The Progression of Nephropathy in Non-Insulin-Dependent Diabetes Mellitus

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i

Dedication

This thesis is dedicated to my wife, Dr. Fiona Mackie, who has shared the many trials and tribulations in its production. She has borne the separation without complaint and offered encouragement and support throughout.

Declaration of originality

I declare that this thesis was composed by myself, and with the exception below, the work here presented is entirely my own:

The assay used to measure 'Mixed Laminin Fragments' in the serum and urine was developed by Dr Sally Taylor and Professor Robert Price of the Biochemistry Department, King's College, London.

Alasdair Mackie

Acknowledgements

Abstract

The natural history and factors affecting the progression of nephropathy in non-insulin-dependent diabetes mellitus are poorly understood. The hypothesis that glomerular filtration declines at a similar rate in NIDDM and IDDM was examined in a cohort of 87 subjects (55 NIDDM and 32 IDDM). The rate of decline of calculated glomerular filtration rate (GFR) {Cockcroft-Gault} was significantly slower in IDDM compared to NIDDM (0.29 vs 0.43 $ml.min.^{-1}month^{-1}$ $p<0.05$), in subjects with a baseline glomerular filtration rate (GFR) of ≤ 80 $ml.min^{-1}$ followed for a median of 6.4 years. The rate of decline of GFR was more rapid in Caucasian than in Afro-Caribbean NIDDM subjects. For all individuals, 24-hour protein excretion proved the most significant variable associated with the decline of GFR. Together with diastolic blood pressure these factors accounted for 34% of the variation of the data. For the NIDDM group, blood pressure treatment at the outset replaced diastolic blood pressure as a significant associate of decline of GFR. The effect of percutaneous renal artery angioplasty was evaluated in nine subjects with NIDDM, nephropathy and renal artery stenosis to determine if this procedure influences the progression of nephropathy. No benefit was demonstrated.

Twenty-six NIDDM individuals from the above cohort were prospectively studied over a two-year period. The rate of decline of GFR was 0.48 $ml.min.^{-1}month^{-1}$. Blood pressure, serum cholesterol and 24-hour protein were all associated with GFR decline, with 38% of the variation in the data accounted for by the first two factors. EDTA clearance was compared to calculated GFR and creatinine clearance for 72 patient episodes to determine the clinical value of these surrogate markers of GFR in NIDDM subjects. Calculated GFR underestimated true GFR by 4%, on average, with significant differences for the Afro-Caribbean, but not the Caucasian or Asian, group.

A reliable non-invasive marker of disease progression would be valuable in established nephropathy. Serum and urine type IV collagen and, using a novel assay, mixed laminin fragments (MLF) were compared in 55 subjects with diabetic nephropathy, 33 control diabetic subjects and 42 normal control subjects. The

median values for serum type IV collagen were 161, 115 and 94 μg.L^{-1} and for serum MLF 940, 1000 and 224 μg.L^{-1} respectively. The level of serum collagen found in the nephropathic group was significantly greater than diabetic or normal controls (p<0.001), whereas for serum MLF the nephropathic and diabetic control group were similar, though both significantly higher than the normal group (p<0.001). Type IV collagen was undetected in the urine in all normal subjects and all but three diabetic controls, compared to 50% of nephropathic subjects, whereas MLF were detected in the urine of 1 of 28 normal, 10 of 28 diabetic control (all $\leq$ 110 μg.mmol Cr^{-1}) and 50 of 51 nephropathic subjects. The level of MLF was greater in individuals with a more rapid decline of GFR and suggests a role for MLF in monitoring of progression in advanced diabetic renal disease.

Abbreviations and definitions

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
ACEID	Angiotensin converting enzyme insertion/deletion gene polymorphism
AER	albumin excretion rate. Measured either as timed overnight collection or as 24-hour collection.
BMI	body mass index. Defined as weight (kg)/ height^2(m)
BMT	basement membrane thickening
CAPD	chronic ambulatory peritoneal dialysis
cGFR	estimated GFR using the Cockcroft-Gault formula.
CL IV	type IV collagen
CSII	continuous subcutaneous insulin infusion
DBP	diastolic blood pressure
DEAE	diethylaminoethyl
EDTA	European Dialysis and Transplantation Authority
EDTA	ethylenediaminetetraacetic acid
ESRD	End-stage renal disease. Also known as end-stage renal failure.
GBM	glomerular membrane thickening
GFR	glomerular filtration rate
HbA_1/HbA_{1c}	glycosylated haemoglobin
HD	haemodialysis
HDL	high density lipoprotein
IDDM	Insulin-dependent diabetes mellitus
ioth	iothalamate clearance
LDL	low density lipoprotein
LP1, LP2	principal pepsin digest fragments of laminin
MAP	mean arterial pressure. Defined as diastolic blood pressure plus one-third of the SBP - DBP
MDRD	modification of diet in renal disease
MLF	mixed laminin fragments
mRNA	messenger RNA
NC1	N-terminal end of type IV collagen
NIDDM	Non-insulin-dependent diabetes mellitus
PBS	phosphate buffered saline
PCTA	percutaneous transluminal angioplasty
PEG	polyethylene glycol
PTH	parathyroid hormone
RAS	renal artery stenosis
RRT	renal replacement therapy
S.Cr.	serum creatinine
SBP	systolic blood pressure
SDS	sodium dodecyl sulphate
TBM	tubular basement membrane
UAE	urine albumin excretion
UKPDS	United Kingdom Prospective Diabetes Study

PREFACE

CHAPTER 1 : Introduction.

CHAPTER 3 : Does renal artery angioplasty influence the progression of renal impairment in diabetic nephropathy?

CHAPTER 4 : A prospective study of the factors influencing the decline of glomerular filtration rate in NIDDM subjects with nephropathy.

CHAPTER 5 : Assessment of the methods of monitoring progression of renal impairment in NIDDM.

CHAPTER 6 : Serum and urinary type IV collagen and mixed laminin fragments as markers of progression of nephropathy in NIDDM.

CHAPTER 7 : Conclusions.

Addenda and corrigenda

Addenda

to **section 1.3.3:**

In the Kumamoto study, analogous to the DCCT, Ohkubo and colleagues (1995) examined the effect of intensive insulin therapy on progression of microvascular complications in NIDDM. Over a six-year period, 110 subjects were randomly assigned to conventional therapy (1 or 2 insulin injections) or intensive therapy (3 or more injections). The cohort was further subdivided into a primary and a secondary prevention group. With reference to nephropathy, those subjects in the former group had a UAE of <30 mg.24 hr^{-1}, whereas the UAE in the latter was <300 mg.24 hr^{-1}. Progression was defined as an increase in one or more nephropathy stages, described as normoalbuminuria, microalbuminuria or albuminuria (>300 mg.24 hr^{-1}). Development of nephropathy was observed in 28% vs. 7.7%, conventional vs. intensive therapy (p = 0.03), of individuals in the primary intervention group and progression in 32% vs. 11.5%, conventional vs. intensive therapy (p = 0.04), in the secondary intervention group. Furthermore the authors suggested the glycaemic threshold for the development and progression of microvascular complications was an HbA1$_c$ of 6.5%.

There are a number of weaknesses in this study. It is not clear exactly how the classification of nephropathy stage was arrived at in individual subjects and patient numbers are relatively small for an intervention study. For example of 55 subjects in the primary prevention group, only 9 showed development of nephropathy. It is quite possible that misclassification of nephropathy stage could significantly affect the conclusions reached. Although a glycaemic threshold of an HbA1c of 6.5% is suggested in the text, examination of Figure 4 suggests the true value should lie between 7% and 7.5%.

Addenda to section 1.3.4 (at foot of page 16):

More recently, Lam and colleagues (1995) examined the influence of lovastatin, an HMG CoA reductase inhibitor, in delaying the progression of nephropathy in Chinese subjects with NIDDM. In a two year study, these workers suggested that lovastatin caused an attenuation in the rate of decline of GFR, and that this effect only became evident after two, rather than one, years therapy. However, whilst the GFR was significantly lower at two years, compared to baseline, in the placebo group, no statistical difference was observed in GFR between the groups at two years, despite comparable baseline values. Furthermore it is unclear if efforts were made to exclude non-diabetic renal disease.

These two additional studies are referenced at the end of the reference section.

Statistical comment

In the prospective study, a single subject - case 5 - exhibited a marked deterioration in GFR over the observation period. Consequently this may have influenced the significance of certain of the statistical associations. Statistical advice was sought and, as documented on page 94, the data re-examined removing this individual. In the univariate analysis, diastolic blood pressure and 24 hour protein excretion were no longer associated with decline of GFR. Removing this individual did not affect the conclusions drawn from the stepwise regression analysis.

Variability in analysis of protein markers of nephropathy progression

There are no data available to determine the intra- and inter-assay coefficient of variation of laminin. Samples were prepared in duplicate and the mean of the two used in comparing results of serum and urine from nephropathic, non-nephropathic diabetic and control subjects. Neither sequential nor large volume samples were available to determine the CV of the assay, when used to determine the concentration of laminin in

these samples. To minimise bias in the assay equal numbers of samples were analysed from each of the above groups within each assay. It is acknowledged that a high CV, if found, would limit the clinical applicability of this assay. The intra and inter assay CV for collagen, determined by the manufacturer, were 3.9% and 5.2% respectively. Because of the limited material available, it was decided not to repeat these measurements but to use it all for the analysis of the samples.

Corrigenda

page 9; end line 3 : should read '**in the United Kingdom**'

page 16; line 24 : -0.25 to -0.21 should read '**0.25 to 0.21**'

page 19; line 9 : (1990) should read '**(1995)**'

page 40; line 15 : unpaired should read '**paired**'

page 41; line 7 : unpaired should read '**paired**'

page 48; table : in line 'Final year' 27 should be **22** and 23 should be **28**

page 91; line 12 : 0.91 should read '**-0.91**'

page 93; table 4.7 : significance for MAP should be '**NS**', not p < 0.001

page 98; table 4.8 : decline in GFR should read **0.04** and not 0.44

page 109; the lines ' **However, whether this.....seen.**' should be removed

page 134; line 7 : should read clearance of **0.73** (slightly stronger in **Caucasians** and...)

page 161; add 'The values are the mean of three samples.'

page 169; line 5 : should read '.. serum MLF does **not** distinguish...'

page 173; line 8 : should read '...nephropathy, **compared to normal controls**, (p<0.005)...

page 174; line 18 : for relate to read '**be explained by**'

page 177; line 1 : for 64% read '**36%**' and for 96% read '**only 4%**'

CHAPTER 1

Introduction

In 1859 Griesenger described the case of a patient with renal failure and non-insulin-dependent diabetes mellitus, well before the advent of insulin and over 75 years before Kimmelsteil and Wilson identified the lesions which characterise diabetic glomerulo-pathy (Griesenger 1859; Kimmelsteil and Wilson 1936). Our understanding of the pathophysiology and progression of nephropathy in NIDDM has, until recently, lagged well behind that of IDDM. More is now understood about the early stages of nephropathy in NIDDM and of those reaching end-stage renal disease, yet of the intervening period much less is known.

1.1   THE SCALE OF THE PROBLEM

1.1.1   Diabetes mellitus and renal impairment

An inexorable march towards end-stage renal disease (ESRD) and renal replacement therapy (RRT) is the fate of many diabetic patients with chronic renal impairment, assuming death from cardiovascular disease does not intervene. Exactly what constitutes ESRD is unclear (Wing 1992) and many texts use the term end-stage renal failure. It is assumed here to represent the phase, of varying duration, immediately before and including RRT (Mogensen 1993). Each year in the United Kingdom 300 to 350 individuals with diabetes commence RRT (Cameron 1992a). However, this figure falls well short of the 580 estimated to develop ESRD in a given year (Joint Working Party 1988 & 1989). Notwithstanding this shortfall, the proportion of diabetic patients treated for ESRD world-wide has increased significantly since the first individual started maintenance dialysis over thirty years ago (Avram 1982). Initial acceptance for those with diabetes mellitus was slow. In 1975 only 1.4% of patients who commenced RRT in the UK had diabetes. By 1985 this had risen to 11.4%, 12.7% by 1988 and in 1992 the figure for all Europe was 17% (Brunner et al. 1988; Cameron 1992a; Vaderrabano 1994). Elsewhere the proportion is higher. The latest available data for the USA indicate an acceptance rate of 33.6% in 1993 (United States Renal Data Service Report 1996). In Finland

the 1985 figure was 34% (almost all IDDM) and for Japan 19% (predominantly NIDDM) for the period 1982-7 (Brunner et al. 1988; Odaka 1990). Currently in New Zealand the proportion is 35 - 40% (Dr. PL Drury personal communication).

1.1.2   The increasing proportion of non-insulin-dependent diabetes mellitus in renal replacement programmes.

An increasing proportion of diabetic patients on dialysis programmes have NIDDM (Rettig and Teutsch 1984; Gonzalez et al. 1985; Cameron and Challah 1986; Friedman 1986; Koch et al. 1989; Catalano et al. 1990a; Grenfell et al. 1992). Between 1983 and 1990 the number of those on the European Dialysis and Transplant Association (*EDTA*) registry with NIDDM increased by 263% against 76% for IDDM (Raine 1993), though in part this may have been due to improved accuracy of classification (see below). In many countries the majority of diabetic patients receiving renal replacement have NIDDM, e.g. in the USA, in the first six months of 1988, 57% were so classed (MMW Report 1989). This proportion may be as high as 86%, depending on the local referral pattern (Friedman 1986).

Misclassification of diabetes type may be responsible for the under-representation of NIDDM in certain of the published figures. Before 1983 there was no separate code for IDDM and NIDDM on the *EDTA* registry (Raine 1993). In France, Zmirou and colleagues (1992) found that more than one third of all patients with NIDDM were misclassified, most commonly on the basis of insulin *treatment* alone. Adjusting for these errors, this group estimated the relative proportion of IDDM and NIDDM patients to be 1.4% and 5.5% respectively compared to the official estimates of 3.2% and 3.7%. Similarly in 1991, Ritz and colleagues in Germany classed only 36% of diabetic patients as insulin-dependent, compared with the figure of 71% published by the EDTA registry, despite using the same population base. Almost identical results were found in Italy by Catalano and colleagues (1990b). Even adjusting for these

discrepancies it is very likely that the proportion of NIDDM subjects receiving renal replacement therapy will show a real increase in the future.

1.1.3 Outcomes on renal replacement therapy

Once commenced on RRT the prognosis for the diabetic patient, though improving, remains poorer than for those with non-diabetic renal disease (McMillan et al. 1990; USRDS 1996). Using data supplied by the EDTA registry, Brunner and Selwood (1990) recorded an overall 5 year survival of 13% in diabetic subjects over the age of 65 years on renal replacement, compared to 34% in an age-matched, non-diabetic, group. Somewhat better survival, 30% at 4 years for all treatment modalities, was recorded in 51 diabetic patients (combined IDDM and NIDDM) aged 55 years and over (Grenfell et al. 1992). The best overall survival following renal replacement in a NIDDM population, 33% at five years, has recently been documented in the Pima Indians (Nelson et al. 1996a).

It is well established that patients receiving dialysis fare less well than those receiving a transplant (Matson and Kjellstrand 1988; Najarian et al. 1989; Hirschl et al. 1992; Nelson et al. 1996a). This is particularly so in diabetes mellitus. Hirschl and colleagues (1992) found an overall 5 year survival of only 14% in 78 NIDDM patients on RRT. The figures for transplantation and haemodialysis (HD) were 59% v 2% respectively. Nelson and colleagues (1996a) recorded a median survival of 92 months in 12 Pima Indians with NIDDM, who received a renal transplant, compared to 35 months in 124 who did not. Recent data suggest that there may be a survival advantage from haemodialysis compared to chronic ambulatory peritoneal dialysis (CAPD) in NIDDM. Nelson and colleagues (1996a) recorded a median survival of 45 months in 107 subjects treated with HD against 29 months in 29 individuals treated with CAPD. However, the small numbers meant the data failed to reach statistical significance. Similar findings were observed by Bloembergen and colleagues (1995), though others, including Nolph (1988) have found no difference. In the UK the

primary treatment for ESRD in diabetes mellitus, particularly NIDDM, is CAPD (Raine 1993).

In general, overall survival rates for those with diabetes are < 50% for those with non-diabetic renal disease (Raine and Ritz 1994). It remains inescapable that the present outlook for diabetic patients, in particular those with NIDDM, is poor once renal replacement is commenced (Vollmer et al. 1983; Held et al. 1990; Brunner and Selwood 1992).

1.1.4   Resource implications of diabetic nephropathy

Healthcare costs for diabetes are estimated at 4-5% of the UK health budget (Department of Health 1991; Williams 1991). A significant proportion of this is spent on the treatment of complications. In 1993 in the US over $1.5 billion was spent on ESRD due to diabetes mellitus (United States Renal Data Service: Annual Data Report 1996). Each person entering a renal replacement programme costs, in 1993 terms, US $43,500 per annum to treat, some US $11,500 more than their non-diabetic counterpart (USRDS 1996). In Germany the estimated annual cost, at 1991 prices, of dialysis ranged from US $35-55,000 and for transplantation US $14-35,000 in the first year and US $7,000 per annum in subsequent years (Borch-Johnsen et al. 1993).

Given the considerable number of patients involved, the significant healthcare costs and the poor outcome on RRT, delaying the start of treatment for ESRD for those with NIDDM and nephropathy presents an attractive alternative, not just to the patient, but also to the physician and to the health economist. Fulfilment of these aspirations requires a greater understanding of the evolution of nephropathy and the factors that govern progression.

4

1.2   DISEASE PROGRESSION

1.2.1   Progression to renal replacement therapy in diabetes mellitus

The frequency with which overt nephropathy (defined as a protein excretion of $\geq$ 0.5g in 24 hours in the absence of other renal disease) leads to the development of renal failure in NIDDM is not well documented for most populations (Nelson et al. 1993; Nelson et al. 1996b). In IDDM, where the position is clearer, diabetic nephropathy previously affected over 40% of individuals after 40 years of diabetes (Anderson et al. 1983). Of these two-thirds progress to end-stage renal disease, the majority within 25 to 30 years of diagnosis (Mogensen 1993). Analyses of IDDM cohorts over successive decades show a decline in incidence (Krolewski et al. 1985; Kofoed-Enevoldsen et al. 1987). The former found that 53% of individuals aged under 21 years when diagnosed in 1939 developed nephropathy in contrast to the 25% projected for those of similar age diagnosed in 1949 and 1959. Recent estimates of the prevalence of micro-albuminuria (albumin excretion of 20 to 200 μg.min^{-1}) of 13-15% (Berglund et al. 1987; Parving et al. 1988) suggest that a further decline can be anticipated.

Accurate data on the prevalence of nephropathy in NIDDM are harder to obtain. Lack of ascertainment, as significant numbers of those with NIDDM are cared for in the community (Olivarius et al. 1993), proteinuria due to non-diabetic renal disease (Parving et al. 1992) and premature death from cardiovascular disease (Deckert 1994) all contribute to this difficulty. Reports that 3-8% of NIDDM patients progress to ESRD (Balodimus 1971) clearly underestimate the true prevalence of nephropathy and, at least in certain populations, the lifetime risk of requiring RRT. Ballard and colleagues (1988) recorded a 20 year cumulative incidence of persistent proteinuria of 24.6% in a group of Europid NIDDM subjects from Rochester, Minnesota. In contrast to the studies in IDDM cited above, this group found no evidence that the incidence was declining with time. In selected populations the proportion is even

higher. Studies in the Pima Indians, a native American tribe living in Arizona, shed considerable light on the evolution of nephropathy in NIDDM, due to the high prevalence of NIDDM and the young age of onset (Knowler et al. 1978). In this group 35% develop proteinuria within 15 years and 50% within 20 years of diagnosis (Nelson et al. 1988; Kunzelman et al. 1989). Similar rates are found in Japan (Sasaki et al. 1989).

High prevalence of ESRD is described in other non-white populations with diabetes (Rostand et al. 1982; Eggers et al. 1984; Pugh et al. 1988). Fifteen percent of Pima Indians develop ESRD {Serum creatinine (S.Cr.) $\geq$ 442 μmol.L^{-1}} within 20 years of diagnosis. Once proteinuria is established, 35% will develop ESRD in 10 years and 54% in 15 years (Nelson et al. 1988). These figures exceed the 11% and 17% seen in individuals in a study in Caucasians (Humphrey et al. 1989), despite a lower threshold (S.Cr. $\geq$ 350 μmol.L^{-1} on two occasions) used to define renal failure.

Few groups have examined the prevalence of ESRD in IDDM and NIDDM patients simultaneously. Nelson and colleagues (1988) compared their NIDDM population to a group of IDDM patients attending the Joslin clinic and found a 1.5-fold difference (15% v 9%) twenty years after diagnosis. Hasslacher and colleagues (1989) observed no difference - of 496 NIDDM patients without proteinuria at baseline, 27% developed proteinuria after 20 years, compared with 28% of 312 with IDDM. Of these 63% and 59% respectively developed renal failure (S.Cr. $\geq$120 μmol.L^{-1}) within five years. Humphrey and colleagues (1989) observed a cumulative incidence of renal failure of 6.2% for NIDDM and 8% for IDDM after 25 years of diabetes.

Persistent proteinuria is already present in a significant number of individuals with NIDDM at diagnosis. Ballard and colleagues (1988) recorded proteinuria in 8.2%, whilst Olivarius and colleagues (1993) found an albumin excretion of > 200 μg.min^{-1} (equivalent to 300 mg.24hr^{-1}) in 6% of 1128 individuals in a General Practice-based

study in Denmark. In the Pima Indians 9% were proteinuric at diagnosis (Kunzelman et al. 1989), whilst the highest recorded prevalence appears in the Nauruan population with 24%. (Collins et al. 1989). The latter used a cut off of 300 μg.ml^{-1} for albumin excretion and the prevalence might be an over-estimate, as it is not clear what efforts were made to exclude non-diabetic causes of proteinuria.

The time to onset of persistent proteinuria is naturally shorter in NIDDM than in IDDM, reflecting the pre-symptomatic period occurring in many individuals (Harris et al. 1992). The average time from diagnosis to proteinuria was 16 years in 46 NIDDM patients and 20 years for 48 IDDM (Hasslacher et al. 1989). In a group of mixed races the duration to proteinuria was 10.1 years in NIDDM and 16.9 years in IDDM subjects (Ordonez and Hiatt 1989); the time interval from proteinuria to the start of RRT was 5.9 years and 6.5 years respectively. Biesenbach and colleagues (1994) found the mean (range) interval from proteinuria to dialysis was 77 (44-133) months in 16 IDDM and 81 (40-124) months in an equal number of NIDDM patients. There is little evidence from these studies that the time interval from proteinuria to ESRD differs between IDDM and NIDDM.

1.2.2   Rate of decline of glomerular filtration rate

Observational studies in IDDM patients with nephropathy suggest a rate of fall of glomerular filtration rate (GFR) of approximately 1 ml.min.$^{-1}$month^{-1} (Mogensen 1976; Mogensen 1982). Individual rates of decline are highly variable, ranging from 0.13 to 2.47 ml.min.$^{-1}$month^{-1} (Jones et al. 1979; Parving et al 1981; Viberti et al. 1983). Intervention, particularly with anti-hypertensive therapy, slows the rate of decline on average to 0.20 ml.min.$^{-1}$month^{-1}, 6 to 10 years after starting therapy (Parving et al. 1993). Nyberg and colleagues (1986) found similar results with a mean decline of 0.38 ml.min.$^{-1}$month^{-1} over a 2 to 3 year period in 29 subjects with a baseline GFR of approximately 50 ml.min^{-1}; a third of the group maintained stable renal function throughout the observation period.

The natural evolution of established nephropathy in NIDDM will remain to a large extent unknown, since the majority of individuals are already on treatment. Prospective studies which use a valid technique to measure GFR are few. Friedman and Gross (1991a) examined seven NIDDM subjects with a baseline GFR of 40-85 ml.min^{-1} over 4 to 25 months. Excluding one subject who developed multiple myeloma, GFR showed a mean improvement of 0.14 ml.min.$^{-1}$month^{-1} (range -0.72 to 0.80) over 20 months. A larger series from Gall and colleagues (1993) reported a mean decline of GFR of 0.48 ml.min.$^{-1}$month^{-1} in 31 proteinuric diabetic subjects. In a four-year study in the Pima Indian population, Myers and colleagues (1995) recorded a more rapid decline of GFR of 0.75 ml.min.$^{-1}$month^{-1} in 30 individuals with clinical proteinuria and a baseline GFR of 108 ml.min^{-1}. Nielson and colleagues (1991) measured GFR at baseline and at follow up (3-4 years later) in 70 NIDDM subjects (mean age 63 years) of whom 45 were normo-albuminuric, 18 micro-albuminuric and 7 proteinuric. The rate of decline varied from 0.08 ml.min.$^{-1}$month^{-1} in the normo-albuminuric group to 0.61 ml.min.$^{-1}$month^{-1} in the proteinuric group. Nelson and colleagues (1996b) examined progression in a group of 34 Pima Indians with macroalbuminuria and a serum creatinine < 115 μmol.L^{-1}. They found that GFR declined by 35% over a four year period, where the baseline GFR was 124 ml.min^{-1}.

1.3 DETERMINANTS OF PROGRESSION OF NEPHROPATHY

1.3.1 Ethnicity and progression of nephropathy

The high prevalence of nephropathy secondary to diabetes mellitus in many non-white populations has already been mentioned (section 1.2.1). The greater risk of these groups requiring RRT is now widely recognised. Cowie and colleagues (1989) found a 2.6-fold increase in black compared to white Americans, despite correcting for the higher prevalence of diabetes in the former. Stephens and colleagues (1990), in a similar analysis, found a 3.6-fold increase, with a slightly greater (non-significant) risk for NIDDM. Such figures are small compared to the 20-fold increase

in risk of ESRD seen in the Ute and Pima Indians of North America (Narva 1985; Nelson et al. 1988). A greater number of Afro-Caribbeans and Asians with diabetes mellitus, relative to their total population are entering renal replacement programmes (Grenfell et al. 1988; Burden et al. 1992).

Though clearly at greater risk of progressing to ESRD, there is divided opinion on whether non-white subjects progress more rapidly once nephropathy is established. Brazy and Fitzwilliam (1990) found no difference in the rate of decline of renal function, using the slope of inverse creatinine, comparing 112 black (racial origin not identified) with 88 white subjects with progressive renal disease of undisclosed aetiology. However, Tierney and colleagues (1989) did identify race as an independent risk factor for progression (along with diabetes and systolic blood pressure). Cowie (1993) found the time taken from a S.Cr. ≥ 159 μmol.L^{-1} to RRT averaged significantly longer in black (23.8 months), compared to white (18.5 months), NIDDM subjects.

Several factors have been advanced to explain the higher prevalence of ESRD in non-white populations, including access to healthcare (Smith et al. 1991), differences of calcium metabolism (Bell et al. 1985) and blood pressure (Dustan et al. 1987; Cowie et al. 1989). In the next section the key role of the latter in determining the progression of nephropathy is considered.

1.3.2   Blood pressure and progression of nephropathy

It is well established that anti-hypertensive therapy is able to reduce the decline of GFR in IDDM and probably delays the onset of ESRD. In 1976 Mogensen drew attention to the association of hypertension and declining GFR in proteinuric IDDM subjects and in a subsequent paper showed apparent therapeutic benefit from reducing blood pressure in six patients with diabetic nephropathy (Mogensen 1976; Mogensen 1982). In the latter study the mean pre-treatment decline of GFR was 1.23

ml.min.$^{-1}$ month^{-1} and mean blood pressure 162/103 mmHg. Following intervention with various anti-hypertensive agents the blood pressure fell to 144/95 mm Hg and the mean decline of GFR to 0.49 ml.min.$^{-1}$month^{-1}. These results were confirmed by Björck and colleagues (1986) and Parving and colleagues (1983; 1987). Benefit also occurs once renal failure is established (Zander et al. 1990). Following these studies the question was no longer whether anti-hypertensive treatment was beneficial, but rather when should it be started, which agent should be used to lower blood pressure and by how much. Mogensen and colleagues (1991) argued the case for a target mean arterial pressure (MAP) of 90 to 95 mmHg. in those with incipient nephropathy and 100 to 105 mm Hg in overt nephropathy in IDDM.

Many studies have examined the relative merits of the different classes of agent often using microalbuminuria as a surrogate marker of progression. All agents slow the rate of progression, though a claim of superiority is made for angiotensin converting enzyme inhibitors (ACEI). In a large multi-centre trial comparing captopril with placebo in addition to other anti-hypertensive therapy, Lewis and colleagues (1993) demonstrated significant slowing of progression (determined by doubling S.Cr., rate of decline of creatinine clearance, reaching RRT and death) with captopril in 207 IDDM subjects with overt nephropathy and further showed the benefit derived not just from control of blood pressure alone. Viberti and colleagues (1994) have shown a beneficial effect of captopril in slowing progression from microalbuminuria to clinical proteinuria in 46 normotensive IDDM patients. A summary of intervention trials of anti-hypertensive therapy is found in Mogensen and co-workers (1993); selected studies in NIDDM are shown in Table 4.10.

The situation in NIDDM is not so clear cut. Hypertension frequently precedes the onset of diabetes (Pugh et al. 1993), and may be present in the absence of microalbuminuria (Schmitz et al. 1991). Furthermore, as discussed below, it may be systolic, rather than diastolic, pressure which is linked to the progression of

nephropathy in NIDDM. Retrospective analyses of NIDDM subjects with renal failure often identify blood pressure as an important determinant of progression. In 16 NIDDM patients, Biesenbach and colleagues (1994) found a slower rate of decline of GFR, as measured by creatinine clearance; 0.78 ml.min.$^{-1}$month^{-1} in those with a systolic blood pressure below 160 mm Hg compared to 1.38 ml.min.$^{-1}$month^{-1} for those above. Both systolic and diastolic pressure were shown to relate to progression, with the former being the stronger associate.

In 47 patients with overt nephropathy studied over a mean period of 57 months, Hasslacher and colleagues (1993) identified systolic blood pressure as the strongest determinant of progression in a multiple regression analysis. Tierney and colleagues (1989), in a study in hypertensive adults of whom 41% had diabetes (most NIDDM), found systolic blood pressure correlated with deteriorating renal function (S.Cr. and estimated GFR by Cockcroft-Gault). In a very short-term study (mean observation period 1.4 years), Dillon (1993) found that diastolic, and to a lesser degree systolic, pressure correlated with decline in estimated GFR in 59 diabetic subjects, of whom 50% were NIDDM.

In the Pima Indians, Nelson and colleagues (1993) found no evidence that the level of blood pressure at the onset of proteinuria predicted ESRD. It is not clear whether the time to ESRD was influenced by blood pressure. Subsequent studies (Nelson et al. 1996b) found no evidence that blood pressure influenced the decline of GFR in 34 macroalbuminuric subjects, suggesting that it may not do so in this population. Pugh and colleagues (1993) found that the time from diagnosis of diabetes to ESRD was significantly shorter in the NIDDM subjects who already had hypertension at diagnosis (15.5 vs 18.2 years). It is not stated whether the blood pressure was the same in both groups after diabetes was diagnosed. In 131 diabetic patients (47 IDDM & 84 NIDDM) recruited from an eye clinic, Walker and colleagues (1990) found systolic blood pressure in excess of 140 mm Hg was associated with a progressive

decline of renal function in both IDDM and NIDDM subjects; the association being more marked in the latter. No significant deterioration in renal function occurred in those with a systolic pressure <140 mmHg over the 3.5 years of observation. The principal criticism of this study rests with the use of serum creatinine alone as the marker of renal function.

Of the prospective studies in NIDDM with overt nephropathy, Friedman and Gross (1991) and Myers and colleagues (1995) found no association with blood pressure, whilst Gall and colleagues (1993) only with systolic pressure. In normo- and micro-albuminuric subjects, Nielsen and colleagues (1993a) demonstrated a correlation of systolic blood pressure with decline of GFR. The same group also showed an association of systolic pressure with albumin excretion in 135 NIDDM subjects who completed six years of follow-up (Schmitz et al. 1994).

Studies in renal disease not due to diabetes reach broadly similar conclusions, although good prospective, let alone interventional, studies are lacking. Using inverse creatinine in a retrospective examination of 102 subjects with renal disease of mixed aetiology, Wight and colleagues (1992) found diastolic, though not systolic, blood pressure correlated with the rate of progression. In a similar retrospective analysis, Hannedouche and colleagues (1993) also showed a strong correlation with blood pressure, this time using estimated creatinine clearance as their end-point. Brazy and Fitzwilliam (1990) demonstrated, in a study of 200 subjects, that in 45 cases where the diastolic pressure fell from > 90 mm Hg to < 90 mm Hg the rate of progression of renal impairment fell from 0.76 ml.min.$^{-1}$month^{-1} to 0.46 ml.min.$^{-1}$month^{-1}. The same group had previously demonstrated that the rate of progression was significantly faster in the upper two quartiles of diastolic blood pressure than the lowest quartile, when all previous clinic blood pressures were stratified (Brazy et al. 1989). Oldrizzi and colleagues (1985) had shown similar results where the diastolic threshold was set at 100 mm Hg.

1.3.3   Blood glucose and progression of nephropathy

Studies under the umbrella of the Diabetes Control and Complications Trial (DCCT) established the case for a permissive role for hyperglycaemia in the development of microalbuminuria in IDDM, and strongly implicated it in the subsequent progression to overt nephropathy, with a 56% risk reduction in the intensively treated group (DCCT 1993, 1995). In so doing the trial confirmed the findings of Feldt-Rasmussen and colleagues (1986) and Dahl-Jørgensen and colleagues (1988), who demonstrated the therapeutic benefit of strict metabolic control on progression in smaller groups. Mathiesen and colleagues (1990) observed a threshold effect of glycaemic control, with only those having an HbA_{1c} greater than 8% progressing from normo- to microalbuminuria. Krolewski and colleagues (1995) have published similar findings. These benefits were challenged by the Microalbuminuria Collaborative Study Group (1995), who found no impact of intensive insulin therapy on progression of albuminuria in individuals with microalbuminuria.

In a prospective study of 6 IDDM individuals with overt nephropathy, Viberti and colleagues (1983) demonstrated no significant benefit from strict glycaemic control. However the mean decline of GFR fell from 1.35 ml.min.$^{-1}$month^{-1} to 0.69 ml.min.$^{-1}$ month^{-1} and the graphic data suggest an attenuated decline of GFR in all cases. However this small study has relatively limited power. In contrast Nyberg and colleagues (1987) followed 18 IDDM patients over a 21 month period and did show a significant association between a higher HbA_{1c} and a faster decline in GFR.

As with blood pressure, studies in NIDDM are fewer. Schmitz and Vaeth (1988) have shown an association between hyperglycaemia and elevated urinary albumin excretion (UAE). In the United Kingdom Prospective Diabetes Study (UKPDS), raised blood glucose was associated with UAE at diagnosis and during the three subsequent years (UKPDS Report 1993). However less than 10% of the variance of

the data was accounted for by blood glucose, systolic blood pressure and serum triglyceride. In a 12-year prospective study into progression of microalbuminuria, Gilbert and colleagues (1993) found that HbA_{1c} correlated with albumin excretion rate (AER) in the 22 subjects (9 NIDDM) who showed increasing albumin excretion. However, when the whole group was divided into 'Progressors' and 'Non-progressors', on the basis of albumin excretion, no significant relationship with glycaemic control was observed.

Retrospective studies of those with ESRD do, in certain cases, show association with raised blood glucose. Higher 2hr blood glucose (post 75g glucose) at the onset of proteinuria in Pima Indians was predictive of progression to ESRD (Nelson et al. 1993). Within-clinic post-prandial blood glucose was found to correlate with decline of GFR in 94 nephropathic NIDDM individuals, although mean HbA_1 performed in the latter stages did not (Hasslacher et al. 1993). No association of glycaemic control with progression was noted in 16 NIDDM subjects followed over 81 (40-120) months (Beisenbach et al. 1994), nor was it seen in the studies of Friedman and Gross (1991) and Gall and colleagues (1993).

On balance the evidence for an effect of blood glucose on progression is strongest in the early stages of nephropathy and less so, if at all, in the latter stages in both IDDM and NIDDM.

1.3.4 Serum lipids and progression of nephropathy

Virchov (1860) is credited with being the earliest to recognise the association between lipid deposition and chronic renal disease. In their seminal paper, Kimmelsteil and Wilson (1936) described the pathological hallmarks of diabetic nephropathy but also noted the presence of widespread lipid deposits in the kidney. However so abundant were these deposits that they were discounted as being of no pathological significance. It fell to Wilens and Elster in 1950 to recognise their

potential importance by describing the presence of lipid in the renal arterioles and what has now come to be known as the mesangium in both hypertensive and diabetic subjects. Thus was the link between mesangial expansion, systemic hypertension, hyperlipidaemia and diabetes mellitus forged, although it was to be 30 years before any further significant advances were made. Moorhead and colleagues (1982), citing studies in patients with nephrotic syndrome, speculated that lipids, rather than the more fashionable dietary protein (Brenner et al. 1982), could be an additional permissive factor influencing the progression of renal disease. Although clinical studies have yet to substantiate this hypothesis there is considerable evidence from animal studies that lipids may play a significant role in the aetio-pathogenesis of glomerular disease and in the subsequent progression of renal impairment.

Dietary supplementation with cholesterol was shown by Al-Shebab and colleagues (1988) to induce glomerular hypercellularity and expansion of the mesangial matrix in the guinea pig, whereas similar dietary supplementation lead to albuminuria and focal glomerulo-sclerosis in the Sprague-Dawley rat (Kasiske et al. 1987). More significantly, micropuncture techniques demonstrated increased glomerular hydraulic pressure despite there being no change of systemic blood pressure. The principal proteins in the expanded matrix were shown to be laminin, fibronectin and type IV collagen (Kasiske et al. 1985). Similar findings have been observed in the Zucker rat, which is not only a model for NIDDM mellitus but also develops hyperlipidaemia spontaneously. Once hyperlipidaemia is established in these animals glomerulo-sclerosis and then albuminuria develop, although without evidence of raised intraglomerular pressure (Kasiske et al. 1985, O'Donnell et al. 1985). Rats in which a renal artery is clipped (Goldblatt model) and have their diet supplemented with lipid develop more severe glomerulosclerosis (Tolins et al. 1992). Conversely treating animals with lipid lowering agents limits mesangial expansion and glomerulosclerosis (Kasiske et al. 1988).

Studies on the mesangial cell also help establish a link between lipids and glomerulosclerosis. Exposure of the mesangial cell in culture to cholesterol rich low density lipoprotein (LDL) leads to cell proliferation (Wheeler and Chana 1993) and may promote monocyte infiltration, a key player in mesangial expansion, through the production of monocyte chemoattractant protein (Rovin et al. 1993). LDL has also been shown to increase the expression of type IV collagen mRNA (Keane 1994). This subject will be examined in Chapter 6.

