg)				
CC	153±2.5	130	0.6	0.5
TC	143±14	3		
SBPd (mmHg)				
CC	88±1.4	130	0.1	0.2
TC	76±3	3		
SBPf (mmHg)				
CC	149.8±2	130	0.3	0.6
TC	143±14	3		
DBPf (mmHg)				
CC	85±1.1	130	0.5	0.03*
TC	70±5	3		

Abbreviations: SBPd: systolic blood pressure at diagnosis; DBPd: diastolic blood pressure at diagnosis; SBPf: systolic blood pressure during follow up; DBPf: diastolic blood pressure during follow-up.

Because of the rarity of this polymorphism it was found that all of the studied pathological sections were of the CC genotype.

4.8 Linkage disequilibium studies

There was significant linkage disequilibrium between alleles at the studied loci. There was a strong linkage disequilibrium between the alleles at the Arg25Pro and T (-509) C polymorphic sites, for example, the G-allele of Arg25Pro and the T-allele of C-509T (Table 4.24). Linkage disequilibrium was also noted between the different alleles of Arg25Pro and Leu10Pro polymorphisms (Table 4.24). Furthermore, linkage disequilibrium was found between C-509T and Leu10Pro (Table 4.24).

The coefficient of linkage disequilibrium (D') was calculated for the studied polymorphisms using the formula given earlier in this chapter (section 4.1).

D' for Leu10Pro and Arg25Pro was –0.57 which means that there is a negative association between the G allele at Arg25Pro and the T allele at Leu10Pro.

D' for C-509T and Arg25Pro was 0.87 which means that there is a strong association between the G allele at Arg25Pro and the T allele at C-509T.

D' was 0.8 for C-509T and Leu10Pro. This means that there is a strong association between the T alleles of both polymorphisms.

Table 4.24. Linkage disequilibrium between alleles of studied polymorphisms

I- C-509T and Leu10Pro	**WithC allele at Leu10Pro**	**Without C-allele at Leu10Pro**	**Total**	**p=0.02**
With C-allele atC-509T	18	14	32	
Without C-allele at C-509T	1	7	8	
Total	19	21	40	
II-C-509Tand Arg25Pro	**Without C-allele at Arg25Pro**	**With C-allele at Arg25Pro**	**Total**	**p=0.0009**
With C-allele at C-509T	71	14	85	
Without C-allele at C-509T	2	5	7	
Total	73	19	92	
III- Leu10Pro and Arg25Pro	**Without C-allele at Arg25Pro**	**With C-allele at Arg25Pro**	**Total**	**p=0.034**
Without C-allele at Leu10Pro	15	11	26	
With C-allele at Leu10Pro	25	5	30	
Total	40	16	56	

Double homozygotes for the G allele at Arg25Pro and the T allele at C-509T were more liable to progress to ESRF than patients with other genotypes (Table 4.25). On the other hand, there was no significant difference in the other studied parameters associated with double homozygosity (OR=3.8, CI: 1 – 9) (Table 4.26).

Table 4.25. Distribution of Arg25Pro and C-509T genotypes among CRF progressors and non-progressors

	Progressors	**Non-progressors**	**Total**
GG + TT	59	8	67
GG + TC	20	8	28
GG + TT	2	0	2
CG + TT	10	4	14
CG + TC	6	4	10
CG + CC	1	4	5
Total	98	28	126

Table 4.26. Arg25Pro and C-509T genotypes among the different parameters

	Double homozygotes (GG + TT) (n=67)	**Other genotypes (n=59)**
CRF progressors	59*	39
CRF non-progressors	8*	20
Proteinuria (g/24hrs)	2.8±0.5	2.1±0.3
BP (mmHg)	151.6±2.1	149.9±2.8
Slope	-2.7E-05±1.2E-05	-8.6±9E-06

*=significant difference (p=0.002)

4.9 Discussion

In this study TGF-B1 gene polymorphisms were screened to investigate susceptibility to, and progression of, CRF in a Caucasian population of patients with CRF.

The calculated allele frequencies at C-509T of the control group were simailar to those reported in European (Sergio, *et al.*, 1997), UK (Lympany, *et al.*, 1998) and Japanese (Yamada, *et al.*, 2001) populations. On the other hand, they were different from those reported in one UK study (Grainger, *et al.*, 1999). The allele frequency at C-509T reported by Grainger and his colleagues (1999) was also different from other published studies (Lario, *et al.*, 1997; Lympany, *et al.*, 1998; Yamada, *et al.*, 2001). The allele frequencies of control and CRF patients at Leu10Pro were similar to other published work (Cambien, *et al.*, 1996, Lympany, *et al.*, 1998, Yamada, *et al.*, 1998, Syrris, *et al.*, 1998, Yasuhiro, *et al.*, 2001). Furthermore, it was noted that there was no difference in allele frequencies at Arg25Pro between this study and other published studies (Cambien, *et al.*, 1996, Lympany, *et al.*, 1998, Li, *et al.*, 1998, El-Gamel, *et al.*, 1998). Moreover, there was no difference between the allele frequency of the studied group and the published frequency at Thr263Ile (Cambien, *et al.*, 1996, Pociot, *et al.*, 1998, Lympany, *et al.*, 1998, Syrris *et al.*, 1998).

Carriage of the C allele at Leu10Pro and the T-allele at C-509T was significantly more frequent in the CRF patient group than in the control group. Furthermore, the same alleles were significantly more frequent in progressors with CRF compared to the control group. This means that carriers of these alleles are at greater risk of progression of CRF.

Subsequently, when the frequency of progressive renal insufficiency amongst different genotypes was analysed, those patients who were homozygous for the G allele (Arg25Pro) and those who were homozygous for the T allele (C-509T) were found to be at higher risk. No such difference was noted for the other studied genotypes including Leu10Pro and Thr263Ile. These findings are novel. Previous authors have noted an association between the G allele at Arg25Pro and early onset of cardiac allograft vasculopathy (Densem, *et al.*, 1999). However, others failed to observe an association

between GG at Arg25Pro or TT at C-509T and atherosclerotic coronary artery disease in native hearts (Syrris, *et al*, 1998, Holweg, *et al.*, 2001). The Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) study on TGF-B1 polymorphism in relation to myocardial infarction and blood pressure observed an increased risk of myocardial infarction and reduced risk of hypertension in patients homozygous for the C allele (Cambien, *et al*, 1996). Homozygosity for the G allele at codon 25 of the leader sequence of TGF-β1 was associated with fibrotic lung disease (El-Gamel, *et al.*, 1998). Furthermore, these authors noted that this genotype was associated with the development of pulmonary fibrosis in lung allografts. The G allele was also more frequent in patients with progressive cystic fibrosis (Arkwright, *et al.*, 2001). Grainger and colleagues (1999) reported a relationship between high levels of circulating TGF-β1 and homozygosity for the T allele at C-509T in normal individuals.

The association between carriage of the G allele (Arg25Pro) and the T alleles (C-509T) and the progression of CRF may be a reflection of the impact of these genotypes on other risk factors associated with the progression of renal diseases. Of these, systemic hypertension and proteinuria are known to be important prognostic factors (Harris, 2000; Locatelli and Del Vecchio, 2000). In this study, no association was observed between levels of systemic blood pressure and disease progression or the studied alleles. On the other hand, proteinuria levels correlated with the rate of decline in renal function. Further, proteinuria at diagnosis was significantly higher in patients homozygous for the G (Arg25Pro) than other genotypes of the same polymorphism. Proteinuria during follow-up was also significantly higher in patients homozygous for the T allele than those homozygous for the C allele at C-509T. These observations may explain the higher frequency of progressors with these genotypes in view of the severity of proteinuria in these patients. It has been postulated that heavy proteinuria is not only a marker of progressive renal diseases, but that it also contributes directly to the renal scarring process (Burton and Harris, 1996; for review, Harris, 2000). This has been attributed to the activation of proximal tubular cells by excessive proteinuria and its reabsorption by these cells, leading to their stimulation and release of pro-inflammatory chemokines and cytokines (Prodjosudjadi, *et al.*, 1995; for review, Harris, 2000; Eddy, 2001). This, in

turn, would contribute to the exacerbation of interstitial cellular infiltration and inflammation leading to renal interstitial fibrosis (For review, Harris, 2000). Of note no correlation between the severity of proteinuria and that of the interstitial cellular infiltrate was observed in this study. However, there was significantly more tubulointerstitial cellular infiltration in homozygotes for the T allele (C-509T). Interstitial inflammation has been previously implicated as an important prognostic factor for the progression of a wide range of renal diseases (Alexopoulos, *et al,* 1989, Eddy 2001).

Bean and colleagues (2000) reported a significant association between the presence of proline at codon 10 and the development of CRF, but this association was found in patients who underwent a heart transplantation and treated by cyclosporin and steroids. It is well established that heart failure affects renal function. In addition to that, the patients studied by Bean and colleagues (2000) were on cyclosporin, which *per-se* induce renal toxicity. So, in the presence of the heart failure, cyclosporin and steroids, Pro10 might potentiate the impairment of renal function. Also, there was a significant association between the presence of Pro10 and the development of end-stage heart failure, caused by cardiomyopathy, rather than by ischaemic heart diseases (Holweg, *et al.,* 2001). So, it might be concluded that the if Pro10 was associated with renal impairment, it was mainly due to other factors rather than primary renal impairment. Pociot and colleagues (1998), however, found a weak significant association between the presence of the T allele at Thr263Ile in exon 5 and the development of nephropathy in diabetic patients. This study revealed no significant associations with other renal functional parameters. This was probably due to the rarity of the allelic variant.

Finally, I found that there was linkage disequilibrium between the promoter marker (C-509T), Arg25Pro and Leu10Pro. This is a reflection of the proximity of these polymorphic regions within the TGF-B1 gene. Furthermore, I noted the presence of both G (Arg25Pro) and T (C-509T) homozygosity conferred the worst risk of decline in renal function.

In conclusion, I describe for the first time in this thesis, a range of associations between TGF-B1 gene polymorphisms and the progression of CRF. More specifically, I report a higher frequency of progressing renal failure in homozygotes for the G (Arg25Pro) and T (C-509T) alleles. These associations may be related to increased proteinuria and glomerulosclerosis in homozygotes for the G allele at Arg25Pro. On the other hand, the T allele at C-509T appears to be associated to proteinuria, tubular TGF-β1 over-expression and interstitial inflammation.

Chapter 5

Circulating and tissue TGF-β1 levels

5.1 Introduction

Transforming growth factor-β1 is a key factor in the regulation of mesangial and interstitial ECM turnover (Peters, *et al.*, 1997, Gaedeke, *et al.*, 2001).

Both circulating and local TGF-β1 may contribute to the development of renal fibrosis (Datta, *et al.*, 1999; Grande, *et al.*, 2002). Increased production of TGF-β1 is essential for induction of progressive renal disease in both animal and human models (Border and Noble, 1994, 1997). Sustained abnormal production of TGF-β1 alters both production and degradation of extracellular matrix and has been associated with the development of glomerulosclerosis (GS) in several models of renal disease (Border and Noble, 1994; Basile, 1999; Yu, *et al* 2002). Furthermore, high plasma levels of active TGF-β1 induce tubulointerstitial fibrosis, which occurs after induction of GS (Kopp, *et al.*, 1996). Transforming growth factor-β1 actions are associated with hypertension. Kopp and colleagues (1996) found that blood levels of TGF-β1 were higher in "Afro-Caribbean" and "Caucasian" with high blood pressure than in those with normal blood pressure. African Americans had higher levels of TGF-β1 than their white counterparts whether or not they had hypertension (Suthanthiran, *et al.*, 1998). Transforming growth factor beta 1 is not the sole cause of high blood pressure, but gene variations could be used to identify individuals who make higher levels of TGF-β1 and might benefit from a more aggressive approach (American Heart Association meeting report September 14, 1999).

Transforming growth factor-β1 levels are markedly increased in the plasma of hypertensive patients. The same study postulated that TGF-β1 contributes substantially to the development of end organ damage in essential hypertension, independently of blood pressure levels (Derhaschnig, *et al.*, 2002). Complications associated with high blood pressure, such as kidney failure, stroke, and heart disease, may be related to a variety of processes that involve overproduction of various proteins, such as angiotensin II and TGF-β1 (August, *et al.*, 2000).

There is no accurate method to assess the biological role of TGF-β1. Also, measurement of absolute levels of TGF-β1 either at protein or messenger RNA (mRNA) level may not reflect biological activity. This is because of the complexities of TGF-β1 release and activation (Jain, *et al.*, 2000). Furthermore, once TGF-β1 has been activated it may be inhibited by local factors, so that measurement of active TGF-β1 is not more informative than measurement of total TGF-β1 in assessing biological activity. Some groups have used deferential antibody staining of active and latent TGF-β1, but there are few data to confirm the accuracy of this method. At present, there is no gold standard method to assess TGF-β1 activities (Jain, *et al.*, 2000). Because there are many factors affecting TGF-β1 after its production (activation and inhibition), it is considered more reliable to measure the natural processed TGF-β1 (Jain, *et al.*, 2000).

In the kidney, TGF-β1 is released mainly from activated mononuclear cells, renal proximal tubule cells (which may contribute to the pathogenesis of tubulointerstitial injury), glomerular cells and platelets (Nathan, 1987, Erwig, *et al.*, 2001) (see general introduction). Transforming growth factor-β1 inhibits renal tubular epithelial cell proliferation, which may promote tubular cell hypertrophy, which is characteristic of tubulointerstitial fibrosis (For review, Jernigan and Eddy, 2000).

The intensity of interstitial TGF-β1 protein expression was significantly related to the degree of interstitial fibrosis, tubular atrophy, interstitial collagen III expression, and serum creatinine values (Goumenos, *et al.*, 2001).

5.2 Methodology

5.2.1 Circulating TGF-β1

Plasma and serum total acid activated TGFβ1 protein levels were measured by using an ELISA [Quantikine Human TGF-β1 Immunoassay kits (R&D Systems, UK)]. The assay was carried out according to the manufacturer instructions (for detail, Materials and Method, Chapter 2 section 2.6).

5.2.1 Renal TGF-β1 (immunostaining)

Renal biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned stained using a standard immunoperoxidase staining technique (Muchaneta-Kubara *et al.*, 1996) (for details see Materials and Method Chapter 2 section 2.6). Two independent observers counted the presence of staining for each section. Omitting adding the primary antibodies (negative control) tested the non-specific binding of the secondary antibody.

5.2.3 Statistical Analysis

Analysis of the means of the studied parameters (blood pressure and proteinuria, creatinine levels and serum lipids) according to the progression status of CRF are carried out using F-tests followed by t-tests (unpaired, two tailed, for equal or unequal variance, as appropriate). Chi square analysis tests the null hypothesis. Yates' correction was applied for the 2x2 contingency table if any of its cell less than 5. Correlation and regression between the different measurements was carried out using ANOVA test on Microsoft Excel. A p value < 0.05 was considered significant.

5.3 Results

5.3.1 Circulating TGF-β1

One hundred and thirty five patients had their circulating TGF-β1 measured both in serum and plasma. Seven samples were excluded from the study because they looked autolysed. The plasma TGF-β1 levels were 18479±981 pg/ml (mean±SEM) and the serum levels were 23739±1149 pg/ml.

5.3.1.1 Validity of the measurement

The inter and intra-assay coeffecients of variations (CV) was tested on patient samples. The results showed that the readings of the absorbance were very close for the same samples in both intra and inter-assay comparisons (Table 5.1).

Table 5.1. Precision of the measurements of plasma TGF-β1

Intra-assay Precision			***Inter-assay Precision***	
Samples	1	2	1	2
n	20	20	10	10
Mean (pg/ml)	28640	677	40200	38600
Standard deviation	1200	1320	1520	1560
CV(%)	4.2	4.8	3.7	3.9

Serum TGF-β1 protein levels (23605±849 pg/ml) were significantly higher (p=0.0002) than the plasma TGF-β1 protein levels (18603±1016 pg/ml). This might be due to the fact that the platelets contribute to the serum levels of TGF-β1. There was a significant

correlation between the serum and plasma measurements (r =0.4, p = 0.000003) (Figure 5.1). Furthermore, except for the significant difference between the serum levels of TGF-β1 and the progression of CRF (see below), both plasma and serum TGF-β1 measurements showed comparable results in relation to the different studied parameters across the study.

So, plasma levels of TGF-β1 protein were chosen to represent the circulating TGF-β1 protein levels to minimise the effects of platelet release of TGF-β1.

Figure 5.1. Correlation between serum and plasma TGF-β1 levels

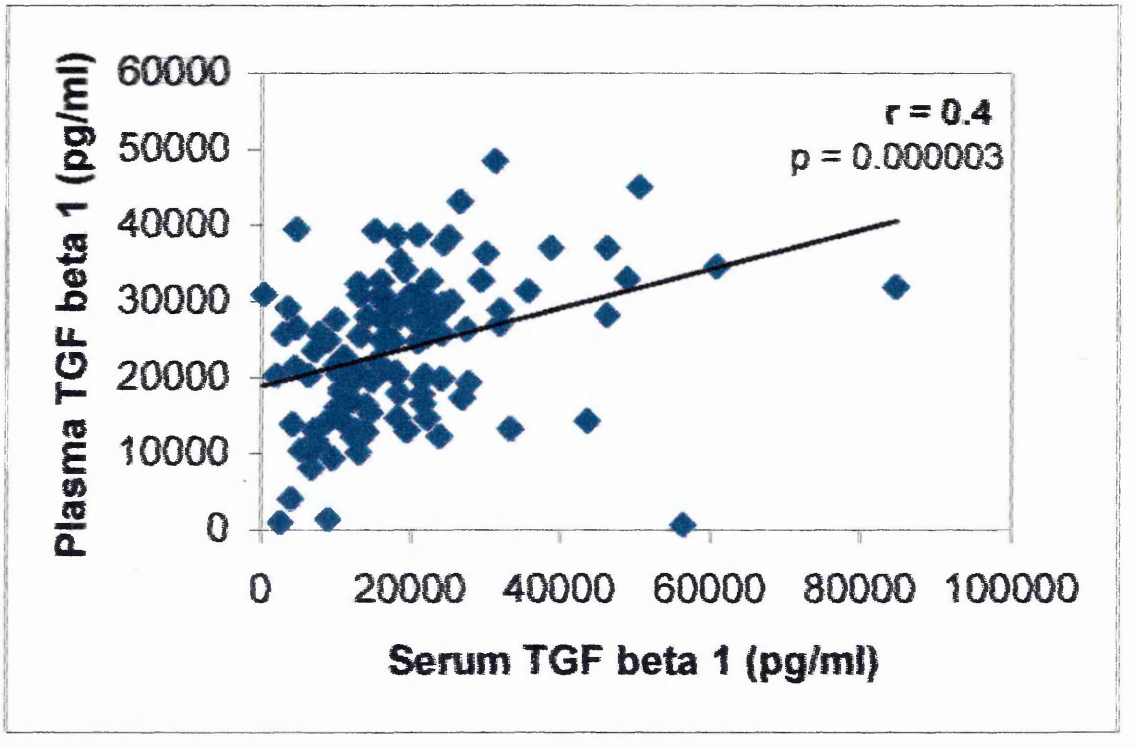


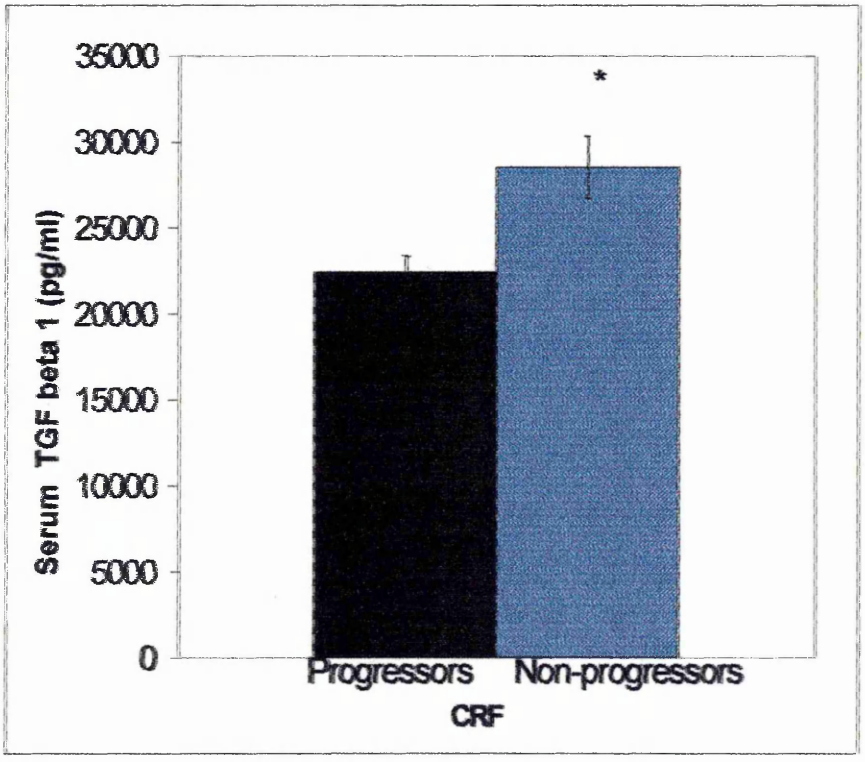
Table 5.2. Plasma TGF-β1 levels according to clinical parameters

Prameters	*Mean±SEM (pg/ml)*	*Observations*	*P(f)*	*P(t)*
I- Clinical parameters				
Gender				
Males	20421±1515	93	0.054	0.37
Females	17785±1606	42		
Proteinuria at diagnosis				
< 3 g/24hrs	17785±1255	86	0.054	0.37
> 3 g/24hrs	20421±2550	26		
Proteinuria during follow up				
< 3 g/24hrs	19023.8±1210	81	**0.01***	0.4
> 3 g/24hrs	19998±2875	35		
SBPd				
< 140 mmHg	18812±2275	46	0.27	0.4
> 140 mmHg	19169±1409	66		
DBPd				
< 90 mmHg	19202±1464	72	**0.007***	0.19
>90 mmHg	18954±1954	40		
SBPf				
< 140mmHg	17592±1938	42	0.18	0.7
> 140 mmHg	19469±1439	70		
DBPf				
< 90 mmHg	17216±986	72	**0.005***	0.4
> 90 mmHg	18948±765	40		
CRF progressors	17444±1909	98	0.1	0.4
CRF non-progressors	19373±1187	37		

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd:diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow-up, DBPf: diastolic blood pressure during the follow-up. *: significant difference in variance.

There was no significant difference between mean plasma TGF-β1 levels in the CRF progressors and non-progressors. On the other hand, the mean serum level of TGF-β1 was significantly higher CRF in non-progressors than progressors (p=0.0028) (Figure 5.3).

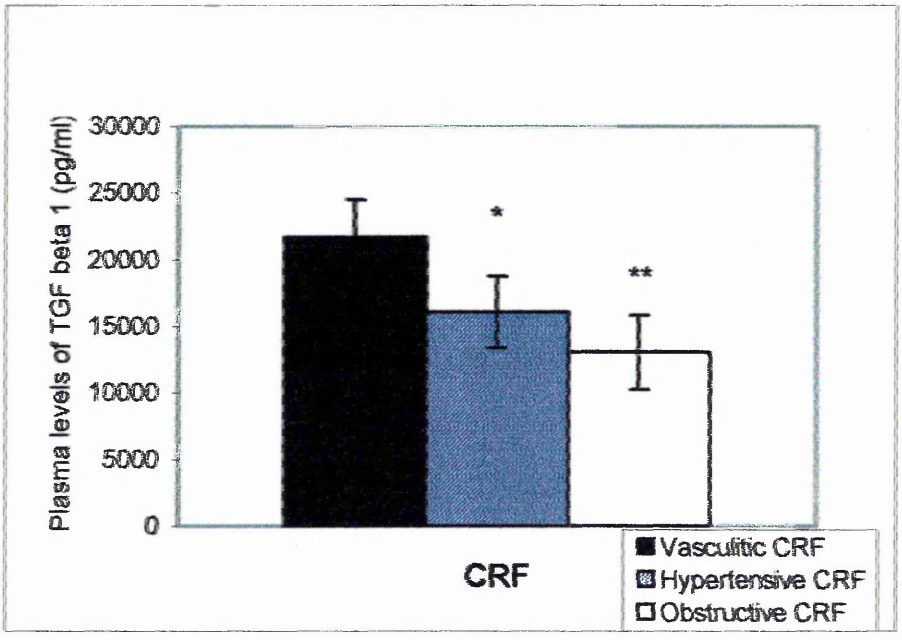
Figure 5.3. Serum TGF-β1 levels (mean ±SEM) and the progression of CRF



**: P= 0.0028*

The highest levels of plasma TGF-β1 were found in the vasculitic causes of CRF (Wegner's granulomatosis, system lupus erythematosus and rheumatoid arthritis). Mean plasma TGF-β1 levels in vasculitic CRF was significantly higher than in patients with hypertensive causes (p=0.05) or obstructive uropathy (p=0.02) (Figure 5.4) (Table 5.3).

Figure 5.4. Plasma TGF-β1 (mean ± SEM) according to different causes of CRF



* : p = 0.05

** : p=0.02

Table 5.3. Plasma TGF-β1 (mean ± SEM) and both clinical diagnosis

Clinical parameters	*n*	*Plasma TGF-β1 (pg/ml)*
Hypertensive glomerulosclerosis	26	16080±3500
Chronic interstitial nephritis	23	15030±2949
Glomerulonephritis	15	20044±2617
Obstructive uropathy	14	13028±2531
Diabetic nephropathy	11	16680±3283
Systemic vasculitis	10	21711±2839
Polycystic kidney disease	7	15095±3336
Unknown	29	19714±2068

5.3.1.2.3 Plasma TGF-β1 protein levels and pathological diagnoses

The highest levels of plasma TGF-β1 were found in mesangiocapillary glomerulonephritis (MCGN) (Table 5.4).

Table 5.4. Plasma TGF-β1 (mean±SEM) and pathological diagnoses

Pathology diagnosis	*n=*	*Plasma TGF-β1 (pg/ml)*
MCGN	6	26469±4190
FSGS	5	16416±4173
MN	2	22472±1110
DN	2	13414±1017
CIN	5	18927±5930

Abbreviations: CIN: chronic interstitial nephritis, MCGN: mesangiocapillary glomerulonephritis, FSGS: focal segmental glomerulosclerosis, MN: membranous nephropathy DN: diabetic nephropathy, CGN: crescentic glomerulonephritis.

There was no significant difference in plasma levels of TGF-β1 in the different levels of severity of glomerulosclerosis or tubulointerstitial fibrosis (Table 5.5).