Although there is strong evidence from these animal studies that lipids have a prominent role to play in the initiation and progression of renal disease, data in humans are scarce and conflicting. Two cross-sectional studies have shown a two-fold greater rate of progression in non-diabetic renal disease in individuals with high lipids (Maschio et al. 1989; Samuelson et al. 1989). These studies were substantiated by the work of Mulec and colleagues (1990) who showed a link between the progression of renal disease and serum cholesterol, although there have been a number of negative studies (e.g. Gall et al. 1993). In a retrospective study in 424 IDDM subjects, Krolewski and colleagues (1994) found that progression of renal disease, defined as the ratio of serum creatinine at three years to baseline level, was linked to serum cholesterol and diastolic blood pressure: the highest bands for these measures showing the greatest proportion of 'Progressors'. Though these data are interesting, a significant number of individuals did not progress, despite elevated cholesterol, whilst the differences in some cases were marginal.

Lipid-lowering agents have been shown to retard progression of renal disease (Rabelink et al. 1990; Bazatto et al. 1992). The claim by the latter that a GFR decline from -0.25 to -0.21 ml.min.$^{-1}$month^{-1} was significant appears questionable. Other groups have failed to demonstrate any benefit in NIDDM and IDDM (Neilsen et al. 1993b; Hommel et al. 1992), although both were short-term studies.

Understanding the link between lipids and progressive renal disease extends beyond causation. The high mortality associated with proteinuria, renal impairment and renal transplantation is primarily due to cardiovascular disease (Held et al. 1990; Brunner and Selwood 1990). Before widespread anti-lipid therapy becomes established practice the effect of lipids on progression of renal disease needs to be examined more closely.

1.3.5 Dietary protein intake and progression of nephropathy

Evidence for protein restriction ameliorating the decline of GFR comes largely from animal studies. Rats subjected to uni-nephrectomy showed progressive renal damage where the diet was supplemented with protein (Moise and Smith 1927). Subsequent studies in a wide variety of models, including streptozotocin-induced diabetes, supported these observations (El-Nahas et al. 1983; Zatz et al. 1985; Hostetter et al. 1986). Despite these findings, benefit is not so clear in humans with renal disease. Although early clinical studies supported the observations in animals (Rosman et al. 1984; Ihle et al. 1989), many used surrogate markers of GFR (in themselves sensitive to changes in dietary protein intake) and were uncontrolled or 'self-controlled'.

The results from the Modification of Diet in Renal Disease (MDRD) were published in 1994. No benefit was observed from restricting protein intake to less than 0.6 g.kg.$^{-1}$ day^{-1} (and in some cases down to 0.3 g.kg^{-1}) in 840 subjects with various renal diseases and baseline GFR of 25 to 55 ml.min^{-1} (Group 1) and 13 to 24 ml.min^{-1} (Group 2) when followed for approximately 2 years (Klahr et al. 1994). A more rapid decline in the first four months of intervention was followed by a slight, though non-significant, benefit for those in Group 1. Only 3% of the participants had diabetes mellitus. Previously several studies have examined the effect of protein restriction on progression in IDDM subjects with nephropathy (Cohen et al. 1987; Walker et al. 1989; Zeller et al. 1991). Zeller and colleagues (1991) examined the decline of GFR in 20 individuals with a mean baseline GFR of 40 ml.min^{-1} and demonstrated

significant benefit from restricting protein to 0.6 g.kg.$^{-1}$day^{-1} over a 37 month period. Similar benefit was shown by Walker and colleagues (1989) in a group of nineteen subjects. Both the above can be criticised on the grounds of confounding variables; the control group had higher urine protein levels at the outset in the former, whereas in the latter blood pressure was slightly lower in the intervention group. Attman and colleagues (1983) found no benefit from dietary restriction in those with more advanced diabetic nephropathy. No intervention studies exist for subjects with NIDDM, save for individuals in multi-disease studies, such as the MDRD study above.

1.3.6   Miscellaneous factors associated with progression of nephropathy

A variety of additional factors have been associated with progressive decline of GFR. Of these, cigarette smoking has received the most attention. Stegmayr and Lithner (1987) showed that cigarette usage was greater in 22 uraemic individuals with diabetic nephropathy compared to a control group. Smoking was associated with an earlier onset of proteinuria. Progression of established nephropathy may be more rapid in smokers than non-smokers with IDDM (Sawicki et al. 1994); similar observations have been made in lupus nephritis (Ward and Studenski 1992).

Haemostatic factors may also influence the progression of renal impairment. Knöbl and colleagues (1993) found significant associations between various thrombogenic factors and albumin excretion in diabetes mellitus. Gordge and colleagues (1991) found that serum thromboxane B$_2$ and von Willebrand antigen levels were significantly associated with progression of nephropathy in both IDDM and NIDDM. It remains to be seen if these factors have a pathogenic role or whether they are a reflection of the disease process.

1.4   **MARKERS OF PROGRESSION OF DIABETIC RENAL DISEASE.**

1.4.1   **Identification of at risk individuals**

1.4.1.i   *Familial pre-disposition*

Familial clustering of renal disease in those with IDDM and NIDDM suggests that genetic pre-disposition plays a role in the development of diabetic nephropathy (Seaquist et al. 1989; Pettitt et al. 1990; Freedman et al. 1995). In the Pima Indians, Pettitt and colleagues (1990) recorded proteinuria in 14.3% of subjects where neither parent had proteinuria; 22.5% if one parent had proteinuria and 45.9% if both parents were affected. In support of these observations, Freedman and colleagues (1990) demonstrated an 8-fold increase in the risk of developing nephropathy where there is a close (up to third-degree) relative with end-stage renal disease. Thirty seven percent of 52 cases with diabetic nephropathy had a close relative with ESRD compared to only 7% in a matched group of 45 with no nephropathy. Parents of IDDM subjects with microalbuminuria have been shown to have higher blood pressures, compared to normo-albuminuric individuals (Viberti et al. 1987a; Krolewski et al. 1988). Thus far no genetic linkage has been found to substantiate these observations, though increased Na^+/Li^+ counter-transport has been proposed to account for the latter observations (Mangili et al. 1988; Krolewski et al. 1988).

Not only may parental hypertension contribute to the development of nephropathy so too may pre-diabetic blood pressure. Nelson and colleagues (1993) measured UAE 5 years after diagnosis and found a close association between the pre-diabetic blood pressure and the subsequent development of elevated UAE. The presence of hypertension prior to the diagnosis of diabetes mellitus has also been shown to influence the time to renal replacement therapy (Pugh et al. 1993).

1.4.1.ii   Renal hypertrophy and hyperfiltration

Glomerular hyperfiltration is a characteristic early feature in IDDM subjects and is argued by many (Mogensen & Christensen 1985; Rudberg et al. 1992), though not all (Lervang et al. 1988), to be a strong independent predictor of subsequent nephropathy. However no such consensus exists in NIDDM. Early work suggested that hyper-filtration was not a feature in NIDDM (Fabre et al. 1982; Damsgaard & Mogensen 1986). Support for this view came from Schmitz and colleagues (1989a) who found no evidence of hyperfiltration in 18 NIDDM subjects with a mean duration of diabetes of 5 years. They did however show a modest, but significant, reduction of GFR in 10 newly-diagnosed patients after three months treatment with diet or diet and an oral agent with a more significant reduction in renal size (Schmitz 1989b). The same group have since shown the ratio of GFR to kidney volume to be reduced once microalbuminuria develops, suggesting loss of functioning renal tissue may occur early in the course of progression to proteinuria (Schmitz 1993).

In contrast, in a large controlled study of newly-diagnosed diabetic patients Vora and colleagues (1992) found hyperfiltration in 45% of 110 NIDDM subjects. In 16% the GFR exceeded 140 ml.min^{-1}. Follow up studies are awaited. A smaller, uncontrolled, cross-sectional study from Brazil observed hyperfiltration (definition not given) in 21.1% of 71 NIDDM patients attending an outpatient clinic (Friedman et al. 1992). Two further studies in non-white groups support these findings. Lebovitz and Palmisano (1990) in a study in black Americans recorded hyperfiltration (>140 ml. min^{-1}) in 7 of 20 newly-diagnosed patients (< 2 years). In the Pima Indians, Nelson and colleagues (1996b) found GFR 16% higher in 30 subjects with newly diagnosed diabetes, though this did not predict subsequent decline of GFR.

1.4.1.iii   Microalbuminuria

Microalbuminuria, defined as an albumin excretion of 20 - 200 µg.min^{-1} (Mogensen et al. 1985-6), is a common finding at diagnosis in NIDDM (Collins et al. 1989;

Schmitz et al. 1989b; Martin et al. 1990; Olivarius et al. 1993). Many cross-sectional studies are now in the literature relating albumin excretion to various parameters, including blood pressure, blood glucose and antihypertensive therapy.

In NIDDM, microalbuminuria predicts cardiovascular mortality (Jarrett et al. 1984; Mogensen 1984). However it is less predictive of subsequent proteinuria and renal failure than has been shown for IDDM (Mogensen and Christensen 1984). Table 1.1 (overleaf) summarises selected follow-up studies of microalbuminuria in NIDDM.

It is uncertain if microalbuminuria will prove as useful an indicator of progression of nephropathy in NIDDM as in IDDM. The hypothesis that it reflects more widespread vascular damage suggests that it may not be the case (Deckert et al. 1988, 1992). Furthermore it remains to be seen if a reduction of microalbuminuria in NIDDM halts the decline in GFR demonstrated recently in IDDM (Rossing et al. 1994a).

1.4.2 Invasive and non-invasive markers of progression
1.4.2.i Structural - functional relationships in diabetic nephropathy

Over 30 years ago Gellman and colleagues (1959) described the relationship between the severity of glomerulosclerosis and interstitial fibrosis and plasma creatinine, blood pressure and proteinuria in a mixed group of those with diabetes mellitus. Subsequent work in IDDM has shown a close correlation between proteinuria and ultra-structural changes in the glomeruli of renal biopsies from subjects with advanced nephropathy (Mauer et al. 1984; Østerby et al. 1990). Recently the latter group have examined glomerular structure and renal function in 20 NIDDM subjects (Østerby et al. 1993). Combining light- and electron- microscopic techniques they demonstrated a significant relationship between estimated filtration surface area of each nephron with current GFR. Furthermore a remarkably close association was observed between structural appearance (quantified as the glomerulopathy index,

Study	Number of cases	Duration (years)	Definition of microalbumin	Outcome measure(s)	Findings*	Reference
1	76	9	30-140µg.ml^{-1}	proteinuria	22% v 5%	Mogensen (1984)
2	503	10	15-200µg.min^{-1}	mortality	76-148% RR=1.5	Schmitz (1988)
3	61	7	30-150µg.min^{-1}	progression of UAE	20%	Cooper (1988)
4	439	4	ACR > 30mg.g^{-1}	proteinuria	9-fold	Nielson (1991)
5	228	9	median value 17.4 µg.min^{-1}	mortality	65%**	Damsgaard (1992)
6	8	6.5	30-150µg.min^{-1}	proteinuria	50%	Jerums (1987)

* increase compared to normoalbuminuric subjects with diabetes mellitus (unless stated).

** excess in group with UAE above median.

Table 1.1 : Selected follow-up studies of microalbuminuria in NIDDM

based on the changes in peripheral basement membrane and the mesangial matrix) and the subsequent decline of GFR. Somewhat surprisingly no correlation was found with albuminuria, though both systolic and diastolic pressure were associated.

In a less detailed study over 4 years in a combined group of 41 diabetic subjects, Taft and colleagues (1994) found significant association of interstitial fibrosis and glomerular morphology with creatinine clearance. As in the previous study no correlation between histology and protein excretion was observed. In 19 who underwent repeat biopsy an increase in fibrosis was seen only in those in whom creatinine clearance declined significantly. These findings support the earlier work of Bader and colleagues (1980) and Lane and colleagues (1993) in IDDM.

The degree of interstitial fibrosis and the expansion of the mesangial matrix are thus closely linked to the decline of GFR. Neither offer a reliable measure of disease severity nor are they predictive of rapid progression of nephropathy. In any case routine renal biopsies are neither ethically justifiable nor practically feasible.

1.4.2.ii   *Measurement of glomerular filtration rate - isotopic and surrogate methods*

In practical terms isotopic methods represent the 'gold standard' for measuring GFR. Of the techniques available ^{51}Cr ethylenediaminetetra-acetic acid (EDTA) clearance is the most widely used (Brøchner-Mortensen 1972, 1985). These methods have also, almost completely, replaced inulin clearance in research studies. In diabetic subjects, albeit with normal renal function, repeated measurements have a coefficient of variation of 5.3% to 8.8% - though in a few patients this may rise to 25% (Mau Pedersen et al. 1992; Mogensen et al. 1993). Referring to diabetes mellitus, Mogensen felt that any study into the progression of renal disease should have a minimum of three isotopic measurements of GFR over two years (Mogensen 1993).

Time and expense limit the use of isotopic methods for the determination of GFR. Surrogate measures, viz. creatinine clearance and estimated GFR (e.g. Cockcroft-Gault), are commonplace in clinical practice (Cameron 1992b) and their use quoted frequently in published work. Whilst the validity of the Cockcroft-Gault formula has been demonstrated in IDDM nephropathy (Sampson and Drury 1992), it has not been assessed in NIDDM. These surrogate measures of GFR are discussed in greater detail in Chapter 5.

1.4.2.iii   *Non-invasive markers of disease progression*

Proteinuria is the hallmark of diabetic nephropathy. Sufferers may be identified by routine urinalysis, though confirmation is required with 24-hour urine collections. Prior to effective anti-hypertensive therapy and RRT the onset of proteinuria often heralded an inexorable decline of GFR and death from renal failure or cardiovascular disease. Williams and colleagues (1988) suggested that proteinuria may prove a useful prognostic indicator of progression of established renal failure. Though several renal diseases, including diabetic nephropathy, were examined this association held only for glomerulo-nephritis and chronic pyelonephritis, confirming the experiences of Idelson and colleagues (1977) and Cameron (1979). These observations are further substantiated by the recent work of Petersen and colleagues (1995) and Maschio and colleagues (1996), also in non-diabetic renal disease. 24-hour urinary protein excretion has not, hitherto, proved reliable in predicting outcome in either IDDM or NIDDM (Sawicki and Berger 1994). However Rossing and colleagues (1994a) have shown that an initial anti-proteinuric effect of antihypertensive therapy predicts an attenuated decline of GFR in IDDM subjects with early diabetic nephropathy (GFR >70 ml.min^{-1}). Similar findings have been demonstrated in non-diabetic renal disease (Aperloo et al. 1992). No directly comparable studies exist in NIDDM, though AER at baseline has been shown

recently to be related to subsequent decline of GFR in 34 Pima Indians with macroalbuminuria (Nelson et al. 1996b).

In section *1.4.1.iii* the predictive value of microalbuminuria in NIDDM was briefly discussed. A variety of other urinary markers of disease progression have been investigated though none so far has proved helpful in clinical practice. Howard and colleagues (1991) measured urinary iron in a small number of NIDDM subjects and demonstrated a progressive increase in both iron and transferrin as albuminuria increased. The latter two were closely associated. In both IDDM and NIDDM, Jerums and colleagues (1989) observed a changing pattern of excretion of IgG and transferrin with progression of nephropathy. The ratio of IgG to transferrin correlated with protein excretion in individuals with NIDDM and overt nephropathy. Thus far no reliable urinary or serum marker of progression in advanced nephropathy is available. This subject will be examined in Chapter 6, where the role of collagen and laminin fragments as markers of disease progression is assessed.

1.4.2.iv *Angiotensin-converting-enzyme gene polymorphisms and progression of nephropathy*

The finding of elevated glomerular capillary hydraulic pressure in experimental rat models of diabetes led to the hypothesis by Hostetter and co-workers (1981) that haemodynamic factors initiate the development and promote the progression of diabetic glomerulopathy. Many factors linking hyperglycaemia and intrarenal hypertension have been proposed (Vora et al. 1994). Of these, angiotensin II, amongst other substances, has been shown to stimulate the growth of glomerular cells (Parving et al. 1996a). Serum ACE, which converts angiotensin I to angiotensin II, has been shown to be higher in those subjects with microvascular complications (van Dyck et al. 1994). Furthermore ACEI retard the progression of nephropathy in both human (Bjorck et al. 1986, Lewis et al. 1993) and experimental diabetes (Kakinuma et al. 1992). The ACE gene, therefore, has been proposed as a candidate

gene predisposing individuals with diabetes to the development and progression of nephropathy.

In 1992, Cambien and colleagues demonstrated that serum ACE levels were related to an insertion/deletion polymorphism (ACEID) of intron 16 of the ACE gene, and furthermore that this polymorphism was linked to an increased risk of coronary artery disease. Subsequently, Tarnow and colleagues (1995) examined the distribution of ACEID in 198 IDDM subjects with nephropathy and 192 normoalbuminuric controls, but found no evidence of segregation, concluding that possession of this polymorphism did not confer susceptibility to the development of nephropathy.

Contrasting results have, however, been found in IgA nephropathy. An association of the homozygous deletion genotype with earlier onset and more severe disease was found by Harden and colleagues (1995). Yoshida and colleagues (1995) found 43% of subjects with declining renal function to be homozygous for the deletion (genotype DD), compared to 16% of those with stable renal function. Furthermore this group suggested that the specific ACEID predicted the response to ACE inhibition. However the number of subjects studied was very small.

Recently, Parving and colleagues (1996b) have examined the relationship between ACE gene polymorphism and therapeutic response to ACEI in 35 IDDM subjects (11 genotype DD; 24 genotype ID or II). Using historical data on the decline of GFR over an average of 7 years, they found the mean decline was 5.7 ml.min.$^{-1}$year^{-1} in the homozygous DD group, compared to 2.6 ml.min.$^{-1}$year^{-1} in the ID and II groups, despite comparable blood pressure lowering. Further studies in IDDM are required before this polymorphism can be adopted as a marker identifying those subjects who will fare badly once nephropathy develops.

1.5 BROAD AIMS OF THE THESIS

1.   'What factors influence the rate of progression of nephropathy in NIDDM subjects with impaired renal function ?' To try to answer this question the historical records of all individuals attending a joint diabetic-renal clinic were examined. Data were available in 87 (32 IDDM and 55 NIDDM) subjects in whom progression could be determined from the serial serum creatinine measurements. In Chapter 2 the factors, including race, which may determine the decline of GFR in this cohort are examined. Furthermore the rates of decline of GFR in NIDDM and IDDM are compared. The study also seeks to determine whether attendance at such a clinic has a beneficial effect on ameliorating the rate of decline of GFR.

2.   In the following chapter the influence of renovascular disease on progression is addressed. All subjects under-going percutaneous renal artery angioplasty in the clinic were assessed to determine whether this procedure has a beneficial effect in halting the progression of nephropathy.

3.   From the above cohort, 26 individuals with NIDDM were selected for a two year prospective study. The aims were to determine the decline of GFR using a validated technique (^{51}Cr EDTA clearance) and to determine the factors which were associated with this decline.

4.   The simultaneous measurement of creatinine clearance and serum creatinine allowed a direct comparison of surrogate measures of GFR with EDTA clearance. The objective here was to examine whether either creatinine clearance or estimated GFR offered a practical alternative to isotopic GFR measurement in NIDDM, as has been shown in IDDM.

5.   Certain individuals with nephropathy appear to progress rapidly to end-stage renal failure, whereas other, apparently similar, individuals fare much better. Clinico-

27

pathological studies using renal biopsy tissue suggest that structural changes in the renal glomerulus bear a direct relationship to decline in renal function in NIDDM. Type IV collagen and laminin are principle components of the basement membrane and mesangial matrix in the glomerulus. The hypothesis that collagen and laminin synthesis are increased in those individuals with more aggressive renal disease is examined in Chapter 6. A recently developed assay for mixed laminin fragments was used to determine whether the measurement of these proteins in the urine of nephropathic subjects offers a reliable non-invasive marker of progression of nephropathy in NIDDM. The claims that type IV collagen could be such a marker were also investigated.

CHAPTER 2

Observational study in individuals with diabetic nephropathy and moderate renal impairment attending a specialist diabetic-renal clinic

2.1 INTRODUCTION

The diabetic-renal clinic was set up at King's College Hospital in 1986 and is one of the largest of its kind in the United Kingdom. All individuals with diabetes mellitus and renal impairment ('cut-off' serum creatinine of 200 μmol.L^{-1}) are referred to this clinic, both from the general diabetes clinic and direct from General Practice. In the majority, diabetes is the cause for the renal impairment, although in a small proportion a different primary renal disease is present. A small number of IDDM subjects, with less severe renal impairment, are also seen. The primary aim of the clinic is to concentrate care on those with renal failure in an attempt to slow the progression of their disease and to respond to the greater clinical need of these individuals, many of whom suffer the full range of complications of diabetes. In addition it facilitates co-ordination with the Nephrologists. The frequency of visits varies from every 1 to 4 months. When the serum creatinine reaches approximately 400-500 μmol.L^{-1}, or when symptoms dictate, individuals (5-8 per year) are considered for RRT. This is usually CAPD; very occasionally haemodialysis or immediate transplantation. It is exceptional for a patient to be turned down for dialysis. Individuals in whom renal function remains stable, or improves, may return to the general clinic.

This study assessed the first seven years experience of this clinic to determine the factors associated with disease progression and to examine how effective the clinic was in delaying RRT. The specific aims were as follows:

2.2 AIMS.

- To determine the present rate of progression of renal failure in patients with diabetes mellitus and renal impairment.

- To determine if racial differences and type of diabetes influence the rate of progression of renal impairment.

- To examine the effect of blood pressure and anti-hypertensive therapy on the rate of decline of glomerular filtration.

- To assess whether regularly measured parameters, e.g. glycosylated haemoglobin, serum lipids and urinary protein excretion are associated with the rate of change of glomerular filtration rate.

- To assess whether attendance at a specialist clinic confers benefit by reducing the rate of deterioration of glomerular filtration.

2.3   PATIENTS AND METHODS

2.3.1   Data collection

150 individuals attended the joint diabetic-renal clinic in the seven years from June 1986 to May 1993, with 80 - 90 current attendees at the end of the period. The notes of all patients, including those who had died, were scrutinized and demographic details, together with all data from each attendance, including the visits prior to commencing the joint clinic, were entered onto computer (Dell Products, Austin, Texas; Excel software). A list of all data entered is given in Table A2.1 in the appendix. In all over 100,000 data entries were made.

Blood glucose was analysed at each visit, whilst HbA_{1c} and serum creatinine were measured at each combined clinic attendance but only intermittently prior to joining. All attendees had at least an annual 24-hour urine collection for protein performed. Only data from those where three collections were available are included in the analysis. Serum lipids were not measured routinely in the early stages of the renal clinic and again data are included only if three or more samples were available. There were few data on HDL and LDL cholesterol. These along with the small

number of samples of urinary urea and electrolytes were not entered into the database.

Inevitably blood pressure was measured by a number of observers over the seven-year period. All were performed, usually at the end of the consultation, using a standard mercury sphygmomanometer with the patient seated. Early measurements were often recorded to 5 mmHg, but more recently all have been to 2 mmHg. (BMJ guidelines - Petrie et al. 1987). Anti-hypertensive and lipid lowering therapy, with any change, were entered. BMI was calculated from weight(kg)/height^2(m) and estimated GFR (cGFR) from the serum creatinine, using the Cockcroft-Gault formula (details as given in Chapter 5).

2.3.2   Data analysis

Only subjects with a cGFR below 80 ml.min^{-1} at the start of the observation period and in whom a minimum of six serum creatinine measurements had been performed over at least a two year period were included in this analysis.

All cGFR data points were plotted against time (as shown overleaf in Figure 2.1) for all individuals. The rate of change of GFR was calculated by linear regression analysis (the least squares method) using all data points. To determine whether the decline of cGFR changed significantly over the observation period, a separate analysis was performed comparing the initial one and two years data with the most recent one and two years. Before carrying out this analysis each graph was inspected visually to identify any in which aberrant cGFR measurements (taken as those $\geq$ 3SD's from the mean) might produce spurious results; such readings were excluded from the analysis. An example of such aberrant values is shown in the appendix (Figure A2.1 - middle graph - cluster of three cGFR measurements excluded). Of the 87 graphs examined, single values were excluded in seven cases (3 IDDM; 4 NIDDM), two values in one patient (NIDDM) and three values, also in one (NIDDM
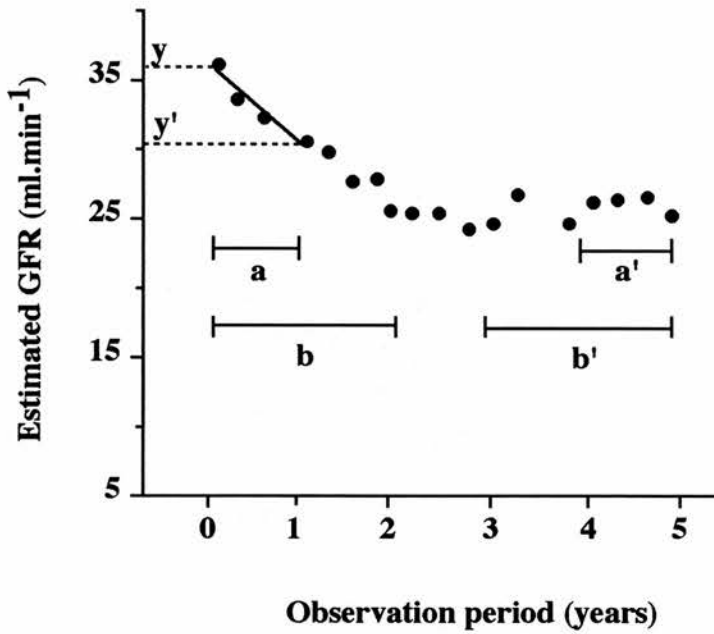
Figure 2.1: Diagramatic representation of decline of estimated GFR. a, a' and b, b' delineate the first and last, and the first two and last two years of observations respectively.

- above example). Thus for each graph the rate of change of estimated GFR {ml.min.$^{-1}$month^{-1}} over the first year is calculated for "a". Similar analyses were performed for a', b, and b', allowing comparison of "a" with "a'", and "b" with "b'" (see Figure 2.1).

An identical exercise was carried to determine cGFR before and after entering the joint clinic. For inclusion each individual must have had at least four visits over a 12 month period both before and after entering the joint clinic.

The data were analysed using the 'Cricketgraph II' programme (Stream Valley Parkway, Malvern, PA, USA) on an Apple Macintosh LC computer.

2.3.3 Statistical analysis

Results are expressed as mean (±SD), unless the data are skewed, where median (range) is given. The values for 24-hour protein were logarithmically transformed and the data presented as geometric mean and range. Comparison of the decline in cGFR between the racial groups and diabetes types over the whole period of observation was by Student's t-test for unpaired data, whilst comparison of the one and two year data periods at the beginning and end of observation was by Student's t-test for paired data. The factors thought to associate with decline in cGFR were examined initially with a univariate analysis, using Pearson's correlation coefficient. A multiple regression analysis was then performed. In the first instance all 87 subjects were included with cGFR the dependent variable. The continuous variables entered were age, duration of diabetes, HbA$_{1c}$, systolic blood pressure (SBP) and diastolic blood pressure (DBP), serum cholesterol, serum triglyceride, and log 24-hour protein as was the dichotomous variable, blood pressure therapy at outset (classed as present/absent). A sub-group analysis of the IDDM and NIDDM subjects was undertaken although, as shown below, this lessened the strength of associations seen for the whole group.

For both IDDM and NIDDM, the groups were divided into 'Responders' and 'Non-responders' on the basis of the median decline of cGFR. An individual with a decline of cGFR of < median was termed a 'Responder', whereas > median a 'Non-responder'. The parameters measured (i.e. the above independent variables) were then compared between the 'Responder' and the 'Non-responder' group, using the Students unpaired 't-test'. Throughout significance is assumed when $p \leq 0.05$ (two-tailed). The statistical packages 'Statview' (Brain Power, Inc., Calabasa, CA. 91302, USA) and 'Statistica' (StatSoft Inc., Tulsa, Oklahoma) were used throughout.

2.4   RESULTS

2.4.1   Patient groups

Of the 150 subjects who attended the clinic, sufficient data meeting the above criteria were available for analysis in 96. A further nine of these were excluded on the grounds of other renal disease {systemic vasculitis 2, amyloidosis 1, HIV nephropathy 1, unknown - bilateral small kidneys - 5}. The data are presented on 87 subjects; 32 IDDM and 55 NIDDM. All were deemed to have diabetic nephropathy on the basis of the definition given in section *4.3.1.iii* of Chapter 4. Table 2.1 shows the demographic data, viz. racial origin, age and duration of diabetes in the two groups.

	IDDM	NIDDM
Subjects	32	55
Race (Cauc:Af:As)	31:1:0	23:26:6
Age (Current)	50.5 (24.3 - 66.6)	63.0 (39 - 79)
Duration of DM	31.1 (15.8 - 51.9)	18.6 (4.1 - 36.7)

Key: Cauc=Caucasian; Af=Afro-Caribbean; As=Asian. Age and DM duration are median (range).

Table 2.1 : Demographic characteristics of the study groups.

Table 2.2 shows the number of patients followed for at least two and four years. 68 subjects (28 IDDM) were observed for at least 4 years. The median duration of follow up (that is from the time cGFR falls to 80 ml.min^{-1}, or below, to the most recent visit) for all subjects was 6.4 years (range 2.0-19.3); for IDDM and NIDDM the values were 7.4 (2.0-19.3) and 6.0 (2.1-17.9) years respectively (see Table 2.3 overleaf). Of this cohort 36 individuals (17 IDDM) commenced RRT during this period. The median duration from diagnosis of diabetes to RRT in the IDDM group was 27 (range 16-48) years and 14 (4-31) years in the NIDDM group.

Observation period	Caucasian	Afro-Caribbean	Asian
IDDM			
Two years	31	1	-
Four years	27	1	-
NIDDM			
Two years	24	25	6
Four years	16	19	5

Table 2.2 : Number of subjects for whom sufficient data are available for two and four year comparisons.

2.4.2 Change in estimated GFR

The median decline of cGFR for all 87 individuals was 0.39 ml.min.$^{-1}$month^{-1} (range 1.92 to -0.22). The rate of fall was significantly faster in NIDDM (0.43 ml.min.$^{-1}$month^{-1} vs 0.29 ml.min.$^{-1}$month^{-1}; unpaired 't-test'; $p<0.05$), despite similar baseline cGFR (57 vs 54 ml.min^{-1} resp.). This is graphically represented in Figure 2.2. (over).

	Caucasian	Afro-Caribbean	Asian	Total
IDDM				
Decline of eGFR (ml.min⁻¹.month⁻¹)	0.29*	0.24#	-	0.29♦
Observation period (years)	7.4 (2.0-19.3)	NA	NA	7.4 (2.0-19.3)
NIDDM				
Decline of eGFR (ml.min⁻¹.month⁻¹)	0.73	0.43**	0.38	0.43
Observation period (years)	5.4 (2.1-13.8)	6.3 (2.6-17.9)	7.2 (2.4-9.5)	5.9 (2.1-17.9)

Key : # single subject; NA = not applicable. Results given as median (range)
♦ $p < 0.05$ (Total IDDM vs NIDDM); * $p = 0.005$ (Caucasian IDDM vs Caucasian NIDDM); ** $p < 0.05$ (Afro-Caribbean NIDDM vs Caucasian NIDDM)

Table 2.3 : Rate of decline in estimated GFR over the total observation period, subdivided by diabetes type and racial origin
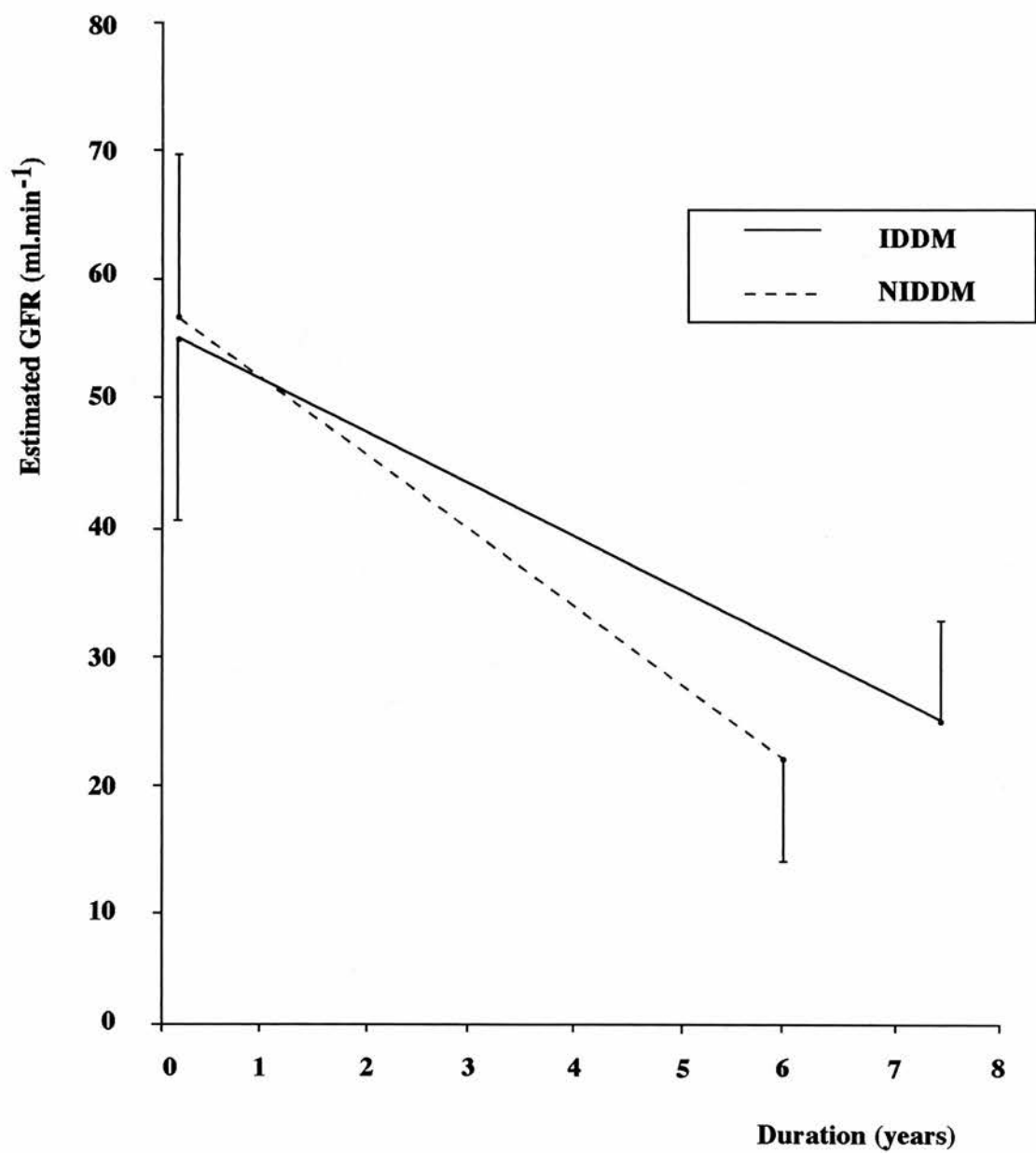
Figure 2.2 : Decline of cGFR in IDDM and NIDDM subjects. SD bars shown.

The difference between IDDM and NIDDM is more striking when the Caucasian subjects are considered alone, with declines of cGFR of 0.29 and 0.73 ml.min.⁻¹month⁻¹, IDDM vs NIDDM respectively (*p*=0.005) {Table 2.3}. Contrary to expectation, cGFR declined faster in Caucasians compared to Afro-Caribbeans with NIDDM.

Not all individuals exhibit a linear decline of cGFR. Table 2.4 segregates subjects by the mode of decline of cGFR. Non-linear progression is defined as more than one significant excursion from the regression line and where the regression coefficient for cGFR v time is ≤0.8 (adapted from Brazy and Fitzwilliam 1990). Individuals with a curvilinear decline of cGFR are included in the above analysis as linear. Eight subjects with a mean baseline creatinine of 170 µmol.L⁻¹ (range 117-244) had stable renal function over the observation period.

	Linear	Non-linear	Stable
IDDM	17	10	5
NIDDM	46	6	3
Total	63	16	8

Table 2.4 : Progression of estimated GFR in diabetic subjects.

Examples of progression are given in the appendix in Table A2.1

The results of comparing the decline of cGFR between the initial and final years are shown in Table 2.5 (overleaf). All subject comparison suggests a trend toward a reduction in the decline of cGFR. The two-year comparison shows an improvement from 0.44 to 0.34 ml.min.⁻¹month⁻¹, (paired 't-test'; NS) whereas the one-year

	One year		Two year	
	First	Last	First	last
IDDM (n=32)				
Caucasian (n=31)	0.37	0.07	0.38	0.19
Afro-Caribbean (n=1)	0.37	-0.07	0.95	0.02
Total	0.37	0.07	0.39	0.18
NIDDM (n=55)				
Caucasian (n=24)	0.64	0.48	0.41	0.48
Afro-Caribbean (n=25)	0.44	0.37	0.49	0.26*
Asian (n=6)	0.38	0.22	0.75	0.23
Total	0.46	0.38	0.48	0.32
All subjects	0.44	0.23**	0.43	0.34

* $p < 0.02$ (Afro-Caribbean subjects, first and last two years comparison)

** $p < 0.05$ (All subjects: first and last one year comparison)

The number of subjects are given for the two year comparisons

Table 2.5 : Rate of decline of estimated GFR - comparison of first and last one
and two years data

comparison shows a significant change from 0.43 to 0.23 ml.min.$^{-1}$month^{-1} (paired 't-test'; $p<0.05$). This improvement appears more marked in IDDM patients, with the two year comparison improving from 0.38 to 0.19 ml.min.$^{-1}$month^{-1}. However this is not statistically significant because of the wide variation in the data (two subjects had a markedly worse decline of cGFR in the most recent year of observation). The virtual lack of decline of cGFR in the most recent year in the IDDM subjects is striking.

Of 32 IDDM subjects, only eight (25%) had a decline of cGFR above 0.5 ml.min.$^{-1}$month^{-1} in the most recent year. This apparent arresting of decline of cGFR is not observed in NIDDM subjects, where the average decline of cGFR in the most recent year of observation is 0.38 ml.min.$^{-1}$month^{-1}.

In NIDDM no difference in the decline of cGFR is seen for Caucasian subjects in either the one or two year comparisons. However in the Afro-Caribbean group there is a significant reduction of decline of cGFR from 0.49 to 0.26 ml.min.$^{-1}$month^{-1} between the initial and final two years (unpaired 't-test'; $p<0.02$) {Table 2.5}. The small number in the Asian group precludes a meaningful interpretation of the apparent improvement seen in their cGFR decline.

2.4.3   Decline of estimated GFR before and after attendance at a joint diabetic-renal clinic.

Sufficient data, i.e. at least four serum creatinine measurements within a minimum of twelve months before and after entering the clinic, were available for 58 individuals (23 IDDM). As shown overleaf (Table 2.6 over) the mean period of observation before entering the joint clinic was 6.8 (range 1.4 - 15.1) years for the IDDM group and 4.4 (1.0 - 12.8) years for the NIDDM group. The respective duration of attendance at the clinic were 2.9 (1.0 - 5.7) and 2.9 (1.0 - 6.2) years respectively.