Table 5.5. Plasma TGF-β1 levels (Mean ±SEM) and scarring parameters

Pathological *parameters*	*TGF-β1 (pg/ml)*	*Observations*	*P(f)*	*P(t)*
GS				
Mild	17702±1553	13	0.4	0.5
Moderate and severe	20995±1988	12		
IF				
Moderate	20378±1790	20	0.09	0.4
Severe	14904±2815	5		

Abbreviations: GS: glomerulosclerosis, IF: interstitial fibrosis.

5.3.1.3 Plasma TGF-β1 protein levels and genotyping

Overall, there was no significant difference in plasma TGF-β1 levels between genotypes. This might be due to the variety of different pathological mechanisms operating in different subjects.

When patients with PKD and obstructive uropathy were excluded, highly significant differences in variance and in mean levels were observed (Table 5.6).

Table 5.6. Plasma TGF-β1 levels (mean ± SEM) and the polymorphisms studied

Genotypes	***plasma TGF-β1* (pg/ml)**	**Observations**	**P(f)**	**P(t)**
C-509T				
TT	20030±1636	63	**0.00031***	0.2
TC+CC	17148±1090	53		
TT	20030±1636	63	0.38	0.5
CC	15719±4424	5		
Leu 10Pro				
CC	19478±1883	22	0.06	0.49
TC+TT	17788±1131	51		
CC	19478±1883	22	**0.04***	0.7
TT	20326±2181	27		
Arg25Pro				
GG	18886±1419	90	**0.00045***	**0.01***
CG + CC	13817±1442	24		
Thr263Ile				
TT	18613±1005	113	0.4	0.5
TC	14694±4272	3		

The data were analysed according to the different causes of CRF (Table 5.7). There was no significant difference between mean plasma TGF-β1 levels in the different genotypes according to different the causes of CRF except for CIN. In CIN, the mean plasma TGF-β1 was higher in the homozygotes for T allele (TT) compared to heterozygotes (TC) plus homozygotes for C allele (CC) at Leu10Pro polymorphism (Table 5.7). Again, significant differences in variance were observed between different genotypes. This was particularly marked in patient with CRF of unknown causes. After division of the patients according to the causes of CRF the scanty numbers of patients might give unreliable data to be analysed.

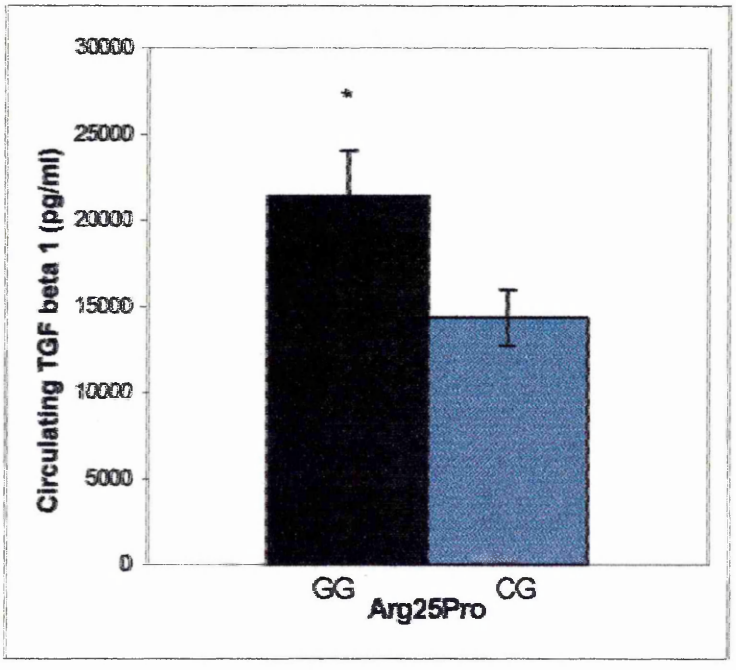
Table 5.7. Plasma TGF-β1 levels (pg/ml) (mean ± SEM) according to polymorphisms and the causes of CRF

Hypertensive nephrosclerosis	*C-509T*		*Leu10Pro*		*Arg25Pro*	
	TT	*TC+CC*	*TT*	*TC+CC*	*GG*	*CG+CC*
(Mean±SEM)	21248±3002	13835±2756	25575±3841	16540±2395	18890±2341	14041±2541
Observations	10	7	4	12	16	8
P(f)	**0.0006***		0.053		0.5	
P(t)	0.18		0.28		0.6	
DN						
Mean±SEM	18360±6836	11282±1942	13772±3690	10559±4868		
Observations	6	4	2	8	10	0
P(f)	0.07		0.1			
P(t)	0.3		0.3			
CIN						
Mean±SEM	17733±2645	17633±1432	9649.6±1231	19856±1432	19587±1133	10633±1621
Observations	5	9	3	11	11	3
P(f)	**0.01***		0.3		0.3	
P(t)	0.3		**0.02***		0.24	
OU + PKD			*CC*	*TC+TT*		
Mean±SEM						
Observations	16952±2160	16234±2654	18723±1342	15097±1543	17177±2153	14319±3019
P(f)	10	7	6	11	13	5
P(t)	0.17		0.14		0.27	
	0.9		0.4		0.5	
GN + Vasculitis						
Mean±SEM	18157±4748	14748±3567	18053±3032	14485±4969	16948±3342	17227±7424
Observations	15	13	19	12	21	11
P(f)	0.1		0.13		0.4	
P(t)	0.36		0.28		0.4	
Unknown causes						
Mean±SEM	22333±3220	17152±1835	22275±6149	16832±1907	21333±2657	14333±1624
Observations	19	15	7	19	27	6
P(f)	**0.0002***		**0.0007***		**0.004***	
P(t)	0.3		0.48		**0.04***	

Abbreviations: DN: diabetic nephropathy, CIN: chronic interstitial nephritis, PKD: polycystic kidney disease; OU: obstructive uropathy

There was a higher plasma TGF-β1 level among patients homozygous for the G allele at Arg25Pro (p=0.04) in unknown causes of CRF (Figure 5.5).

Figure 5.5. Plasma TGF-β1 levels (mean ± SEM) according to Arg25Pro in unknown causes of CRF



*=P < 0.05.

Furthermore CIN patients were found to have lower TGF-β1 plasma levels in T-homozygous at Leu10Pro compared to the other genotypes at that locus (Table 5.5). On the other hand, I failed to find any significant relationship between any of the other studied genotypes and plasma TGF-β1 levels according to causes of CRF (Table 5.5).

5.5 Correlations between plasma TGF-β1 levels and the different CRF parameters

It was found that there were no significant correlation between the plasma TGF-β1 levels and the different clinical parameters (Table 5.8).

Table 5.8. The correlations between the plasma TGF-β1 levels and the different clinical parameters

	n =	*r* =	R^2 =	*P (r)*
A) Clinical parameters 1) Proteinuria (g/24hrs) at diagnosis	116	0.003	0.000	0.97
2) Proteinuria (g/24hrs) during follow up	114	0.009	0.000	0.92
3) SBPd (mmHg)	112	0.03	0.001	0.75
4) DBPd (mmHg)	112	0.002	0.000	0.98
5) SBPf (mmHg)	112	0.08	0.006	0.39
6) DBPf (mmHg)	112	0.003	0.000	0.98
7) Serum cholesterol (mmol/l)	53	0.27	0.07	0.2
8) Serum triglycerides (mmol/l)	49	0.19	0.04	0.4
B) Progression parameters 1) CrCl (ml/min)	49	0.05	0.002	0.7
1) Serum creatinine (μmol/l)	59	0.04	0.001	0.8
2) 1/Creatinine slope	59	0.09	0.007	0.5

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during the follow up, CrCl: creatinine clearance.

5.4 Renal immunostainable TGF-β1

The control renal sections (three normal kidney donors) were obtained from the Pathology Department of Northern General Hospital, Sheffield, UK. The control sections were processed as the CRF patient's sections.

Slides were scored independently and subsequently, I reviewed my scoring with my supervisor who finalized the scoring with me.

The intra-observers variations were tested. The intra-observers correlation coefficients were 1.2% for glomerular (Mean±SD: 3.15±0.005%), 1.7% for tubular (Mean±SD: 9.4±0.23%), immunostaining and 1.2 (cell/field) for ICI (Mean±SD: 21.6 ±0.35).

The control renal tissues did not stain for TGF-β1 (Figure 5.10&5.12). On the other hand, the tubules and glomeruli of the CRF patients (23 patients renal sections) stained with TGF-β1 (bright red staining). The glomerular percentage of TGF-β1 staining ranged from 0 to 13.7 % (median = 0). The tubular percentage of TGF-β1 staining ranged from 0.05 to 20.9 % (median = 12.1%) (Figure 5.11).

Photomicrographs of Immunostaining (red) of TGF-β1 in normal (Figure 5.6) and diseased glomeruli (Figure 5.7 & Figure 5.8)

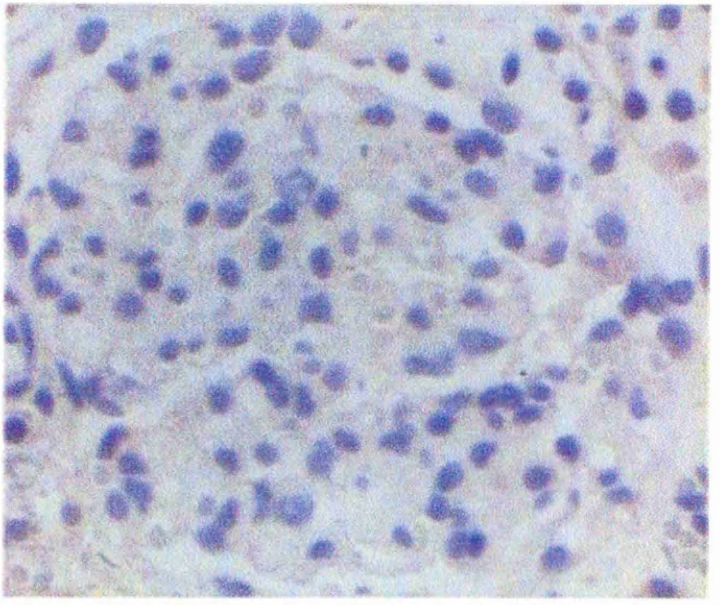


Figure 5.6. Glomerulus control (Magnification X 400)

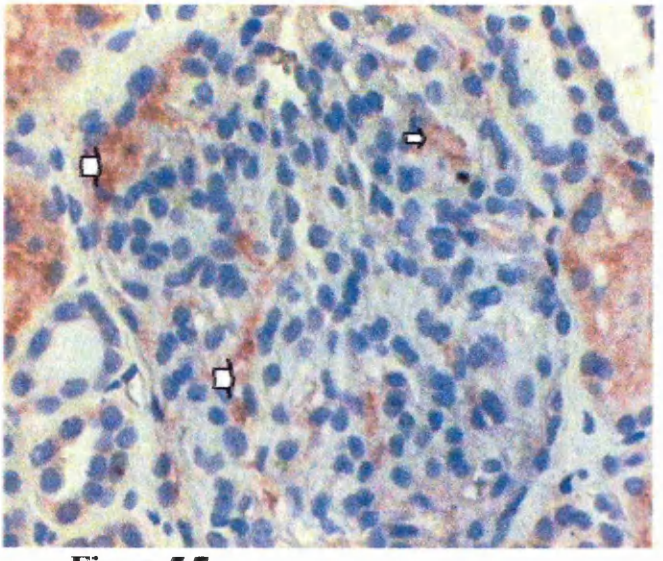


Figure 5.7.

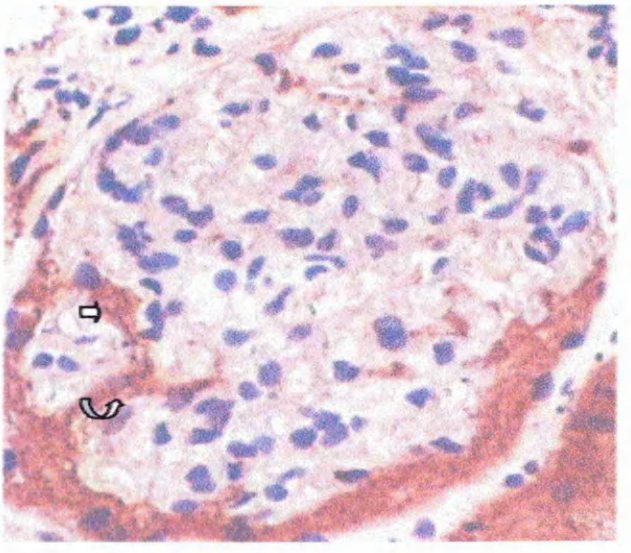


Figure 5.8.

Diseased glomerulus stained with TGF-β1 (arrows) (Magnification X 400).

5.5.1 Relationship of glomerular (G) immunostainable TGF-β1 and the clinical parameters

There was no significant difference in glomerular immunostaining of TGF-β1 (gTGF-β1) between the progressors (gTGF-β1 = 2.2%) (mean±SEM) and non-progressors (gTGF-β1=4.8%) (mean±SEM) CRF (Table 5.10). Also, there was no significant correlation with serum creatinine, proteinuria levels, blood pressure or the 1/serum creatinine slope (Table 5.9). Furthermore, there was no significant difference in glomerular TGF-β1 immunostaining between different pathological categories.

Table 5.9. Immunostainable gTGFβ1 and clinical CRF parameters (mean ±SEM)

CRF parameters	*gTGF-β1 + (n=9)*	*gTGF-β1 – (n=11)*	*P(f)*	*P(t)*
Proteinuria at diagnosis (g/24hr)	4.94±1.6	5.15±1.7	0.9	0.8
Mean of proteinuria (g/24hr)	3.7±1.2	5.8±1.8	0.2	0.34
SBPd (mmHg)	149.6±9	149.7±5	0.06	0.3
DBPd (mmHg)	88±6.5	84.2±5	0.2	0.67
SBPf (mmHg)	142.8±6	147.3±5	0.4	0.5
DBPf (mmHg)	89.5±5	81.7±4	0.24	0.2
Plasma TGF-β1 (pg/ml)	17474±3804	17778±3258	0.4	0.2

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow-up, DBPf: diastolic blood pressure during the follow-up; gTGF-β1+: patients whose glomeruli stained with TGF-β1; gTGF-β1-: patients whose glomeruli did not stained for TGF-β1.

Table 5.10. Immunostainable gTGF-β1 and progression of CRF

	gTGF-β1-	gTGF-β1+	*Total*	χ^2 *Yates' correction (at one degree of freedom)*	*P value*
CRF Progressors	6	9	15	2.73	0.098
CRF Non-progressors	6	2	8		
Total	12	11	23		

Abbreviations: gTGF-β1+: patients whose glomeruli stained with TGF-β1; gTGF-β1-: patients whose glomeruli did not stained for TGF-β1.

5.5.1.7 TGF-β1 polymorphisms

There was no significant difference between the mean of glomerular TGF-β1 and the different studied polymorphisms (Table 5.11). On the other hand, there were significant differences in variance.

Table 5.11. Glomerular TGF-β1 and the studied polymorphisms

	n =	Mean±SEM	P(f)	P(t)
C-509T				
TT	12	3 ± 1.3%	0.0017*	0.19
TC+CC	9	1.4 ± 0.5%		
Lue10Pro				
TT	8	3.7± 1.9%	0.015*	0.2
TC+CC	12	1.5 ± 0.75%		
Arg25Pro				
GG	15	3.1 ± 1.1%	0.007*	0.13
CG	7	1.2 ± 0.6%		
Thr263Ile				
TT	23	2.9±1.3%		
TC	0	0		

5.5.1 Tubular (T) TGF-β1

Photomicrographs of Immunostaining (red) of TGF-β1 in normal (Figure 5.9) and diseased tubules (Figures 5.10&5.11)

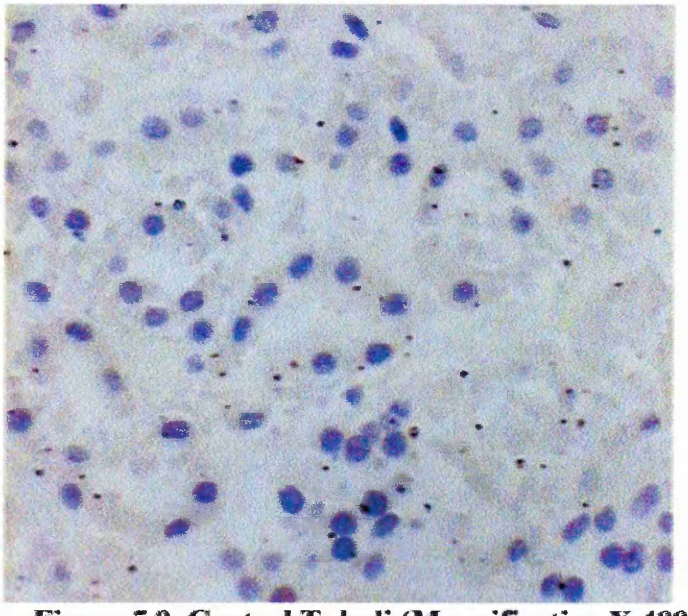


Figure 5.9. Control Tubuli (Magnification X 400)

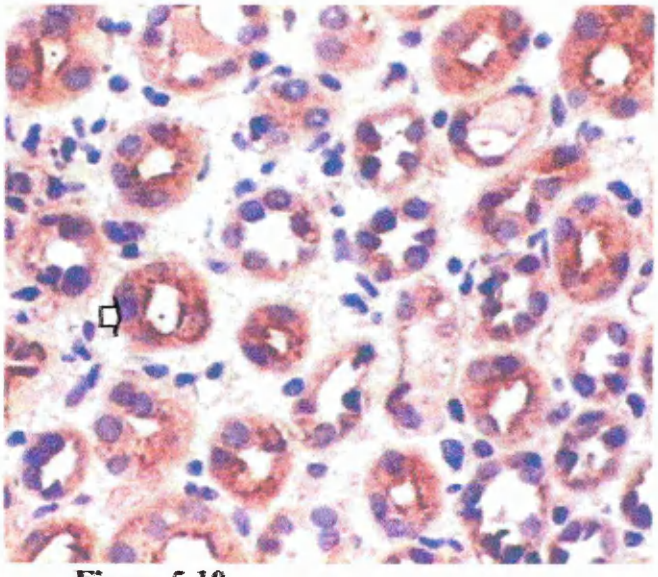


Figure 5.10.
immunostaining TGF-βI (arrow) in tubules (Magnification X 200)

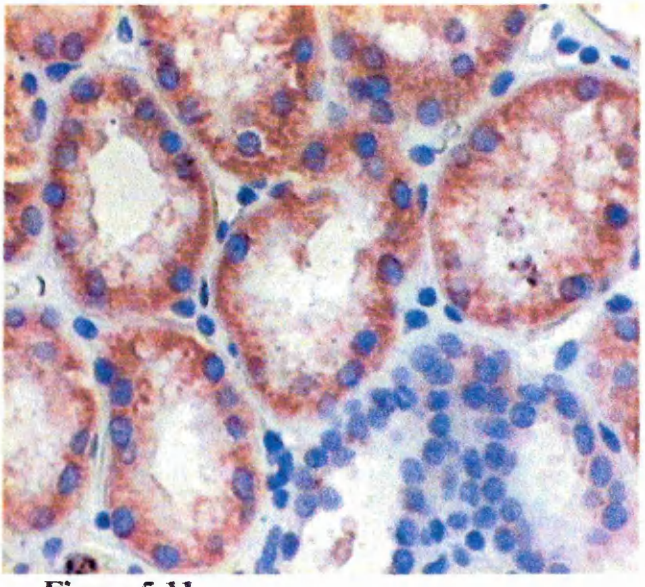


Figure 5.11.

5.5.2.2 Proteinuria

There was no significant difference between tubular TGF-β1 in patients with different proteinuria levels (Table 5.12). There was also no significance difference with the other CRF parameters (Table 5.12).

Table 5.12. Levels of tubular immunostainable TGF-β1 according to the severity of different clinical parameters

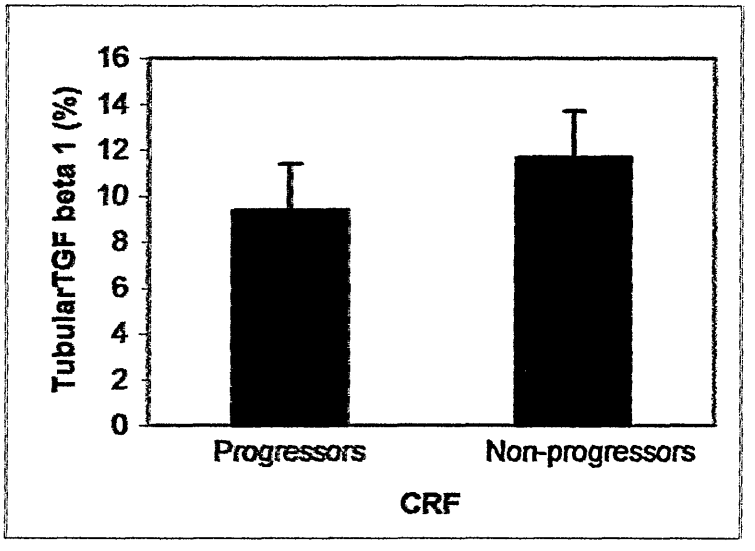
CRF parameters	*n=*	*Tubular TGF-β1 (Mean±SEM)*	*P(f)*	*P(t)*
Proteinuria at diagnosis (g/24hr)				
<3g/24hr	8	10.4±2.2%	0.2	0.9
>3g/24hr	13	10.6±2.3%		
Proteinuria during follow-up (g/24hr)				
<3g/24hr	11	9.3±2%	0.4	0.5
>3g/24hr	10	11.7±2%		
SBPd (mmHg)				
<140mmHg	7	7.9±2.3%	0.37	0.5
>140mmHg	13	10.7±2%		
DBPd (mmHg)				
<90mmHg	9	8.8±2.2%	0.3	0.6
>90mmHg	11	10.5±2.5%		
SBPf (mmHg)				
>140mmHg	7	8.5±2.2%	0.3	0.46
<140mmHg	13	10.9±2%		
DBPf (mmHg)				
<90mmHg	10	10.6±2.2%	0.4	0.5
>90mmHg	10	9.2±2%		

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during the follow up.

5.5.2.6 Progression of CRF

There was no significantly difference in tubular immunostainable TGF-β1 (tTGF-β1) (mean±SEM) between the progressors (tTGF-β1 = 9.4%) and non-progressors (tTGF-β1 = 11.7%) patients groups (Figure 5.12).

Figure 5.12. Tubular TGF-β1(tTGF-β1) (mean ± SEM) immunostaining and the progression of CRF



5.5.2.5 TGF-β1 polymorphisms

There was no significant difference between tubular TGF-β1 and both Arg25Pro and Lue10Pro. On the other hand, there was a statistical significant difference between tubular TGF-β1 and C-509T (P = 0.001) (Table 5.13).

Table 5.13. TGF-β1 polymorphisms according to tubular TGF-β1

	n =	Mean ± SEM	P(f)	P(t)
C-509T				
TT	12	14.3 ± 1.6%	0.5	0.001*
TC+CC	9	5.3 ± 1.7%		
Leu10Pro				
TT	8	8.7 ± 2.9%	0.3	0.5
TC+CC	12	10.6 ± 1.8%		
Arg25Pro				
GG	15	7.8 ± 1.1%	0.3	0.25
CG	7	10.8 ± 0.6%		
Thr263Ile				
TT	23	2.9±1.3%		
TC	0	0		

5.5.3 Renal Inflammatory cellular infiltration (ICI)

The patients (n = 23) with ICI were divided into those with (n = 13) and those without (n = 10) moderate/severe ICI.

Photomicrographs of the diseased kidneys showing the inflammatory cellular infiltration (ICI)

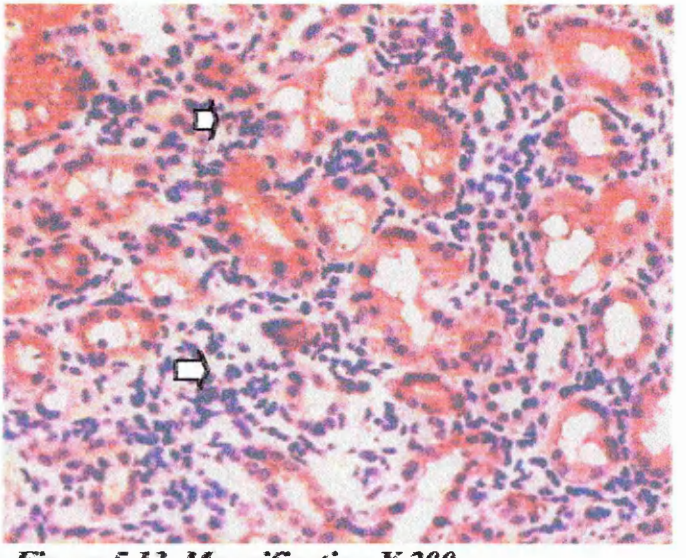


Figure 5.13. Magnification X 200

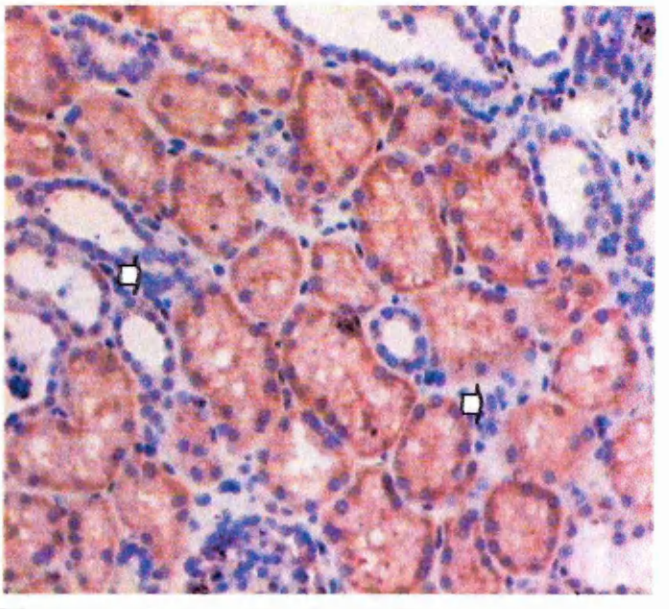


Figure 5.14. Magnification X 400

There was no difference in the clinical parameters of patients with or without ICI (Table 5.14).

Table 5.14. The associations of inflammatory cellular infiltration and the clinical parameters

Parameters	*ICI- (n = 9)*	*ICI+ (n = 11)*	*P(f)*	*P(t)*
Proteinuria at diagnosis (g/24hr)	3.58±1.4	5.95±1.3	0.5	0.3
Proteinuria during follow up (g/24hr)	4.1±1.4	5±1.3	0.6	0.7
SBPd (mmHg)	147.5±2.4	150±5	0.1	0.8
DBPd (mmHg)	92±2.5	84±5	0.01*	0.2
SBPf (mmHg)	146±5	145±4	0.4	0.9
DBPf (mmHg)	83±3.2	86±4	0.1	0.8

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during the follow up, ICI-: without inflammatory cellular infiltration; ICI+: with inflammatory cellular infiltration.