The mean (SD) decline of cGFR for IDDM was 0.49 (0.32) ml.min.$^{-1}$month^{-1} before

and 0.18 (0.32) ml.min.$^{-1}$month^{-1} after entering the clinic (paired 't-test'; $p<0.001$).

The respective figures for the NIDDM group were 0.48 (0.36) and 0.41 (0.31)

ml.min.$^{-1}$ month^{-1} (paired 't-test'; p=NS). Systolic blood pressure did not differ in

either group following attendance at the joint clinic. However diastolic blood

pressure fell significantly, from 90 to 84 mmHg in the IDDM group and 94 to 89

mmHg. in the NIDDM group (unpaired 't-test'; both $p<0.005$). In both groups the

use of anti-hypertensive medication increased significantly, from 9 to 22 out of 23 in

IDDM and 17 to 31 of 35 in NIDDM (Chi-squared test: $p<0.001$). As shown below

duration of attendance at the clinic per se does not appear to influence the decline of

cGFR in either IDDM or NIDDM subjects.

	Duration pre-clinic (years)	Duration post -clinic (years)	Decline of cGFR pre-clinic	Decline of cGFR post-clinic
IDDM (n=23)	6.8 (1.4-15.1)	2.9 (1.0-5.7)	0.49 (0.32)	0.18 (0.32) *
NIDDM (n=35)	4.4 (1.0-12.8)	2.9 (1.0-6.2)	0.48 (0.36)	0.41 (0.31)

Key : decline of GFR given as ml.min.$^{-1}$month^{-1}. Data given as median (range) for duration of attendance and mean (SD) for decline of estimated GFR. * $p<0.001$.

Table 2.6 :   Decline of estimated GFR in IDDM and NIDDM subjects before and after commencing attendance at the joint diabetic-renal clinic.

2.4.4   Factors responsible for decline in GFR

Figures 2.3 and 2.4 (overleaf) show the results of univariate analysis between cGFR

and selected clinical parameters for IDDM and NIDDM subjects respectively. For

both groups, only 24-hour protein excretion is significantly associated with cGFR

decline.
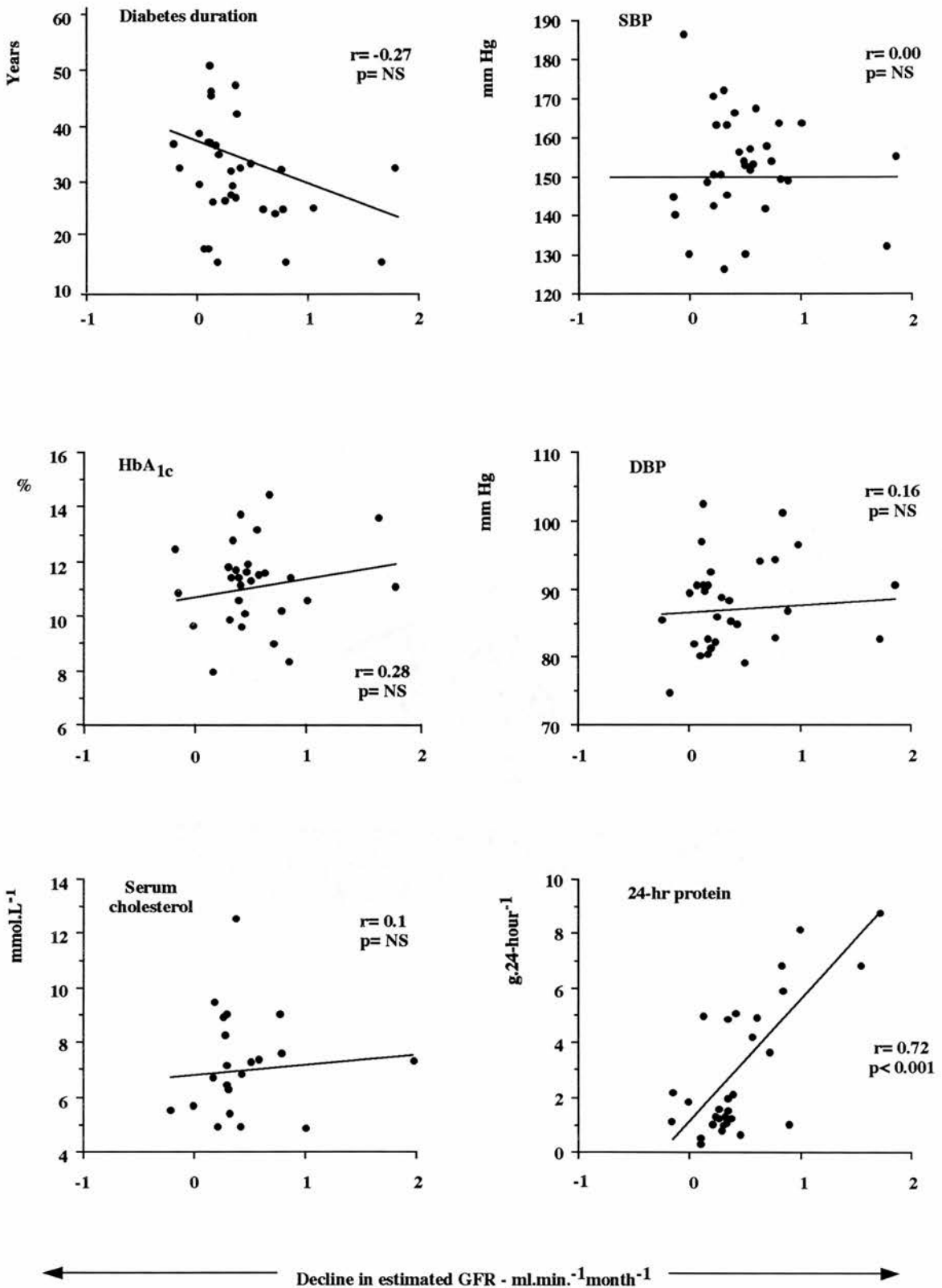
Figure 2.3 : Association between the rate of decline of estimated GFR and measured clinical parameters in insulin-dependent subjects. Univariate analysis.
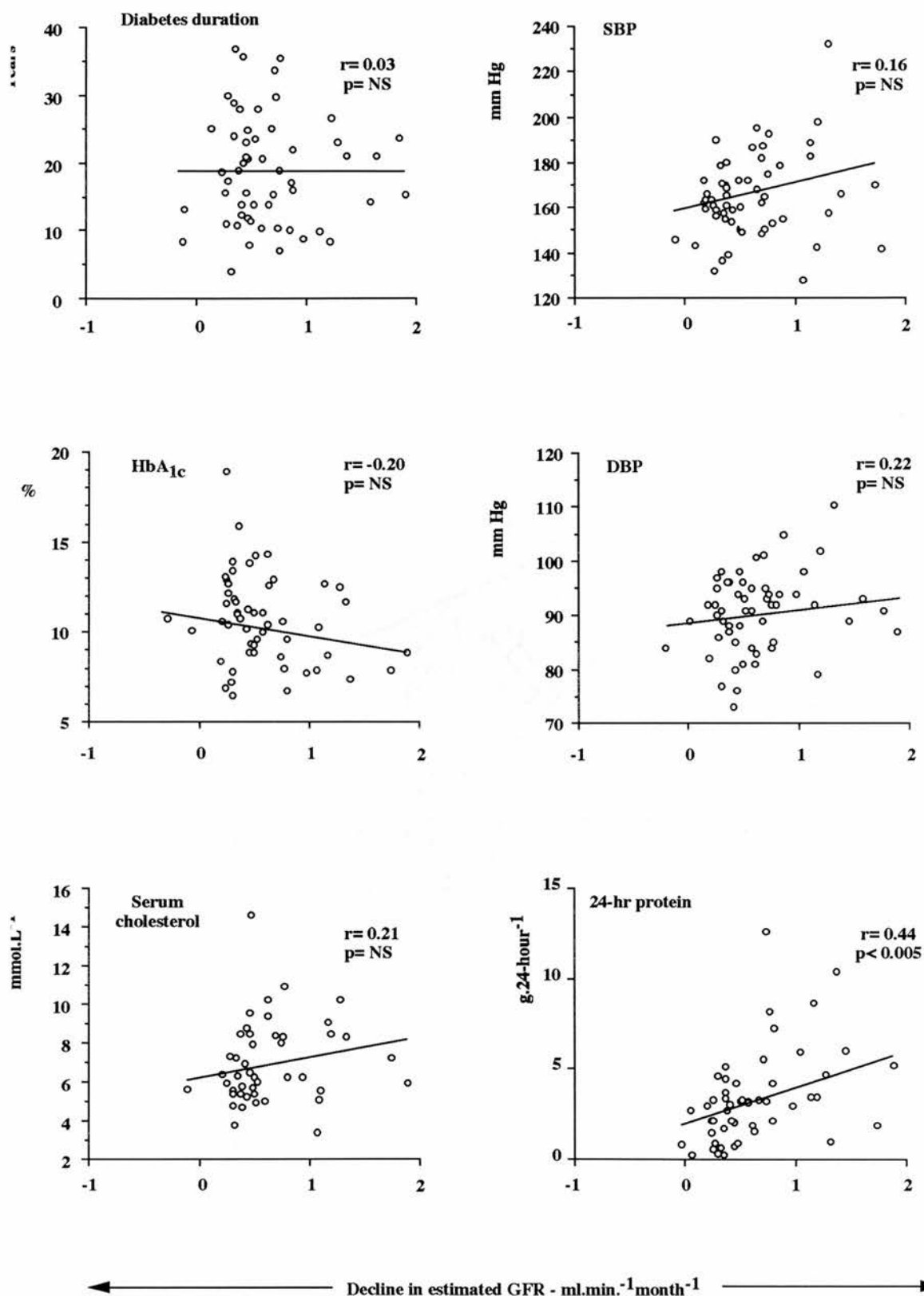
Figure 2.4: Association between the rate of decline of estimated GFR and measured clinical parameters in non-insulin-dependent subjects. Univariate analysis.

Multiple regression analysis reveals a highly significant association of 24-hour protein excretion ($p<0.00005$) with the decline of cGFR when all subjects are grouped together. Diastolic blood pressure is the only other variable to emerge as significantly associated with decline of cGFR ($p<0.05$). No significant association is seen with BMI, age, duration of diabetes, HbA_{1c}, serum cholesterol, systolic blood pressure and anti-hypertensive therapy. Proteinuria and DBP together account for 34% of the variation in the decline in cGFR in this group. The following regression equation (with a standard error of 4.3) links decline of cGFR with these variables:

Annual rate of change of cGFR = 10.2 - 1.1xlog 24-hr protein - 0.5xDBP
($ml.min.^{-1}year^{-1}$)

If the groups are subject to a separate multiple regression analyses, being on anti-hypertensive therapy at the outset is significantly associated ($p<0.05$) with decline of cGFR in NIDDM, but not in IDDM. Diastolic blood pressure is no longer a significant variable when the group is subdivided by diabetes type.

To determine the factors that may be responsible for the more rapid decline of GFR in NIDDM the measured parameters were compared between the groups. IDDM patients are naturally younger than NIDDM and have a longer duration of diabetes. Table 2.7 (overleaf) shows prevailing systolic and, to a lesser degree diastolic, blood pressure to be significantly higher in those with NIDDM. When the final two years are examined separately these differences are less pronounced. As would be expected BMI is significantly greater for NIDDM, but no differences exist for HbA_{1c}, serum cholesterol or 24-hour protein excretion.

2.4.5   Is there a difference between those who fare well and those who fare badly ?

To identify any factor which has a determining effect on renal function both IDDM and NIDDM subjects have been divided into equal subgroups on the basis of cGFR.

Parameter	IDDM (n=32)	NIDDM (n=55)	Difference	Significance
Age (years)	50.0 (11.0)	62.8 (9.1)	12.8	$p<0.001$
Duration of DM	32.2 (9.5)	18.7 (8.1)	13.5	$p<0.001$
cGFR	0.41 (0.42)	0.60 (0.41)	0.29	p=0.03
SBP (mmHg)	154 (14)	165 (19)	11	p=0.05
DBP (mmHg)	87 (7)	91 (7)	4	$p<0.05$
BMI	25.1 (4.3)	28.8 (4.2)	2.7	p=0.001
HbA_{1c}	11.2 (1.5)	10.7 (2.5)	-0.5	NS
Cholesterol ($mmol.L^{-1}$)	7.0 (1.8)	7.0 (2.1)	0	NS
24-hour protein (g)	2.9 (2.5)	3.4 (2.6)	0.5	NS
SBP (2 year)	153 (21)	167 (23)	14	p=0.005
DBP (2 year)	86 (8)	89 (9)	3	NS

Data are given as mean (SD). cGFR given as $ml.min.^{-1}month^{-1}$

Key : SBP = systolic blood pressure, DBP = diastolic blood pressure
2 year = last two years of blood pressure recordings

Table 2.7 : Comparison of the measured variables between IDDM and NIDDM. The data are compared using unpaired 't-test'.

Table 2.8a: IDDM

	Responders	Non-responders	Difference	Significance
Decline of cGFR	0.14 (0.15)	0.67 (0.44)	- 0.53	-
Number (M:F)	16 (7:9)	16 (9:7)	-	-
Age	55.1 (8.3)	44.2 (10.8)	10.9	$p<0.01$
Duration of DM	35.6 (9.0)	28.9 (9.0)	6.7	$p<0.05$
BMI	25.1 (4.4)	25.1 (4.2)	0	NS
Systolic BP (mmHg)	156 (15)	152 (13)	4	NS
Diastolic BP (mmHg)	88 (7)	87 (6)	1	NS
HbA$_{1c}$ (%)	10.8 (1.2)	11.6 (1.8)	-0.8	NS
Cholesterol (mmol.L^{-1})	6.7 (1.2)	7.5 (2.1)	-0.8	NS
24-hour protein (g)	1.5 (1.1)	4.2 (2.6)	-2.7	$p=0.01$

Table 2.8b: NIDDM

	Responders	Non-responders	Difference	Significance
Decline of cGFR	0.30 (0.12)	0.93 (0.36)	-0.63	-
Number (M:F)	28 (19:9)	27 (15:12)	-	-
Race (Cauc:Af:As)	10:14:4	11:14:2	-	-
Age	67.3 (6.1)	58.2 (9.4)	9.1	$p<0.001$
Duration of DM	18.7 (8.3)	18.7 (8.0)	0	NS
Systolic BP (mmHg)	162 (13)	168 (2.3)	-6	NS
Diastolic BP (mmHg)	88 (7)	93 (7)	-5	$p<0.02$
HbA$_{1c}$ (%)	10.9 (2.8)	10.5 (2.1)	0.4	NS
Cholesterol (mmol.L^{-1})	6.7 (2.2)	7.4 (2.0)	-0.7	NS
24-hour protein (g)	2.1 (1.4)	4.7 (2.9)	-2.6	$p<0.001$

Key : GFR = glomerular filtration rate (ml.min.$^{-1}$month^{-1}) as estimated by the Cockcroft-Gault formula. Results as mean (SD)

Table 2.8: Characteristics of the 'Responders' and 'Non-responders' (for definition - see text). Insulin-dependent subjects (Table 2.8a) and non-insulin-dependent diabetic subjects (Table 2.8b). The data are compared by unpaired 't-test', significance as shown.

Those with a lower rate of decline of cGFR are termed 'Responders' and those with the greater rate of decline the 'Non-responders'. The results of these analyses are presented in Tables 2.8a and 2.8b (preceding page).

2.4.5.i   IDDM subjects

In IDDM the division was a median decline of cGFR of 0.29 ml.min.$^{-1}$month^{-1}, giving a mean decline for 'Responders' and 'Non-responders' of 0.14 and 0.67ml.min.$^{-1}$month^{-1} respectively. Surprisingly age was a significant factor with 'Responders' being, on average, 11 years older than the 'Non-responders' (unpaired 't-test'; p<0.01). Perhaps as a consequence, duration of diabetes was some 6.5 years longer in the 'Responders' (p<0.05). The only other factor which differed between the two groups was 24-hr protein excretion, being some three-fold greater in the 'Non-responders' (p=0.01). Blood pressure (all readings) was not significantly different between the two groups.

Neither the number of patient visits per year nor the duration of attendance at the renal clinic differed between the groups. Those attending more frequently were, not surprisingly, mostly to be found in the Non-responder group. The number of subjects on anti-hypertensive therapy at the start of the observation period was similar, approximately 50%, in both groups, whilst at the end the figure was close to 100%.

2.4.5.ii   NIDDM subjects

In NIDDM the division was a median decline of cGFR of 0.43 ml.min.$^{-1}$month^{-1}, giving a mean decline of 0.28 vs 0.91 ml.min.$^{-1}$month^{-1}, 'Responders' vs 'Non-responders'. In this group the 'Responders' were again significantly older than the 'Non-responders' by some nine years on average (unpaired 't-test'; p<0.001). In contrast the duration of diabetes was identical between the groups at 18.7 years; some 15 years on average shorter than for IDDM patients. As was the case for the latter mean 24-hour protein excretion is significantly higher in the 'Non-responders',

averaging 4.67g against 2.14g for the 'Responders' (p<0.001). Mean diastolic and systolic blood pressure are lower in the 'Responders' but to a significant degree only in the former (p<0.02). As in IDDM no difference exists with HbA_{1c}, serum cholesterol nor with visit frequency or duration of clinic attendance. Fewer 'Responders' than 'Non-responders' were treated for hypertension at the start, whereas the reverse was true at the end of the study.

2.4.6   Anti-hypertensive usage

Tables 2.9, 2.10a and 2.10b (overleaf) show the usage of anti-hypertensive medication in IDDM and NIDDM subjects. Two factors emerge for both groups. Many subjects with renal failure were on no anti-hypertensive medication in the first year of observation despite a cGFR of $\leq$ 80ml.min^{-1} - only 18/32 (56%) IDDM and 32/55 (57%) NIDDM. By the final year of observation this figure was 1/32 (3%) and 5/55 (9%) respectively.

	IDDM		NIDDM	
	Responders	Non-responders	Responders	Non-responders
	(n=16)	(n=16)	(n=27)	(n=28)
First year	7	8	14	18
Final year	16	15	27	23

Table 2.9 :   Number of patients receiving anti-hypertensive therapy in the first and last year of observation.

During this last year of observation each patient was taking on average two anti-hypertensive drugs in contrast to the first year for the IDDM group of 0.72 and 0.64 drugs/patient for the NIDDM group. The rise in usage of the combination of an

Table 2.10a: IDDM

	First year	Second year	Penultimate year	Final year
ACE inhibitor	4	7	18	24
Beta-blocker	10	8	3	4
Ca^{++} channel antagonist	2	2	7	11
Diuretic	6	9	16	23
Other	1	1	0	0
No therapy	18	14	4	1
Average no. drugs/person	0.72	1.00	1.63	1.94

Table 2.10b: NIDDM

	First year	Second year	Penultimate year	Final year
ACE inhibitor	9	13	33	41
Beta-blocker	8	12	5	4
Ca^{++} channel antagonist	5	10	25	29
Diuretic	12	15	32	40
Other	1	1	2	2
No therapy	32	22	7	5
Average no. drugs/person	0.64	1.02	1.96	2.07

Table 2.10: Anti-hypertensive usage in insulin-dependent (Table 2.10a) and non-insulin- dependent (Table 2.10b) diabetic subjects

ACEI and frusemide mirrors the decline in the use of beta-blockade in both groups. Five of 28 NIDDM subjects received no drug therapy in the final year of observation (four were normotensive, BP ≤ 135/80 mmHg, and one unreliable with medication). The median decline of cGFR for these individuals was 0.26 ml.min.$^{-1}$month^{-1} as against 0.43 ml.min.$^{-1}$month^{-1} for all NIDDM subjects.

2.5   DISCUSSION

The natural progression of renal impairment in untreated patients is unknown for NIDDM nephropathy and is likely to remain so given the established role of anti-hypertensive therapy in chronic renal failure. This study has examined the evolution of renal impairment, and the factors associated with its progression, in a cohort of 87 subjects with diabetic nephropathy under routine clinic conditions.

2.5.1   The progression of renal impairment.

The median decline of cGFR of all subjects, with an initial mean cGFR of 56 ml. min^{-1}, followed for an average of 6.4 years, was 0.39 ml.min.$^{-1}$month^{-1}. The rate of decline was significantly faster in NIDDM, even though baseline cGFR was similar. No directly comparable published study exists. The closest, by Gall and colleagues (1993), found a fall of 0.48 ml.min.$^{-1}$month^{-1} in 26 Caucasian NIDDM subjects with biopsy-proven diabetic glomerulosclerosis, though they had slightly better baseline renal function (mean creatinine 95 vs 135 μmol.L^{-1} in this study). In a recently-published work in the Pima Indians, Myers and associates (1995) recorded a decline of GFR from 108 to 71 ml.min^{-1} over a four-year period (equivalent to 0.77 ml.min.$^{-1}$ month^{-1}).

It remains to be determined if the rate of decline of GFR is similar for IDDM and NIDDM once nephropathy is established. Similar rates of progression were observed in 32 pre-dialysis subjects by Biesenbach and associates (1994). The rates of decline

of cGFR at 1.05 and 0.91 ml.min.$^{-1}$month^{-1} respectively are significantly more rapid than seen in this study. Ordonez and colleagues (1989) drew the same conclusion, based on the time from proteinuria to ESRD. However such a deduction may not be valid since proteinuria is more likely to be associated with normal GFR in NIDDM. Others, including Pugh and colleagues (1993) and Friedman and Gross (1991), suggest that progression may indeed be slower in NIDDM. The reasons for the more rapid rate of progression for NIDDM subjects in this study are discussed below.

Rutherford and colleagues (1977) were among the first to suggest that the type of primary renal disease did not influence the rate of disease progression. Their study included eight individuals with diabetes mellitus. This view has been challenged (Williams and Mallick 1993) and recent studies indicate disease specific rates of decline of cGFR, though with considerable individual variation. Diabetes mellitus compares unfavourably with other conditions and appears to show the greatest variation.

Williams and colleagues (1988) observed more rapid progression in 14 diabetic individuals (unclassified for IDDM and NIDDM) compared to 13 with chronic pyelonephritis and 9 with polycystic kidney disease. In a retrospective study Hannedouche and colleagues (1989) assessed the progression of renal failure in 167 patients (only four with diabetes - all NIDDM) already on dialysis; all had a creatinine in excess of 500 μmol.L^{-1} at baseline. Using the Cockcroft-Gault equation the mean decline for the four diabetic subjects was 1.06 ml.min.$^{-1}$month^{-1} comparable to chronic glomerulonephritis (n=55) at 1.07 ml.min.$^{-1}$month^{-1}, though significantly greater than chronic pyelonephritis, polycystic kidney disease and nephrosclerosis. Only a small group of individuals with Alport's syndrome progressed more rapidly. In a dated study, Ahlmén and colleagues (1975) also observed more rapid progression in those with diabetes. The mean duration from 'elevated creatinine' to terminal renal failure and dialysis (death for the diabetic subjects) varied from six

months in 24 subjects with diabetes to 18.1 months for 11 with polycystic kidney disease. The validity of this study must be questioned as data on 'baseline creatinine' is lacking.

In a recent retrospective study of 159 subjects with non-diabetic renal disease, Jungers and colleagues (1995) concluded that primary renal disease and the level of protein excretion were the principal determinants of progression. The baseline creatinine clearance was comparable to the current study, yet of the various groups only chronic tubulo-interstitial nephritis showed a slower decline of GFR than seen in our diabetic group. This supports the assertion that the overall progression in this cohort is encouragingly slow.

Early studies suggested that the decline of GFR in renal failure was linear (Mitch et al. 1976). Jones and colleagues (1979) gave substance to this view observing a linear fall of reciprocal creatinine in 9 subjects with IDDM and a serum creatinine over $200\mu mol.L^{-1}$. However, Williams and colleagues (1988), in an analysis of 108 patients with various renal diseases (14 with diabetes mellitus), recorded linear progression in 70 (65%), non-linear progression in 15 (14%) and no progression in 23 (21%). For those with diabetes mellitus linear progression occurred in twelve and non-linear in two. No subject maintained stable renal function. In miscellaneous renal conditions, though not including diabetic nephropathy, Stenvinkel and colleagues (1989), in contrast, found stable renal function in 17 of 108 (16%) subjects. Despite significant renal impairment, eight individuals (3 with NIDDM) in our cohort show stable renal function even after 7.5 years of follow-up. This raises the tantalising prospect of halting the progression of renal failure in diabetes mellitus, at least in certain individuals, where previously an inexorable slide towards dialysis was deemed inevitable.

2.5.2   Factors associated with the progression of renal impairment.

2.5.2.i   *Gender and age and progression of renal impairment*

A male preponderance in renal disease is well established both for diabetes mellitus (Krolewski et al. 1985; Kofoed-Enevoldsen et al. 1987) and non-diabetic renal disease (Finn and Harmer 1979). A significant male excess was seen only in NIDDM in this study. No difference was observed in the rate of decline of GFR between the sexes (0.41 ml.min.$^{-1}$month^{-1} vs 0.43 ml.min.$^{-1}$month^{-1}; male vs female). This contrasts with the studies of Hannedouche and colleagues (1993) and Jungers and associates (1995), where the rate of decline is significantly slower in females. The study of Gall and colleagues (ibid.) included only one female subject. Contrary to other published studies (Mallick et al. 1987; Gall et al. 1993) increased age was associated with a slower rate of decline of GFR in this study. In this study the 'Responders' in both IDDM and NIDDM were approximately ten years older than the 'Non-responders'.

2.5.2.ii   *Ethnic background and progression of renal impairment*

The increased prevalence of diabetic nephropathy and other renal disease amongst non-white populations in ESRD programmes is the subject of considerable interest (Easterling et al. 1977; Rostand et al. 1982; Cowie et al. 1989; Pazanias et al. 1991). It has been variously related to the increased prevalence of diabetes (Cowie et al. 1989; Nelson et al. 1989); to the greater incidence of hypertension (Tierney et al. 1989); to social deprivation; to abnormalities of parathyroid and vitamin D metabolism (Bell et al. 1985; Rostand et al. 1982) and to a more rapid decline of GFR, possibly related to hypertension (Smith et al. 1991). Our data do not support this latter observation. Here Caucasian NIDDM subjects progress more rapidly than Afro-Caribbean subjects (0.73 vs 0.43 ml.min.$^{-1}$month^{-1}; p<0.05). No difference was seen in blood pressure or its treatment, protein excretion, glycosylated haemoglobin or serum cholesterol between the two groups. The recent work of Cowie (1993)

supports this finding. She found that the time taken from a serum creatinine of 159 $\mu mol.L^{-1}$ to RRT was five months shorter in white, compared to black, NIDDM subjects. A significant decrease in the rate of decline of cGFR in this present study is also seen in Afro-Caribbean, but not Caucasian, subjects, when observed over four or more years (see Table 2.4).

2.5.2.iii   *Protein excretion and the progression of renal impairment*

This study demonstrates a significant association of 24-hour protein excretion with decline of cGFR both for IDDM and NIDDM subjects. Multiple regression analysis identifies proteinuria as the foremost factor accounting for variation of cGFR decline. This agrees with previous findings of Williams and colleagues (1988) and Wight and colleagues (1992) in non-diabetic renal disease, but is in contrast to the study of Gall and colleagues (1993) who found no association in NIDDM in a stepwise multiple linear regression analysis, though it did associate in a univariate analysis.

Williams and colleagues (ibid.) found that proteinuria was significantly correlated with progression of renal disease, particularly so in chronic glomerulo-nephritis and chronic pyelonephritis, although not so marked with diabetes mellitus. Proteinuria was significantly less in those individuals with stable renal function compared to those with linear progression, and is in agreement with the findings for both IDDM and NIDDM in this study where there is a two-fold greater protein excretion in the 'Non-responders' compared to the 'Responders'. Wight and colleagues (1992) using breakpoint analysis found proteinuria to be the most important factor governing progression of 102 patients with moderate renal impairment, although only six had diabetes. Increasing proteinuria suggests progression of renal failure is likely (Williams and Mallick 1993). However, Sawicki and Berger (1994) doubted the clinical value of 24-hour protein excretion as a predictor of renal outcome in diabetic nephropathy. Other researchers suggest a reduction of proteinuria may improve

prognosis in IDDM (Rossing et al. 1994a). The prospective study reported in Chapter 4 indicates a more modest association of protein excretion with progression.

2.5.2.iv   *Blood pressure and progression of renal impairment*

Multiple regression analysis identifies a modest association of diastolic blood pressure with progression in all subjects in this study. The association is lost when the cohort is sub-divided by diabetes type. Furthermore neither systolic or diastolic blood pressure in IDDM nor systolic pressure in NIDDM differs significantly between 'Responders' and 'Non-responders'. This may at first glance appear surprising given the acknowledged importance of blood pressure control on slowing progression. As will be discussed below, perhaps it is the type of therapy together with a 'threshold effect', rather than the prevailing blood pressure which are critical. In addition how long a subjecthas been treated with anti-hypertensive therapy when studied may be crucial.

Many early studies identified control of blood pressure to be linked to progression in non-diabetic renal disease (Pohl et al. 1974; Kajiwara et al. 1975; Van der Peet et al. 1977; Oldrizzi et al. 1985) and, more recently, in diabetic nephropathy (Mogensen 1982; Parving et al. 1987; Björck et al. 1992; Lewis et al. 1993). Such an association is not always found e.g. Williams and colleagues (1988), Malangone and colleagues (1989), Stenvinkel and colleagues (1989) and Wight and colleagues (1993). Malangone and colleagues (ibid.) reported a retrospective analysis of 402 patients entering dialysis, of whom 74 (61 IDDM) had diabetic nephropathy. Reciprocal creatinine was not correlated with either systolic or diastolic pressure in the group as a whole, nor specifically in those with diabetes. Also using reciprocal creatinine Stenvinkel and colleagues (ibid.) examined progression in 108 patients with various underlying renal diseases, though not diabetes. The baseline creatinine ranged from 200-350 $\mu mol.L^{-1}$ and subjects were followed for 8-63 months. No correlation was observed between progression and blood pressure. However when the group was

subdivided on the basis of rate of progression mean arterial pressure at 110 mmHg was significantly greater in the 91 subjects defined as 'Progressors' compared to the 17 defined as 'Non-progressors', where MAP was 103 mmHg. The patients showing progression were also taking more anti-hypertensive drugs, mean 2.5 vs 1.2. A similar threshold effect was shown by Biesenbach and associates (1994) where the rate of decline of cGFR was almost two-fold greater in those individuals with a systolic pressure >160 mmHg, (1.38 ml.min.$^{-1}$month^{-1} vs 0.78 ml.min.$^{-1}$month^{-1} resp.).

Fröhling and colleagues (1989) found a negligible relationship between the rate of progression and blood pressure in subjects with advanced renal failure comparable to the degree of renal impairment seen in this study. Wight and colleagues (1993) in a longitudinal study of 18 patients with advanced renal failure, due to various underlying diseases, observed no correlation of decline of GFR with systolic or diastolic pressure. However when the same group was studied prospectively an association did emerge. Furthermore no improvement in the rate of decline of GFR occurred when the diastolic pressure was subsequently lowered from 84 to 77 mm Hg. This suggests that lowering blood pressure below a certain level may confer no additional benefit in ameliorating the rate of decline of GFR.

2.5.2.v   *Anti-hypertensive therapy and progression of renal impairment*

An earlier report in many fewer patients suggested that the use and choice of anti-hypertensive therapy rather than the level of achieved blood pressure is a greater determinant of the decline of cGFR (Drury et al. 1993). Twice the number of subjects examined together with extension of the observation period in this study indicates that this finding holds only in NIDDM. It is perhaps surprising that a significant proportion ($\approx$50%) of all subjects were not taking anti-hypertensive therapy at the start of observation in 1986. This reflects previous local policy, although undoubtedly a heightened awareness of the importance of anti-hypertensive therapy in slowing progression of renal impairment in diabetic nephropathy followed

the studies of Mogensen (1982), Parving and co-workers (1983, 1987), Björck and colleagues (1986, 1992) and most recently Lewis and colleagues (1993). The change in prescribed anti-hypertensive medication follows recent trends and emphasises the increasing reliance on the use of ACEI in diabetic nephropathy. The ACEI class of drugs have been shown by some to be more beneficial than conventional therapy in slowing progression of renal impairment (Björck et al. 1992; Kamper et al. 1992; Lewis et al. 1993). Such studies are small in scale and of short duration. It remains to be established whether such agents confer greater longevity or reduced morbidity. A greater use of anti-hypertensive drugs together with a switch to ACEI from beta-blockade is observed in this study.

Brazy and Fitzwilliam (1990) examined the effect of blood pressure and anti-hypertensive therapy (amongst other factors) on the decline of GFR in 200 subjects of whom 57 had diabetes mellitus. In addition to the level of diastolic blood pressure, specific agents were associated with a slower decline of renal function (reciprocal creatinine). Those on no therapy, minoxidil, clonidine and calcium channel antagonists progressed more slowly than the group on diuretic therapy alone, although only those treated with minoxidil showed a significant reduction of blood pressure. No data on patients prescribed ACEI were presented in this study. Claims of benefit for ACEI exceeding the anti-hypertensive effect alone have been made (Lewis et al. 1993). Whether these agents do so by reducing intraglomerular pressure (Zatz et al. 1985), reducing proteinuria (Taguma et al. 1985), reducing the accumulation of mesangial matrix (Remuzzi et al. 1990) or by some other mechanism is not yet established.

Normotensive subjects on no anti-hypertensive medication have been shown to fare better than treated subjects with equivalent blood pressures (Jungers et al. 1995). The cGFR was lower in the five NIDDM individuals in our study compared with the treated group, although the difference was not significant due to the small numbers.

Blood pressure differences may contribute to the faster rate of decline of cGFR seen in the NIDDM group. Systolic blood pressure was significantly greater throughout the observation period as well as during the most recent two years in this group, though diastolic pressure was significantly higher only over the whole period. The presence of an elevated blood pressure prior to beginning anti-hypertensive therapy may have a deleterious effect on subsequent disease progression, particularly in NIDDM. In a mixed racial group, Pugh and colleagues (1993) observed the time from diagnosis of diabetes to ESRD was on average 2.7 years shorter in individuals hypertensive at the onset of diabetes.

Haemodynamic factors may contribute to the greater rate of progression seen in NIDDM, given that atheromatous renovascular disease appears more common than in IDDM (Ritchie et al. 1988; Brown et al. 1992). The influence of renovascular disease on progression of renal impairment in NIDDM is discussed in the following chapter.

2.5.3　　Has the specialist diabetic renal clinic conferred benefit on the attendees?

Comparing the rate of decline of cGFR before and after entering the joint clinic reveals a significant reduction only in IDDM. However the average duration of follow-up of three years in both groups is relatively short. The reduction in the rate of decline of cGFR in Afro-Caribbean subjects, followed-up for four years or more, suggests that the benefit of clinic attendance may yet emerge in NIDDM. The reason for improvement may be due to improved blood pressure with a significant fall in diastolic pressure in both IDDM and NIDDM groups.

No clear association was observed with prescribed anti-hypertensive medication, although the results of the multiple regression analysis presented above suggest it may be important. The number of prescribed drugs is greater at the end compared to

the start of the period of observation. Improved drug compliance cannot be discounted as a cause for the improvement in cGFR. The benefit of specialised, as compared to routine, hypertension care has been demonstrated recently in diabetic nephropathy. In a 5 year follow-up study comparing 46 IDDM patients receiving routine care with 45 receiving specialised care the latter fared considerably better (Sawicki et al. 1993). Survival to death or dialysis was significantly longer for those receiving specialised care. Whilst blood pressure was significantly improved in the specialist clinic attendees, it is not clear whether the groups were evenly matched for diabetic complications. Bergström and colleagues (1986) have demonstrated the beneficial effect of more frequent clinic reviews in 17 subjects with chronic renal failure. However the reduction of the slope of reciprocal creatinine was associated with a modest, though significant, drop in diastolic blood pressure. They demonstrated an overall improvement in 50% of cases, although in two cases there was an accelerated decline. There was no evidence in this current study, either in IDDM or NIDDM, that more frequent clinic attendance or indeed duration of attendance conferred greater benefit. Rather the converse; the individuals with the faster progression were seen more frequently. This observation is not entirely unexpected.

Although one might feel that attendance at a clinic devoted to patients with specific long-term complication of diabetes would be an advantage the evidence suggests only limited benefit to the group as a whole. The lack of a control group and the use of individuals as their 'own controls' makes it difficult to quantify the factors responsible for the improvement observed. One may speculate however that the benefits of the specialist clinic are likely to extend beyond reducing the rate of decline of GFR.

2.6   CONCLUDING REMARKS

- The median rate of decline of cGFR for all subjects was 0.39 ml.min.$^{-1}$month^{-1}. The rate for IDDM subjects was 0.29 ml.min.$^{-1}$month^{-1} compared to 0.43 ml.min.$^{-1}$month^{-1} in NIDDM ($p<0.05$).

- In the most recent year of observation the median rate of decline of cGFR for the IDDM subjects was 0.07 ml.min.$^{-1}$month^{-1}.

- Only Afro-Caribbean NIDDM subjects showed a statistically significant reduction of the rate of decline of GFR when observed over four years or more, improving from 0.49 ml.min.$^{-1}$month^{-1} to 0.26 ml.min.$^{-1}$month^{-1} ($p<0.02$), comparing the first and last two years.

- 24-hour protein excretion emerged as the most significant associate of the rate of decline of cGFR in both IDDM and NIDDM

- Diastolic blood pressure showed a modest association with the rate of decline of cGFR in all subjects, though not when sub-divided by diabetes type. Systolic pressure, glycosylated haemoglobin and serum cholesterol were not significant factors.

- Antihypertensive treatment at the start of the observation period was associated with a slower rate of decline of cGFR in NIDDM, though not in IDDM subjects.

- A significant slowing of the rate of decline of cGFR was observed following attendance at the joint diabetic renal clinic in IDDM, though not in NIDDM.

That a number of patients, particularly those with IDDM, may have stable renal function over six or more years despite significant renal impairment should provide

encouragement not just to those patients but also to their carers and others who see renal replacement therapy as the inevitable consequence of diabetic nephropathy.

Chapter 3

Does renal artery angioplasty influence the progression of renal impairment in diabetic nephropathy ?

3.1   INTRODUCTION

Autopsy studies indicate a prevalence of renal artery stenosis (RAS) of 8-10% in subjects with diabetes mellitus compared to 4% in non-diabetic individuals (Sawicki et al. 1991). In selected groups, however, the prevalence may be significantly higher. Shapiro and colleagues (1965) found 44% of a group of 55 subjects with RAS and hypertension had diabetes mellitus, although in the majority the latter diagnosis was made only at the time of angiography. A more recent survey of diabetic clinic attendees with hypertension screened for RAS by digital subtraction angiography revealed 5 of 24 non-insulin-treated subjects to have a unilateral RAS, compared to none in an insulin-treated group (Ritchie et al. 1988). The degree of stenosis in this study was not stated. The only published study confined to diabetic nephropathy showed significant RAS (defined as a stenosis > 50%) in 10 (30%) of 33 NIDDM subjects (Brown et al. 1992). Renal artery stenosis was not found in 7 IDDM subjects in this study, highlighting the relative rarity of this finding in these patients.