There was a significant difference in TGF-β1 immunostained tubules in sections with inflammatory cellular infiltration (ICI+) compared to those without inflammatory cellular infiltration (ICI-) (Table 5.15).

Table 5.15. The associations between inflammatory cellular infiltration and TGF-β1

	ICI-	***ICI+***	***P(f)***	***P(t)***
Plasma TGF-β1 (pg/ml)	18551±4603 $n = 8$	17035±1704 $n = 12$	0.17	0.7
gTGF-β1 (%)	2.3±1 ($n = 9$)	2.6±0.5 ($n = 14$)	**0.03***	0.8
tTGF-β1 (%)	5.6±1.1 ($n = 9$)	13.7±0.8 ($n = 14$)	0.5	**0.0048***

Abbreviations: ICI-: without inflammatory cellular infiltration; ICI+: with inflammatory cellular infiltration. gTGF-β: glomeruli TGF-β1; tTGF-β1: tubular TGF-β1.

*=significance <0.05.

5.5.3.6 TGF-β1 polymorphisms

There was a higher ICI in homozygous patients for the T allele at the promoter site (C-509T) (TT) 44.6 ± 7cells/field) compared to the heterozygous (TC) plus the homozygous for the C allele (CC) 16.3 ± 8.5cells/field) ($p = 0.01$). On the other hand, there was no significance difference ($p > 0.05$) between ICI and genotype either at Arg25Pro (GG 37.1± 6.7cells/field, CG .17.5 ± 11 cells/field) or at Leu10Pro (TT 34.1 ± 9 cells/field, CC + TC 27.7 ± 8.7 cells/field) (Table 5.16).

Table 5.16. The distribution of the different polymorphisms according to whether there is inflammatory cellular infiltration or not

Genotyping	ICI+	ICI-	Chi square at two degree of freedom (Yate's correction)	P value
C-509T				
TT	2	10	5.6	0.018*
TC+CC	6	3		
Leu10Pro				
TT	2	6	0.97	0.3
TC+CC	4	5		
Arg25Pro				
GG	4	12	3.28	0.07
CG	4	2		

Abbreviations: ICI+: with inflammatory cellular infiltration, ICI-: without inflammatory cellular infiltration

5.6.1 Correlations between immunostainable TGF-β1 and different parameters

There was no significant correlation between glomerular TGF-β1 and renal ICI. Of note when I divided the patients to those who had inflammatory cell infiltration (ICI) and those who did not, there was a significantly higher tubular TGF-β1 in the former (13.2 ± 1.7%) compared to the latter (5.6 ± 2%) ($p = 0.0047$). There was also a significant correlation between tubular iTGF-β1 and renal ICI ($r = 0.447$, $p = 0.03$) (Table 5.18). Furthermore it was noted that there was a significant correlation between the tubular TGF-β1 and proteinuria during the follow up (Table 5.18). Although there was no significant correlations between the ICI and the severity of tubulointerstitial fibrosis, we noted that there was a significant correlation between the ICI and the severity of glomerulosclerosis (Table 5.19).

Table 5.17. Correlations between glomerular TGF-β1 (gTGF-β1) and different CRF parameters

gTGF-β1 with	n	r	R^2	P(r)
Proteinuria at diagnosis (g/24hr)	20	0.04	0.002	0.8
Proteinuria during follow up (g/24hr)	20	0.2	0.04	0.4
SBPd (mmHg)	20	0.08	0.006	0.8
DBPd (mmHg)	20	0.226	0.05	0.4
SBPf (mmHg)	20	0.2	0.04	0.4
DBPf (mmHg)	20	0.35	0.119	0.2
Plasma TGF-β1 (pg/ml)	20	0.223	0.05	0.4
GS	22	0.002	0.000	0.9
IF	22	0.008	0.000	0.9

Abbreviations: gTGF-β1: glomerular TGF-β1; SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during follow up, GS: glomerulosclerosis, IF: interstitial fibrosis.

Table 5.18. Correlations between tubular TGF-β1 (tTGF-β1) and different CRF parameters

tTGF-β1 with	**n =**	**r**	**R^2**	**P(r)**
Proteinuria at diagnosis (g/24hr)	20	0.25	0.06	0.7
Proteinuria during follow up (g/24hr)	20	0.47	0.223	**0.048***
SBPd (mmHg)	20	0.04	0.0015	0.17
DBPd (mmHg)	20	0.17	0.03	0.5
SBPf (mmHg)	20	0.046	0.002	0.85
DBPf (mmHg)	20	0.064	0.004	0.8
Plasma TGF-β1 (pg/ml)	20	0.025	0.06	0.29
GS	22	0.16	0.26	0.49
IF	22	0.025	0.000	0.9

Abbreviations: tTGF-β1: tubular TGF-β1 SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during follow up, GS: glomerulosclerosis, IF: tubulointerstitial fibrosis.

*= significance < 0.05.

Table 5.19. Correlations between inflammatory cellular infiltration (ICI) (cell/field) and different CRF parameters

ICI with	n =	r	R^2	P(r)
Proteinuria at diagnosis (g/24hr)	20	0.158	0.025	0.5
Proteinuria during follow up (g/24hr)	20	0.14	0.019	0.6
SBPd (mmHg)	20	0.06	0.003	0.8
DBPd (mmHg)	20	0.25	0.06	0.36
SBPf (mmHg)	20	0.103	0.11	0.7
DBPf (mmHg)	20	0.15	0.021	0.5
Plasma TGF-β1 (pg/24hr)	20	0.002	0.000	0.99
gTGF-β1 (%)	23	0.02	0.0003	0.9
tTGF-β1 (%)	23	0.404	0.164	0.06
GS	22	0.46	0.21	0.037*
IF	22	0.025	0.0006	0.9

Abbreviations: SBPd: systolic blood pressure at diagnosis, DBPd: diastolic blood pressure at diagnosis, SBPf: systolic blood pressure during follow up, DBPf: diastolic blood pressure during follow up, G.S: glomerulosclerosis, IF: interstitial fibrosis. gTGF-β1: glomerular TGF-β1; tTGF-β1: Tubular TGF-β1.

*= significance < 0.05.

5.5 Discussion

In this chapter I have measured the circulating and tissues levels of TGF-β1.

Firstly, I have found that there was no significant difference between plasma TGF-β1 protein levels and any of the clinical parameters (proteinuria, progression of CRF, blood pressure and pathological processes). On the other hand, plasma TGF-β1 protein levels were significantly higher in patients homozygous for the G allele at Arg25Pro compared to heterozygotes and homozygotes for the C allele. This finding is in agreement with the data of Li and co-workers (1999) who found that there was a significant difference between high TGF-β1 plasma protein levels and systolic blood pressure in G-homozygotes allele at Arg25Pro. Of relevance, high circulating TGF-β1 levels are associated with atherogenesis both in mice (Grainger, *et al.*, 1994) and humans (Grainger, *et al.*, 1999). There is a significant difference between the C allele and increased risk of myocardial infarction, although the C allele was associated with lower diastolic blood pressure (Langdahl, *et al.*, 1997). These contradictions make the role of TGF-β1 in hypertension controversial and unsettled. Futhermore, there was a significant correlation between the presence of the T allele at –509 promoter and high levels of plasma TGF-β1 (Grainger, *et al.*, 1999). This may be explained by the observations made in this thesis (Chapter 4) of a linkage disequilibrium between the T allele at –509 promoter and the G allele at codon 25. On the other hand, there was no significant difference between plasma TGF-β1 protein levels and the other studied polymorphisms.

There were no significant difference between serum TGF-β1 protein levels and clinical parameters. Patients with progressive CRF had lower serum TGF β1 levels compared to non-progressors (NP). Although this finding was at first surprising, many explanations could account for it. While TGF-β1 is thought to be a fibrogenic growth factor, a growing body of evidence suggests that this growth factor has anti-inflammatory and anti-proliferative functions (Park, *et al.*, 2000; Cheng, *et al.*, 2001). The progression of chronic renal failure is associated with a brisk inflammatory interstitial response. Low TGF-β1 may, therefore, facilitate such interstitial inflammation which is known to lead to fibrosis. Also, glomerular, tubular as well as interstitial cell proliferation may go

uninhibited in the absence of TGF-β1. Low TGF-β1 have been recently reported to be associated with atherosclerosis (Grainger, *et al.*, 1995). Glomerular and interstitial fibrosis pathways are similar to those of atherosclerosis, involving inflammation, proliferation and fibrosis. In atherosclerosis it has been argued that TGF-β1 initially inhibits the proliferation of smooth muscle cells, but later on in the process may encourage the production of ECM (Grainger, *et al.*, 1995). A similar explanation can be advanced for the progression of renal scarring. Other studies have reported that high circulating TGF-β1 levels were associated with higher blood pressure (Grainger, *et al.*, 1999). I failed to observe such an association. This may be due to two factors, firstly, most patients in the present study have secondary rather than primary hypertension. Further, the majority of our patients with secondary hypertension were treated with antihypertensive drugs with adequate blood pressure control, making any difference of genotype with hypertension difficult to ascertain.

Plasma TGF-β1 levels were significantly higher in patients with primary and secondary glomerulonephritis compared to the other groups. This is of interest, since TGF-β1 has been implicated in the injury and repair of experimental and clinical glomerulonephritis. Furthermore, there are reports of elevated urinary TGF-β1 in membranous glomerulonephritis (Hellmich, *et al.*, 2000). Also, reports of high circulating levels of TGF-β1 have been published in systemic vasculitis including ANCA-associated vasculitis (AAV) (Kekow, *et al.*, 1997). In SLE, however active disease was associated with decreased circulating TGF-β1 levels (Robak, *et al.*, 2001).

There was a close association between levels of immunostainable tubular TGF-β1 and proteinuria. This might due to the fact that proteinuria can be considered a marker of renal damage. Also, a link between proteinuria and the progression of renal scarring has been suggested (For review, Harris, 2000). Incubation of proximal tubular epithelial cells with high levels of proteins (albumin, transferin) is capable of stimulating the induction of TGF-β1 by these cells (For review, Harris, 2000). This thesis is the first report linking proteinuria to elevated tubular TGF-β1 in human nephropathies.

On the other hand, there was no association between immunostainable tubular TGF-β1 and plasma TGF-β1 levels or blood pressure levels. Overall, there were no significant associations between any of the studied polymorphisms and either glomerular or tubular TGF-β1 immunostaining. This might be attributable to the small number of available sections, as there was a trend (albeit not significant) between glomerular and tubular TGF-β1 immunostaining levels and the presence of the G allele at Arg25Pro and T allele at C-509T.

Homozygosity for the G allele (GG) at Arg25Pro was found to have a weak but significant association with severity of renal inflammatory cellular infiltration. Also, there was also a significantly higher renal inflammatory cellular infiltration in patients homozygous for the T (TT) allele at C-509T compared to heterozygotes (TC) or C-homozygotes (CC). The severity of interstitial inflammation in chronic renal diseases is a major prognostic factor (For review, Jernigan and Eddy, 2000). A genetic association or predisposition to inflammatory renal changes would be a major detrimental influence on the progression of renal disease. As discussed in Chapter 4, the number of patients with progressive CRF was significantly higher in the patients homozygous for the G allele. The predisposition to interstitial inflammation may explain this association. Surprisingly, this polymorphism was not associated with more severe interstitial fibrosis. Similarly, an association has been described in Chapter 4 between the presence T allele on the C-509T and the progression of progressive CRF and, as mentioned above interstitial inflammation. Here again, this allele was not directly associated with interstitial fibrosis.

Furthermore, there was a strong association between the severity of renal inflammatory cell infiltration and tubular TGF-β1 levels. This would suggest interactions between tubular cells and cellular infiltration involving TGF-β1. Transforming growth factor-β1 may be increased in tubular cells through increased production or increased uptake. Tubular cells have receptors for TGF-β1. It may be that the TGF-β1 released by inflammatory cells is taken up by the adjacent tubular cells. On the other hand, it could be that tubular cells release TGF-β1 in response to peritubular interstitial inflammation.

TGF-β1 production in this context would be the natural response of the kidney to interstitial inflammation.

In conclusion, the measurements of TGF-β1 in the circulation and renal tissue have identified potential links with interstitial inflammation as well as proteinuria. Whether TGF-β1 is pathogenic or a reaction to these factors is difficult to ascertain from this work.

Discussion

There is a large number of studies published on the association of TGF-B1 polymorphisms and a variety of renal diseases. For example, there is an association between increased frequency of the C allele at Leu10Pro and development of renal failure in patients who have undergone heart transplantation (Baan, *et al.*, 2000), the T allele at Thr263Ile and the development of nephropathy in diabetic patients (Pociot, *et al.*, 1998), and the G allele at Arg 25Pro and increased blood pressure in ESRF (Li, *et al.*, 1999).

Most of the studies are case control and population-based rather than pedigree-based analysis of families. This is because genetic analysis in case control studies can be used directly to identify functional SNPs contributing to a particular phenotype. Furthermore, population screening might conceivably be useful in identification of the minority of people whose environment or lifestyle could be changed. On the other hand, family-based studies (case-sibling and case-parents design) have a reduced statistical efficiency due to overmatching and reduced sample size on the studied genotype.