Few data are available on the effect of percutaneous transluminal angioplasty (PCTA) in renal failure due to atherosclerotic RAS. Benefit has been shown by certain groups (Martin et al. 1988; O'Donovan et al. 1992; Pattison et al. 1992), but not by others (Luft et al. 1983; Textor et al. 1992). No study has systematically examined the effect of PCTA on RAS in subjects with diabetic nephropathy. The impression was gained that such patients in our clinic undergoing PCTA did not appear to benefit from the procedure. A retrospective analysis of subjects with RAS and diabetic nephropathy who underwent PCTA was therefore carried out. The data from these subjects are included in a more comprehensive survey of PCTA in atherosclerotic RAS, pre-dominantly of non-diabetic origin (Connolly et al. 1994). The latter study examined 94 patients of whom 24 had diabetes mellitus. The subjects presented here are confined to those with NIDDM and diabetic nephropathy.

3.2   AIM

To examine the effect of percutaneous transluminal angioplasty on renal function
and blood pressure in subjects with NIDDM and nephropathy who were found to
have renal artery stenosis.

3.3   PATIENTS AND METHODS

3.3.1   Patients

Nine subjects with NIDDM, fulfilling the criteria of diabetic nephropathy, were
selected from a group of 14 who underwent PCTA for RAS between 1988 and 1992.
Of the remaining 5, two had no retinopathy, in two the diagnosis of RAS preceded
the diagnosis of diabetes and one had IDDM. All were regular attendees at the
diabetic renal clinic as described in Chapter 2.

The clinical details of these nine cases are given in Table 3.1 (overleaf). NIDDM and
diabetic nephropathy are defined as in sections *4.3.1.ii and 4.3.1.iii* respectively. No
subject underwent percutaneous renal biopsy. The values given for serum creatinine
and blood pressure in Table 3.1 are those of the visit immediately preceding
angioplasty. Neither protein intake nor excretion were measured systematically and
dietary protein restriction was not advised throughout the period of observation
despite the advanced nature of renal impairment in several cases. However a cohort
of subjects, including several examined here, followed prospectively showed no
change in 24-hour urea excretion over the period of observation (see Chapter 4).

3.3.2   Indications for angiography

Angiography was carried out in these subjects for the clinical indications as outlined
in Table 3.2 (overleaf). Uncontrolled hypertension was defined as a blood pressure
$\geq 160/95$ mmHg. despite maximal anti-hypertensive medication, or, as in case 3,
accelerated-phase hypertension. Subjects with renal failure in whom RAS was

Case	Gender	Race	Duration DM	Retinopathy		24-hour protein (g)	PVD	Creatinine (μmol.L^{-1})	BP	BP therapy
				Type	Laser					
1	F	Caucasian	12	BGR	no	1.80	yes	188	164/96	CD
2	M	Caucasian	4	Prol	yes	1.61	yes	267	160/100	D
3	F	Afro-Caribbean	7	Mac	yes	1.55	no	138	270/134	D
4	F	Caucasian	9	Mac	yes	7.44	no	276	170/98	ACD
5	M	Caucasian	15	Mac	yes	5.50	no	190	190/96	AD
6	F	Afro-Caribbean	23	Prol	yes	1.10	yes	489	184/94	AD
7	F	Afro-Caribbean	15	Mac	yes	3.30	no	340	160/100	ABD
8	F	Afro-Caribbean	19	Prol	yes	2.20	no	280	242/98	AD
9	M	Caucasian	13	Mac	yes	2.40	yes	229	156/94	CD

Key : Retinopathy   BGR = Background

Mac = Maculopathy

Prol = Proliferative

BP therapy

A = ACE inhibitor   C = Calcium channel antagonist

B = Beta-blocker   D = Diuretic

Table 3.1: Clinical details of the subjects prior to renal artery angioplasty

Case	Indication for angiography	Findings
1	Asymmetric kidneys	(L) RAS > 90%
2	Fluid overload	Multiple bilateral stenoses
3	Hypertension	(L) RAS - 30%
4	Resistant hypertension	(L) RAS > 90%
	Renal failure	(R) - occluded
5	Hypertension	(L) RAS > 50%
6	Renal failure	(L) RAS - 30%
7	Renal failure	(L) RAS - 30%
8	Hypertension	Bilateral stenoses
		(R) > 50%; (L) - 40%
9	Fluid overload	Bilateral stenoses
		(R) >50%; (L) - 30%

Table 3.2: Indications for and findings at angiography

thought to be a contributory factor, or who showed a worsening of GFR with the introduction of an angiotensin converting enzyme inhibitor (ACEI), were considered for angiography. A size difference exceeding 1.5 cm between kidneys on renal ultrasound prompted investigation to exclude RAS. Cardiac failure, both acute and chronic, is a recognised, though rare, presentation of RAS (Pickering et al. 1988; Missouris et al. 1993). Peripheral vascular disease was recorded as present in those with a history of intermittent claudication, absent foot pulses or the presence of stenosis of the iliac, femoral or tibial arteries on peripheral angiograms.

Table 3.2 indicates the findings at angiography. Bilateral stenoses were present in three cases (2, 8 & 9). In one (Case 4) stenosis in one renal artery was associated with occlusion of the contralateral artery. Two subjects had numerous peripheral stenoses in addition to the more proximal stenosis (Cases 1 & 8).

3.3.3   Angioplasty technique

All angioplasties were carried by an experienced interventional radiologist, using a 5-gauge French catheter inserted via the femoral artery. The balloon was dilated up to 5-7mm in diameter for 30 seconds on average, repeated if necessary, under cover of an isosorbide dinitrate infusion. All subjects not already receiving aspirin were commenced on it.

3.3.4   Renal function and blood pressure

Data on renal function were available for a minimum of three (Case 7) to a maximum of eighteen months before and after angioplasty. Serum creatinine measurements in the three weeks after angioplasty were excluded from analysis, as contrast may transiently worsen renal function (Spångberg-Viklund et al. 1989). Estimated GFR was derived from these creatinine measurements using a modification of the Cockcroft-Gault formula (Cockcroft and Gault 1976; Hull et al.

1981). The equation is given below (section 5.3.1) and the subject discussed at greater length in Chapter 5. The formula has been validated for IDDM (Sampson and Drury 1992; Rossing et al. 1994b), whilst a strong association is shown between EDTA GFR and GFR estimated from Cockcroft-Gault (r=0.78; p<001) in NIDDM subjects (see Chapter 5). A minimum of three creatinine measurements were recorded before and after angioplasty in all subjects and the mean estimated GFR compared over equivalent time periods before and after the procedure. The means of estimated GFR, systolic and diastolic blood pressure were compared over equivalent time periods before and after angioplasty.

Blood pressures (phase I and V) was recorded in the sitting position at each visit using a standard mercury sphygmomanometer, reading to the nearest 2 mmHg, by one of three observers. Anti-hypertensive therapy was recorded at each visit. All subjects gave informed consent to the procedure.

3.4   RESULTS

All angioplasties were deemed technically successful at the end of the procedure as judged by angiographic appearances after dilatation. Four subjects had repeat angiograms, 6-12 months later. Only one (Case 8) required a further angioplasty. The only complications occurred in case 1 with a large groin haematoma and an intimal tear to the renal artery; neither required specific intervention.

3.4.1   Changes in renal function

The mean pre- and post- angioplasty values for cGFR are given in Figure 3.1 (below). No benefit from PCTA in attenuation of renal function decline is seen in this group with estimated GFR higher before angioplasty than afterwards (29.0±12.7 ml.min^{-1} vs 23.3±12.7 ml.min^{-1}, mean±SD, p=0.02: paired 't-test'). The relatively short duration of observation precludes a meaningful comparison of the rates of

decline of GFR before and after PCTA for the whole group. However in the six subjects observed for ≥ 10 months an approximate fall in GFR can be calculated. Again there is no significant improvement in GFR; mean fall pre-angioplasty 0.36 ml.min^{-1}.month^{-1} vs 0.41 ml.min^{-1} month^{-1} post angioplasty (p=0.64; paired 't-test'), although two of the subjects might show a very modest slowing of decline of GFR (Cases 4 & 8). These figures differ little from the results previously shown with a the median decline of 0.43 ml.min.$^{-1}$ month^{-1} seen for all 55 NIDDM subjects recorded in the observational study (see Chapter 2). The mean follow-up for these nine cases was 12 months. Extrapolating from the median decline of cGFR (above), the average expected decline of cGFR over the period following angioplasty would be 5.2 ml.min^{-1}, compared to the observed of 5.7 ml.min^{-1}.

In all subjects, except Case 9, the procedure itself appeared to have a slight adverse effect on renal function. Individual profiles showing the effect of angioplasty on renal function are shown in Figure 3.2 (overleaf).

3.4.2   Changes in blood pressure

Figure 3.3 (overleaf) shows mean systolic and diastolic blood pressure before and after angioplasty, demonstrating no benefit from intervention in this group. Both systolic (178±36 vs 188±44; p=0.37) and diastolic blood pressure (90±14 vs 95±15; p=0.12) rose slightly, albeit non-significantly, after angioplasty. In only two or three cases could there be described a modest fall of blood pressure. In addition there was no reduction in the need for anti-hypertensive medication in any individual following the procedure.
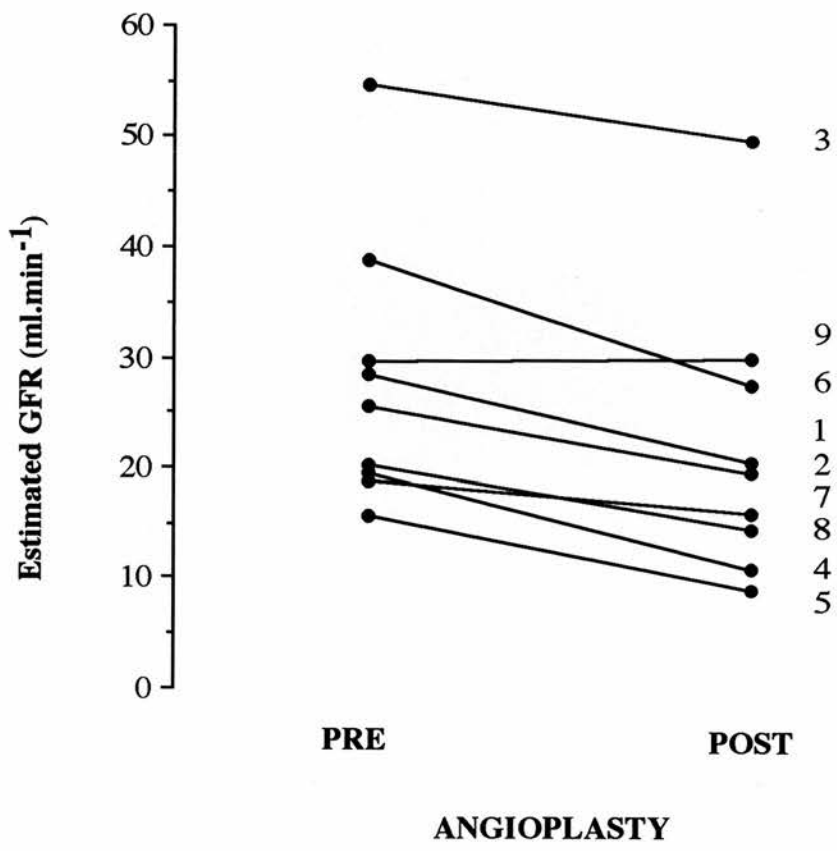
Figure 3.1 : Mean estimated GFR before and after angioplasty.
Values are means over equivalent time periods.
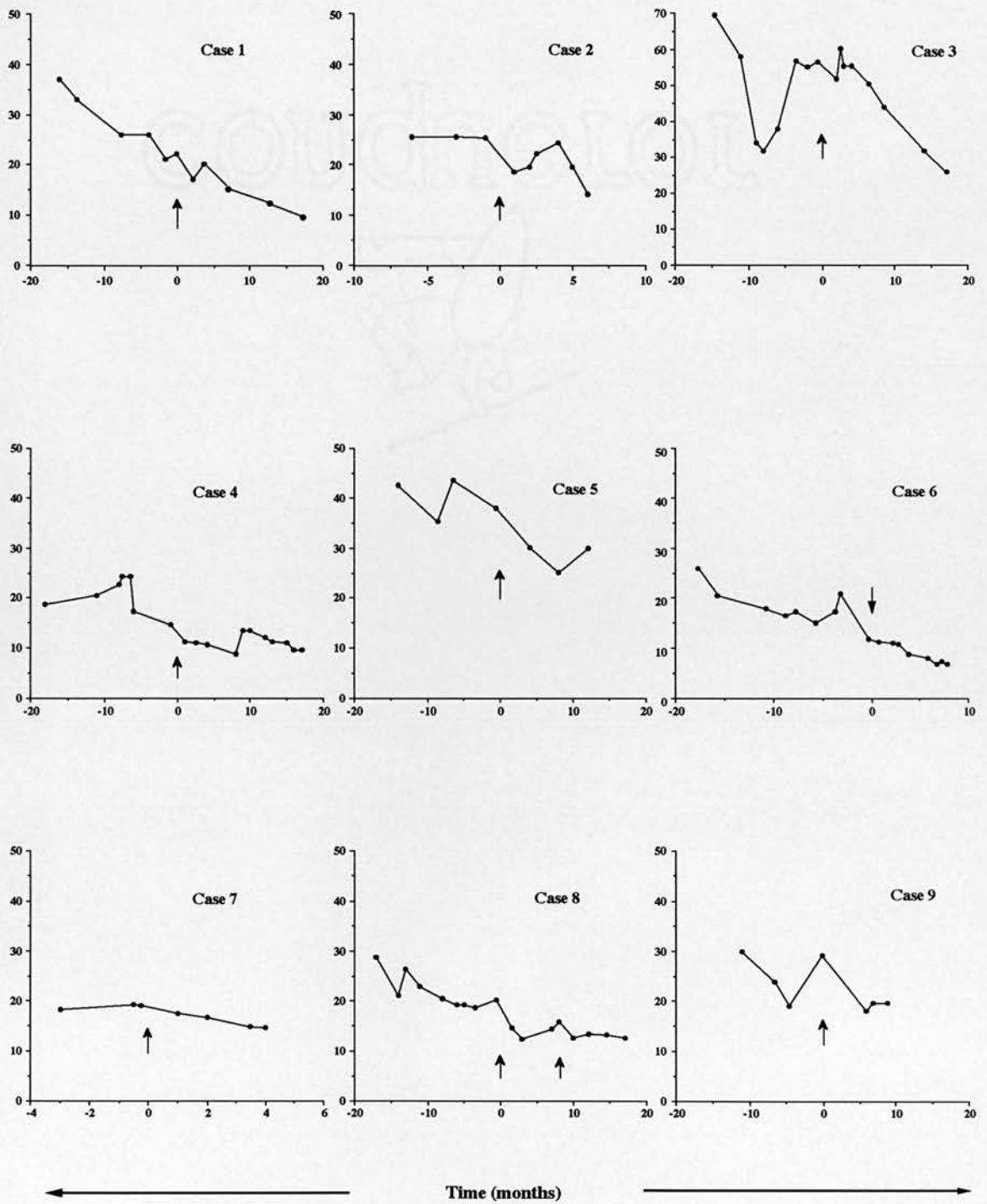
Figure 3.2 : Decline in estimated GFR in individual patients. Angioplasty denoted by arrow. Case 8 had repeat angioplasty.
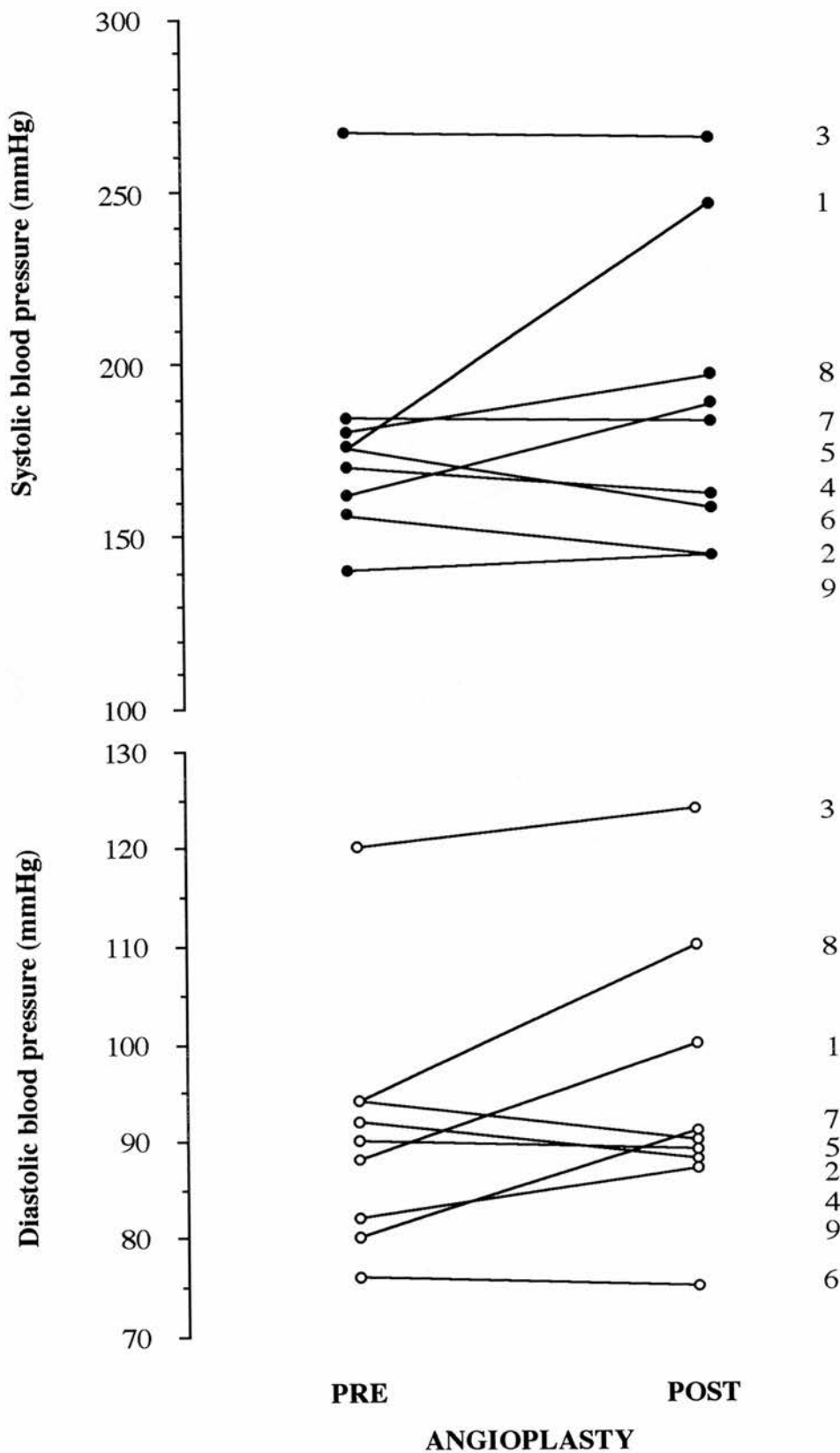
Figure 3.3 : Mean blood pressure before and after angioplasty. Values are means over equivalent time periods.

3.5   DISCUSSION

It is eighteen years since the original description by Grüntzig and colleagues (1978) of renal artery angioplasty in a patient with renovascular hypertension due to atheromatous RAS. Fostered by a series of reports advocating its benefits it has become both an attractive and commonplace procedure. A critical analysis of the ten largest series published prior to 1987 challenged this approach (Ramsey and Waller 1990). These authors considered that one in three of all subjects with RAS did not benefit from the procedure. Even fewer of those with RAS due to atheroma benefit when compared to those with fibromuscular hyperplasia. Most studies of PCTA have examined the effect of angioplasty on blood pressure; studies in diabetic subjects are rare. An exception is the recent study of Martin and colleagues (1992) who demonstrated improved blood pressure control following angioplasty for ostial RAS in four of six patients with NIDDM and three of eight with IDDM (no data on the effect of angioplasty on renal function are given).

Fewer studies have examined the effect of angioplasty on renal function, and none exclusively so in diabetic nephropathy. Table 3.3 (over) summarises the results of these studies. Direct comparison between them is difficult because different criteria for success are used. For instance Bell and colleagues (1987) used a fall in creatinine of 50μmol.L^{-1}, Pattison and colleagues (1992) and Connolly and colleagues (1994) a fall of 20% from the pre-angioplasty value, while Luft and colleagues (1982) considered benefit occurred only if the post-angioplasty creatinine bettered the pre-angioplasty level.

It is clear from these studies, however, that a number of patients appear to benefit from the procedure in terms of improved renal function. Furthermore sub-groups of patients do better than others. Martin and colleagues (ibid.) demonstrated the greatest benefit in those with bilateral stenoses. In contrast Pattison and colleagues

No. of subjects	Improved	Stable	Deteriorated	Reference
10	2 (20)	7 (70)	1 (10)	Schwarten (1980)
2	2 (100)	-	-	Sos (1983)
12	3 (25)	NS	4 (33)	Luft (1983)
20	7 (35)	10 (50)	3 (15)	Bell (1987)
79*	34 (43)	NS	NS	Martin (1988)
9	3 (33)	2 (22)	4 (45)	McDonald (1988)
9	3 (33)	6 (67)	-	Kim (1989)
17	7 (41)	4 (24)	6 (35)	O'Donovan (1992)
60	24 (60)	NS	6 (10)	Pattison (1992)
52	2 (4)	36 (70)	14 (26)	Connolly (1994)**

Key : NS = not stated; figures in parenthesis are percentages

*- includes 7 non-atheromatous subjects ** - data given for 12 month follow-up

Table 3.3 : Effect of PCTA on renal function in previous studies of patients
with atheromatous renal artery stenosis

(ibid.) found the greatest improvement in those with stenosis in one renal artery, with a non-functioning or absent contra-lateral kidney (in a number of these the benefit may have been due to the correction of additional reversible renal disease), while limited benefit was seen in those with bilateral stenosis and none for unilateral stenosis. In addition they found that the greatest benefit was found in individuals showing the most rapid reduction in GFR prior to angioplasty.

Few authors indicate the diabetic status of the patients studied. Of 17 elderly subjects examined by O'Donovan and colleagues (1992), 8 had diabetes. Whether or not these individuals fared differently from the remainder is not stated. Bell and colleagues (1987) suggested that patients with end-stage renal disease or severe intra-renal atheroma (as seen in two of our cases) may not benefit from angioplasty. The majority of these studies are, however, relatively short-term and all are uncontrolled. Examined over a longer time period outcome from angioplasty may not prove so successful (Textor et al. 1992).

Definition of what constitutes a successful angioplasty is disputed. As indicated above end-points in the various studies differ. The observation that renovascular disease is progressive (Schreiber et al. 1984, Dean et al. 1981) led Connolly and colleagues (1994) to conclude that intervention leading to stable renal function represented success. It could be argued that since none of the subjects presented here showed an accelerated decline of GFR some degree of benefit accrued. In practice it appears that angioplasty had neither a beneficial nor a detrimental effect on renal function in these subjects. Certainly the individual patient profiles of decline of estimated GFR presented in Figure 3.2 do not suggest that intervention has altered the natural progression of the condition. It is clear from the data presented in Chapter 2 that an amelioration in the decline of estimated GFR may occur in certain individuals (and certain groups). This further emphasises the lack of benefit observed

from angioplasty in this group. However the absence of direct imaging or functional studies makes is difficult to draw firm conclusions on the influence of renovascular disease (and intervention) on the progression of diabetic nephropathy.

Failure to demonstrate any benefit from angioplasty in this study may have arisen due to a number of factors. The lesion may not have been haemodynamically significant, particularly those with 30% stenoses. The decision to perform angioplasty was made by the radiologist performing the angiogram. Neither venous sampling for renal vein renin nor a captopril renogram was performed to demonstrate a functional stenosis. Restenosis may have occurred, as in Case 8. However three others showed no evidence of restenosis on repeat angiography. The presence of intrarenal arterial stenosis may have a greater influence on the outcome of angioplasty than the successful dilation of the proximal lesion (Dean et al. 1985). Similarly it is possible that renal parenchymal disease may determine the outcome of angioplasty. Studies have suggested that the degree of glomerular hyalinisation may determine whether or not renal function is recoverable after revascularisation (Vidt et al. 1972; Zinman et al. 1977). If this is the case then subjects with diabetic nephropathy may not benefit from dilation of an associated RAS.

3.6   CONCLUDING REMARKS

In this small study no individual appeared to benefit from angioplasty in terms of an improvement in renal function or significant slowing in its decline, nor in the level of blood pressure control or reduction in anti-hypertensive therapy. As all nine individuals failed to show a sustained improvement in renal function and blood pressure a type 2 error is unlikely. Where diabetic nephropathy is established either clinically or by renal biopsy then it may be that dilation of an associated RAS does not alter the natural decline of renal function. Given the increased usage of ACE inhibitor therapy, particularly in subjects with diabetic nephropathy, a rise in renal

failure due to renovascular disease seems likely. The beneficial effect of PCTA in RAS in such individuals remains to be established.

Chapter 4

A prospective study of the factors influencing the decline of glomerular filtration rate in NIDDM subjects with nephropathy

4.1   INTRODUCTION

Prospective studies of the progression of nephropathy in NIDDM are few. Although there are a number of studies of NIDDM subjects with micro-albuminuria (Cooper et al. 1988; Schmitz and Vaeth 1988; Vora et al. 1992), only four, using a validated technique for the measurement of GFR exist in those with more advanced nephropathy. The earliest, by Friedman and Gross (1991), included only seven subjects, one of whom had multiple myeloma, and the majority had near-normal GFR. Gall and colleagues (1993) examined almost exclusively male Caucasian subjects with less severe renal impairment than in this present study. The two most recent, by Myers and colleagues (1995) and Nelson and colleagues (1996b) in the Pima Indians, used iothalamate clearance. Again the renal impairment was modest; in the former the subjects had a mean baseline GFR of 108 ml.min^{-1} whereas in the latter the value was 124 ml.min^{-1}.

In the retrospective analysis presented above in Chapter 2 a remarkably slow rate of progression of GFR was witnessed, particularly in IDDM. However the study was limited both by the retrospective nature, and by the use of a surrogate measure of GFR. Thus a prospective study was conducted in 26 NIDDM subjects to determine whether the rate of decline of GFR was comparable when an isotopic clearance method was used and to identify the principal factors associated with the recorded decline. Inevitably such a study was time-limited, with the individuals being followed for up to two years.

4.2   AIMS

- To determine the rate of decline of renal function in a well characterised group of subjects with NIDDM and nephropathy.

- To examine whether racial differences exist in the rate of decline of glomerular filtration rate.

- To assess the relationship between the rate of decline of glomerular filtration rate and several measured variables, including glycaemic control, blood pressure, 24-hour protein excretion and serum lipid concentration.

4.3   PATIENTS AND METHODS

4.3.1   Patients

4.3.1.i   Patient selection

The 26 subjects selected were attendees at the diabetic outpatient clinic at King's College Hospital. Twenty-three attended the joint diabetic-renal clinic as previously described (details in Chapter 2), while three were recruited from the general diabetic clinic. Of 38 NIDDM patients attending the joint clinic potentially suitable for study 15 were excluded; 9 (5 Afro-Caribbean; 3 Caucasian; 1 Asian) declined to participate, 3 (all Afro-Caribbean) were near end-stage failure or were on intermittent dialysis and 3 had a history of erratic clinic attendance. At the time of selection all subjects had a serum creatinine of ≥ 140 μmol.L^{-1} (normal range 50-105 μmol.L^{-1}), although in one case this had improved to 129 μmol.L^{-1} when the study commenced. All subjects were deemed to have NIDDM (*4.3.1.ii*) and to fulfil the criteria for diabetic nephropathy (*4.3.1.iii*).

Of the 26 subjects within the group 21 were male. Eleven were Caucasian, ten Afro-Caribbean and five of Asian extraction. The median age at entry was 65.5 years (range 43-78) with a duration of diabetes of 13.5 (3-24) years at the start of the study. See Table 4.1 (overleaf) for details.

4.3.1.ii   Definition of non-insulin-dependent diabetes mellitus

Patients were recognised as having NIDDM where treatment was with diet alone or diet together with an oral hypoglycaemic agent for at least two years following

Case	Gender	Race	Age	Duration DM (years)	BMI	Creatinine (μmol.L^{-1})	24-hour protein (g)
1	M	AF	67	25	29	195	224
2	M	C	65	16	34	129	1650
3	M	C	69	3	30	156	1150
4	M	AS	61	11	22	154	805
5	M	C	66	8	31	154	9730
6	F	C	46	14	48	296	6270
7	F	AF	43	9	23	216	7440
8	M	AF	60	6	34	263	1760
9	F	C	72	17	30	226	308
10	M	AS	56	8	27	187	1420
11	M	AF	74	17	25	191	1400
12	M	C	76	18	21	220	1570
13	M	AS	78	27	27	257	4930
14	M	C	71	18	30	141	1440
15	M	C	49	9	30	351	7350
16	M	AF	59	13	27	241	10890
17	M	AF	67	10	21	336	4800
18	F	AF	64	16	32	337	15700
19	M	AS	67	10	28	218	5010
20	M	AF	74	6	31	159	1250
21	M	C	70	10	26	236	2250
22	F	AF	58	20	26	302	1680
23	M	C	64	13	29	224	1960
24	M	C	61	21	23	361	2290
25	M	AF	60	14	30	165	3850
26	M	AS	47	14	30	286	6780
Median			65.5	13.5	29	222	2110
Range			43 - 78	3.0 - 24.0	21 - 48	129 - 361	224 - 15700

Table 4.1 : Characteristics of the nephropathic subjects entering the study

diagnosis. In no subject was there a history of ketoacidosis. All subjects were aged thirty years or more at the time of diagnosis with a median body mass index (BMI) at entry was 29 (range 21-48). Measurement of basal or stimulated C-peptide was not carried out. These criteria take account of the published guidelines for the diagnosis of diabetes mellitus {WHO Technical report (1985); Høther-Nielson and colleagues (1988)}.

4.3.1.iii   *Criteria for the diagnosis of diabetic nephropathy.*

Diabetic nephropathy is here defined as a urinary protein excretion of greater than 500 mg in 24 hours in the absence of urinary tract infection, cardiac failure or other renal disease. In most cases diabetic retinopathy and hypertension are present but neither are pre-requisites for the diagnosis, although the lack of the former should prompt a search for an alternative renal diagnosis. This definition is based on that given by Viberti and colleagues (1992).

Non-diabetic renal disease was excluded by the absence of serological markers (see below), by the finding of a normal renal ultrasound and by the absence of red cell casts, frank haematuria or significant pyuria in a freshly voided urine specimen (see below). Percutaneous renal biopsies were not routinely performed.

4.3.1.iv   *Exclusion of other renal disease*

All subjects had a renal ultrasound performed before entry into the study. Subjects were excluded if bilateral small kidneys (<10 cm.), asymmetric kidneys (size difference ≥1.5 cm.), evidence of urinary obstruction (hydronephrosis or pelvicalyceal dilatation) or a mass lesion within the kidney were demonstrated.

All subjects had blood taken for taken for auto-antibodies (anti-nuclear factor [ANF] and anti-neutrophil cytoplasmic antibody [ANCA]), complement [C3, C4] and serum protein electrophoresis. Subjects were excluded if positive ANA or ANCA found, unless a renal biopsy confirmed glomerulosclerosis (Cases 1 & 6 in Table 4.2). A

sterile midstream specimen was examined microscopically and cultured and a random sample of urine was examined for Bence-Jones protein where clinically indicated.

Five subjects underwent a percutaneous renal biopsy to establish the diagnosis, where doubt was raised from preliminary clinical and laboratory assessments. One further subject (Case 6) had a second biopsy four months after commencing the study on account of a very rapid decline in renal function and was found to have amyloidosis in addition to diabetic glomerulosclerosis. A previous renal biopsy had shown only diabetic glomerulosclerosis with no evidence of amyloid deposition. The indications for and the findings at biopsy are given in Table 4.2 (below).

Case	Indication for biopsy	Findings
1	ANF positive	Diabetic glomerulosclerosis No evidence of SLE
2	No retinopathy	Diabetic glomerulosclerosis
3	No retinopathy	Diabetic glomerulosclerosis
4	No retinopathy	Diabetic glomerulosclerosis
5	Rapid rise in creatinine	Diabetic glomerulosclerosis Amyloidosis
6	ANCA positive	Diabetic glomerulosclerosis

Table 4.2 : Indications for and findings in subjects undergoing renal biopsy.

4.3.1.v *Non-renal diabetic complications*

All but two individuals had diabetic retinopathy at the start of the study (in both, renal biopsy confirmed diabetic glomerulosclerosis). Background retinopathy was present in 6 (23%) whilst 20 (77%) had undergone laser photocoagulation, 5 for proliferative retinopathy and 15 for maculopathy. Of the two subjects not initially

exhibiting retinopathy one developed maculopathy (VK) and one proliferative (LR) during the study period; both required laser photocoagulation.

Nine of the twenty-six (35%) subjects had initial evidence of peripheral vascular disease as documented by absent foot pulses or by significant stenoses on peripheral angiography. None had undergone a significant amputation, although two subjects (AR and MT) had toes amputated. Fourteen (54%) had known ischaemic heart disease, defined as a myocardial infarction, substantiated by ECG changes and/or cardiac enzyme elevation, angina with documented ECG changes at rest or on exercise, or cardiac failure. Six (23%) had cerebrovascular disease, either a completed stroke or a transient ischaemic attack.

Twelve (46%) had clinical evidence of a peripheral neuropathy, as judged by an appropriate history with absent ankle reflexes; of these eight had confirmatory nerve conduction studies. Only one subject was known to have autonomic neuropathy, although few had the standard cardiovascular autonomic function tests to exclude this complication. Details of non-renal complications are given in Table 4.3 (overleaf).

4.3.1.vi   Treatment regimens

At the start of the study fourteen of the twenty-six patients were receiving insulin and the remainder sulphonylurea therapy. No additional subject was converted to insulin during the study. Twenty-three of the twenty-six were receiving anti-hypertensive drug therapy at entry. One patient commenced anti-hypertensive treatment after twelve months. The details of the date of diagnosis of hypertension, its relationship to the diagnosis of diabetes and the individual treatment regimens are given in Table 4.4 (overleaf).

Case	PVD		IHD	CVD	Retinopathy	
	Present	Amputation			Status	Treatment
1	+		+	+	BGR	
2	+				Maculopathy	Laser
3			+		Maculopathy	Laser
4			+		Maculopathy	Laser
5			+		BGR	
6			+	+	Maculopathy	Laser
7					Maculopathy	Laser
8					BGR	
9					Maculopathy	Laser
10			+		Maculopathy	Laser
11	+		+	+	Maculopathy	Laser
12	+		+		Maculopathy	Laser
13			+		BGR	
14	+	+	+		Proliferative	Laser
15	+		+		Proliferative	Laser
16					Maculopathy	Laser
17			+		BGR	
18			+		Maculopathy	Laser
19					Maculopathy	Laser
20					Maculopathy	Laser
21				+	Proliferative	Laser
22				+	Proliferative	Laser
23	+	+	+	+	Proliferative	Laser
24					Maculopathy	Laser
25					Maculopathy	Laser
26					BGR	

Key : BGR = Background retinopathy

Table 4.3 :   Associated complications in patients with diabetic nephropathy

Case	Hypertension year of diagnosis	Interval (years)	Initial BP therapy	Final BP therapy	Diab Rx.
1	1989	23	E(5)	E(15),F(20)	I
2	1981	6	E(15),B(2),H(100)	E(15),B(2),H(100)	S
3	1985	-2	C(37.5),F(40)	died	S
4	1982	2	M(100),C(50),F(40)	M(50),F(40)	S
5	1982	-1	E(15),F(40),N(40)	E(20),F(40),V(240)	S
6	1990	13	E(15),F(40)	E(15),F(80)	I
7	1992	10	none	V(240)	I
8	1985	-1	E(20),F(80),A(100),V(240)	E(20),F(40),V(240)	S
9	1975	1	E(10),F(40)	E(10),F(40)	I
10	1984	1	E(5)	E(5),(40)	S
11	1986	12	E(5),F(200)	Q(5),F(200)	I
12	1989	16	N(40)	N(40),E(5)	I
13	1986	22	E(20),F(80)	E(10),F(40),N(40)	S
14	none	-	none	died	I
15	1986	4	C(50),N(40)	N(40)	S
16	1990	12	A(50),C(100),F(40)	dialysis	I
17	none	-	none	none	I
18	1990	15	C(37.5),F(40)	dialysis	I
19	1985	4	L(5),F(40)	L(20),F(80)	S
20	1984	-1	N(40),A(50)	A(50),N(40)	S
21	1981	0	N(40),B(5)	E(2.5), F(40)	I
22	1989	18	E(20),F(40)	E(20),F(40)	I
23	1983	5	N(40)	N(60)	I
24	1990	18	L(10)	L(10),B(1),V(120)	S
25	1985	6	L(2.5),V(240)	L2.5),V(240)	I
26	1986	7	E(10),F(40)	E(10),F(40)	S

' :   A = Atenolol   E = Enalapril   L = Lisinopril   Q = Quinapril
     B = Bumetanide   F = Frusemide   M = Metoprolol   V = Verapamil
     C = Captopril   H = Hydralazine   N = Nifedipine
     Diab. Rx. : I = Insulin; S = Sulphonylurea

ble 4.4 : Duration of hypertension, prescribed anti-hypertensive medication at the start and
.sh of the study period. The interval between the diagnosis of diabetes and hypertension is
en in Column 3: negative indicates hypertension preceded the diagnosis of diabetes.
ɔ figures in parenthesis are drug dosages given in mg.

The aim of anti-hypertensive treatment for NIDDM patients attending the joint-diabetic clinic was to maintain blood pressure below 160/90 mmHg. Efforts to achieve these targets were tailored to the individual patient, taking account of age, drug-induced side-effects (principally postural hypotension) and the rate of decline of renal function. Towards the end of the study period more rigorous guidelines (Mogensen 1991) were introduced aiming to achieve a pressure of 140/80 mmHg. However this did not extend to the patients participating in the study. The preferred anti-hypertensive regimen was an ACEI, supplemented where necessary with a loop diuretic followed by a calcium channel antagonist. At the start of the observation period of the 23 subjects treated, 2 (9%) were on ACEI alone, 13 (56%) ACEI and diuretic, 3 (13%) triple therapy (ACEI, diuretic and calcium channel antagonist) and 5 (22%) on miscellaneous regimens (Table 4.4).

All subjects were maintained on their normal diabetic diet. Attempts were made to improve glycaemic control when the HbA_{1c} exceeded 8% (N range 4.3-6.0%). Protein intake was not significantly restricted although efforts were made to keep intake below 1 mg.kg.$^{-1}$day^{-1}. Potassium intake was restricted where the serum level exceeded 5.5 mmol.L^{-1} (Reference range 3.5-5.0 mmol.L^{-1}).

4.3.1.vii   Ethical approval

All subjects gave informed consent for the study, which was approved by the hospital ethics committee in accordance with the Helsinki guidelines (World Medical Association 1985).

4.3.2   Methods

4.3.2.i   Study protocol

The study protocol is outlined in Figure 4.1 (overleaf). EDTA clearance was performed on an annual basis. At the same visit subjects had a full clinical examination to assess progression of established complications or identify any newly
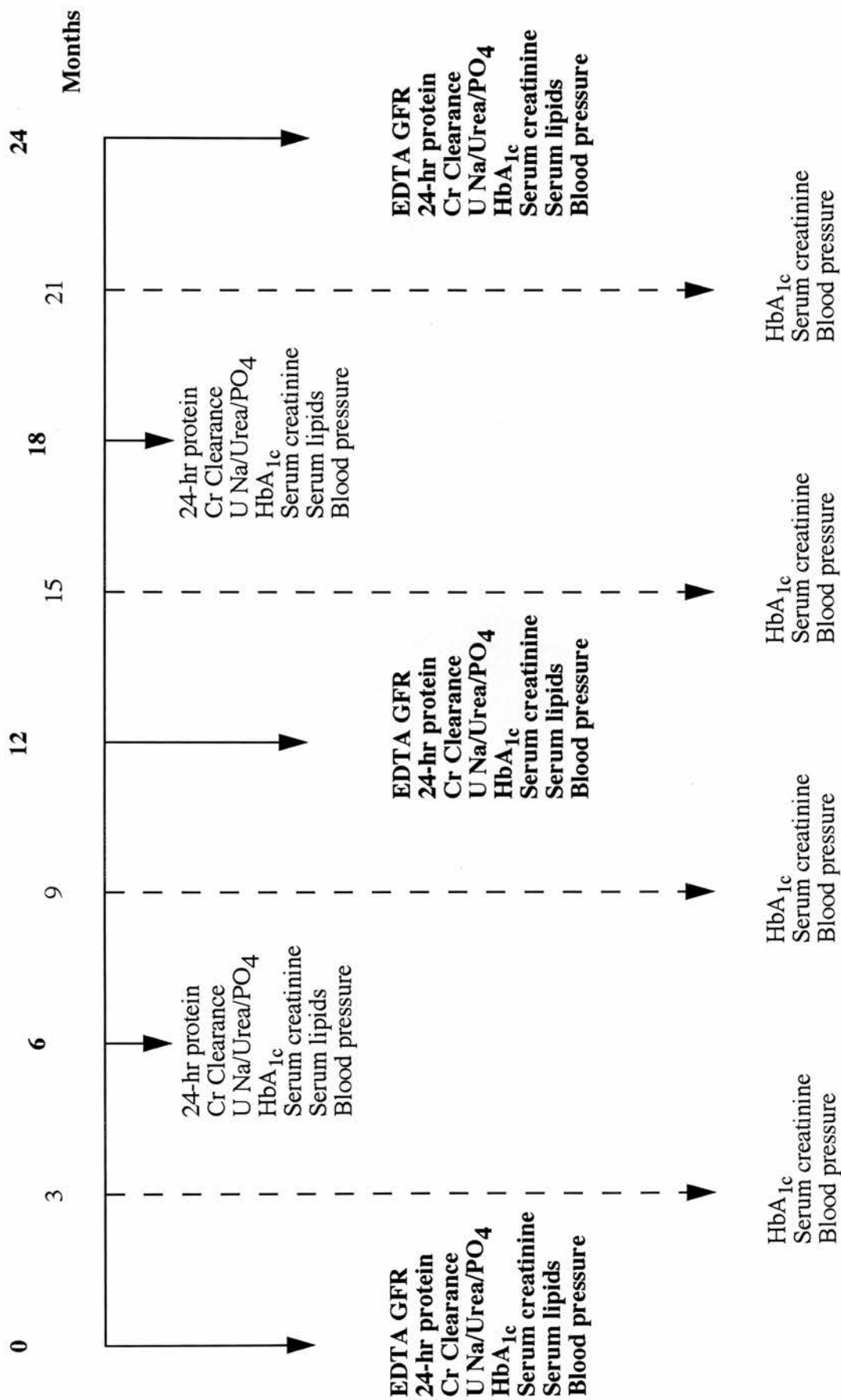
Figure 4.1 : Study protocol for the assessment of progression of nephropathy in non-insulin-dependent diabetes mellitus

emerged. Additional measurements performed at this visit are shown in Figure 4.1. and described below (Sections *4.3.2.iii* and *4.3.2.iv*). Figure 4.1 also shows the measurements performed at the three and six monthly visits. As all the EDTA clearance studies were personally performed only two subjects were seen for the annual visit each week. Thus it took three to four months to complete the group.

4.3.2.ii   *Isotope measurement of GFR*

GFR was measured using the 'single shot' ^{51}Cr EDTA method of Brøchner-Mortensen (1972). All subjects attended at 0930 hrs. in a non-fasted state (insulin administered as usual) and for the duration of the clearance study were requested to refrain from smoking, drinking coffee, tea or alcohol and consuming meat.

70µCi (≈3MBq) of ^{51}Chromium labelled Ethylenediaminetetraacetic acid (EDTA) were injected through a butterfly needle (Venisystems) placed in an ante-cubital fossa vein and the syringe flushed with the patient's own blood. The time of injection was accurately recorded. Ten ml of venous blood were drawn at regular intervals from an in-dwelling cannula or butterfly needle in an opposite forearm vein (flushed with heparinised saline) between two and four hours after the isotope was injected. Accuracy of timing rather than exact spacing of samples is crucial. The drawn blood was placed in a lithium heparin tube and gently mixed by hand. Within the hour samples were centrifuged at ≈1200 G (260 rev.min^{-1} Clandon T52 centrifuge) for six minutes. Duplicate 2 ml samples were then aliquoted (Gibco 1ml pipette) into labelled plastic sample tubes. These together with duplicate 2ml 'Standards' and empty tubes (background count) were counted on a Gamma counter ('Cobra').

Data on the sampling times, mean counts from samples, 'standard' and background together with patient surface area (obtained from nomogram of height and weight) were entered into the standard computer programme for the determination of GFR in the department of Nuclear Medicine. Measurement of the rate of loss of the isotope

from the blood after equilibration with the extra-vascular fluid gives a clearance rate constant and extrapolation of the clearance data back to the time of injection gives a measure of the volume of distribution. The GFR is calculated from these two values. The result is corrected for a standard body surface area and to an inulin equivalent clearance rate. Where the estimated error in the calculation exceeded 10% the data were re-entered omitting sample 1.

Due to a policy change in the department of Nuclear Medicine midway through the study two different methods were used to calibrate the isotope used to measure GFR. For the first twelve months the procedure involved counting the emitted radioactivity from a syringe pre-filled with a known weight of ^{51}Cr EDTA on a scintillation counter before and after injection of the isotope. Each study therefore required a fresh sample and standard to be made up. For the remainder of the study batches of 20 samples with a single standard were made up and could be used for up to one month. This obviated the need to count the radioactivity as the standard and the samples decayed in proportion and the single standard could be used for all 20 studies. Samples were also used for clinical studies. Both methods gave virtually identical GFR results (y=1.00x - 0.01). All the GFR studies were personally performed by myself whilst holding an Administration of Radioactive Substances Advisory Committee (ARSAC) licence.

4.3.2.iii   Laboratory measurements

All subjects participating in the study were familiar with the collection of 24 hour urine. Subjects were however given written instructions with each collection and asked to perform the collection the day prior to their visit. Table A4.1 (in appendix) indicates the methods used to determine urine protein, urea, calcium, phosphate and sodium. Also shown in Table A4.1 are the methodologies for measuring plasma glucose, creatinine, calcium, phoshate, alkaline phosphatase, serum cholesterol (total, HDL and LDL) and triglycerides. HDL and LDL cholesterol could not be measured

where the serum triglyceride exceeded 3.6 mmol.L^{-1}. Haemoglobin was also measured at each visit. The method used to measure glycosylated haemoglobin was changed by the laboratory during the study. For the first year HbA$_1$ was measured using the Corning method (Corning electrophoresis; CIBA-Corning Diagnostics Ltd., Halstead, UK.). Thereafter HbA$_{1c}$ was measured by DCA 2000 system (Abbots Diagnostics Limited). For the purposes of analysis all data are presented as HbA$_{1c}$ using the formula, derived in-house, to convert HbA$_1$ to HbA$_{1c}$ as follows:

$$HbA_{1c} = 0.61 \times HbA_1 + 0.90$$

4.3.2.iv   *Blood pressure measurement*

Blood pressure was measured using a Hawkesley random-zero sphygmomanometer (Wright and Dore 1970). All subjects had the pressure measured in the right arm in the seated position after at least five minutes rest. The mean of three readings taken to nearest 2 mmHg at two minute intervals was recorded. Height and weight were recorded at each visit enabling body mass index (BMI) to be calculated.

4.3.3   Statistical analysis

The analyses were performed on the 19 subjects who completed the study. The results are expressed as mean ±SD, unless otherwise stated when median (range) is given. Prior to analysis the data for 24-hour protein were logarithmically transformed, with the data presented as geometric mean and range. However this transformation did not alter the significance of the results compared to 24-hour protein alone. The decline of GFR was determined from the measurements of EDTA clearance using the least squares method in a linear regression analysis and the results expressed as ml.min.$^{-1}$ month^{-1}.

The data at the start and finish of the study were compared using paired Student's 't-test'. The Pearson's correlation coefficient was used in the univariate analysis of measured variables against decline of GFR. These variables, namely age, duration of

diabetes, BMI, systolic and diastolic blood pressure, HbA_{1c}, serum cholesterol and triglyceride, serum phosphate, calcium-phosphate product and albumin, urinary protein, sodium, phosphate and urea were then entered (F factor set at 3 for inclusion in the model) into a forward stepwise linear regression analysis to determine those exerting a significant influence on the decline of EDTA GFR.

On the basis of median decline of GFR the subjects were subdivided into 'Progressors' and 'Non-progressors'. Each independent variable was compared between the 'Progressors' and the 'Non-progressors' using the Student's unpaired 't-test'. Significance throughout is assumed when $p \leq 0.05$ (two-tailed).

The statistical packages 'Statview' (Brain Power Inc., Calabasa, USA) and Statistica (StatSoft Inc., Tulsa, Oklahoma) were used throughout.

4.4   RESULTS

4.4.1   Progression of nephropathy

4.4.1.i   Subject follow-up

Nineteen of the 26 subjects who entered the study were followed for a mean of 21 months (range 20-24). Of the remaining seven, two (Cases 3 and 14) died from cardio-vascular disease within one year, two proceeded rapidly to dialysis, whilst three completed only 6 - 12 months of follow-up (Cases 24-26). Both subjects who started dialysis did so within one year of commencing the study, despite an initial GFR exceeding 25 ml.min^{-1} Although both had biopsy proven diabetic glomerulosclerosis, one (Case 16) had a 'horseshoe kidney' and the other (Case 18) a repeat biopsy demonstrated amyloid deposition in addition to the previously reported glomerulosclerosis.

4.4.1.ii   Baseline renal function

Data on renal function in individual subjects are presented in Table 4.5 (overleaf). The median GFR of all subjects at baseline was 42.6 ml.min^{-1} (range 11.4 - 105.9). In only one subject (JE) did the GFR exceed 80 ml.min^{-1}. By the end of the study the GFR had fallen significantly to 30.1 ml.min^{-1} (8.3 - 58.4), $p<0.05$. The comparable creatinine value rose from 225 μmol.L^{-1} (139-351) to 279 μmol.L^{-1} (129 - 567), $p<0.05$. 24-hour protein excretion varied widely at baseline, ranging from 0.22 to 15.7 g (median 2.3 g). The protein excretion did not change significantly during the follow-up period (see Table 4.5). Notably the two subjects who commenced dialysis had protein excretions exceeding 10 g.24 hr^{-1}.

4.4.1.iii   Rate of decline of GFR

The median decline of GFR for the 19 subjects was 0.48 ml.min.$^{-1}$month^{-1} (range - 0.91 to 3.98). The values for the individual subjects are shown in Table 4.5. Racial differences existed in the rate of decline of GFR as shown in Table 4.6 (below).

	Caucasian	Afro-Caribbean	Asian
Nos	8	7	4
Decline of GFR	1.12 (1.24)**	0.56 (0.53)*	-0.28 (0.5)

Table 4.6 : Rate of decline of GFR in different racial groups. * p<0.03 v Asian; ** p=0.06 v Asian. Caucasian v Afro-Caribbean NS.

Despite the small numbers the four Asian subjects declined at a significantly slower rate than the Afro-Caribbeans, whilst the comparison with Caucasians just fails to achieve significance.

Case	GFR (ml.min^{-1})		Decline of GFR (ml.min.$^{-1}$month^{-1})	Creatinine (μmol.L^{-1})		Protein excretion (g.24-hr^{-1})		Study period (months)
	Start	Finish		Start	Finish	Start	Finish	
1	51.2	56.2	-0.06	142	140	224	702	20
2	57.0	43.0	1.52	139	129	1650	618	21
3*	66.0	-	-	159	-	1150	-	8
4	25.2	43.4	-0.91	154	151	805	1020	20
5	105.9	17.4	3.98	154	412	9730	3590	21
6	28.1	17.2	0.48	296	425	6270	6630	21
7	31.5	10.3	1.06	252	421	7440	4600	20
8	43.9	32.1	0.59	263	248	1760	2600	20
9	22.5	21.3	0.09	226	235	308	907	21
10	54.2	58.4	-0.44	186	189	1420	2320	24
11	32.5	36.6	-0.18	191	198	1400	3170	20
12	43.0	16.7	1.14	220	321	1570	4750	24
13	26.7	24.8	0.02	257	286	4930	3290	24
14*	74.5	-	-	151	-	1440	-	12
15	26.4	8.3	0.81	351	567	7350	2670	20
16**	34.1	-	-	241	-	10890	-	11
17	31.2	19.3	0.48	336	435	4800	1040	24
18**	27.1	-	-	337	-	15700	-	7
19	32.6	29.4	0.20	218	275	5010	2530	20
20	77.4	52.4	1.22	159	188	1250	1610	20
21	33.4	21.4	0.40	236	297	2250	478	24
22	41.2	25.9	0.80	302	430	1680	2280	20
23	39.3	25.9	0.55	224	261	1960	957	24
24	11.4	21.3	-0.83	361	282	2290	1460	12
25	63.8	49.5	2.39	165	216	3850	3380	6
26	28.2	30.6	-0.4	286	314	6780	6710	6
Median	34.1	25.9	0.48	224	275	1760	2320	
	p<0.02			*p<0.03*		NS		

Table 4.5 : Measures of renal function at the start and end of the study in all 26 subjects. (2 died* and 2 started dialysis**). Cases 24 - 26 were excluded from the analysis (see text)

The rate of decline of GFR does not correlate with initial GFR when considering all subjects with a GFR < 100 ml.min^{-1}. If subject 5 is included in this analysis then a higher baseline GFR is significantly associated with a more rapid fall in GFR.

4.4.2   Clinical parameters and the decline of GFR.

4.4.2.i   Characteristics of subjects at baseline and follow-up.

The mean values for the measured variables at baseline and at the final visit for the 19 subjects who completed the study are to be found in Table 4.7. Systolic blood pressure fell significantly from 162 to 150 mmHg. Both diastolic and mean arterial blood pressure fall during the study, but in neither case was this significant.

Parameter	Baseline	End of study	Difference	Significance
GFR (ml.min^{-1})	42.3 (20.6)	29.5 (15.0)	12.8	*p*<0.02
BMI	29 (6)	29 (5)	-	NS
SBP (mmHg)	162 (30)	150 (25)	12	*p*=0.05
DBP (mmHg)	88 (13)	82 (9)	6	NS
MAP (mmHg)	113 (15)	104 (11)	*9*	*p*<0.001
HbA$_{1c}$	8.0 (1.6)	7.4 (2.6)	0.6	NS
Cholesterol (mmol.L^{-1})	7.2 (2.4)	6.0 (1.4)	1.2	*p*<0.01
Triglyceride (mmol.L^{-1})	3.8 (3.4)	2.6 (1.4)	1.2	NS
UProtein (g.24hr^{-1})	3.2 (2.8)	2.4 (1.7)	0.8	NS
UPO4	22.3 (8.7)	20.2 (8.4)	2.1	NS
UNa	148 (30)	122 (41)	26	*p*<0.01
UUrea	346 (102)	334 (124)	12	NS

Table 4.7 : Measured variables at baseline and follow-up. Data as mean (SD).

Comparison is by paired 't-test', significance as shown.

Of the remaining factors only serum cholesterol and 24-hour urine sodium excretion are significantly different at the end of the study. The reduction in urinary sodium does not correlate with the drop in blood pressure in individual subjects. Prescribed anti-hypertensive medication did not change significantly during the study (Table 4.3), although the more frequent clinic attendance may have ensured greater drug compliance.

4.4.2.ii   Factors which associate with the decline in GFR.

Univariate regression analysis comparing the decline of GFR with the measured clinical variables identifies a significant correlation of systolic blood pressure, diastolic blood pressure and mean arterial pressure with decline of GFR. Also significantly associated are serum cholesterol and 24-hour protein excretion. However if Case 5 (JE) is excluded from the analysis (as being an extreme value) only systolic blood pressure, mean arterial pressure and serum cholesterol remain significant. This result highlights the influence such a value can have on data analysis where the numbers studied are small. These data are presented graphically in Figures 4.2 to 4.4 and summarised in Table A4.2 (appendix).

Forward stepwise regression analysis identifies seven variables with an F to enter value above 3. Of these systolic blood pressure ($p<0.005$) and serum cholesterol ($p<0.005$) are highly associated with the decline of GFR. Diastolic blood pressure is also associated, although to a less significant degree ($p<0.05$). The remaining four factors, namely 24-hour protein, age, BMI and serum albumin have p values between 0.05 and 0.1. The decline of GFR is related to SBP, DBP and serum cholesterol in the following regression equation:

Decline of GFR ($ml.min.^{-1}month^{-1}$) = -12.3 + 0.02 SBP + 0.15 DBP + 0.22 cholesterol

These three factors account for 38% of the variability of the decline of GFR.
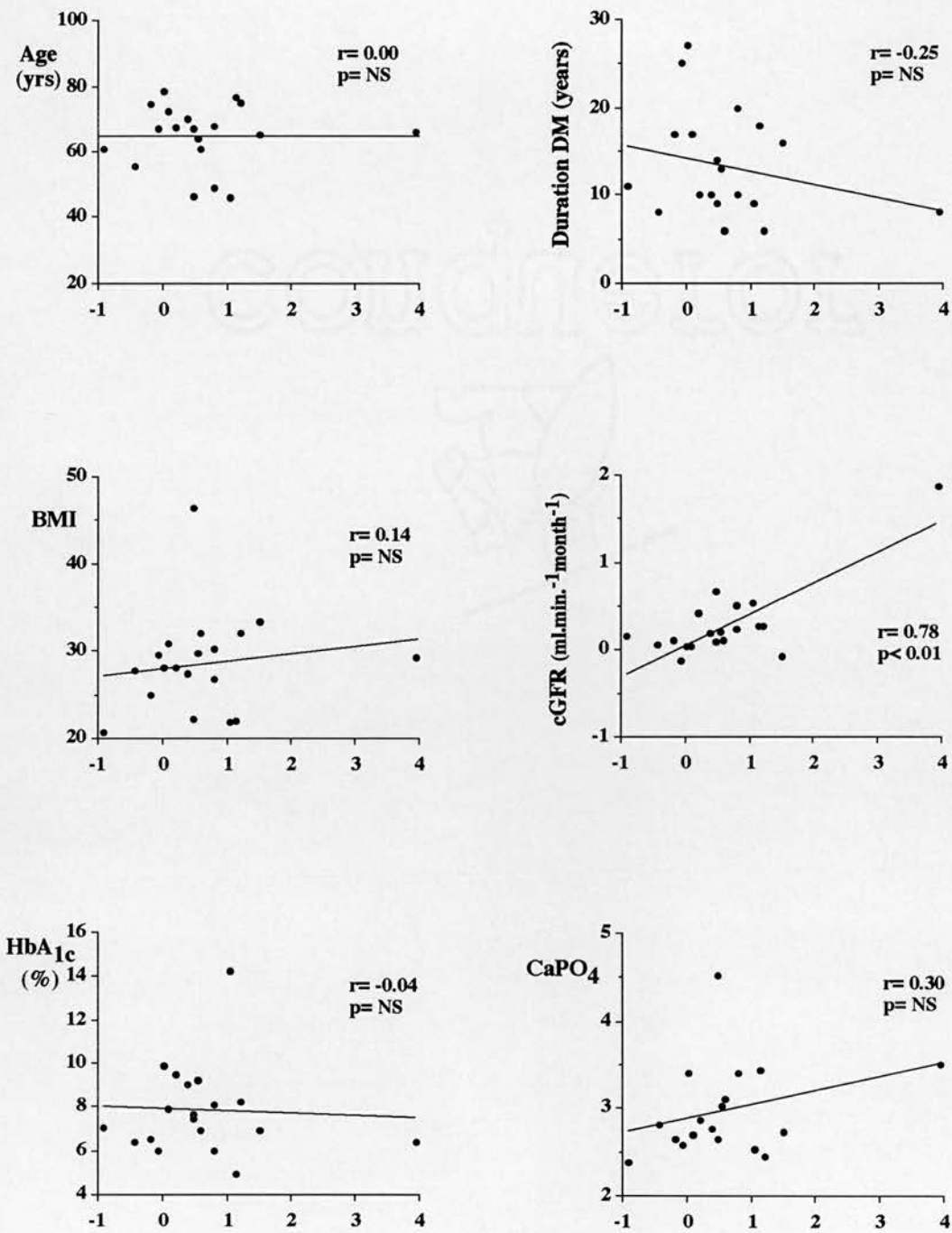
Rate of decline of EDTA GFR (ml.min.$^{-1}$month^{-1})

Figure 4.2 : Relationship between decline of EDTA GFR and the measured parameters of age, duration of diabetes, BMI, cGFR, HbA$_{1c}$ and CaPO$_4$ product. Univariate analysis.
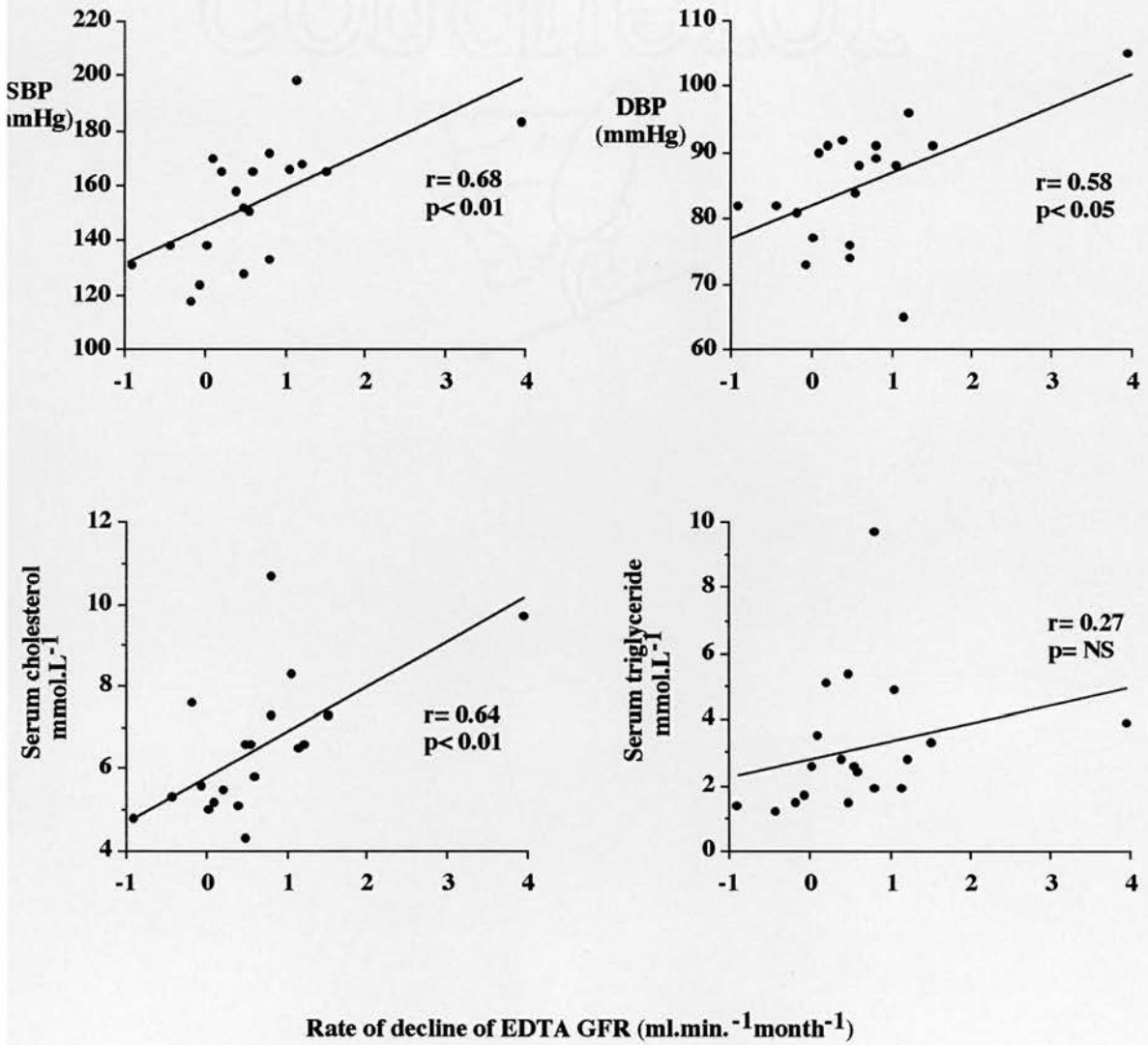
Figure 4.3: Relationship between the decline of EDTA GFR and blood pressure and serum lipids. Data presented as mean of all values obtained during study. Univariate analysis.

Figure 4.4 : Relationship between the rate of decline of EDTA GFR and the urinary excretion of protein, sodium, phosphate and urea. Univariate analysis.

Parameter	Progressors n = 10	Non-progressors n = 9	Difference	Significance
Age (years)	63.5 (10.5)	66.0 (9.5)	-2.5	NS
Duration of DM	11.8 (5.2)	14.8 (6.7)	-3.0	NS
BMI	29 (4)	29 (7)	0	NS
SBP (mmHg)	167 (18)	142 (18)	25	$p < 0.01$
DBP (mmHg)	89 (11)	82 (7)	7	NS
HbA_{1c} (%)	7.9 (2.7)	7.7 (1.4)	0.2	NS
Serum cholesterol	7.6 (1.6)	5.5 (1.0)	2.1	$p < 0.01$
Serum triglyceride	3.7 (2.5)	2.7 (1.6)	1.0	NS
$CaPO_4$	3.06 (0.41)	2.93 (0.62)	1.03	NS
24-hr protein	3.78 (3.15)	2.46 (1.92)	1.32	NS
24-hr phosphate	22.9 (7.5)	20.1 (6.4)	2.8	NS
24-hr sodium	142 (22)	140 (32)	2	NS
24-hr urea	352 (132)	320 (100)	32	NS
Decline in GFR	1.22 (1.03)	0.44 (0.43)	0.78	-

Table 4.8 : Measured variables in 'Progressors' and 'Non-progressors' (see text for definition. The data are presented as mean (SD). Comparison between the groups is by unpaired 't-test'; significance as shown. Decline in GFR in ml. min.$^{-1}$month^{-1}

4.4.2.iii   *Differences between the 'Progressors' and the 'Non-Progressors'*

The subjects were then divided into two groups, 'Progressors' and 'Non-progressors' on the basis of decline of GFR (Table 4.8 preceding page). The mean decline of GFR in the 'Progressors' (n=10) was 1.22(1.03) ml.min.$^{-1}$month^{-1}; mean(SD) compared to -0.04 (0.43) in the 'Non-progressors' (n=9). Of the variables measured only systolic blood pressure {167(18) vs 142(18) mmHg; $p<0.01$} and serum cholesterol {7.6(1.6) vs 5.5(1.0) mmol.L^{-1}; $p<0.01$} were significantly different between the 'Progressors' and 'Non-progressors'. 24-hour protein excretion was slightly higher, but not significant, in the 'Progressors' (3.78 v 2.46g; p=NS), whereas HbA$_{1c}$ and BMI were almost identical in both groups.

4.5   DISCUSSION

4.5.1   General comments

GFR declined at a rate of 0.48 ml.min.$^{-1}$month^{-1} (equivalent to 5.76 ml.min.$^{-1}$year^{-1}) in the 19 subjects who completed the two years of follow-up in this prospective study. This figure is identical to that found by Gall and co-workers (1993), and very similar to the decline seen for NIDDM subjects in the retrospective analysis presented in Chapter 2. The range in decline for individual subjects is wide, from -0.91 to 3.98 ml.min.$^{-1}$month^{-1} (-10.1 to 47.8 ml.min.$^{-1}$year^{-1}) a feature common to all studies. The published series in diabetic subjects in whom progression is assessed by isotope clearance methods are shown below (Table 4.9 overleaf). These indicate that the observed decline of GFR was $\approx$10ml.min.$^{-1}$year^{-1}, some ten-fold greater than the natural decline of $\approx$1.3ml.min.$^{-1}$year^{-1} (Davies and Shock 1950a; Wesson 1969; Rowe et al. 1986). The studies in IDDM subjects (Mogensen 1982; Parving et al. 1983; Björck et al. 1986) indicate that following the introduction of anti-hypertensive treatment there was an $\approx$50% reduction in the decline of GFR to levels currently demonstrated in NIDDM subjects. No data exist on the rate of progression in untreated NIDDM subjects. The data in treated patients suggest that the limit to

	Subjects	Method	Follow-up (months)	Initial GFR (ml.min^{-1})	Rate of decline (ml.min.$^{-1}$month^{-1})	Comment
IDDM						
Pre intervention						
Mogensen (1976)	10	Ioth	32	95	0.91	Proteinuria
Mogensen (1982)	5	Ioth	22	100	1.23	pre-BP Rx.
Parving (1983)	10	EDTA	29	105	0.91	pre-BP Rx.
Bjorck (1986)	15	EDTA	32	60	0.86	pre-BP Rx.
Walker (1989)	19	EDTA	29	62	0.61	Normal diet
Post intervention						
Mogensen (1982)	5	EDTA	73	70	0.49	on BP Rx.
Parving (1983)	10	EDTA	39	75	0.39	on BP Rx.
Bjorck (1982)	15	EDTA	24	35	0.46	on BP Rx.
Walker (1986)	19	EDTA	33	46	0.12	Restricted protein
NIDDM						
Friedman (1991)	6	EDTA	19	83	No change	-
Gall (1993)	26	EDTA	62	60	0.48	-
Myers (1995)	48	Ioth	48	108	0.77	-
Nelson (1996)	34	Ioth	48	124	0.93	-
This study	26	EDTA	20	34	0.48	-

Key : EDTA = Ethylenediaminetetraacetic acid clearance; Ioth = Iothalamate clearance

Table 4.9 : Studies in diabetic subjects where decline in GFR was measured isotopically

arresting the decline of GFR may have been reached with the current treatment strategies. It is not yet certain whether the observed reduction of the rate of decline can be sustained in the long-term although the findings in this study would suggest that this may indeed be the case.

Studies based on isotope clearance methods (see Table 4.9) suggest that the rate of progression of nephropathy is similar in IDDM and NIDDM, although that of Friedman and Gross (1991) is a clear exception. These authors found no decline of GFR in six subjects with a baseline GFR of $\approx$ 60 ml.min^{-1}month^{-1} over a period of six months. On the basis of this small study and the findings from the study of Fabre and associates (1982) these authors considered the rate of decline of GFR to be slower in NIDDM than IDDM. The results of the more recent studies suggest these claims are unjustified. This subject is discussed at greater length in Section 2.6.1.

A weakness of this prospective study is the short observation period. This was inevitable given the constraints of time. Mogensen (1992) regarded three isotopically determined GFR measurements performed over a two year period as the minimum necessary for the reliable determination of the decline of GFR. Though these criteria have been met, a longer observation period with more EDTA clearance measurements would have been desirable. The same author (Mogensen 1976) however demonstrated that short-term studies could predict long-term decline of GFR (r=0.96, $p<0.01$). Comparing the data from this present study for the 16 subjects where adequate follow-up data were available (first two years predicted GFR - data presented in Chapter 2 vs isotope GFR) showed a significant, albeit less strong, correlation (r=0.62, $p<0.01$).

The striking feature, however, as demonstrated in the retrospective analysis, is the minimal progression seen in certain individuals. This observation alone should give encouragement to those with renal impairment. If the reasons for this slow

progression can be identified treatment might be better targeted for those who progress more rapidly.

4.5.2 The factors associated with progression of nephropathy

4.5.2.i Ethnic origin and progression of nephropathy

The reason for the higher prevalence of non-Caucasoid subjects entering renal replacement programmes has exercised many (Grenfell et al. 1988; Pugh et al. 1988; Cowie et al. 1989; Stephens et al. 1990). Broadly speaking there could be three explanations - (1) a higher prevalence of diabetes; (2) a higher prevalence of renal disease in those with diabetes mellitus (often ascribed to hypertensive nephrosclerosis); or (3) a more rapid progression once nephropathy is established. This subject is discussed at greater length and referenced in Section 2.6.2. Of the above this study has examined only the last mentioned and has found no significant difference in progression between Afro-Caribbean and Caucasian subjects. The progression if anything was more rapid in the latter and confirms the impression gained in the retrospective survey (Section 2.4.2). The wide variance and small numbers studied combine to ensure that these differences are not statistically significant. That the four Asian subjects decline at a more modest rate than the Caucasian group may be atypical, though this again is in agreement with the findings in the retrospective survey.

Generally speaking the studies performed in nephropathy have used surrogate markers of progression and as demonstrated below (Chapter 5) should be interpreted with caution. In subjects with nephropathy secondary to essential hypertension, for example, Rostand and colleagues (1989) recorded black race (unspecified) as being associated with more rapid progression. Given that all the subjects in this study had a baseline creatinine of < 135 $\mu mol.L^{-1}$ and progression was defined as a rise of > 35 $\mu mol.L^{-1}$ the legitimacy of this claim must be questioned. Brazy and Fitzwilliam (1990) found no racial differences in progression as determined by reciprocal

creatinine. In a recent study, Cowie (1993) has shown that the time taken from a serum creatinine of approximately 160 μmol.L^{-1} to renal replacement is significantly longer in black compared to white subjects, supporting the above findings. That the prevalence of renal disease is higher in non-white populations is generally accepted, although whether such subjects progress more rapidly to ESRD remains contentious. There are no studies to my knowledge which have examined progression of renal impairment using isotope clearance in non-white populations.

4.5.2.ii   Blood pressure and progression of nephropathy

In contrast to the findings reported in Chapter 2, stepwise regression analysis shows that elevated blood pressure, both systolic and diastolic, is associated with a more rapid decline of GFR. However, when the group is divided into 'Progressors' and 'Non-progressors' only systolic pressure is significantly different, being higher in the former. Given that control of blood pressure has an established place in the management of patients with progressive renal disease it is surprising that not all studies identify blood pressure as a significant variable.

Systolic, rather than diastolic, blood pressure is emerging as the more significant factor in the progression of nephropathy, although recently published data from Rossing and colleagues (1994a) in IDDM subjects suggests the converse. Nielsen and co-workers (1993a), in 24 normoalbuminuric and 13 microalbuminuric NIDDM subjects, found a more rapid decline of GFR in those with higher baseline and mean systolic blood pressure throughout study. These results should be interpreted with caution since GFR was measured only twice, at the beginning and again at the end of the study. The same group found no effect of blood pressure in a previous study of seven proteinuric subjects (Nielsen et al. 1991), perhaps due to the small number of subjects examined.

In a retrospective analysis of hypertensive Japanese subjects with NIDDM, Babo and colleagues (1990) observed a more rapid decline of GFR in those with systolic hypertension (Table 4.10 overleaf). Hasslacher and associates (1993) and Biesenbach and associates (1994) also identified systolic blood pressure as an important determinant of decline of GFR, as did Gall and colleagues (1993). The latter demonstrated it to be the dominant influence over the decline of GFR. No association with blood pressure was observed in the other two published prospective studies in NIDDM {Friedman and Gross (1991); Myers et al. (1995)}.

An increase in urinary albumin excretion has been suggested by some as a marker of progression of nephropathy (Mogensen 1993), although this is questioned (Lane et al. 1992; Tsalamandris et al. 1994). Conversely a decrease in albumin excretion is equated with a reduction in progression of diabetic (Rossing et al. 1994a) and non-diabetic renal disease (Apperloo et al. 1992). In a six-year follow-up study of 278 NIDDM subjects with varying degrees of protein excretion, Schmitz and colleagues (1994) observed that progression of UAE was associated with systolic, though not diastolic, blood pressure. Furthermore when the subjects were divided into 'Progressors' (n=46) and 'Non-progressors' (n=132) on the basis of protein excretion, the former had higher systolic, but not diastolic, blood pressure. Unfortunately no assessment of renal function was made to complement these findings. By contrast Cooper et al. (1988) found no association with blood pressure.

It is argued that a raised UAE precedes the development of hypertension in IDDM (Mogensen 1993), but that the reverse may be the case for NIDDM (Nelson et al. 1993). In this present study in only three subjects did the diagnosis of hypertension precede diabetes, although this is most probably an underestimate. In the remainder, eight years on average elapsed before hypertension was diagnosed. If it is the case, as has been suggested (Pugh et al. 1993), that diabetic subjects developing hypertension before diabetes have more rapid progression of subsequent renal disease then greater

Reference	Subjects	Renal disease	Method	Rate of decline (ml.min.$^{-1}$.month^{-1})	Associated BP*
Baba (1990)	6	-	Endogenous CrCl	1.15	BP $\geq$ 150/90
Baba (1990)	9	-	Endogenous CrCl	0.33	BP < 150/90
Nielsen (1991)	7	proteinuria	EDTA	0.61	SBP
Nielsen (1993)	13	microalbuminuria	EDTA	0.13	SBP
Hasslacher (1993)	47	proteinuria	Estimated GFR	0.48	SBP
Gall (1993)	26	renal impairment	EDTA	0.48	SBP
This study	26	renal impairment	EDTA	0.48	SBP

Key : CrCl = creatinine clearance; EDTA = Ethylenediaminetetraacetic acid; Estimated GFR from Cockcroft-Gault formula

* BP as shown or as determined in multiple stepwise regression

Table 4.10 : Studies in NIDDM subjects highlighting the effect of blood pressure on the rate of decline of GFR

attention to the diagnosis and treatment of hypertension will be required. Nelson and colleagues (1996b) found no association between baseline blood pressure and subsequent rate of decline of GFR. It is not clear whether anti-hypertensive therapy if started early, as suggested by Marre and colleagues (1988) and Mathiesen and colleagues (1991), will have an effect greater than that suggested by short-term studies.