In this thesis, genotypic polymorphisms of the TGF-B1 gene were analysed in relation to susceptibility and progression of CRF in Caucasian patients with renal diseases. I noted that carriers of the T allele at C-509T and the C allele at Leu10Pro were significantly more common in CRF patients (particularly those with progressive disease) than in the control population. The implication of this is that carriers of these alleles are more susceptible to chronic renal diseases (CRD). This observation is original to the best of my knowledge.

Furthermore, I analysed the frequency of progressive renal insufficiency amongst different genotypes and noted that G-homozygotes (Arg25Pro) and T-homozygotes (C-509T) were significantly more common among patients with progressive renal disease and in those who reached ESRF when compared to those with non-progressive disease and stable renal function. No such difference was noted for the other studied genotypes including Leu10Pro and Thr263Ile. Again, this is, to the best of my knowledge, the first observation of such associations in renal diseases. I have therefore identified both

susceptibility markers (-509T and 10Pro) for the development of CRD and progression markers (-509T and 25Arg) that identify patients who have progressive CRF and those who reached ESRF. This study indicated that there is increase risk of carriers of the C allele (Leu10Pro) developing CRD. Other investigators reported that there was a significant association between the T allele (Leu10Pro) and the development of CRF in heart-transplanted patients (Baan, *et al.*, 2000) as well as in those with end-stage heart failure due to cardiomyopathy (Holweg, *et al.*, 2001).

The deleterious effect of the -509T allele on the progression of CRF might be due to its direct effect or indirectly through many factors involved in progressive renal scarring.

Firstly, proteinuria was higher in the T-homozygotes at C-509T than the C-homozygotes. Also, proteinuria was higher in the progressors compared to the non-progressors. It is now well known that proteinuria levels are significantly higher in patients with progressive CRF compared to those with stable renal function (For review, Locatelli and Del Vecchio, 2000). In support of that observation, I have observed that proteinuria levels in the patients I studied correlated with the rate of decline in renal function as measured by the reciprocal of serum creatinine against time slope. Proteinuria is now thought to be a cause as well as the consequence of progressive renal damage (For review see Harris, 2000). Proteinuria induces mesangial cells proliferation, infiltration and activation of macrophages, deposition of ECM and, consequently, glomerulosclerosis (For review see Harris, 2000). Glomerular damage leads to the appearance of protein in the glomerular ultrafiltrate and its subsequent uptake by the tubular epithelium. This leads to release of pro-inflammatory cytokines and chemokines, chemo-attraction of inflammatory cells and stimulation of ECM protein production (For review see Harris, 2000). Of relevance, I confirmed that there is a significant correlation between proteinuria and renal inflammatory cellular infiltration in patients with a variety of causes of CRF. I found that proteinuria was significantly higher in males than females. However, there was no significant association between a particular allele at the studied polymorphisms and different gender. This indicates that gender is not a confounding in the allele association with proteinuria.

Secondly, renal inflammatory cellular infiltration was significantly associated with the T-allele at C-509T. The severity of the interstitial inflammatory infiltrate has been shown to correlate with the rate of decline in renal function both in experimental and in human nephropathies. Inflammatory cellular infiltration plays an important role in renal fibrosis. This is because the inflammatory cells are a source of fibrosis-promoting growth factors, vasoactive molecules and ECM proteins (Nathan, 1991). Circulating monocytes migrate into the renal interstitium when inflammation is present. The chemotactic factors that involve monocytes recruitment are becoming better known (For review, Jernigan and Eddy, 2000). Many cytokines and chemokines have been implicated. These include interleukins, tumour necrosis factor-α, monocyte chemo-attractant protein 1 (MCP-1), macrophage inhibitory factor-2 (MIF-2) as well as RANTES. Many of these factors have been shown to be up-regulated in scarred and inflamed kidneys. They have also been shown to be released by proximal tubular epithelial cells when activated by injury of proteinuria (For review, Jernigan and Eddy, 2000).

Thirdly, there was a significant increase in immunostainable tubular TGF-β1 in patients who were homozygous for the T allele. Increased expression of TGF-β1 has been associated with progressive renal fibrosis in experimental and human nephropathies (Border and Noble, 1997). TGF-β1 is a fibrogenic molecule and it has a role in the potentiation of interstitial monocyte recruitment. Of relevance, I found that there is a significant correlation between tubulo-interstitial inflammatory cellular infiltration and tubular immunostainable transforming growth factor-β1. TGF-β1 also stimulates the synthesis of ECM proteins and decreases matrix degradation (Border and Noble, 1994). The sources of TGF-β1 are infiltrating monocytes, fibroblasts and myofibroblasts (Yamamoto, *et al.*, 1994; Pankewycz, *et al.*, 1996). In addition, proximal tubular epithelial cells are capable of the synthesis and release of TGF-β1. In my study, I noticed the presence of immunostainable TGF-β1 predominantly in the cytoplasm of tubular epithelial cells. Some TGF-β1 was also present in the peritubular interstitium. TGF-β1 also stimulates cells at the sites of tissue injury resulting in increase of its own production

(Wei, *et al.*, 1998). This leads to a vicious cycle of TGF-β1 production, inflammation and fibrosis.

The G allele (Arg25Pro) is associated with increased blood pressure in patients with ESRF (Grainger, *et al.*, 1999). Similar observations were made in cardiac allograft vasculopathy (Densem, *et al.*, 1999), fibrotic lung disease (El-Gamel, *et al.*, 1998) and progressive cystic fibrosis (Arkwright, *et al.*, 2001).

The deleterious effect of the G-allele (Arg25Pro) on the progression of CRF might be due to its influence of some of the risk factors associated with progressive renal insufficiency. Firstly, proteinuria was higher in the G-homozygotes at Arg25Pro than the C-homozygotes or heterozygotes. The effect of proteinuria was discussed above. Further, I noted that proteinuria was correlated with diastolic blood pressure at the time of diagnosis. Again as discussed earlier, both blood pressure and proteinuria affect renal function.

In patients with hypertensive nephrosclerosis, carriage of the G-allele was significantly more common in the progressors than non-progressors. I failed to find a significant association between carriage of the G allele and increased blood pressure. This might be due to the fact that patients were treated with anti-hypertensive agents and had a fairly uniform and well-controlled blood pressure during the follow-up period. Elevation in systolic blood pressure is a strong predictor of ESRF (Klag, *et al.*, 1996). Also, I noted that systolic blood pressure during follow-up was significantly higher in progressors than non-progressors. Also elevation in systolic blood pressure accelerates the rate of loss of renal function (Brazy, *et al.*, 1989). In systemic hypertension associated with the loss of renal function, the decrease in resistance of the afferent glomerular arteriole allows the transmission of the elevated blood pressure to the glomerular capillaries (For review see Dworkin and Weir, 2000). Therefore, deficient arteriolar auto-regulation renders damaged kidneys particularly sensitive to systemic hypertension. Intraglomerular hypertension promotes proteinuria, which further activates the renin-angiotensin system (RAS). Angiotensin II, apart from its vasoconstrictor effects, induces local pro-

inflammatory and pro-fibrotic signaling molecules resulting in renal scarring (Soergel and Schaefer, 2002).

Homozygosity for the G allele was significantly more frequent in patients with severe glomerulosclerosis. The other two risk factors associated with the G allele (proteinuria and hypertension) lead to glomerulosclerosis as described earlier in this chapter. I also noted a significant correlation between inflammatory cellular infiltration and glomerulosclerosis.

Increased plasma levels of TGF-β1 was associated with the presence of the G allele. This is in agreement with the observation of Li and co-workers (1999) who found that circulating TGF-β1 levels were significantly higher in G-homozygotes with ESRF. Grainger and co-workers (1999) found an association between T-homozygotes at C-509T and increased TGF-β1 levels in the normal population. Circulating TGF-β1 levels are higher in African-Americans with ESRF compared to Caucasians (Suthanthiran, *et al.*, 2000). Surprisingly, in this study I noted that there are significantly lower levels of serum TGF-β1 in progressors compared to non-progressors. The progressor group in this study included patients with ESRF on renal replacement therapy. Stefoni and co-workers (2002) found that TGF-β1 was significantly reduced in haemodialysis patients particularly in those with severe cardiovascular disease. The same authors concluded that low TGF-β1 serum levels are a risk factor for atherosclerotic disease in ESRF patients. I found that plasma TGF-β1 levels were significantly higher in patients with vasculitic cause of CRF compared to other causes of CRF. This is the first time such an association has been described and may be attributable to the release of TGF-B1 from inflamed vascular endothelium.

The frequency of the rarer, T-allele at Thr263Ile was very low to be analysed as a marker for CRF in this sample group. The allele frequencies of this polymorphism were similar to the published frequencies in the normal population. However, one study has reported a significant association between the T allele at this site and the development of diabetic

nephropathy (Pociot, *et al.*, 1998). In this study, the number of the studied diabetic patients was too small for meaningful analysis with this marker.

Finally, I found that the deleterious alleles (T at C-509T, C at Leu10Pro and G at Arg25Pro) were in linkage disequilibrium. This reflects the fact that these polymorphic regions located in proximity to each other within the TGF-B1 gene on the long arm of chromosome 19. Of interest, this region of chromosome 19 has been linked to numerous genetic abnormalities related to the development of proteinuria and consequently glomerulosclerosis. For instance, mutations in the NPHS1 gene (coding for nephrin) as well as in the gene coding for β-actinin4 are located on the long arm of chromosome 19 and have been associated with steroid resistant forms of glomerulosclerosis (Patrakka, *et al.*, 2002).

Genetic associations may either result from a direct functional effect of the sequence variation, or may simply reflect linkage disequilibrium in the region with the tested alleles acting as markers for a nearby functional variation. In the case of C-509T and coding dequence variations, there is reason to suspect a possible direct functional effect. The polymorphism at the promoter site may disrupt a consensus half-site for the binding of the nuclear factor cAMP response element binding protein (CREB). The -509T may be involved in the modulation of expression of the TGF-β1 gene (Syrris, *et al.*, 1998).

The signal sequence of which functions to translocate the synthesized TGF-β1 protein across the membrane of the endoplasmic reticulum consists of three regions: a positively charged NH2-terminal region, a central hydrophobic core and a polar-terminal region (Randall and Hardy, 1989). The Leu10Pro (amino acid 10 polymorphism) is located in the hydrophobic core. Both leucine and proline are non-polar and polymorphism at this site may not significantly affect the function of the single peptide (Cambien, *et al.*, 1996 and Syrris, *et al.*, 1998). Conversely, Yamada (1998) postulated that the 10Pro amino acid increased export of TGF-β protein in extra-cellular matrix.

In the case of the Arg25Pro polymorphism, arginine is a bipolar amino acid replaces the proline, which is small apolar amino acid. This replacement is reported to affect the export and post-translational modification of the pro-TGF-β protein (Densem, *et al.*, 2000). The Thr263Ile polymorphism is located in the part of TGF-β proprotein that is cleaved from the active part at amino acid 278 (Langdahl, *et al.*, 1997). The substitution of threonine which has one hydroxyl group by isoleucine which is non-polar, could affect the stability of latent TGF-β1 and/or the activation of TGF-β1 (Pociot, *et al.*, 1998).

In conclusion, this study has revealed a number of associations between TGF-B1-gene polymorphisms and susceptibility to as well as progression of CRF. More specifically, higher rate of carriage of the T (C-509T) alleles in patients with CRD compared to the normal population. Also, carriage of the C allele at Leu10Pro appeared to be a susceptibility marker. On the other hand, homozygosity for both the G allele at Arg25Pro and the T allele at C-509T were found to be risk factors for a faster rate of progression of CRF. These associations may be related to changes in proteinuria, elevated circulating TGF-β1 levels and glomerulosclerosis in those with the high-risk genotypes. On the other hand, the T allele at C-509T appears to be associated with proteinuria, tubular TGF-β1 over-expression and interstitial inflammation.

The clinical implication of these findings could be that genotyping of CRF patients for the TGF-β1 polymorphisms at C-509T and Arg25Pro may be indicated, in order to identify those at risk of progressive CRF and, ultimately, ESRF. Patients with high risk genotypes would consequently warrant additional attention to prevent the progression of their underlying kidney disease.

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A.I. Consent form

After approval of this study by Ethical committee from the South Sheffield Ethics Committee, Northern General Hospital, all the patients gave informed consent, which included:

Ethics No:

Brief Title:

Prosper:

Patient consent form

The study's title is the effect of the growth factors polymorphisms on the progression of the chronic renal impairment.

To be completed by the patient:

Have you read the information sheet about this study?	YES/NO
Have you been able to ask questions about this study?	YES/NO
Have you received answer to your entire question?	YES/NO
Have you received enough information about this study?	YES/NO
Do you understand that you are free to withdraw from this study at any time	
Without diving the reason for withdrawing?	YES/NO
Without affecting your future medical care?	YES/NO
Do you agree to take part in this study?	YES/NO

Signed

Name (Block Letters)

A.II. Clinical data form

Patient's name:

Hospital No.	Research No.	
Age:	Gender: M F	
Weight:	Height:	BMI:
Race:	Smoking:	Diet:

Diagnosis:

.....

.....

Other conditions:

.....

.....

Family history:

Renal biopsy:	Date:	Results:
Blood pressure	At onset:	Mean blood pressure:
Serum Creatinine	Serial reading:	Time:
	.....	.....
	.....	.....
	.....	.....
	.....	.....
	.....	.....

Slope 1/Creatinine:	Creatinine Clearance:	
Proteinuria	At onset:	Mean of proteinuria:
Hematuria	Yes:	No:

Serum Cholesterol:

Serum Triglyseride:

Treatment:

.....