Understanding of the role of systemic hypertension in progressive renal disease has been facilitated by studies of animal models. The remnant kidney and diabetes-induced rat models provide insights into the mechanisms of initiation and progression of glomerular injury. Micropuncture techniques reveal a compensatory increase in GFR in the remaining nephrons after 5/6th ablation of renal tissue (Olson et al. 1979; Hostetter et al. 1981). Amongst various histological sequelae of this procedure is an increase of mesangial matrix. Steffes and co-workers (1978) performed a uni-nephrectomy in streptozotocin-induced diabetic rats and found that the degree of mesangial deposition was greater in these rats compared to control diabetic animals.

Hostetter and colleagues (1982) showed subsequently that hyperfiltration occurred in single nephrons in such animals. Also using streptozotocin-induced diabetic animals, Mauer et al. (1978) clipped one renal artery (Goldblatt model) and demonstrated increased glomerulosclerosis in the unclipped kidney of the hypertensive diabetic animals compared to contralateral clipped kidney and the control diabetic animals. Indeed the clipped kidney showed less glomerulosclerosis than seen in these control animals. Similar findings were observed in a patient with diabetic nephropathy and a unilateral renal artery stenosis (Berkman and Rifkin 1973). The role of mesangial matrix expansion is discussed further in Chapter 6.

The studies of Andersen et al. (1985, 1986) demonstrate the effect of anti-hypertensive therapy on glomerulosclerosis and proteinuria in Munich-Wistar rats who underwent 5/6th renal ablation. Animals treated with the ACE inhibitor captopril showed a reduction of glomerulosclerosis and proteinuria. In a second study, triple anti-hypertensive therapy failed to reduce both transcapillary hydraulic pressure in the nephrons and glomerulosclerosis compared to captopril, despite comparable systemic blood pressure levels. Similar findings were recorded by Zatz and coworkers (1986) in diabetes-induced rats.

This study was not designed to examine the benefits of specific anti-hypertensive agents, although the value of ACEI in diabetic nephropathy is emerging (Lewis et al. 1993). Only four individuals, two 'Progressors' and two 'Non-progressors' were not on ACEI at the start of the study. By the end one from each of these groups had been commenced on that agent. The usage of anti-hypertensive drugs is discussed at greater length in Chapter 2.

It is no longer the case of whether to use anti-hypertensive therapy and which agent in diabetic nephropathy. The question is now when to start treatment and at what level of blood pressure? These remain to be answered but in general a MAP of approximately 100 mmHg is advocated in microalbuminuric and proteinuric IDDM subjects (Mogensen et al. 1991). Given the mean blood pressure of the 'Non-progressors' in this study of 142/82 mmHg, a MAP of 100 mmHg may also be a reasonable target in NIDDM.

4.5.2.iii   *Proteinuria and the progression of nephropathy*
The relationship of proteinuria to progression of renal disease has been examined at some length in Chapter 2, both for diabetic and non-diabetic renal disease, and brief comment only is made here. This present study has identified 24-hour urinary protein as being associated with progression both for univariate and stepwise regression

analysis, albeit not statistically significant ($p=0.07$) in the latter. However when the groups are subdivided, there is no significant difference between 'Progressors' and 'Non-progressors', although protein excretion is greater in the former. Similar results were found by Gall and colleagues (ibid). The reasons for the difference between the retrospective and the prospective studies are not clear. The influence of blood pressure appears greater in the latter and may as a consequence lessen the importance of protein excretion. ACEI have been shown to diminish proteinuria and, in theory, could limit the influence of this factor in multiple regression analysis. The published studies which show a significant association of proteinuria with progression of nephropathy are older and, in most cases, retrospective, thus supporting this suggestion. The protein excretion in the NIDDM group in the retrospective analysis at 3.4 g.day^{-1} is little different from the 2.4 g.day^{-1} observed at the end of the prospective study.

4.5.2.iv   *Serum lipids and the progression of nephropathy*

A strong association between serum cholesterol and decline of GFR has been demonstrated in this study both in univariate analysis ($r=0.65$, $p<0.01$) and in stepwise regression. Further, by subdividing the group into 'Progressors' and 'Non-progressors' the serum cholesterol is found to be significantly higher in the former. Interest in the role of lipids in progressive renal disease has been rekindled recently (Moorhead et al. 1982; Klahr and Harris 1989). Whilst there is strong evidence for a causative association in animal models (French et al. 1976, Kasiske et al. 1985, Kasiske et al. 1988) and suggestive evidence from *in vitro* studies (Wheeler et al. 1993), clinical studies are few.

In non-diabetic subjects with proteinuria, Maschio and colleagues (1989) found the rate of decline of GFR in subjects with high serum cholesterol and triglyceride levels to be twice that of subjects with normal lipid levels. In African-American and Hispanic children with nephrotic syndrome, Ingulli and associates (1991) observed a

more rapid progression to renal failure in those with higher lipid levels. Most studies in diabetes have shown no association between lipids and progression of renal disease. The strongest evidence comes from Mulec and colleagues (1990) and Krolewski and colleagues (1994). In the former study, primarily designed to compare two different anti-hypertensive regimens in IDDM subjects, the authors observed a significant association of decline of GFR with serum cholesterol. When the group was divided on the basis of serum cholesterol throughout the study, the mean decline of those with a total cholesterol ≤ 7 mmol.L^{-1} was significantly lower at 0.19 ml.min.$^{-1}$month^{-1}, compared to 0.7 ml.min.$^{-1}$month^{-1} in those with a value >7 mmol.L^{-1}. In addition baseline cholesterol was significantly associated with decline of GFR. A similar analysis performed on the participants in this study is shown in Table 4.11, subdivided on the basis of a serum cholesterol of 6 mmol.L^{-1}.

	≤ 6 mmol.L^{-1}	> 6 mmol.L^{-1}
Subjects	9	10
Decline of GFR	0.04	1.14*
MAP	105	107
24-hour protein	2.05	4.02

Table 4.11 : Decline of GFR (ml.min.$^{-1}$month^{-1}) in subjects with low and high cholesterol levels. (* p<0.01; MAP, 24 hour protein NS).

This indicates that a significantly slower decline of GFR occurs in those with a serum cholesterol under 6 mmol.L^{-1}. However whether this is a direct effect or a consequence of the association between cholesterol and protein excretion (Figure 4.5 overleaf) remains to be seen.

No association was observed between serum triglyceride and progression in this study. Others have recorded similar findings (Gall et al. 1993), whereas Hasslacher and colleagues (1993) did demonstrate triglyceride, but not cholesterol, as a significant factor determining progression (predicted by Cockcroft-Gault) in proteinuric NIDDM subjects. Attman and colleagues (1992) also showed levels of total cholesterol, LDL and HDL cholesterol, and triglyceride to be directly related to decline of GFR in a cross-sectional study in a study of 24 IDDM subjects. Apolipoprotein CIII and E levels were significantly higher in subjects with a GFR < 20 ml.min^{-1}.

Published studies examining the effect of lipid-lowering agents in the progression of renal disease are equally scarce. Two uncontrolled studies, one in nephrotic syndrome secondary to non-diabetic renal disease (Rabelink et al. 1990) and the other in NIDDM (Sasaki et al. 1990), using HMG-CoA reductase inhibitors observed a reduction of albuminuria. The lack of a control group weakens these studies significantly. In a 12-week randomised placebo-controlled study, Hommel and co-workers (1992) found no change of GFR (baseline 70 ml.min^{-1}) in 10 IDDM subjects given simvastatin. In a comparable, though longer, study in 18 NIDDM subjects also using simvastatin Nielsen and colleagues (1993b) drew similar conclusions. Whilst total and LDL cholesterol were significantly lowered, no significant effect was demonstrated on GFR and albumin excretion. It should be noted, however that the mean GFR in the placebo group fell by 8.3 ml.min^{-1} compared to 0.6 ml.min^{-1} in the treated group over the six month period.

The role of lipids (and their treatment) remains to be established in progressive renal disease. It is important to define whether the current empiric administration of lipid lowering agents to patients with renal impairment confers benefit to the patient by slowing disease progression or by protecting against cardiovascular disease and, if so, at what stage should they be introduced.

4.5.2.v   Glycaemic control and progression of nephropathy

This study has demonstrated no association of glycaemic control with decline of GFR. Furthermore the mean HbA_{1c} is virtually identical in the 'Progressors' and the 'Non-progressors'. Opinion differs as to the effect of glycaemic control on progression once clinical nephropathy is reached. In an early interventional study in 6 IDDM subjects Viberti and colleagues (1983) demonstrated no statistical benefit using continuous subcutaneous insulin infusion (CSII) over 24 months on the decline of GFR. However if the graphic data are examined in 5 cases there is an apparent arresting of the decline of GFR. By contrast, Nyberg and co-workers (1987) found better control was associated with slower progression in 21 subjects followed for 30 months. In NIDDM Gall and colleagues (1993) and Hasslacher and colleagues (1993) found no association of HbA_{1c} with decline in GFR thus supporting the finding in the present study. It appears likely that improving glycaemic control does not influence the progression of established nephropathy, although no intervention studies have been undertaken in this group. It cannot be recommended however that clinicians ignore glycaemic control once this stage is reached, since to do so may accelerate other microvascular and macrovascular complications and prejudice the health of a transplanted kidney were this to be the outcome.

4.5.2.vi   Miscellaneous factors and progression of nephropathy

Protein intake was not assessed directly in this study. Urea excretion measured here is a surrogate marker from which protein intake may be derived (Isaksson et al. 1982; Maroni et al. 1985). Neither 24-hour urinary urea nor calculated protein ingestion was associated with decline of GFR nor did levels differ between 'Progressors' and 'Non-progressors'. Gall and colleagues (1993) and Jameel and colleagues (1992) also found no association, although small intervention studies in IDDM have demonstrated benefit (Walker et al. 1989; Zeller et al. 1991). In the former six subjects followed for 24 months showed a 75% reduction in the rate of

decline of GFR on a diet of 0.6 mg.kg.$^{-1}$ day^{-1}, although the study was not controlled throughout for blood pressure. However this level of protein restriction is severe and is unlikely to gain widespread acceptance by the majority.

In the wider context of all renal disease controversy on the benefit of protein restriction has reigned for sometime. The early clinical studies of El Nahas and colleagues (1984) (though benefit was shown only for tubulo-interstitial disease), Rosman and colleagues (1984) and Oldrizzi and colleagues (1985) espoused the value of reducing dietary protein, conclusions supported by similar studies in the 'Remnant kidney' rat model (Hostetter et al. 1986) and in rats with experimental diabetes mellitus (Wen et al. 1985; Zatz et al. 1985; Mauer et al. 1989). A meta-analysis of low protein diets and progression of renal disease found in favour of restriction (Fouque et al. 1992), although several studies including that of Williams and colleagues (1991) demonstrated no benefit from either protein or phosphate restriction in renal failure of various aetiologies. The recent evidence from the MDRD study (Klahr et al. 1994) indicating no advantage of protein restriction in moderate renal impairment (25 to 55 ml.min^{-1}) should spare many from the deprivations of a low-protein diet. Here 840 subjects with chronic renal disease of diverse aetiology were subject over a two year period to a standard, low (0.58 g.kg.$^{-1}$ day^{-1}) or very low (0.28 g.kg.$^{-1}$day^{-1}) protein diet, depending upon their baseline GFR. Only in those with severe renal impairment (13 to 24 ml.min^{-1}) did severe restriction show a modest, non-significant slowing of the rate of decline of GFR. Thus individuals with diabetic renal disease should no longer be subjected to rigid protein restriction, although no intervention study exists in NIDDM. The American Diabetes Association and National Kidney Foundation recommendation is 0.8 g.kg.$^{-1}$ day^{-1} (Consensus Statement).

There is no evidence from this study that serum calcium, phosphate or the $CaPO_4$ product is related to the decline of GFR. Unfortunately parathyroid hormone levels

were not measured, as Massry (1977) and Fröhling and colleagues (1989) have suggested the PTH level may accelerate progression of renal disease. The latter group considered a "moderately-low" protein diet supplemented with ketoacids could partially offset the adverse effect of hyperparathyroidism.

Of other putative factors influencing progression smoking behaviour was not assessed. Stegmayr (1990) and Sawicki and associates (1994) have highlighted the adverse effects of smoking on progression of nephropathy in retrospective and prospective studies in IDDM subjects. However the number of subjects progressing in each study was small and other factors, e.g. glycaemic control and non-statistical differences in blood pressure may have contributed. In theory smoking may advance progression by accelerating macrovascular disease. Ritz and colleagues (1991) have suggested that ischaemic nephropathy secondary to large vessel disease may accelerate progression in NIDDM subjects. This subject is addressed in Chapter 3.

That renal disease may progress remorselessly to end-stage failure despite removal of the original insult argues for continuing tissue destruction at the cellular level. The detail of this process remains to be worked out for many renal diseases including diabetes mellitus. This subject is considered in greater detail in the Chapter 6 along with an evaluation of whether mesangial matrix products have a role as markers of disease progression.

4.6   CONCLUDING REMARKS

In this prospective study of progression of nephropathy in NIDDM 19 of the 26 subjects who entered the study were followed for an average of 20 months.

- The median rate of decline of GFR for all subjects was 0.48 ml.min.$^{-1}$month^{-1}.

- Despite the small numbers the rate of decline was slower in Asians (-0.28 ml.min.$^{-1}$month^{-1}) compared to either Afro-Caribbeans (0.56 ml.min.$^{-1}$ month^{-1} $p<0.03$) or Caucasians (1.12 ml.min.$^{-1}$month^{-1}; $p=0.06$).

- In a univariate analysis systolic and diastolic blood pressure, serum cholesterol and 24-hour urinary protein were shown to be significantly associated with decline in GFR in all subjects(all $p<0.01$). Stepwise regression analysis identified SBP and serum cholesterol as the most important factors ($p<0.005$).

- When the group was subdivided equally into 'Progressors' and 'Non-progressors' systolic blood pressure ($p<0.01$) and serum cholesterol ($p<0.01$) were the only measured variables significantly different between the two groups.

- The rate of decline of GFR in NIDDM was virtually identical in the retrospective and prospective studies, yet a clearer association of blood pressure and serum cholesterol emerged in the latter.

Chapter 5

Assessment of the methods of monitoring progression of renal impairment in NIDDM

5.1   INTRODUCTION

A reliable indicator of progression is a prerequisite for the monitoring of a chronic disease and for the evaluation of any therapeutic intervention. To be effective, such an indicator should be accurate, precise and convenient to measure (Levey 1989). Inulin clearance has been the 'gold-standard' for renal disease since its development (Shannon and Smith 1935; Smith 1951), however it is not only complicated to perform and therefore impractical for clinical use but inulin is limited in its availability and technically difficult to measure (Levey 1990). As a consequence isotopic methods of measuring glomerular filtration rates have evolved with ^{51}Cr ethylenediaminetetra-acetic acid (EDTA) being the most widely used in Europe (Brøchner-Mortensen 1985). These too have drawbacks. They are also time-consuming to perform, involve exposure of the patient to radiation (with the exposure increasing as renal function worsens), and are expensive. Thus they are unlikely to be widely adopted in routine clinical practice, but will remain applicable for clinical research studies.

Prior to the establishment of inulin and isotopic clearance methods clinicians relied on either plasma clearance of creatinine (Miller and Winkler 1938) or serum creatinine to monitor progression of renal disease. The latter is not a reliable measure of renal function, given that 70% of functioning renal tissue may be lost before creatinine rises (Brøchner-Mortensen 1985; Mitch 1986). The measurement of creatinine clearance is the most widely used assessment of glomerular filtration in clinical practice (Cameron 1992b), although its validity is also questioned. Those who discredit it do so largely on the grounds of lack of repeatability (Bauer et al. 1982; Payne 1986). Differences in diet and muscle mass, incorrect urine collection and lack of precision at low GFR serve to reduce the accuracy of this measurement. Despite these reservations Gabriel (Editorial, 1986) concluded that 'creatinine

clearance with urine collected under control conditions remains the simplest, cheapest and most useful measure of renal function'.

In 1976 Mitch and colleagues suggested that the reciprocal of serum creatinine plotted against time provided a simple method for monitoring the progression of chronic renal impairment. Whilst this approach has been criticised (Modena et al. 1991) as over 20% of subjects do not show a linear decline of GFR (for further discussion see chapter 2), it is still widely employed in clinical practice. In the same year that Mitch and colleagues demonstrated the predictive value of reciprocal creatinine, Cockcroft and Gault (1976) published their formula for estimating GFR based on serum creatinine, corrected for gender, age and body weight. This surrogate marker has recently been shown to estimate GFR accurately (Sampson and Drury 1992) and the decline of GFR (Rossing et al. 1994b) when compared to isotopic methods in IDDM subjects with proteinuria and GFR <100 ml.min^{-1}. The validity of GFR determined by the Cockcroft-Gault formula has not, to our knowledge, been tested in NIDDM subjects with nephropathy.

This study compares several measures of renal function namely reciprocal serum creatinine, creatinine clearance and estimated GFR (Cockcroft-Gault) with true GFR as measured by ^{51}Cr EDTA in NIDDM subjects with renal impairment. The specific aims of this study were as follows:

5.2   AIMS

- To compare ^{51}Cr EDTA GFR with surrogate measures of GFR, namely reciprocal creatinine, creatinine clearance and GFR estimated by the Cockcroft-Gault formula in a cohort of patients with NIDDM and diabetic nephropathy with renal impairment.

- To determine whether age, body mass and racial origin affect the validity of GFR, estimated by Cockcroft-Gault, when compared to true GFR.

- To determine whether the Cockcroft-Gault formula can be used to provide a reliable index of decline of GFR.

5.3   STUDY DESIGN AND DATA ANALYSIS

5.3.1   Patients and methods.

The patients studied were the participants in the prospective study described in Chapter 4. An additional five subjects who had a single EDTA clearance carried out but who did not subsequently enter the study are included. These NIDDM subjects also fulfilled the criteria for diabetic nephropathy. The measurement of ^{51}Cr EDTA has previously been described (Chapter 4). Creatinine clearance was measured on a 24-hour urine sample collected the day prior to attendance for the EDTA GFR. Serum creatinine was sampled with the first (2 hour) sample drawn during the measurement of EDTA clearance. Estimated GFR (cGFR) was calculated from the serum creatinine using the formula of Cockcroft-Gault (1976) as follows:

$$(140\text{-age\{years\}} \times \text{body weight \{kg\}} \times K/\text{ serum creatinine } (\mu mol.L^{-1}),$$
where K = 1.23 for men and 1.05 for women.

As previously, estimated GFR is denoted by cGFR and EDTA clearance by EDTA GFR.

5.3.2   Data analysis

EDTA GFR was compared to estimated GFR and creatinine clearance for all subjects, and by racial origin, gender and body mass index. Mean values were compared by Students paired 't-test'. Data for all subjects were examined for agreement by Pearson's univariate linear regression analysis and the slope and 'y'

intercept determined. Similar analyses were also carried out comparing the decline of GFR as determined by EDTA clearance and creatinine clearance, estimated GFR and reciprocal creatinine. A better measure of agreement is obtained by plotting the average of the two methods (horizontal axis) against the difference between the two methods (vertical axis), (Bland and Altman 1986). Limits of agreement were drawn on the same graph as mean ±2SD of the difference. What represents acceptable limits to the degree of agreement is a matter of personal judgement. All the calculations were performed using the Statview software package (BrainPower Inc., Casablasas, CA, USA). Data are given as mean ±SD and a p value of <0.05 was considered significant.

5.4   RESULTS

Data were available for 72 patient episodes, comprising 19 subjects measured thrice, 3 subjects twice and four subjects once in the study with the additional five individuals who did not subsequently enter the study measured once only. Only one subject had an EDTA GFR above 100 ml.min^{-1} and this value was excluded from further analysis.

	cGFR	EDTA GFR	Significance
Afro-Caribbean (n=28)	32.9 (18.5)	39.8 (15.7)	$p<0.02$
Asian (n=14)	32.7 (10.6)	35.9 (13.6)	NS
Caucasian (n=29)	31.5 (16.7)	33.6 (21.3)	NS
All subjects	32.3 (16.3)	36.5 (17.8)	$p<0.01$

Key : NS = not significant

Table 5.1: Comparison of estimated GFR (cGFR) and EDTA clearance for
all measurements of GFR, subdivided by racial group.

The mean EDTA GFR for the 71 measurements was 36.5 (17.8) ml.min^{-1} {mean (SD)} compared to 32.3 (16.3) for cGFR giving a significantly higher EDTA GFR of 4.2 ml.min^{-1} on average (Table 5.1 preceding page). The difference is greatest in Afro-Caribbean subjects where EDTA GFR exceeds cGFR by 6.9 ml.min^{-1} ($p<0.02$). Although EDTA GFR is on average higher for Asian and Caucasian subjects the differences are not significant.

The values for EDTA GFR and cGFR measured simultaneously are plotted for all subjects in Figure 5.1 (overleaf) and are significantly associated (r=0.77; $p<0.001$). Figure 5.2 (overleaf) shows the same analysis subdivided by race with the r values ranging from 0.80 to 0.85. The data for reciprocal creatinine (r=0.73; $p<0.001$) and creatinine clearance (r=0.82; $p<0.001$) against EDTA GFR are plotted on Figures 5.3 and 5.4 respectively (overleaf). Figure 5.5 (overleaf) shows cGFR plotted against creatinine clearance, indicating these also are associated (r=0.78; $p<0.001$). No improvement in the degree of correlation follows correcting the predicted GFR data for surface area (corrected to 1.73 m^2), although the data lie closer to the identity line (see Figure A5.1 in the appendix). Figure 5.6 (overleaf) shows the same data subdivided by gender. All the observations on the female subjects have a EDTA GFR < 45 ml.min^{-1}. Racial differences exist in the degree of correlation between the various measures of renal function, although these differences are, not surprisingly, slight (Table 5.2 - see page 129). Somewhat unexpectedly creatinine clearance appears more closely correlated with EDTA GFR than either reciprocal creatinine or cGFR, although the data are more widely scattered. Regression equations comparing the different measures of renal function in the racial groups are given in the appendix (Table A5.1).

The influence of gender, body weight and serum creatinine on the degree of correlation between the measures of renal function is shown in Table A5.2 in the appendix. The poor correlation in female subjects may reflect the small numbers

Figure 5.1: Relationship between the simultaneously measured plasma clearance of ^{51}Cr EDTA and glomerular filtration rate predicted from the Cockcroft-Gault formula for all subjects (n=71 obsevations; $y = 0.82x + 10.2$; r=0.78; p<0.0001). The line of identity (dashed) is indicated.

Figure 5.2 : Relationship between the simultaneously measured plasma
clearance of ^{51}Cr EDTA and glomerular filtration rate pre-
dicted by the Cockcroft-Gault (cGFR) formula subdivided
by racial group. Pearson's coefficient; significance as shown.
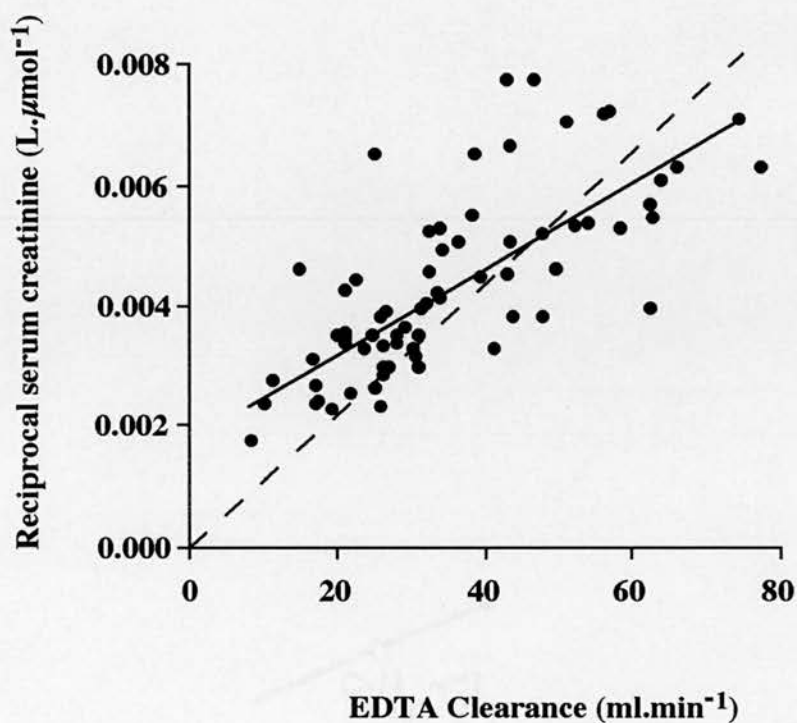
Figure 5.3 : Relationship between simultaneously measured plasma clearance of ^{51}Cr EDTA and the reciprocal of serum creatinine for all subjects (n=71 observations; y = 0.78x + 1.35; r=0.74;p<0.0001). The line of identity (dashed) is indicated
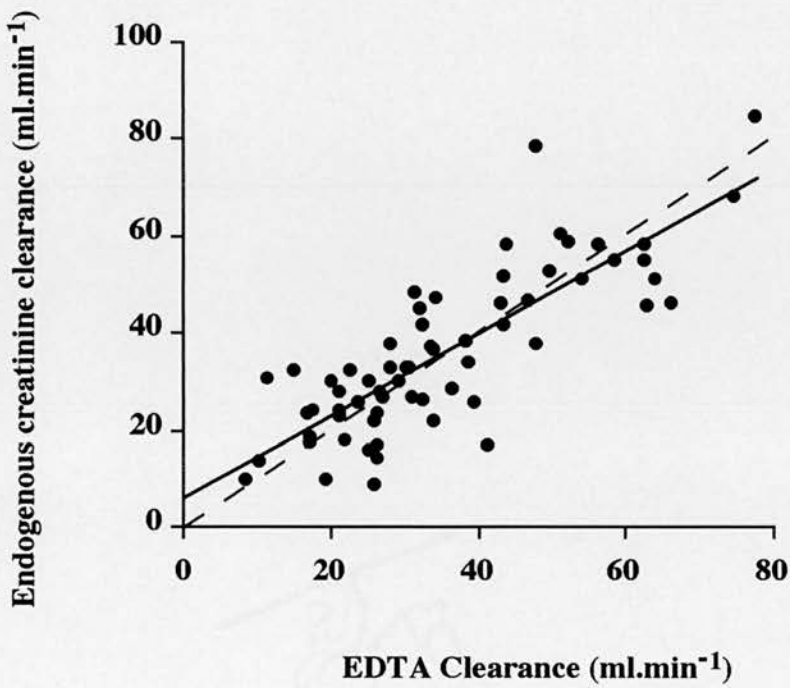
Figure 5.4 : Relationship between simultaneously measured plasma clearance of ^{51}Cr EDTA and endogenously measured creatinine clearance in all subjects (n=71 observations; y=0.79x + 6.8; r=0.82; p<0.0001). The line of identity is denoted by the dashed line.
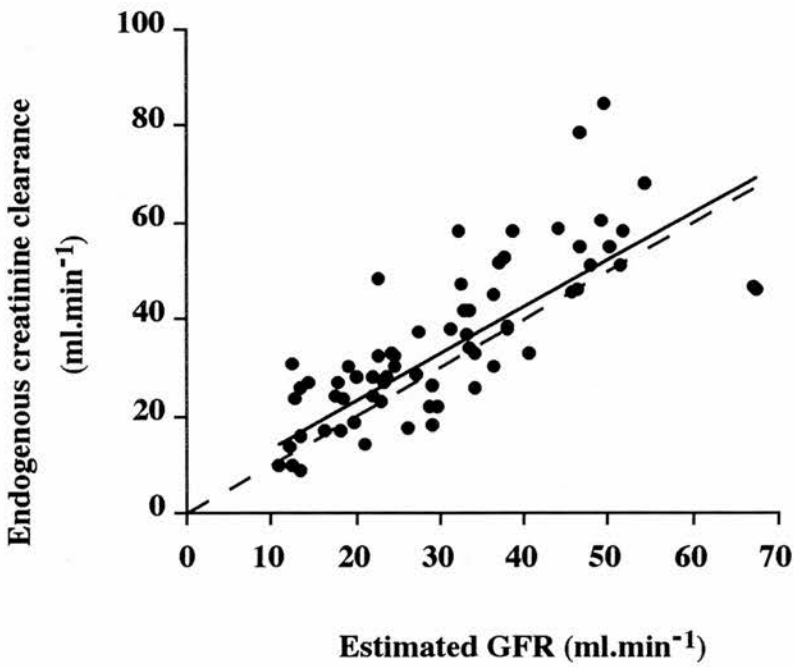
Figure 5.5 : Relationship between simultaneously measured endogenous creatinine clearance and glomerular filtration rate predicted from the Cockcroft-Gault formula (cGFR) in all subjects (n=71 observations; y = 0.93x + 7.6; r=0.78; p<0.0001). The line of identity is denoted by the dashed line.
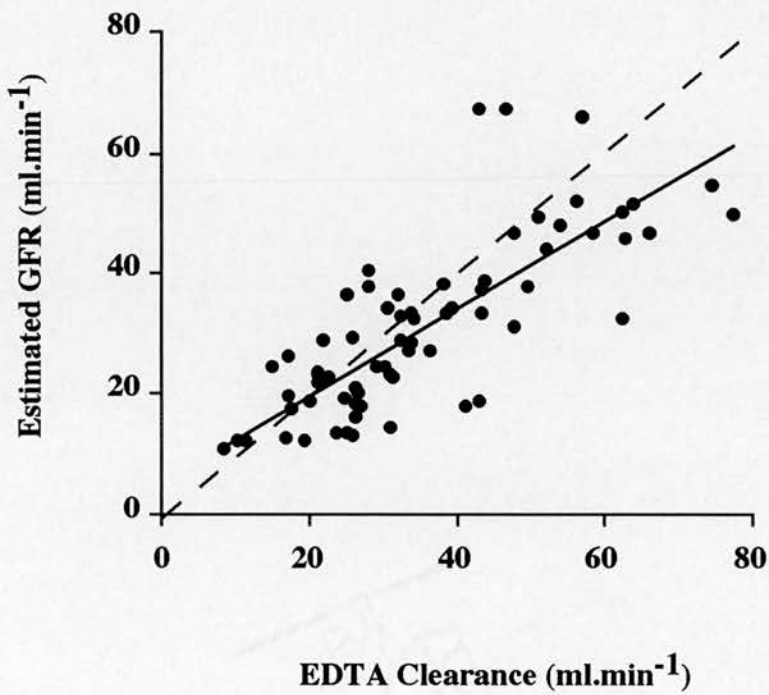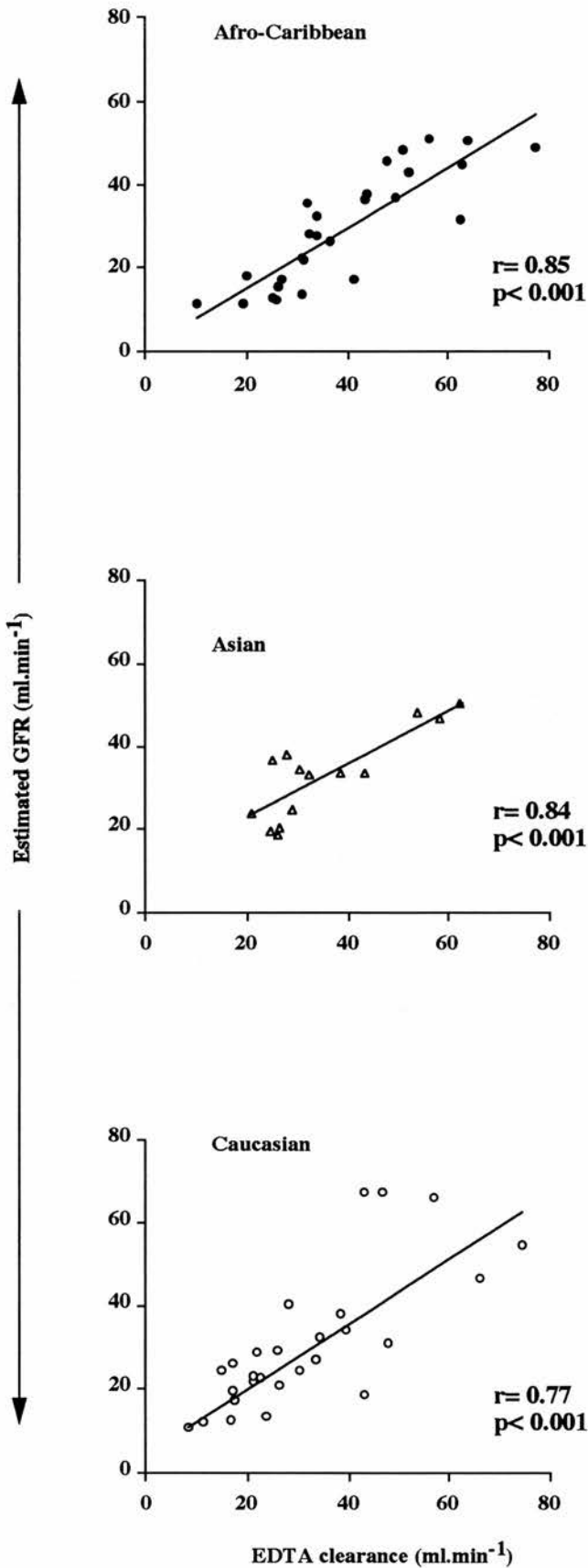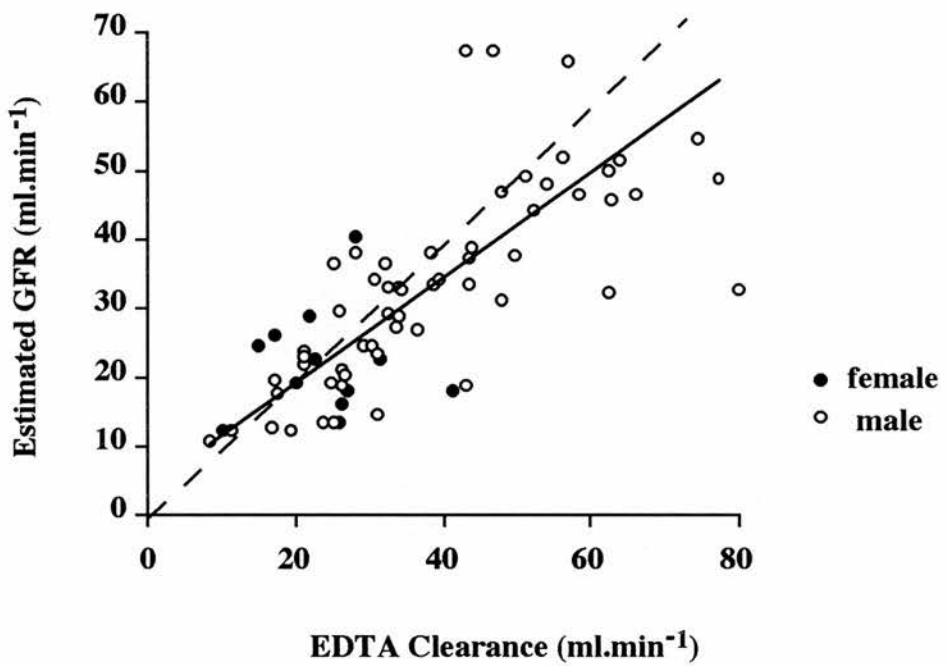
Figure 5.6 : Relationship between simultaneously measured plasma clearance of ^{51}Cr EDTA and glomerular filtration rate predicted from the Cockcroft-Gault formula. Gender as anotated. The line of identity (dashed) is indicated.
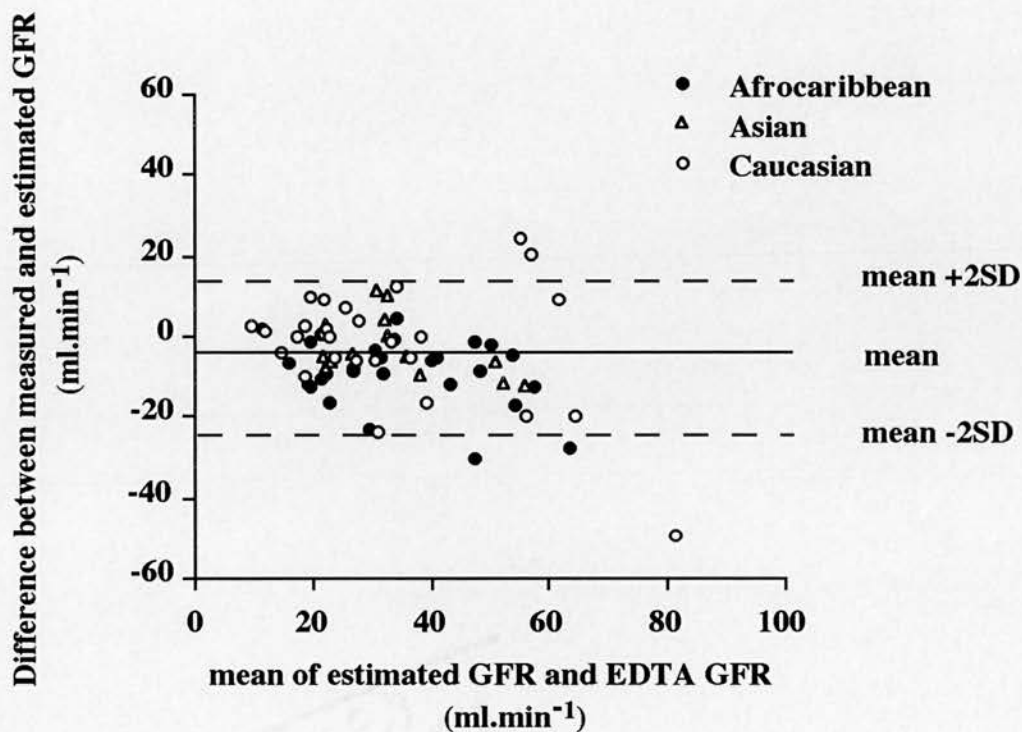
Figure 5.7: Difference between EDTA clearance (EDTA GFR) and GFR
estimated by the Cockcroft-Gault formula (cGFR) vs mean of
the two methods for all observations (n=71). The mean and SD
as shown. Bland-Altman plot. Individual graphs are shown in
Figure A5.2 (appendix).
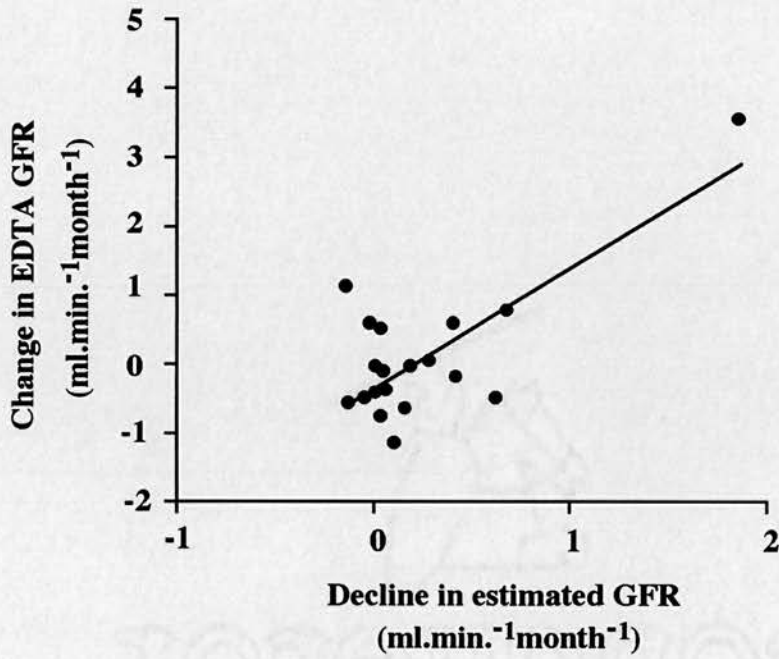
Figure 5.8 : Relationship between the rate of decline of glomerular filtration rate as measured by EDTA clearance and that estimated by the Cockcroft-Gault formula (n=19; r=0.78; p<0.001).
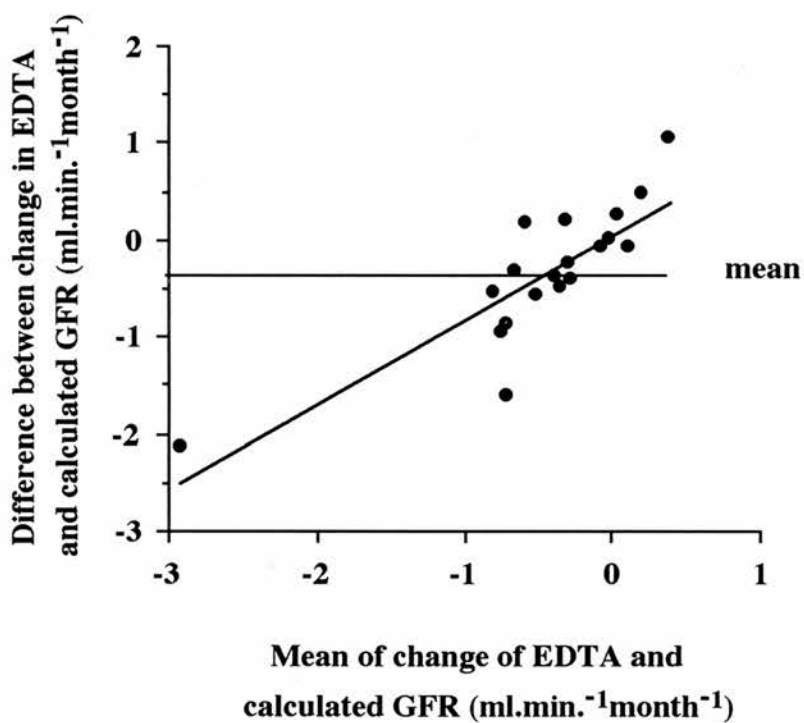
Figure 5.9 : Difference between the rate of change in measured GFR (EDTA clearance) and GFR predicted from Cockcroft-Gault (calculated GFR) vs mean of the two methods. Regression line shown (r=0.84; p<0.001)

	cGFR vs EDTA	1/Sc vs EDTA	CrCl vs EDTA	cGFR vs CrCl
Afro-Caribbean	r = 0.85	r = 0.76	r = 0.81	r = 0.87
Asian	r = 0.84	r = 0.56	r = 0.93	r = 0.91
Caucasian	r = 0.80	r = 0.80	r = 0.83	r = 0.73
Total	r = 0.78	r = 0.73	r = 0.82	r = 0.78

Key : Sc = serum creatinine; EDTA = EDTA clearance; CrCl = creatinine clearance

Table 5.2 : Correlation matrix between the measures of renal function in the different racial groups

129

studied. For subjects in excess of 80 kg reciprocal creatinine and creatinine clearance agree more closely with EDTA GFR than cGFR. The latter measure is undoubtedly biased by the weight correction. The correlation of cGFR with EDTA GFR is surprisingly poor where creatinine exceeds 300 μmol.L^{-1}.