.....

.....

A.III. Buffers for DNA extraction:

1-Buffer A:

For 1 litre of buffer:

Chemical	*Final concentration*	*Amount*
Tris Base (Sigma)	0.01M	1.2 g
Sucrose (Sigma)	0.32M	108.5 g
$MgCl_2$ (Sigma)	0.15M	1g
Distilled water		to 990ml

The pH was adjusted to 8.0 by using hydraulic acid.

The buffer was autoclaved and 10ml of Triton X 100 (Sigma) was added.

2-Buffer B:

For 1 litre of buffer:

Chemical	*Final concentration*	*Amount*
Tris Base (Sigma)	0.4M	48.44g
EDTA (Sigma)	0.06M	22.33g
$NaCl_2$ (BDH)	0.150M	8.766g
Distilled H_2O		to 900ml

The pH was adjusted to 8.0 in a similar manner to buffer A.

The buffer was autoclaved. SDS was added to give a final concentration of 1%.

3- Sodium perchlorate (Sigma) at final concentration of 5M.

A.IV. Equipment and reagent supplier

Bio-rad

Bio-Rad laboratories, ltd.

Bio-Rad House

Mayland Ave

Hemel Hempstead

Hertfordshire, HP2 7TD (GB)

Bioline

16 The Edge Business Centre

Humber Road

London NW2 6YP

Gibco BRL,

Life Technologies Ltd,

3 Fountain Drive,

Inchinnan Business Park,

Paisley, PA4 9RF.

Perkin Elmer

Parkin Elmer Life Sciences

BRU/BRU/40349

Hounslow, TW5 9RT

Promega UK,

Enterprise Road

Chillworth Research Center,

Southampton,

DO16 7NS.

R&D Systems,

R&D System Europe, Ltd.

4-10 The Quadrant,

Barton Lane,

Abingdon,

Oxon, OX14 3YS.

Sigma,

Sigma Aldrich Company Ltd.

Fancy Road,

Poole,

Dorest, BH12 4QH

SORVALL

SORVALL (UK) ltd,

International Centre

Boulton Road, Stevenage,

Hertfordshire SG1 4QX

Wallace,

Wallac Oy,

P.O. Box 10,

FIN-20101,

Turku,

Finland

A.VI. Clinical data of CRF patients

Entrant ID	Gender	SBPd	SBPf	DBPd	DBPf
1-	M				
2-	F	170	155	100	85
3-	M	170	169	110	100
4-	F	120	120	80	75
5-	M	155	152.5	100	92
6-	M	110	110	70	72
7-	M	175	160	85	83
8-	F				
9-	M	120	119	80	60
10-	M	150	158.3	80	78
11-	M	120	118	70	71
12-	M				
13-	F				
14-	M	160	155	82	81
15-	M	130	125	90	76
16-	F	120	125	60	62.5
17-	M	170	164	90	78
18-	M	100	115	50	70
19-	M	178	166	82	70
20-	M	210	200	120	105
21-	M	130	125	95	87.5
22-	M	100	117.5	80	85
23-					
24-	M	180	156.5	80	72
25-	M	132	131	90	85
26-					
27-	M	210	180	140	140
28-	M	150	140	90	85
29-	F	160	140	97	83.5
30-	M	120	135	70	80
31-	F	160	160	90	95
50-	M	140	130	70	65
51-	M	190	175	100	90
52-	F	105	102.5	50	62.5
53-	M				
54-black					
55-	M	200	175	100	95
56-	M	175	170.8	75	75.8
57-	F	180	180	100	100
58-	F	170	160	110	100
59-	F	200	200	100	100
60-	F	190	187.5	90	80
61-	F	150	135	100	85
62-	M	125	152.5	85	90

Entrant ID	Gender	SBPd	SBPf	DBPd	DBPf
63-	F	170	168.5	90	91.5
64-	M	160	179.5	100	103
65-	M	150	162.5	90	92.5
66-	M	150	147	100	95
67-	M	140	142	80	75
68-	F	150	150	85	82.5
69-	M	160	160	70	70
70-	M	180	180	100	90
71-	F	140	142.5	85	70
72-	F	170	176	100	110
73-	F	210	210	100	104
74-	F	160	160	100	91.67
75-	F				
76-	F	140	144	75	72
77-	M	150	150	110	112
78-	F	220	200	110	110
79-	F	200	200	120	114
80-	M	130	130	84	84
81-	M	140	140	95	95
82-	M	150	150	100	90.8
83-	M	130	130	90	90
84-	M	140	140	80	91.6
85-	M	120	120	70	71
86-	F	150	170	90	109
87-	M	160	160	80	88
88-	F				
89-					
90-	M	130	130	90	90
91-	M				
92-	M				
93-	M	204	204	99	99
94-	M	159	159	88	88
95-	M	190	190	85	85
96-	M	140	140	70	78.8
97-	M	130	130	60	60
98-	F	100	120	60	70
99-	M	155	155	105	99.6
100-	M	180	135	80	78
101-	M	180	172.2	86	84.2
102-	F	120	134.2	80	90
103-	F	160	160	95	95
104-	M	156	155	115	100
105-	M	170	170	95	95
106-	F				
107-	F				
108-					

Entrant ID	Gender	SBPd	SBPf	DBPd	DBPf
109-	M	150	146	80	98
124-	F				
125-					
126-	F				
127-	F				
129-	M	190	167.5	80	85
133-					
142-	M	180	159	80	70.25
143-	M	132	132	74	74
144-	M	163	171	67	75.67
145-	F	120	111	80	78.5
146-	M	180	182.5	100	100.83
147-	M				
148-	F	150	152.6	100	95.8
149-	F	170	163	95	90
150-	M	160	162.5	90	92.75
151-	M	170	170	70	70
152-	F	150	145.4	70	84.8
153-	M	173	173	67	67
154-	M	140	140	90	90
155-	M	140	126.67	100	94.3
156-	F	140	144.5	80	77.4
157-	F	180	146.67	110	110
168-					
178-	M	190	143.8	110	81.2
181-	M	160	140	90	73
182-					
184-	F				
185-	M	150	141	80	82.5
186-	F	120	160.67	77	91.3
187-	M	148	148	94	93
188-	F	120	120	80	85
189-	M	150	150	95	96
202-	M	140	140	70	74
203-	M	140	140	80	79
204-	F	130	130	80	89
211-	M	140	110.5	90	72.8
212-	M	100	136	70	75
213-	M	160	140	110	93
214-	F	150	150	97	100
215-	M	120	122	80	89
216-	M	140	140	70	72
217-	M	140	148.33	90	96.7
218-	F	190	190	100	95
219-	F	138	138.4	90	89.2
220-	M	158	158	75	75

Entrant ID	Gender	SBPd	SBPf	DBPd	DBPf
221-	F				
223-	M	140	142	95	96
225-	F	190	176	90	84.5
226-	M	165	137.5	100	80
227-	M	110	112.5	75	75
228-	F	160	170	100	100
229-	M				
230-	M	140	130	80	75
231-	F	130	105	70	55
232-	F	80	100	50	55
233-	M	118	118	60	70
234-	M				
235-	M	160	170	80	80
236-	M	150	150	80	80
237-	F	160	159	85	86
238-	M	155	140.5	90	85
239-	M	214	214	109	98.5
240-	M				
241-	F	140	140	80	89
242-	M	130	134.5	80	79.75
243-	M				
244-	M	140	142	80	83
245-	M	160	165	90	83.75
246-	M	160	147.4	82	83
247-	M	150	136	95	83.8
248-	M				
249-	M	140	136.8	80	82.5
251-	F	170	139.5	110	92.7
252-	M	170	168	90	87
253-					
254-					
255-	F	130	132	85	86.8
256-	M	200	169.2	110	85.8
257-	M	140	147.5	80	77.5
258-	M	85	145	55	80
259-	M	132	102.5	76	67.5
260-	M	173	102.5	105	100
261-	F	140	140	75	86.7
262-	F				
270-	M				
271-	M	140	127.7	75	67
272-	M	125	148.33	80	80
273-	M	150	152.8	90	91
274-	M	170	178.2	90	92.2
276-	M	150	161.75	85	79.25

Entrant ID	Gender	SBPd	SBPf	DBPd	DBPf
277-	M	130	152.2	80	81.25
300-	F				
301-	M				
302-	F				
303-	F				
305-	F				
306-	M				
307-	F				
321-					
322-	M				
323-	M				
324-	M				
325-	M				
341-					

Entrant ID	Proteinuria at onset	Proteinuria during follow up	Creatinine Slope	Progression of CRF
1-	0.2	1.05	0.000094	NP
2-	3.57	2.09	-0.0000013	NP
3-	1.6	2.01	-0.0000078	NP
4-	1	2.41		ESRF
5-	10.7	10.68		ESRF
6-	4.2	3.84	-0.000027	P
7-				ESRF
8-	4.3	2.47	-0.000067	P
9-	0.5	1.03	0.000019	NP
10-	1.83	1.75		ESRF
11-	0.9			ESRF
12-				
13-	1.29	1.25	-0.00008	P
14-	0.36	0.27	-0.000048	P
15-	3.3	2.4	-0.000039	P
16-	0.3	0.34	0.000011	NP
17-	2.3	1.31	-0.000021	NP
18-	0.3	0.133	0.000053	NP
19-	1.5	2.47		ESRF
20-	2.8	7.6		ESRF
21-	0.5	0.26	0.000005	NP
22-			-0.000003	NP
23-				
24-	0.2	0.32		ESRF
25-	2.8	3.07	-0.000076	P
26-				ESRF
27-	2.6	2.22	0.0000412	NP
28-	1.34	1.16	0.0000532	NP
29-	0.22	0.49	0.000013	NP
30-				
31-	0.3	0.3	0.0000007	NP
50-				ESRF
51-	5.1	3.58		ESRF
52-	3	3		ESRF
53-				
54-black				
55-				ESRF
56-	7.8	7.8		ESRF
57-	1	1		ESRF
58-	0.4	0.4		ESRF
59-				ESRF
60-				ESRF
61-	1.5	1.4		ESRF
62-	2.95	2.95		ESRF
63-	2	2		ESRF

Entrant ID	proteinuria at onset	Proteinuria during follow up	Creatinine Slope	Progression state
64-	0.9	0.73		ESRF
65-	5.9	4.7		ESRF
66-	1.8	1.8		ESRF
67-	0.6	0.83		ESRF
68-	0.7	1.73		ESRF
69-				ESRF
70-	1.7	1.7		ESRF
71-	1.5	1.5		ESRF
72-	4.1	4.1		ESRF
73-	2.53	2.35		ESRF
74-	1.4	1.4		ESRF
75-	1.3	1.63		ESRF
76-				ESRF
77-	1.6	0.58		ESRF
78-	0.1	0.19		ESRF
79-	0.25	0.25		ESRF
80-	3.5	2.13		ESRF
81-	3.5	2.87		ESRF
82-	5.1	3.28		ESRF
83-	3.16	1.87		ESRF
84-	2.53	2.53		ESRF
85-	3.1	3.1		ESRF
86-	1.1	1.1		ESRF
87-	16.7	15.87		ESRF
88-	5.4	4.78		ESRF
89-				
90-	1.6	2		ESRF
91-			-0.0000065	NP
92-	1.9	1.9		ESRF
93-	7.8	7.8		ESRF
94-	7	7		ESRF
95-	2.7	2.32		ESRF
96-	0.3	0.3	-0.000018	NP
97-	0.2	0.15	-0.0000048	NP
98-				
99-	2.8	2.61		ESRF
100-	0.8	0.88	0.0000007	NP
101-	0.9	1.14	-0.00008	P
102-	5.6	6.63		ESRF
103-				ESRF
104-	12.8	12.8		ESRF
105-	6.6	6.6		ESRF
106-	3.3	2.17		ESRF
107-				ESRF

Entrant ID	proteinuria at onset	Proteinuria during follow up	Creatinine Slope	Progression state
108-	0.74	0.57		
109-	3.1	2.07		ESRF
124-	5.85	4.1		ESRF
125-				
126-				
127-	3.57	2.64	0.0000307	NP
129-	3.6	2.17		ESRF
133-				
142-	0.2	0.3		
143-	0.78	1.32	-0.000061	P
144-	1.5	2.97		ESRF
145-	0.29	0.3	-0.000011	NP
146-	0.1	0.1	-0.00002	NP
147-	0.37			
148-	0.2	0.15	-0.00008	P
149-	0.2	0.2	-0.0000027	NP
150-	3.6	2.56		ESRF
151-	2.8	2.8	-0.000037	P
152-	0.1	1.6	-0.000054	P
153-	1.79	1.5	-0.0000048	NP
154-	2.1	1.33	-0.000044	P
155-	0.43	0.4		ESRF
156-				
157-				
168-				
178-	3.74	2		ESRF
181-	3.5	2.87		ESRF
182-				
184-	0.29	0.4		ESRF
185-	3.1	3		ESRF
186-				ESRF
187-	0.92	0.92		ESRF
188-	1.1	1		ESRF
189-	0.4	2.24		ESRF
202-	0.2	0.3	-0.00004	P
203-	8.3	7	-0.000006	NP
204-	0.72	1	0.00006	NP
211-	0.06	0.07	-0.00005	P
212-				
213-	3.9	2.57	-0.0000026	NP
214-	1.2	1.2	-0.00002	NP
215-	0.9	0.9		ESRF
216-	2.8	3.07	-0.00006	P
217-	7.2	9.6	-0.0001	P
218-			0.000022	NP