The data can be examined in another way. The Bland-Altman plot (Bland and Altman 1986) demonstrates the agreement rather than the strength of the association (denoted as r) between two variables. Such a plot comparing cGFR against EDTA GFR is presented in Figure 5.7 (above). This shows that the agreement between the two methods is weaker at the higher GFR measurements, where the Cockcroft-Gault underestimates true GFR. Figure A5.2 in the appendix shows this to be the case for Afro-Caribbean (r=0.3; p=NS) and Asian subjects (r=0.44; p=NS) but not for Caucasians.

The rate of decline of EDTA GFR against cGFR for the subjects studied in Chapter 4 is plotted in Figure 5.8 (above). The relatively good correlation between the methods (r=0.78; p<0.001) is weighted by one subject with a marked decline of GFR. When the same data are plotted by the Bland-Altman method (Figure 5.9 above) an unexpected result is obtained. This suggests that the faster the GFR declines the greater the under-estimate as determined by cGFR (r=0.84; p<0.001). There are too few data to examine racial effects.

5.5   DISCUSSION

5.5.1   Is predicted GFR a suitable alternative to EDTA clearance ?

This study has demonstrated a significant correlation between isotopically determined GFR (EDTA clearance) and GFR predicted by the Cockcroft-Gault formula in NIDDM subjects with diabetic nephropathy where the GFR is < 80 ml.min^{-1}. The degree of association, however, is weaker than that observed in two comparable studies in IDDM subjects with nephropathy, though both were almost

exclusively in Caucasian subjects (Sampson and Drury 1992; Rossing et al. 1994b). In the study of Sampson and Drury, the authors found a strong correlation between predicted GFR (Cockcroft-Gault) and true GFR (EDTA clearance) in 20 subjects. The correlation was improved by correcting cGFR for surface area. No such finding was observed in this study. Although Figure A5.1 (in appendix) shows that correction for surface area brings the data closer to the identity line no improvement is seen in the degree of association (r=0.76 vs 0.78). The agreement between paired observations in Sampson and Drury's subjects was strengthened by the exclusion of GFR measurements >100 ml.min^{-1}. In a more recent study in IDDM subjects, Rossing and colleagues (1994b) observed a correlation of 0.91 between measured GFR (EDTA clearance) and estimated GFR (Cockcroft-Gault) in 43 subjects, with a GFR ranging from 35 to 150 ml.min^{-1}. Others do not observe such strong associations. Gross and colleagues (1993) performed 96 assessments in 43 normoalbuminuric IDDM subjects and 61 assessments in 55 normoalbuminuric NIDDM subjects and compared them to GFR estimated by Cockcroft-Gault and found a significant, though relatively poor, correlation between the measures (r=0.53 and r=0.56 for IDDM and NIDDM respectively). The degree of discrepancy between these measurements may reflect the high GFR of many subjects. Similar findings were observed by Waz and colleagues (1993) in 72 children with IDDM. They found a poor correlation (r=-0.46; p<0.05) between isotopically measured GFR (^{125}I-iothalamate) and predicted GFR (Cockcroft-Gault). For all levels of GFR, cGFR consistently underestimated true GFR and this underestimate increased as GFR increased. This is supported by the findings of Trollfors and colleagues (1987) who demonstrated a stronger association between EDTA clearance and predicted GFR, where GFR was in the range 20 to 90 ml.min^{-1}. In their study EDTA GFR was consistently 10 ml.min^{-1} greater than cGFR. In our present study the difference between the two measures was not so marked and averaged 3-4 ml.min^{-1}. Only in the Afro-Caribbean group was this difference significant. Lemann and colleagues (1990)

found a positive correlation (r=0.82) between iothalamate clearance and predicted GFR (Cockcroft-Gault) in 136 IDDM subjects over a wide range of GFR measurements (16 to 175 ml.min^{-1}) as part of a prospective study of angiotensin converting enzyme inhibition in diabetic nephropathy.

The Bland-Altman plot (Figure 5.7 above) indicates a good measure of agreement between true and predicted GFR, particularly where GFR is below 50 ml.min^{-1}. As GFR increases the discrepancy between true GFR and predicted GFR becomes more pronounced, a finding common to many studies (Sampson and Drury 1992; Trollfors et al.1987; Rossing et al. 1994b). This discrepancy is evident in Asian and Afro-Caribbean subjects (Figure A5.2 in appendix). It may, however, be a gender rather than a racial effect since all the Asian subjects and the Afro-Caribbean subjects with a GFR > 40 ml.min^{-1} were male. Against this hypothesis is that this feature was not seen in Caucasian males.

The poor correlation between cGFR and EDTA GFR for females (see Table A5.2 in appendix) almost certainly reflects the small number of subjects studied, especially since three of the observations were in one woman whose weight exceeded 120 kg. There is no theoretical reason why there should be a gender difference. Somewhat surprisingly a weaker correlation is observed in subjects with a serum creatinine >300µmol.L^{-1} (Table A5.2). This is contrary to what would be expected given the greater degree of agreement between GFR, predicted from Cockcroft-Gault, and EDTA GFR as GFR worsens as shown in Figure 5.7 (above). Again this may reflect the small numbers in this group. That weight influences the degree of association between predicted and measured GFR is of no surprise. However the correlation is not improved by correcting the data for surface area. Rolin and colleagues (1984) found that renal function, predicted by Cockcroft-Gault, was inaccurate in the elderly and very obese in a study of 500 paired observations; although the prediction in this case was improved by correcting for surface area. Similar observations were made by

Sawyer and colleagues (1982), leading Gault and colleagues (1992) to recommend adjusting all predicted GFR measurements to ideal weight to correct for obesity, oedema and muscle wasting.

The comparison of the decline of GFR as measured by EDTA clearance and predicted GFR is shown in Figure 5.8 (above). Interpretation of the results should be cautious due to the influence on the results of the outlier with the most rapid decline of GFR. Furthermore, as Rossing and colleagues (1994b) argue, reduced precision of the results occurs where follow-up is less than 3 years. This study has only two years of data. The observation that the more rapid the decline of GFR the greater cGFR underestimates the decline as measured by EDTA clearance (Bland-Altman plot) is puzzling (Figure 5.9 above). There is no obvious explanation for this finding. Whilst serum creatinine may take time to stabilise if perturbed this would not be expected on this timescale. Mitch and Walser (1978) have shown that it takes urine creatinine approximately three weeks to stabilise after a change in muscle mass. This finding underlines the importance of using EDTA clearance to measure GFR, where the loss of function is marked.

5.5.2   Is there a role for serum creatinine ?

Serum creatinine is the measure of renal function most commonly performed in the clinic, and is used directly or indirectly by many as both an indicator of (dys)function and serially as a marker of progression. However serum creatinine is very insensitive in the detection of early renal impairment and as such is of little value (Brøchner-Mortensen 1985; Levey et al. 1988). It is equally insensitive for monitoring progression at least in the early stages of diabetic renal disease (Parving et al. 1985; Viberti et al. 1987b). This is due largely to the day-to-day variation of serum creatinine and to enhanced tubular secretion of creatinine which compensates for reduced filtration at this stage. Serum creatinine may also change throughout the day (Sirota et al. 1950).

Reciprocal serum creatinine has been used to monitor progression and to predict the time to renal replacement therapy in non-diabetic (Mitch et al. 1976) and diabetic subjects (Jones et al. 1979) with chronic renal impairment. Such measures rely on the decline of GFR being linear, although this often not the case. Few studies compare reciprocal creatinine against isotopically-measured GFR. Those that do record broadly similar experiences. This study found an all subject correlation between reciprocal creatinine and EDTA clearance of 0.77 (slightly stronger in Asians and Afro-Caribbeans), whilst Norden and colleagues (1987) observed a similar association (r=0.81) in 50 IDDM subjects measured on a total of 128 occasions. Although the degree of association was fairly strong for the group, GFR could not be predicted in individual subjects from serum creatinine. A poorer correlation (r=0.63) was found by Lemann and colleagues (1990) in 136 subjects. Gretz and colleagues (1983) concluded that reciprocal creatinine was a more reliable indicator of progression where serum creatinine exceeded 240 μmol.L^{-1}. By contrast Walser and colleagues (1989) who studied progression in 17 subjects with severe renal impairment due to a variety of diseases concluded that the use of reciprocal creatinine gave false readings in 40% of cases. Based on a number of assumptions and the work of Steinman and colleagues (1989), Davies and Shock (1950b) and Brøchner-Mortensen and Rodbro (1976), Levey (1990) argues that serum creatinine (and by inference GFR derived from creatinine) is a more precise measure of renal function where GFR < 50 ml.min^{-1} whilst the converse is true where renal function is normal. Unfortunately the lack of agreement between reciprocal creatinine and measured GFR, due to variability of creatinine production, secretion and extrarenal clearance in individual subjects reduces the value of inverse creatinine as a marker of progression.

5.5.3   What of endogenous creatinine clearance ?

There are several practical reasons why creatinine clearance may not be the ideal measure of renal function in subjects with renal insufficiency, particularly those with diabetic nephropathy. It is reliant on accurately-performed urine collection, which may be not be feasible due to diabetic cystopathy (Frimodt-Møller 1980). Whilst the subjects in this present study were not routinely assessed for autonomic neuropathy nor specifically for bladder emptying, all had renal and bladder ultrasound examination. None had a residual urine volume in excess of 50 ml. The subjects studied here were familiar with 24-hour urine collections. Consecutive 24-hour urine collections, as advocated by some (Brøchner-Mortensen and Rodbro 1976; Wibell and Björsell-Ostling 1973), were not performed. Even supposedly 'reliable' subjects may have a wide coefficient of variation of measurement (up to 22%) on consecutive samples over a one month period (Edwards et al. 1969). Furthermore urine collections are less likely to be accurate in the elderly (Goldberg and Finkelstein 1987). Despite these reservations a significant association (r=0.82; $p<0.001$) was observed between EDTA GFR and endogenous creatinine clearance (Figure 5.4 above) although the scatter of paired observations was wide. A similar association (r=0.70) was observed by Lemann and colleagues (1990) in 136 IDDM subjects with a creatinine < 221 μmol.L^{-1}, although again the degree of scatter was wide.

Endogenous creatinine clearance overestimates EDTA clearance due to tubular secretion of creatinine, the difference increasing with progression of renal disease (Miller et al. 1952; Carrie et al. 1980; Shemesh et al. 1985; Norden et al. 1987). In most studies this is in the order of 10-15 ml.min^{-1}, although there was surprisingly no significant difference in this study (Figure 5.4 above). Heavy proteinuria has been found to significantly increase endogenous creatinine clearance when compared to measured GFR (Brod and Sirota 1948; Shemesh et al. 1985). There was no evidence of this in this study (see Figure A5.3 in appendix). Friedman et al. (1991) advocated

abandoning the 24-hour urine collection for assessing renal function based on their finding of a poor correlation between endogenous creatinine clearance and EDTA clearance in 30 diabetic subjects.

Guillausseau and colleagues (1988) compared endogenous creatinine clearance against GFR derived from serum creatinine in 33 IDDM and NIDDM subjects with a serum creatinine < 130 $\mu mol.L^{-1}$ and surprisingly concluded that such methods were suitable for routine clinical practice. A reasonable correlation was obtained between derived GFR (several methods) and endogenous creatinine clearance ($r=0.73$-0.75; $p<0.001$) despite wide coefficients of variation for creatinine clearance (28%) and estimated GFR (17%) on simultaneous samples taken on consecutive days. A stronger association between estimated GFR and creatinine clearance was observed in this study ($r=0.78$; $p<0.001$) with less scatter of the data points than the other comparisons (Figures 5.5 cf. 5.2 - 5.4 above). In an analysis of ten published studies (that of Guillausseau and colleagues was not included) comparing endogenous creatinine clearance to cGFR, Gault and colleagues (1992) found a mean correlation coefficient of 0.83 (range 0.60 - 0.95) between these measures. The number of paired observations ranged from 28 to 722. The difficulties inherent in accurate 24-hour collections in elderly patients with diabetes mellitus probably precludes its use as a reliable marker of progression, although there may be a role in obese individuals (Table A5.2 in appendix).

5.5.4   Isotopic methods of measuring GFR - can they be relied upon ?

The drawbacks to using isotopic methods for measuring GFR have already been alluded to (see Introduction to this chapter). Despite these concerns they remain central to the monitoring of GFR progression in research studies and are advocated for clinical practice. In Europe the single injection technique, using ^{51}Cr EDTA (Brøchner-Mogensen et al. 1985-6) is the most widely used. Due to extra-renal clearance plasma clearance overestimates renal clearance by 3-5% (Brøchner-

Mortensen, Giese and Rossing 1969). In effect this means 4 ml.min^{-1} with normal renal function and 0.5 to 1 ml.min^{-1} where renal function is significantly impaired (Jagenburg et al. 1978; Brøchner-Mortensen and Freund 1981). This discrepancy is probably of no clinical importance since most studies demonstrate that inulin clearance exceeds the renal clearance of isotopes (5% of chelate is reabsorbed by the tubule) (details see Brøchner-Mortensen 1985). The programme used to calculate EDTA clearance in this study corrected for this discrepancy in inulin clearance.

The accuracy of GFR determination using isotopes depends upon a constant rate of clearance over the sampling period. However plasma clearance of the isotope may be biphasic thus making calculation of clearance difficult (Mitch 1986). Furthermore where renal function is severely impaired or in the presence of significant oedema with or without ascites, the time to achieve a steady state may exceed the normal sampling period (Maisey et al. 1969; Manz et al. 1977; Brøchner-Mortensen and Freund 1981). No allowance was made for this in the present study which may account for the large percentage error (up to 12.6%) seen in a small number of subjects with more severe renal impairment. The development of oedema as often seen in NIDDM subjects approaching renal replacement therapy would also be expected to lead to inaccuracies in predicted GFR.

It is tacitly assumed that a prime objective of monitoring the progression of renal disease is to define with accuracy the point at which renal replacement will be needed. Such end-points are determined not by absolute GFR levels or by serum creatinine, but rather by symptoms and the personal judgement of the supervising physician. This limits the need for a precise and accurate measure of GFR. The accurate monitoring of GFR by isotopic methods is helpful in determining benefit from intervention (e.g. Parving et al. 1987) but may be less valid for the individual in the clinic. The greatest value is to identify the individual in whom there is a poor

correlation between serum creatinine and true GFR and who exhibits a greater than average decline of GFR.

5.6   CONCLUDING REMARKS

- Glomerular filtration rate predicted by the Cockcroft-Gault formula is significantly associated with EDTA clearance (r=0.79; p<0.0001). The correlation is increased where EDTA clearance is < 50 ml.min^{-1}.

- Predicted GFR underestimates EDTA clearance on average by approximately 4 ml.min^{-1}. This underestimate is significant in Afro-Caribbean subjects (p<0.02), but not in Caucasian or Asian subjects.

- Reciprocal serum creatinine and endogenous creatinine clearance are similarly correlated (r values, 0.74 and 0.82 resp.; both p<0.0001) with EDTA clearance in all subjects.

- The measurement of endogenous creatinine clearance may have a role in monitoring progression in obese subjects.

- The more rapid the rate of decline of EDTA clearance the greater the discrepancy between the rate of decline measured by EDTA clearance and that by predicted GFR.

- Predicted GFR may be valuable to monitor progression in certain individuals but is probably too imprecise to be applied universally.

Chapter 6

Serum and urinary type IV collagen and mixed laminin fragments as markers of progression of nephropathy in NIDDM

6.1 INTRODUCTION

6.1.1 Background

Histological examination of renal tissue from subjects with progressive renal disease reveals a variety of changes in both the glomerulus and tubule. These include tubule atrophy, capillary closure, cell infiltration and expansion of the extracellular matrix. Although not exclusive to diabetes mellitus, basement membrane thickening (BMT) and expansion of the mesangium characterise this condition.

BMT was first demonstrated in diabetes mellitus in the retinal epithelium (Friedenwald 1950). Detailed analysis by Østerby (1975) demonstrated similar appearances in the glomerular basement membrane (GBM) and by showing the thickening developed some 12 to 24 months after diagnosis refuted the previously held view that these changes preceded, or coincided with, the onset of diabetes. Observations that the GBM increased in thickness with the duration of diabetes (Lazarow 1969) were not sustained upon closer examination (Mauer et al. 1984). The latter group found that the volume of mesangial matrix, but not basement membrane thickening, correlated with clinical measures of progressive renal disease including hypertension, protein excretion and diminished glomerular filtration rate in insulin-dependent diabetic individuals.

6.1.2 The composition and function of extracellular matrix

The extracellular matrix is composed of the basement membrane and the mesangium. The mesangium forms the interstitial portion of the glomerular lobule and comprises the mesangial cells and associated matrix. The mesangial cells not only contribute to the synthesis of the mesangial matrix but also play an important role in the remodelling of the basement membrane (Striker et al 1989). Type IV collagen is the principal constituent of the mesangial matrix and together with laminin and heparan sulphate proteoglycans is synthesised by visceral epithelial cells also known as podocytes (Abrahamson et al. 1987). Both resident and circulating (monocytes)

macrophages and lymphocytes are found within the mesangium and may contribute to progressive glomerulo-sclerosis either by degradation of collagen within the mesangium (Nathan 1987) or by promoting collagen synthesis (Klahr et al. 1988b). A variety of substances, including platelet-derived growth factor (Williams 1986), Interleukin I (Werber et al. 1987) and transforming growth factor β (Roberts et al. 1992) are known to stimulate the production of collagen by mesangial cells and fibroblasts.

The principal components of the GBM, as in the mesangium, are glycoproteins, collagens and proteoglycans; specifically type IV collagen, laminin and heparan sulphate proteoglycans. Other minor components include nidogen, also termed entactin (Carlin et al. 1981), fibronectin (Courtoy et al. 1982) and amyloid P-component (Dyck et al. 1980) with doubtless others still to be identified. The basement membrane is a specialised form of extracellular matrix in which the above macromolecules self-assemble to produce discrete laminated structures, with the main framework provided by type IV collagen (Kefalides 1973; Timpl et al. 1980).

Monomeric type IV collagen is a 400 nm triple helical structure with two non-collagenous domains, a C-terminal globular domain (NC1) and the N terminal end (7S) (Trüeb et al. 1982). These monomers self assemble by forming central tetrameric structures that resemble 'chicken-wire', based on the alignment of the 7S terminals of four monomers (Yurchenco and Furthmayr 1984). The NC1 ends join to form dimers as indicated in Figure 6.1 (overleaf). The full complexity of type IV collagen is only now emerging. Originally conceived as formed from two distinct alpha chains ({alpha1 (IV)2} and {alpha2 (IV)1}), three further chains {alpha 3 to 5 (IV)}have now been added (Weber 1992). In addition to providing structural support type IV collagen also provides a matrix for cell adhesion (Grant et al. 1990) and controls the passage of macromolecules, e.g. plasma proteins, within the glomerulus
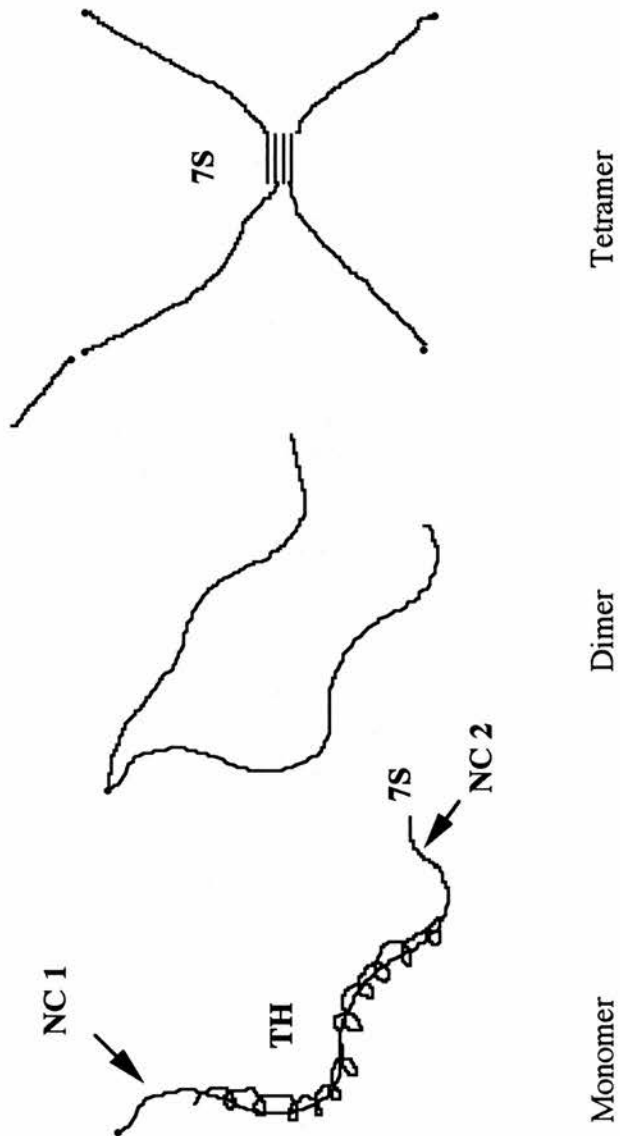
Figure 6.1 : Monomeric, dimeric and tetrameric forms of type IV collagen (schematic representation). The triple helical element (TH) is thought to be the antigen site of intact collagen. NC = non-collagenous domain.

141

(Weber 1992). Type IV collagen is found almost exclusively within the lamina densa (Laurie et al. 1984).

Laminin, the principal glycoprotein in the basement membrane, is composed of three polypeptide chains, connected by disulphide bonds to type IV collagen, nidogen and heparan sulphate proteoglycans. Whilst intact laminin is yet to be extracted from normal tissue (Timpl and Dziacek 1986) it is easily obtained from tumour tissue and the principal laminin fragments, LP1 and LP2, are readily measurable in serum (Risteli et al. 1981). Laminin is thought to act as a bridge between cells, e.g. glomerular podocytes, and the basement membrane (Fukatsu et al. 1988) and as a link between the various macromolecules, e.g. type IV collagen, etc. In addition it also has a modulating effect on cell behaviour (Kleinmann et al. 1985). In contrast to type IV collagen it is located principally within the lamina rara (Madri et al. 1980).

6.1.3 Localisation of type IV collagen and laminin in diabetic nephropathy

Immunohistochemical analysis of renal tissue from those with diabetic nephropathy and animal models confirm the localisation of type IV collagen in the basement membrane and mesangial matrix, particularly the nodular lesions, of the glomerulus. Falk and colleagues (1983), using a semi-quantitative method examined, expansion of basement membrane constituents in renal biopsies from IDDM subjects and found that type IV collagen was increased in both the mesangium and the GBM in the early stages of nephropathy, but was decreased in the late stages. In contrast the level of collagen remained high in the tubular basement membrane (TBM) throughout all stages of nephropathy. Bendayan (1985), using a protein A-gold staining technique in diabetic rats confirmed the findings of Falk and colleagues (ibid) that collagen was deposited in the sub-endothelial region of the GBM. Kim and associates (1991) have recently suggested that the collagen components differ in the mesangium and GBM in diabetic nephropathy. They localised the 'classical' collagen antigens (α_1 and α_2) to the mesangial matrix (and to a lesser degree the GBM), whereas the 'M28' antigens

(including Goodpasture's) and the Alport antigen were confined to the GBM. Furthermore with progressive glomerular sclerosis they observed a loss of the 'classical collagen' antigens in the glomerulus, whereas the M28 and Alport's antigens persisted, echoing the findings of Falk and colleagues (1983) and Shimomura and Spiro (1987). The differential expression of these antigens in diabetic nephropathy closely resembles that found in Alport's nephritis and has also been described in normal renal tissue (Butkowski et al. 1989).

Scheinman and associates (1980) observed laminin in the mesangium and both the sub-endothelial and sub-epithelial surfaces of the GBM. As they had shown for collagen, Falk and colleagues (1983) found the amount of laminin increased in the mesangium and GBM early in the course of nephropathy but decreased in the latter stages and again constantly increased throughout the disease in the TBM. A similar reduction of laminin content in the GBM with disease progression was observed by Shimomura and Spiro (ibid).

6.1.4   Measurement of extracellular matrix components in disease states

Once reliable quantitative assays became available to measure type IV collagen and laminin (Risteli et al. 1981; Dixit et al. 1981) it was only a matter of time before they were used in the investigation of progressive renal disease. The original assays were based on polyclonal antibodies to the 7S and NC1 antigens of type IV collagen and the LP1 and LP2 fragments of laminin. Elevated levels of 7S collagen were found in diabetic subjects with and without microangiopathy (Risteli et al. 1982; Hogemann et al. 1986; Torffvit et al. 1989). Interestingly the latter study did not find elevated levels in IDDM subjects with nephropathy. Similar findings were recorded in animal models with elevated 7S collagen in streptozotocin-diabetic rats (Risteli et al. 1982; Brocks et al. 1985). Hypertension augmented this rise (Hasslacher et al. 1987) and insulin returned the levels to normal (Hasslacher et al. 1984; Brocks et al. 1985). The production of 7S collagen was enhanced by increased glucose concentration in rats

(Hasslacher et al. 1987) supporting observations on the influence of glucose on the production of 7S collagen by mesangial cells (Ayo et al. 1991). Animal studies have demonstrated that the production of collagen precedes basement membrane thickening (Hasslacher et al. 1984). Serum and urine levels of NC1 are also elevated in glomerulonephritis in humans (Keller et al. 1990).

The levels of 7S and NC1 collagen measured in the serum probably represent both newly synthesised monomers and dimers and the collagenase breakdown products of type IV collagen. A one-step sandwich enzyme immunoassay against intact type IV collagen (central triple helical domain) has recently been developed (Obata et al. 1989). It is thought to measure newly-synthesised collagen. Elevated levels of type IV collagen have been demonstrated in the serum and urine of diabetic subjects (Matsumoto et al. 1990; Hayashi et al. 1992) with higher levels recorded in those with more advanced complications (Matsumoto et al. 1990). This finding led the authors to suggest that assay of intact collagen might be helpful in monitoring the progression of renal disease in subjects with diabetic nephropathy. In this chapter I examine whether high levels of Type IV collagen are indeed found in the serum and urine of individuals who have more advanced complications and investigate whether the claim for their use as a disease marker is justified.

The measurement of laminin fragments, principally LP1, has proved of some value in malignant disease and liver fibrosis (Price et al. 1993), but has proved unreliable in renal disease. Högemann and colleagues (1986) found no difference in the levels of LP1 in the serum of diabetic as compared to normal subjects, whilst Pietschmann and co-workers (1988) found elevated levels only in subjects with advanced nephropathy. Elevated serum LP2 levels have also been observed in streptozotocin diabetic rats (Risteli et al. 1982). However it has proved impossible to measure LP1 and LP2 (the principal pepsin digests of laminin) successfully in urine (Price and Taylor 1993). Recently these researchers have created a new assay to detect laminin fragments in

the urine (Price et al. 1994) and have found measurable quantities of laminin fragments in individuals with diabetes and those exposed to perchlorethylene and other hydrocarbons.

The method of preparation of the laminin fragments and subsequent steps in the assay are described in detail in Price and co-workers (1994). In brief, this involved the pepsin digestion of human placenta after the method of Dixit (1985). The digested placenta was precipitated and resuspended before running it through a DEAE-sephacel column. The laminin rich material subsequently eluted was purified with collagenase before running through a gel-filtration column. Eight specific peaks were identified. SDS-polyacrylamide gel electrophoresis identified bands ranging in size from > 500 to < 10 Kd. Three of MW 150, 100 and 50 Kd (coded N_3U, N_3L and N_5) were selected for further study. Suspensions of these proteins were then injected into rabbits and all induced a significant antibody response. There was no evidence of cross reaction to intact laminin, either intact or the NC1 fragment of Type IV collagen or human albumin. However cross-reaction of the antibody raised to N_3U did occur to N_3L, N_4 and N_5. These four laminin fragments are collectively described as 'mixed laminin fragments' (MLF).

This assay has been used to examine the levels of MLF in the urine of subjects with NIDDM and nephropathy and to determine whether they have a role in monitoring disease progression.

6.2   AIMS

- To measure intact type IV collagen in the serum and urine of subjects with NIDDM and nephropathy.

- To determine if subjects showing more rapid disease progression have higher serum levels of type IV collagen

- To ascertain whether urine type IV collagen could be used a marker of disease progression in more advanced diabetic nephropathy.

- To measure the levels of mixed laminin fragments (MLF) in the serum and urine of subjects with NIDDM and nephropathy using a newly developed assay.

- To determine whether the measurement of MLF has a role as a disease marker in diabetic nephropathy.

6.3   PATIENTS AND METHODS

6.3.1   Patients

Serum and urine samples were obtained from 55 subjects with NIDDM and nephropathy. Serial samples were available for those subjects who participated in the prospective study of progression of renal impairment (see Chapter 4). Samples were also obtained from 33 'diabetic controls' and 42 'normal controls' (clinic staff, hospital visitors and partners of diabetic patients). Whilst diabetic and normal control subjects were excluded if urinalysis was positive for protein, no effort was made to exclude those with evidence of microangiopathy, although data on presence/absence of complications were recorded for each subject. In all groups individuals with a history of cancer or liver disease were excluded. All non-diabetic subjects had a random blood glucose and serum creatinine measured. In addition all diabetic subjects had a glycosylated haemoglobin measured. Details of the clinical parameters for each group are presented in Table 6.2 (page 156).

6.3.2   Ethical approval

Informed consent was obtained from all subjects recruited. The study received approval from King's College Hospital medical ethics committee.

6.3.3 Assay of human type IV collagen

The assay for type IV collagen was based on an enzyme immunoassay kit supplied by Fuji Industries Ltd., Japan. A schematic representation of the solid phase assay is shown in Figure 6.2 (overleaf). The epitope thought to represent the antigen of intact type IV collagen is shown in Figure 6.1. The sample is allowed to react simultaneously with anti CL-IV antibody immobilised on a bead and with enzyme labelled anti CL-IV antibody directed at distinctly different antigen sites on the CL-IV molecule. For composition of the solutions see Table A6.1 in the Appendix.

Immunoreaction:

All samples and standards were prepared in *duplicate*. 50 µl of patient/control serum and urine were pipetted into Nunc polystyrene maxisorp immunotubes (4ml; Gibco BRL Ltd.). 300µl of enzyme-labelled anti CL-IV antibody solution {Horseradish peroxidase labelled monoclonal antibody} were then added to each tube and a single anti-CL-IV antibody coated polystyrene bead {Mouse anti-human CL-IV antibody} added to the mixture, taking care to permit excess solution to drain from the bead. The tubes were then incubated at 10 - 30 °C for 60 minutes without shaking. This reaction was then stopped by adding 1ml of washing solution {100ml concentrated buffer (100mM phosphate buffer, pH 7, containing 1M NaCl}. The contents of the tube were then aspirated and the bead washed a further four times with 3.5 ml of the washing solution. The bead was then aspirated to dryness.

Enzyme reaction:

300 µl of the freshly prepared substrate solution {0.4 ml of TMDZ-DMF added to 40 ml of 100 mM acetate buffer (pH 5.5) and mixed gently} were then added to the bead in each tube followed by 100µl of substrate {10 mM acetate buffer, pH 5.5, containing 0.015% hydrogen peroxide}. TMBZ is 3,3',5,5' -tetramethyl benzidine, the chromogen, and is dissolved in N,N dimethyl formamide (details see appendix A6.1). The tubes were then incubated at 10 - 30 °C for 30 minutes again without
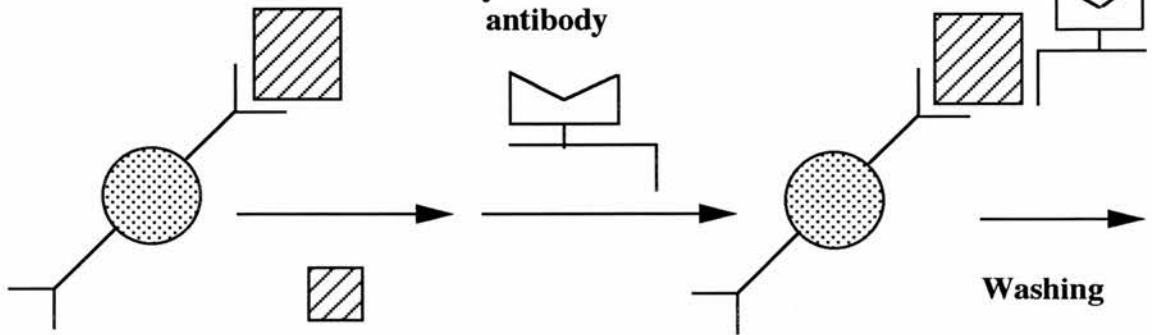
147
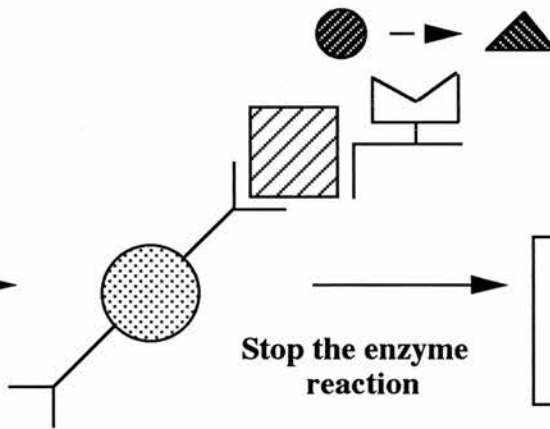
Figure 6.2 : Diagramatic representation of assay of human Type IV collagen

Figure 6.3 : Assay of Type IV collagen in serum and urine

149

shaking. The reaction was halted by adding 1ml of stopping reagent (1.33N sulphuric acid) to each tube. Figure 6.3 (above) outlines the assay procedure. The specimens and the standards (see below) were then read at 450 nm on an Atom 388 Spectrophotometer (Biotron SA Spain). The tubes containing the beads were read directly in the spectrophotometer. De-ionised water was used as the blank.