Entrant ID	proteinuria at onset	Proteinuria during follow up	Creatinine Slope	Progression state
219-	1.47	1.9	-0.000003	NP
220-	3.14	1.8	-0.00008	P
221-	1.28	1.3	0.00004	NP
223-	3.42		-0.00003	P
225-	0.2	0.2	-0.000003	NP
226-	0.2	0.3	-0.000032	P
227-	0.49	0.5	-0.0004	P
228-	0.2	0.15	-0.00003	P
229-	5.4	5	-0.00003	P
230-	1.34	1		ESRF
231-	0.4	0.4		ESRF
232-	0.6	0.5	0.000027	NP
233-				
234-	0.34	0.34		
235-	1.1	1.1	-0.00003	P
236-	2	2		ESRF
237-				ESRF
238-	4	4	-0.0000042	NP
239-	4.4	6.25		ESRF
240-	1.3	1.3	0.000003	N.P
241-	8.9	6.3		ESRF
242-	2.71	2.71	-0.000022	P
243-	2.7	2.3		ESRF
244-	8.1	5		ESRF
245-	0.9	1	-0.000012	NP
246-	0.36	0.3	-0.000036	P
247-	1.62	1.62	-0.000072	P
248-	0.1	0.1	-0.00003	P
249-	2.8	2.6	-0.00006	P
251-	1.3	1	-0.00002	NP
252-				ESRF
253-				
254-				
255-	1.47	1.79	-0.0000006	NP
256-	0.4	0.5		ESRF
257-	0.1	0.1	0.00001	NP
258-	0.08	0.08		ESRF
259-	0.1	0.3	-0.000007	NP
260-	1.04	0.93	0.000008	NP
261-	3.94	3.94	-0.00004	P
262-				
270-			-0.00001	NP
271-				ESRF
272-	0.53	0.58		ESRF
273-	0.1	0.2		ESRF

Entrant ID	proteinuria at onset	Proteinuria during follow up	Creatinine Slope	Progression state
274-	0.81	0.81	0.00008	NP
276-				ESRF
277-	0.3	0.25	0.00002	NP
300-	0.2		-0.000001	NP
301-	1.23		0.00004	NP
302-	0.79		0.000004	NP
303-	2.18		0.00005	NP
305-	0.93		-0.0003	P
306-	19		-0.00034	P
307-	0.28		-0.00025	P
321-				ESRF
322-	5.9			ESRF
323-	5.1			ESRF
324-	9.6			ESRF
325-				ESRF
341-			-0.00015	P

A.VII. TGF beta 1 polymorphisms

Entrant ID	C-509T	Leu10Pro	Arg25Pro	Thr263Ile
1-	CC	TC	CG	CC
2-	TC	TT	CC	CC
3-	TC	TC	CG	
4-	TC	TC		
5-	TC	TC	GG	CC
6-			GG	CC
7-	TC	TC	GG	CC
8-	TT		GG	CC
9-	TC	TC	GG	TC
10-	TT	CC	CC	CC
11-	TT	TC	GG	CC
12-				
13-	TT	TC	GG	TC
14-	TT	TC	GG	CC
15-	TT	TC	GG	CC
16-		TT	GG	CC
17-	CC	CC	CG	CC
18-	TC	TT	GG	
19-	TC	CC		
20-	TT	TC	GG	CC
21-	TT	TT	CG	CC
22-	TC	CC	CG	CC
23-				
24-	TC	TT	GG	CC
25-	TC	TC	GG	CC
26-				
27-	TC	TT		CC
28-	CC	TC	CG	CC
29-	TT	CC	CG	CC
30-				
31-				
50-	TC	CC	GG	CC
51-	TT	CC	GG	CC
52-	TC	TT	GG	CC
53-	TC			
54-black				
55-	TT	TC	GG	CC
56-	TC	TT	GG	CC
57-	TC	TT	CG	CC
58-	TC	TC	GG	CC
59-	TC	TC	GG	CC
60-	TT	CC	GG	CC
61-		TT	GG	CC
62-	TC	TC	CG	CC
63-	TT		GG	CC

Entrant ID	C-509T	Leu10Pro	Arg25Pro	Thr263Ile
64-	TC	TT	CG	CC
65-				
66-	TT	CC	GG	CC
67-	TT	CC	GG	CC
68-	TC	TT	GG	CC
69-	TC	TT	CG	CC
70-	TC	TT	GG	CC
71-	TT		GG	CC
72-	TT	TT	GG	CC
73-	TC	CC	GG	CC
74-	TT	TC	GG	CC
75-	TT	TC		CC
76-	CC	TC	CG	CC
77-	TT	TC	GG	CC
78-	TT	TC	GG	CC
79-	TT	CC	GG	CC
80-	TT	TC	GG	CC
81-	TC	CC	CG	CC
82-	TT	CC	CG	CC
83-	TC	TC	GG	CC
84-	TT	CC	GG	CC
85-	TT	CC	GG	CC
86-	TT	CC	CG	CC
87-	TT		GG	CC
88-	TT		GG	CC
89-				
90-	TT	TT	GG	CC
91-	TC	TC	GG	CC
92-	TT	TT	GG	TC
93-	TT		GG	CC
94-	TC	TC	GG	CC
95-	TC	TC	CG	CC
96-	TC	TC		
97-	TC	TT	GG	CC
98-				
99-	TC		GG	CC
100-	TC	CC	CG	CC
101-	TC	TC	GG	CC
102-	TC	TC	GG	CC
103-	TT		GG	CC
104-	TT	TC	GG	CC
105-	TT		GG	CC
106-	TT	TC	GG	CC
107-	TT		GG	CC
108-				
109-	TC	TT	GG	CC

Entrant ID	C-509T	Leu10Pro	Arg25Pro	Thr263Ile
124-			GG	CC
125-				
126-				
127-				
129-	TT	TC	GG	CC
133-				
142-				
143-	TC		GG	CC
144-	TT	TT	GG	CC
145-	TT	TT	GG	CC
146-	TT	CC	CG	CC
147-				
148-	TT	CC	GG	CC
149-	TC	TC	GG	CC
150-	CC	TC	GG	CC
151-	TT	TC	GG	TC
152-	TT	CC	GG	CC
153-	TT	TT	GG	CC
154-	TC	TC	GG	CC
155-	TT	TC	GG	CC
156-				
157-				
168-				
178-	TT	TC	GG	CC
181-		TC	CG	CC
182-				
184-	CC	TC	GG	CC
185-	TC	CC	GG	CC
186-	TT		GG	
187-	TT		CG	
188-	TC		GG	
189-	TT	TT	GG	CC
202-	TT	TC	GG	CC
203-	TT	TC	GG	TC
204-	TT	TC	GG	CC
211-	TC	TC	GG	CC
212-				CC
213-	TT	TC	CG	CC
214-	TC		GG	CC
215-	TT	TC	GG	CC
216-				
217-	TT	TC	CG	CC
218-				
219-	TC	TC	GG	CC
220-	TT	TT	GG	CC
221-	TT	TC	GG	

Entrant ID	C-509T	Leu10Pro	Arg25Pro	Thr263Ile
223-	TT	TC	GG	CC
225-				
226-	TT	TT	GG	CC
227-	TT	TC	GG	CC
228-	TT	TC	GG	CC
229-	TT	TT	GG	CC
230-	TT	TC	CG	CC
231-	TT	TC	GG	CC
232-	TT	TC	GG	CC
233-				
234-				
235-	TT	CC	GG	CC
236-			GG	CC
237-	TT	TC	CG	CC
238-	TT	TT	GG	CC
239-	TT	TC	CG	CC
240-	TT	TC	GG	CC
241-	TC		GG	CC
242-	TC		GG	CC
243-		TC	GG	CC
244-	TT	TC	GG	CC
245-				
246-	TC		GG	CC
247-	TT		GG	CC
248-				
249-				
251-	TC			CC
252-	TT	TC	GG	CC
253-				
254-				
255-	CC	TC	CG	CC
256-	TC		GG	CC
257-	TT	CC	GG	CC
258-	TC	TC	GG	CC
259-	TC	TC		
260-	TT	TC		CC
261-		TT	GG	CC
262-				
270-			CG	CC
271-	TT	TC	CG	CC
272-	TT	TC	GG	CC
273-	TT		CG	CC
274-	TT	TT	GG	CC
276-				
277-				
300-				

Entrant ID	C-509T	Leu10Pro	Arg25Pro	Thr263Ile
301-		TC		
302-				
303-				
305-				
306-				
307-				
321-	TT	CC	GG	CC
322-	TT		GG	CC
323-	TT	TC	GG	CC
324-	TT	TC	GG	CC
325-	TC		GG	CC
341-				

A.VIII. Pathology and TGF beta 1 immunostaining data

Entrant ID	GS	IF	Cellular Infiltration (cell/field)	% of Glomeruli stained with TGF beta 1	% of tubules stained with TGF beta 1
3-	1	1			
5-	3	4	0	3	3
8-	4	3	60	0	20
11-	3	3	0	8	15
16-	3	3	25	10	15
18-	3	3	58	0	5
28-	3	3	0	0	5
51-	4	4	60	0	14
56-	3	3	0	2	1
68-	2	3	0	3	4
76-	2	4	0	2.8	16
81-	2	3	0	0	0.5
87-	3	4	60	9.4	17
104-	3	3	60	0	12
109-	4	4	60	0	2.5
154-	3	3	20	0	5
157-	3	3	15	0	22
168-			0	0	0.5
217-	2	3	60	2.5	1.3
220-	4	3	60	0.5	5
221-	4	3	60	0	21
238-	3	3	55	14	22
271-	2	4	45	0	7
273-	2	3	0	2	6

A.XI. Circulating TGF-βI levels

Entrant ID	Plasma TGF level	Serum TGF beta
1-		40074
2-	152724	39303
3-	7080	23610
4-		
5-	22471	27555
6-	24105	25710
7-		20517
8-	38663	37092
9-	4687	39474
10-	4682	26574
11-	46170	37014
12-	2580	997
13-	4459	21322
14-	1924	20298
15-	3508	29142
16-	24343	37533
17-	24092	19824
18-	25355	29949
19-	15437	22128
20-	10406	20517
21-	21002	38724
22-	24218	
23-	21310	29874
24-		16305
25-	16403	27417
26-		
27-	26207	
28-	6371	20310
29-	10850	18117
30-		
31-		
50-	21966	25410
51-	24309	29628
52-	14790	19599
53-		15549
54-black		
55-	21574	19728
56-	10099	14850
57-	11072	22740
58-	6795	8124
59-	4129	13842
60-	13767	12624
61-	6699	10353
62-		13524

Entrant ID	Plasma TGF level	Serum TGF beta
63-		22917
64-	7327	11874
65-	6917	
66-	31845	27054
67-	7583	11067
68-	11681	17124
69-	10316	16110
70-	9939	21525
71-	14046	16299
72-	17580	24903
73-	21030	30417
74-	27474	26385
75-	20950	
76-	18459	17889
77-	13033	
78-	31081	
79-	24363	
80-		
81-	24742	
82-		
83-		27912
84-	16020	32667
85-	13116	20553
86-	7777	25710
87-	10689	18024
88-	18431	35367
89-	19985	30885
90-	16225	30924
91-	14122	28074
92-	18296	14685
93-	84664	31899
94-	14542	15399
95-	18039	20760
96-	21404	26910
97-	26593	43167
98-		
99-	27757	19317
100-	18658	29424
101-	18336	27867
102-	9592	9414
103-	12382	12135
104-		18249
105-	20599	27378
106-	7128	13128
107-	13026	10167
108-		

Entrant ID	Plasma TGF level	Serum TGF beta
109-	12397	19599
124-		
125-	16105	24903
126-	22004	30378
127-		1560
129-		29985
133-	21621	16503
142-		
143-		
144-		
145-		
146-		
147-		
148-	16579	
149-	22525	32724
150-	16910	29517
151-	20922	24792
152-	19235	34053
153-	35553	31428
154-	3932	4017
155-		26574
156-	56407	660
157-		1017
168-		
178-	13141	25503
181-		24435
182-	31922	28824
184-	29319	32799
185-	17274	24717
186-		51660
187-	21074	31165
188-	8969	1353
189-	60789	34635
202-	14013	12852
203-	25107	38469
204-	23984	28698
211-	16134	29994
212-	50524	45012
213-	48842	32973
214-	30035	36273
215-	46170	28137
216-	33380	13194
217-	10677	18612
218-		33960
219-	16276	
220-	18128	20412

Entrant ID	Plasma TGF level	Serum TGF beta
221-	12835	13119
223-	21851	20319
225-		23748
226-	9943	27687
227-	16416	
228-	19465	13044
229-	14336	
230-	12151	16962
231-	22134	14655
232-	21460	17823
233-	11424	13812
234-		
235-	26864	17244
236-	11154	22437
237-	17969	38670
238-	14430	
239-	23903	12312
240-	2981	25749
241-	13163	30849
242-	8942	24885
243-	10919	22524
244-	15799	30717
245-		44460
246-		1972.5
247-	7776	25803
248-	17199	28428
249-		21135
251-		
252-	13828	
253-		
254-		
255-	354	30960
256-	31175	48453
257-	43593	14274
258-	10556	
259-	14305	
260-	12039	
261-	25360	
262-		
270-	11703	21180
271-	8661	14055
272-	19313	
273-	8411	
274-	16518	21144
276-		
277-		

Entrant ID	Plasma TGF level	Serum TGF beta
300-		
301-	25402	
302-		
303-		
305-		
306-		
307-		
321-	5333	
322-		
323-		
324-		
325-		
341-		