Preparation of Standard curve:

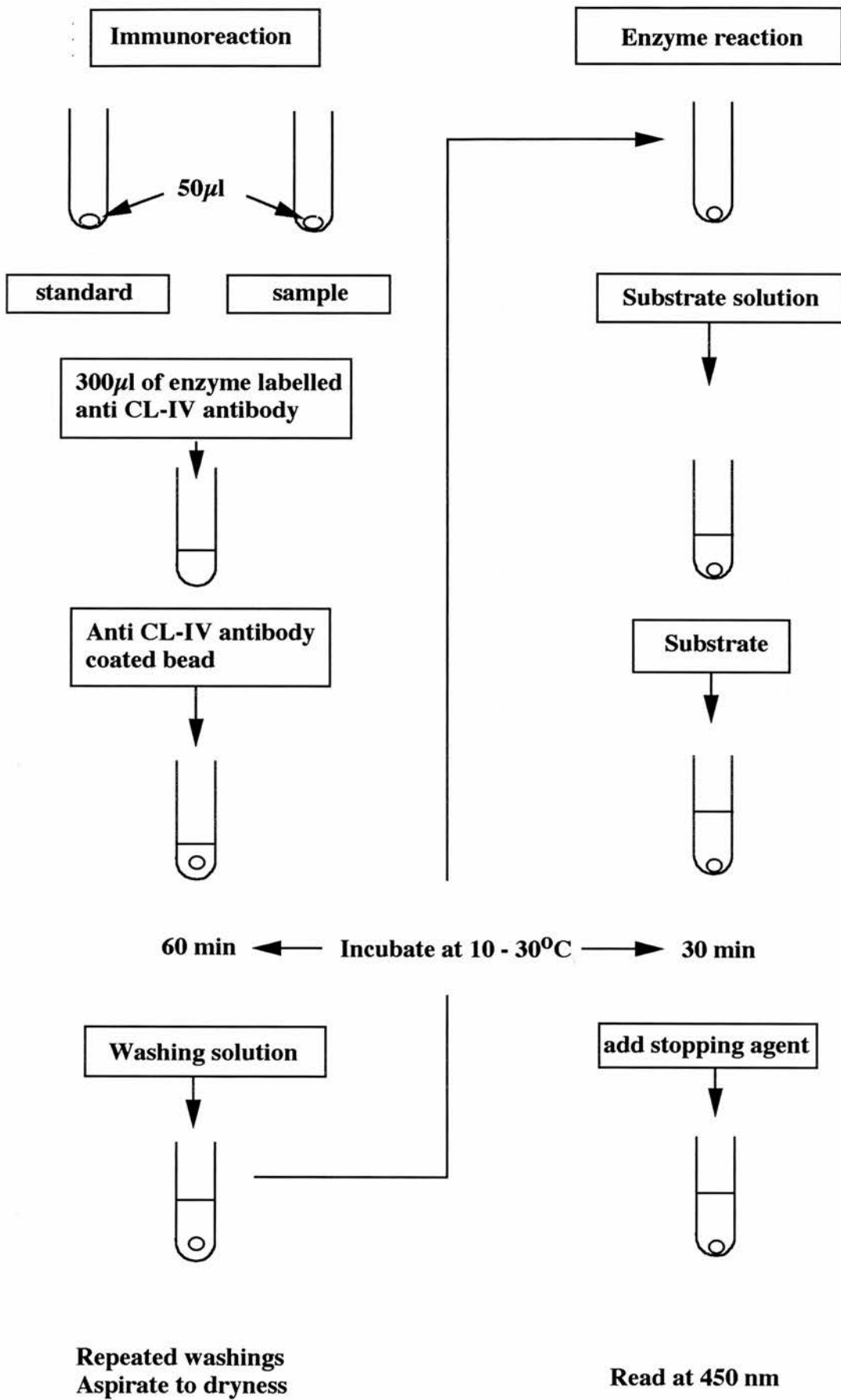The standard curve was prepared with the use of CL-IV standard {human collagen 2μg.ml^{-1}}. 150 μl of dilution buffer {10mM phosphate buffer, pH 7, with 1% BSA and 10% horse serum} is added to 150 μl of the CL-IV standard giving a concentration of 1000 μg.L^{-1}. Subsequent standards were prepared by removing 150 μl of the previous standard and adding 150 μl of dilution buffer to produce the standards given in Table 6.1 (below). 50 μl of each standard are then assayed as in the reaction outlined above and as in Figure 6.3.

CL-IV concentration (μg.L^{-1})	1000	500	250	125	62	31	16	0
CL-IV standard solution	150	150	150	150	150	150	150	0
Dilution buffer	150	150	150	150	150	150	150	300
Standard solution {volume (μl)}	300	300	300	300	300	300	300	300

Table 6.1 : Collagen assay - standard curve concentrations.

The standard curve for Type IV collagen (Figure 6.4) is to be found on the following page. The data were corrected for absorbence at 0 μg.L^{-1}. The data for urinary collagen are corrected for urine flow and are presented as μg.mmol Cr^{-1}.
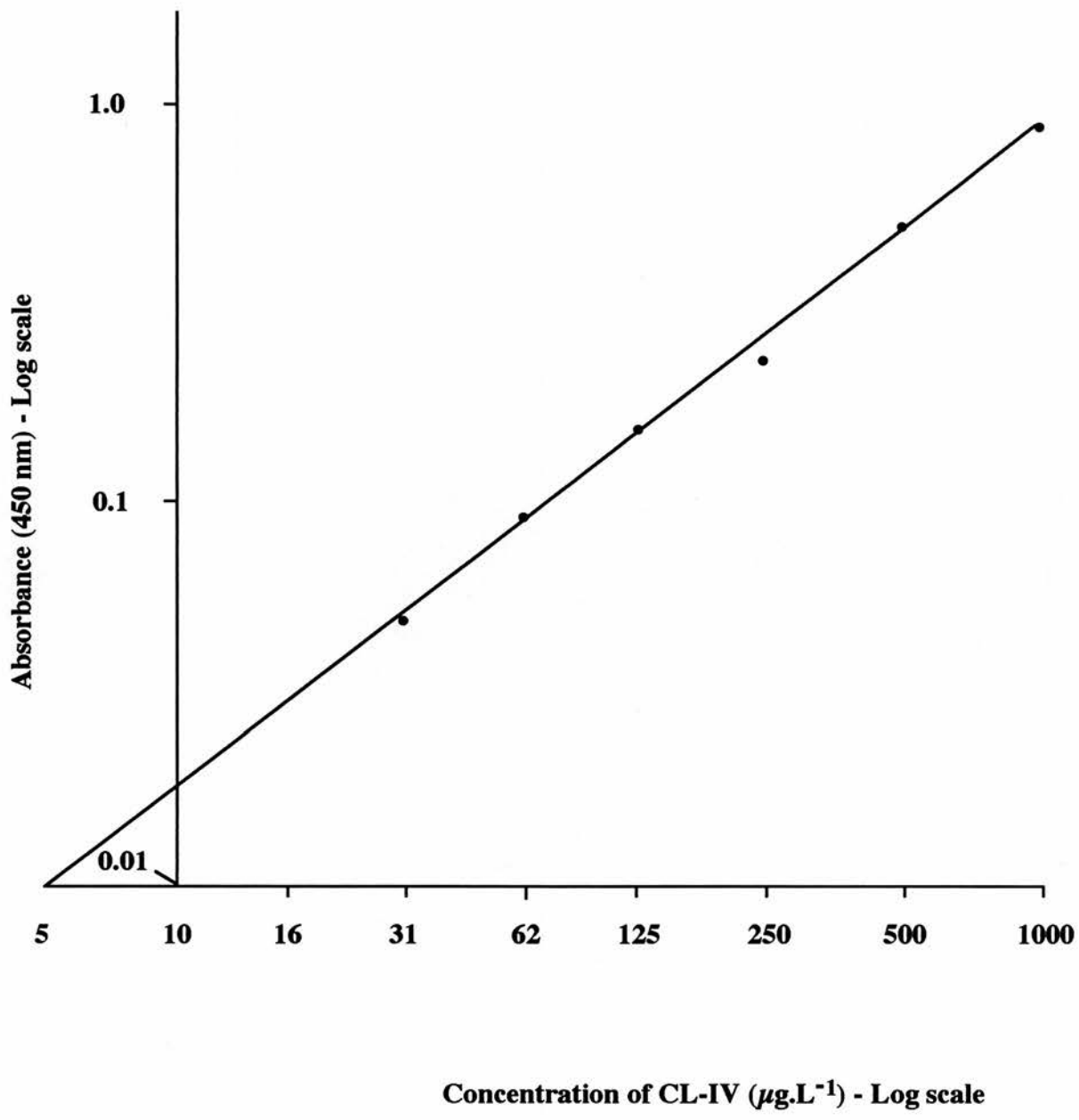
Figure 6.4 : Standard curve for Type IV collagen (CL-IV)

6.3.4 Assay of mixed laminin fragments.

All the assays were performed in Nunc Polystyrene Maxisorp Immunotubes (4 ml Gibco BRL Ltd.) and Pyrex (6ml) glass tubes. Each sample (and standard) was analysed in duplicate.

Step 1:   Preparation of the antigen coated tubes.

A stock solution of mixed laminin fragments (1 mg.ml^{-1} in PBS/0.04%) was first prepared and divided into 500 µl aliquots which were then stored at -70 °C. The stock solution was diluted with 0.05M sodium carbonate buffer (pH 9.6) to give a concentration of laminin fragment of 400 ng.ml^{-1}. 1000 µl of this solution were added to each tube, which were then stored at 4°C for 48 hours. The tubes were then washed twice with 1600 µl of the 'washing' solution (PBS/0.04% Tween 20) before adding 1200 µl of the 'blocking' solution (0.5% casein in PBS/0.04% Tween 20) and incubating at 37 °C for two hours.

Step 2:   Preparation of standard curve and sample tubes.

The standard curve was produced by preparing a 1:10 dilution of the stock laminin fragment solution (above) in glass tubes and thence by doubling dilution to obtain a standard curve ranging from 100 µg.ml^{-1} to 100 ng.ml^{-1}. The volume in each tube was 600µl (preparation as shown in Table 6.1, allowing for the different volumes). 120 µl of sample (serum or urine) were then added to a clean labelled glass tube and diluted with 480 µl of PBS/0.04% Tween 20. The tubes were covered with 'cling-film' and left at room temperature until the addition of the primary antibody. 600 µl of the primary antibody (anti-N3U laminin fragment diluted 1:500 in PBS/0.04% Tween 20 containing 0.1% casein) was added to all the tubes to give a total volume of 1200 µl. The samples were then incubated at 37 °C for 90 minutes timed to coincide with the completion of incubation of the antigen tubes (step 1). Duplicate samples of diluted primary antibody (600 µl with 600 µl of PBS/0.04% Tween 20) and blanks (1200 µl of PBS/0.04% Tween 20) were prepared and incubated as per standard samples.

Step 3:   **Addition of the second antibody.**

After incubation the Nunc tubes (step 1) were washed a further time with 1600 µl of PBS/0.04% Tween 20 and 1000 µl of the primary antibody mixture (prepared in step 2) transferred to each tube and the samples incubated for a further 90 minutes at 37°C. The contents were then discarded and the tubes washed thrice with 1600 µl of PBS/0.04% Tween 20. 1 ml of the second antibody (alkaline conjugated goat anti-rabbit IgG, Sigma Ltd., No. A-8025 diluted 1:1000 with PBS/0.04% Tween 20) was then added to each tube and all incubated once more at 37 °C for 60 minutes. The tubes were then washed three times with PBS/0.04% Tween 20. The sample tubes were then transferred to a 37 °C water bath and 1000 µl of 1mM SLPr-phosphate substrate   (Pyridium   4-[2-{4-phos-phoroyloxy-3,5-dimethoxyphenyl}-vinyl]-1-propyl-quinolinium iodide; PPR Diagnostics Ltd., London, UK. in 1 M diethanolamine buffer {pH 9.6} - pre-incubated to 37 °C - was added to each tube. The samples were left for 15 minutes.

Step 4:   **Reaction termination and sample analysis.**

The reaction was terminated by the addition of 250 µl 'stopping' buffer {0.2 M dipotassium hydrogen phosphate with 100 mM EDTA (pH 9.6)}. A green-blue colour developed and the samples were read at 545 nm on an Atom 388 Spectrophotometer (Biotron SA, Spain). The optical density values obtained were then converted to percentage activity by comparing each to the antigen-free tubes (100% activity).

For each sample the percentage inhibition was calculated as :

% inhibition = 100 - % activity

The standard curve of % inhibition plotted against concentration of MLF is shown in Figure 6.5 (overleaf). The concentration of MLF in each serum and urine sample is
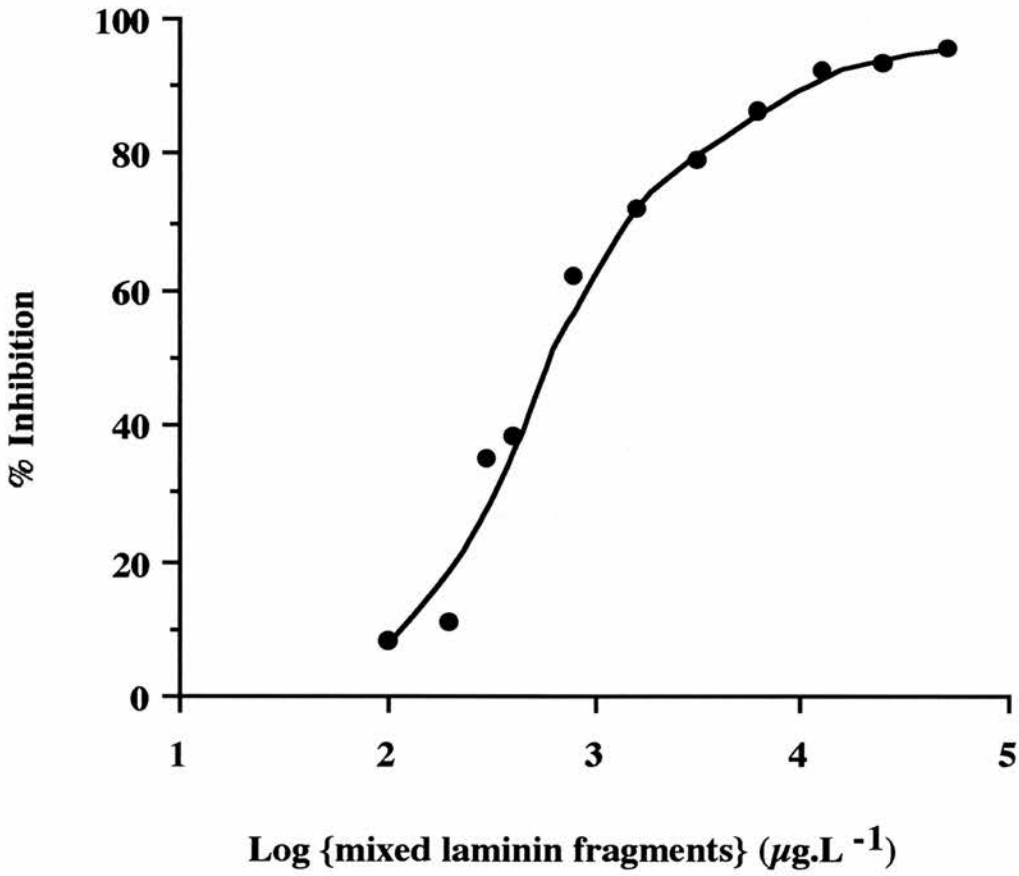
Figure 6.5 : Standard curve with mixed laminin fragments as antigen.
Data points are mean of three samples.

then read from this graph. The same standard curve was used for both serum and urine. However to adjust for urine volume the data for the urine concentration of MLF were corrected for urinary creatinine concentration. Thus the data for urinary laminin are presented as $\mu g.mmol\ Cr^{-1}$.

6.3.5   Statistical analysis:

Median and interquartile ranges and mean and standard deviation were calculated as appropriate for the groups. Comparison between groups was by the Mann-Whitney test for non-parametric data and unpaired Student's 't-test' for normally distributed data. The Pearson correlation coefficient was used to examine the relationship between the serum and urine levels of type IV collagen and MLF and between these variables and serum creatinine and glycosylated haemoglobin. P values < 0.05 were deemed significant.

6.4   RESULTS:

6.4.1   Sample and clinical data

Serum was analysed for type IV collagen and mixed laminin fragments in all 42 normal control and 33 diabetic control subjects. 96 samples were obtained from 55 diabetic subjects with nephropathy and comprised 3 from each of the 19 individuals studied prospectively (Chapter 4), single samples from the four who ended the study prematurely and two from three who were followed for one year. Single samples were obtained from a further 29 individuals with NIDDM and nepropathy. The number of urine samples processed in the three groups was fewer due to an undetected failure of a storage freezer.

	Normal controls (n=42)	Diabetic controls (n=33)	Diabetic nephropaths (n=55)
Gender (M:F)	21:21	19:14	42:13#
Age	61.5 (9.3)	63.3 (10.6)	63.6 (9.8)
Random glucose (mmol.L^{-1})	5.9 (1.6)	11.8 (4.1) *	11.7 (6.9) *
HbA$_{1c}$	-	8.1 (1.9)	7.8 (2.2)
Serum creatinine (μmol.L^{-1})	91 (20)	87 (20)	261 (94) †
Type IV collagen (μg.L^{-1})	94 (71-126)	115 (83-140)	161 (119-217) ††
Laminin fragments (μg.L^{-1})	224 (154-590)	1000 (580-1300)***	940 (220-2240) **

Key : All data are presented as mean (SD), with the exception of the collagen and laminin values, which are given as median and interquartile range *= $p < 0.01$, **= $p < 0.005$, *** $p < 0.0001$ vs normal controls; †= $p < 0.001$, ††= $p < 0.0001$ vs normal and diabetic controls (Mann-Whitney). # - two or more samples from each individual.

Table 6.2 : Serum Type IV collagen and mixed laminin fragments in subjects with diabetic nephropathy, normal and diabetic control subjects.

The mean age of the three groups was similar (Table 6.2 above). There was a male preponderance in the group with nephropathy. However no significant differences in either type IV collagen or the levels of MLF were observed when the data were analysed separately by gender. Neither glycosylated haemoglobin nor glucose taken simultaneously differed between diabetic controls and nephropaths. Predictably serum creatinine, at 261 μmol.L^{-1}, was significantly greater in the nephropathic group.

6.4.2 Type IV collagen

The median (interquartile range) for serum type IV collagen in normal subjects was 94 (71-126) μg.L^{-1} (Table 6.2). The level in nephropathic subjects was significantly higher than both the normal and diabetic control groups ($p < 0.0001$; Mann-Whitney test). Examination of Figure 6.6 (page 158) reveals a marked overlap of the

individual values between the three groups. Of the 96 samples in the nephropathic group only 11 exceed the upper limit observed in the normal group.

In contrast the urine levels are consistently higher in the nephropathic group. Figure 6.7 shows that type IV collagen is undetectable in all normal controls and all but three diabetic controls, whereas it is detected in 40 of 81 (49%) nephropathic samples. The levels of type IV collagen in the serum, but not urine, are slightly higher in those with more advanced renal disease, though the differences are slight (Figure 6.8 overleaf). Neither serum nor urine type IV collagen appears related to glycosylated haemoglobin (Figure 6.8 overleaf).

In the group of 19 individuals studied prospectively there is no difference in the serum level of type IV collagen in those who progress and those who do not (Figure 6.9 overleaf). However in only 3 of 10 'Non-progressors' was collagen detectable in the urine, in contrast to all bar one of the 'Progressors' (Figure 6.9 overleaf). In these 19 subjects there is a suggestion that both serum and urine type IV collagen are lower in those showing a more gradual decline of GFR (Figure 6.10 overleaf), though the association is not statistically significant, and again influenced by the same subject as previously mentioned.
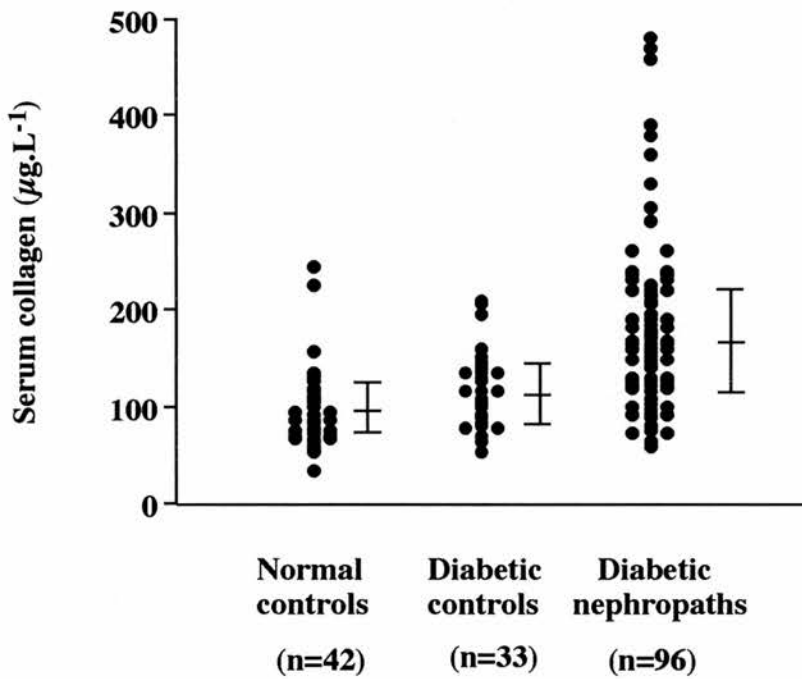
Figure 6.6 : Level of Type IV collagen in the serum of individuals
with diabetic nephropathy together with diabetic and
normal controls. Median (interquartile ranges as shown).
Type IV collagen is higher in the nephropathic group
compared to the diabetic and the normal control group
(p<0.0001; Mann-Whitney).
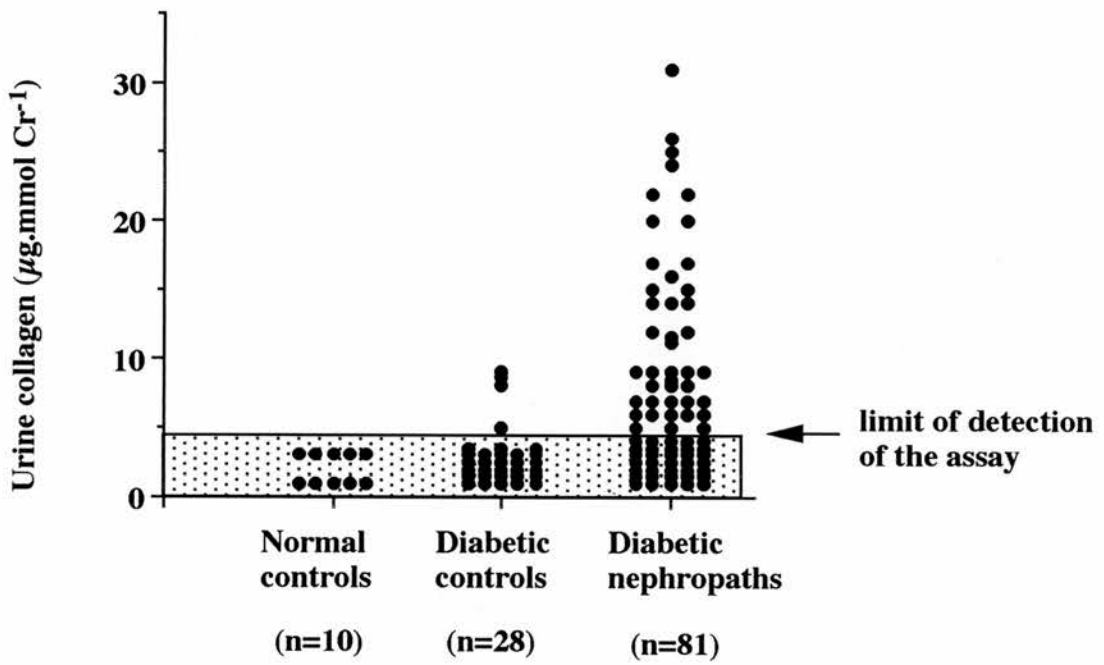
Figure 6.7 : Level of Type IV collagen in the urine of individuals with diabetic nephropathy together with diabetic and normal controls. The sample number is denoted by 'n'.
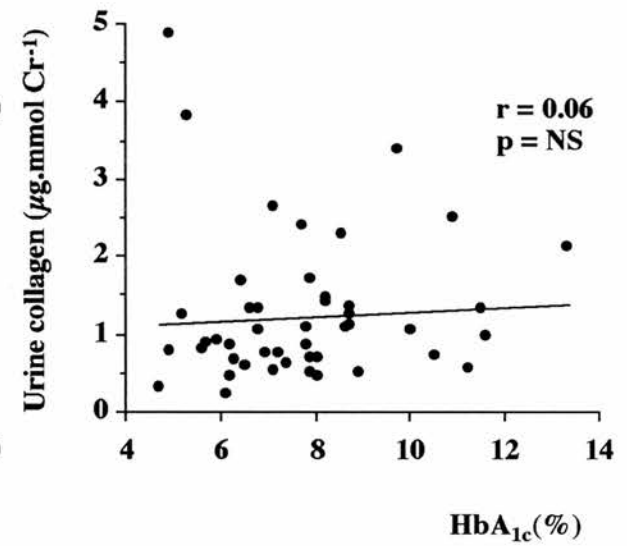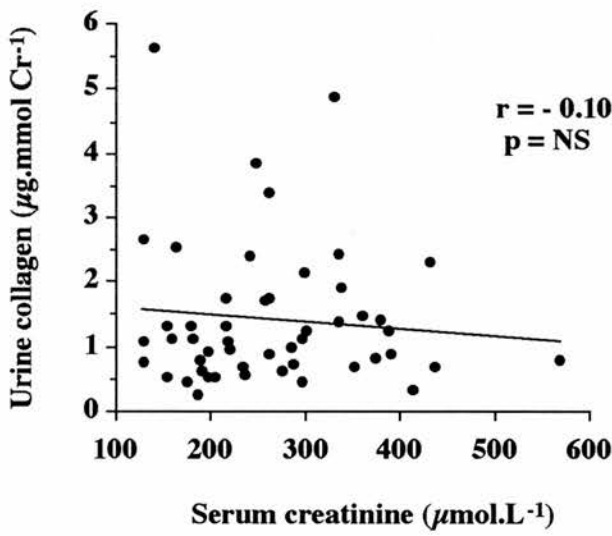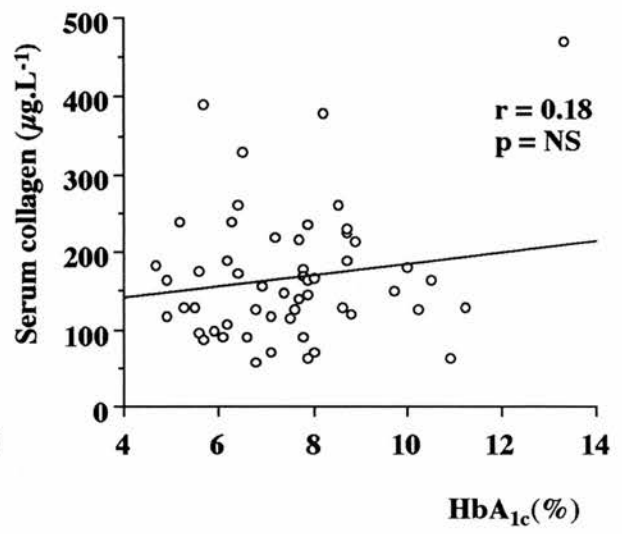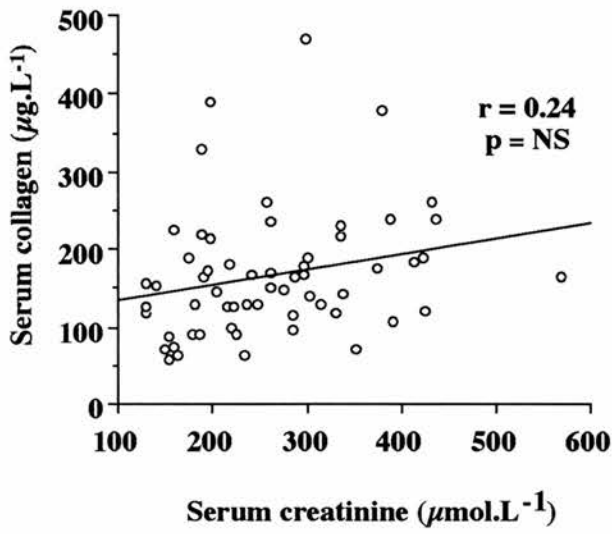
Figure 6.8 : Relationship between serum creatinine, glycosylated haemoglobin and serum and urine type IV collagen in diabetic individuals with nephropathy. Correlation coefficients as shown.
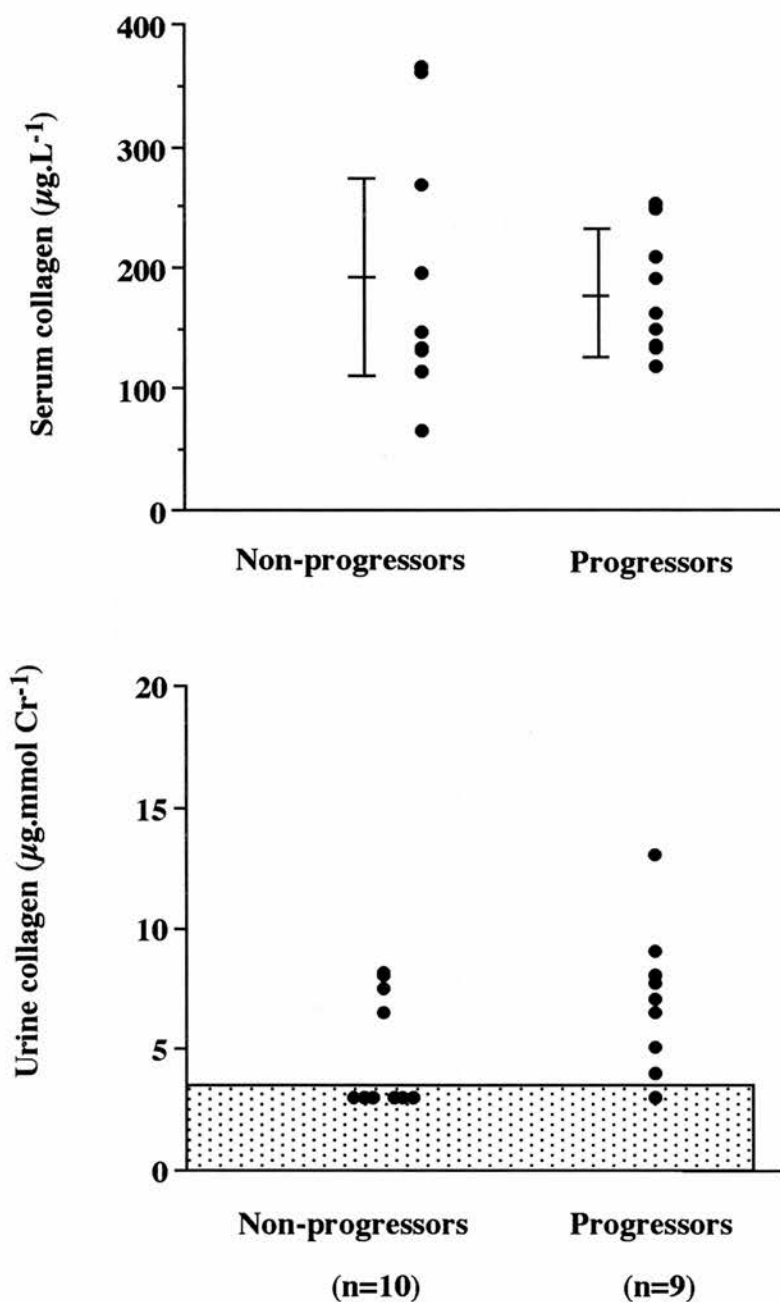
Figure 6.9 : Serum and urine type IV collagen in individuals with NIDDM nephropathy, subdivided by rate of progression. Median (IQ range) as shown for serum, p=NS. Shaded area - limit of detection in urine.
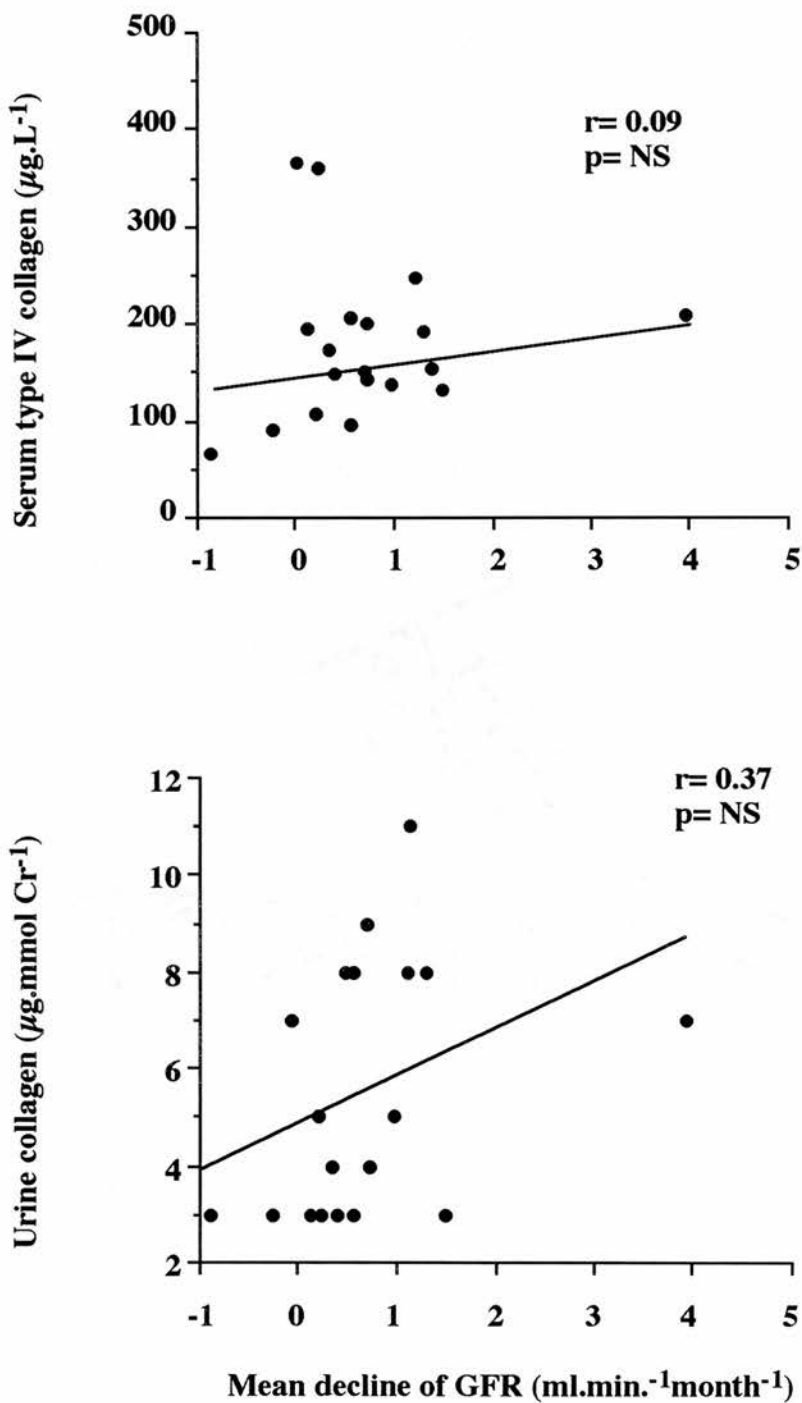
Figure 6.10 : Relationship between the decline in GFR and serum and urine Type IV collagen in all subjects. Each point represents the mean of the samples for each individual.

6.4.3   Mixed laminin fragments

Twenty-eight samples were available from normal controls, 51 from diabetic controls and 84 from nephropathic subjects. The median (interquartile range) for serum MLF in the normal subjects was 224 (154 - 590) μg.L^{-1}. Both the diabetic control (1000 μg.L^{-1}; p<0.0001) and nephropath (940 μg.L^{-1}; p<0.005) groups had significantly higher MLF levels compared to the normal control group (Table 6.2 above). However no difference in the level of MLF was observed between the control group with diabetes and those with nephropathy (Figure 6.11). In only a few cases in the nephropathic group does the level exceed 5000 μg.L^{-1}.

The levels of MLF in the urine are shown in Figure 6.12. Laminin was detected in the urine of only one normal subject, whereas in the nephropathic group in all save one was laminin detectable. 10 out of 28 diabetic controls had measurable MLF, but all were < 110 μg.mmol Cr^{-1}.

As with serum type IV collagen, MLF in the serum was not associated with serum creatinine (Fig 6.13). A modest rise in urine MLF was observed with increasing creatinine and with poorer glycaemic control (same figure).

In contrast to the situation for collagen there is a suggestion that a more rapid decline of GFR is associated with greater levels of urine laminin (Figure 6.14). This figure shows only two of ten 'Non-progressors' with urine MLF in excess of 200 μg.mmol Cr^{-1}. Of these one subject had the largest decline of GFR (0.5 ml.min.$^{-1}$month^{-1}) in the 'Non-progressors' group. However Figure 6.15 shows that this association is not perhaps as strong as is suggested in Figure 6.14.

Figure A6.1 (in appendix) shows that there is no relationship between serum and urine collagen, serum and urine laminin, and serum levels and urine levels of collagen and laminin respectively.
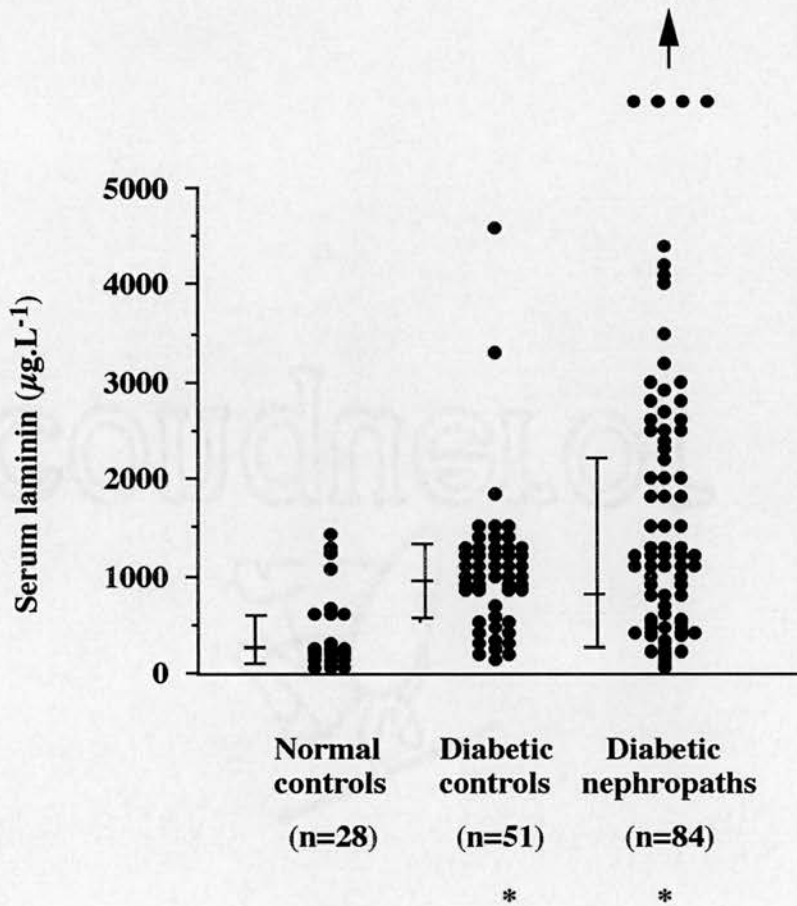
Figure 6.11 : Concentration of mixed laminin fragments in the serum of diabetic subjects with and without nephropathy and that of normal controls. Median (interquartile range) displayed. *p<0.005 nephropathic and diabetic control group vs normal control group (Mann-Whitney U test).
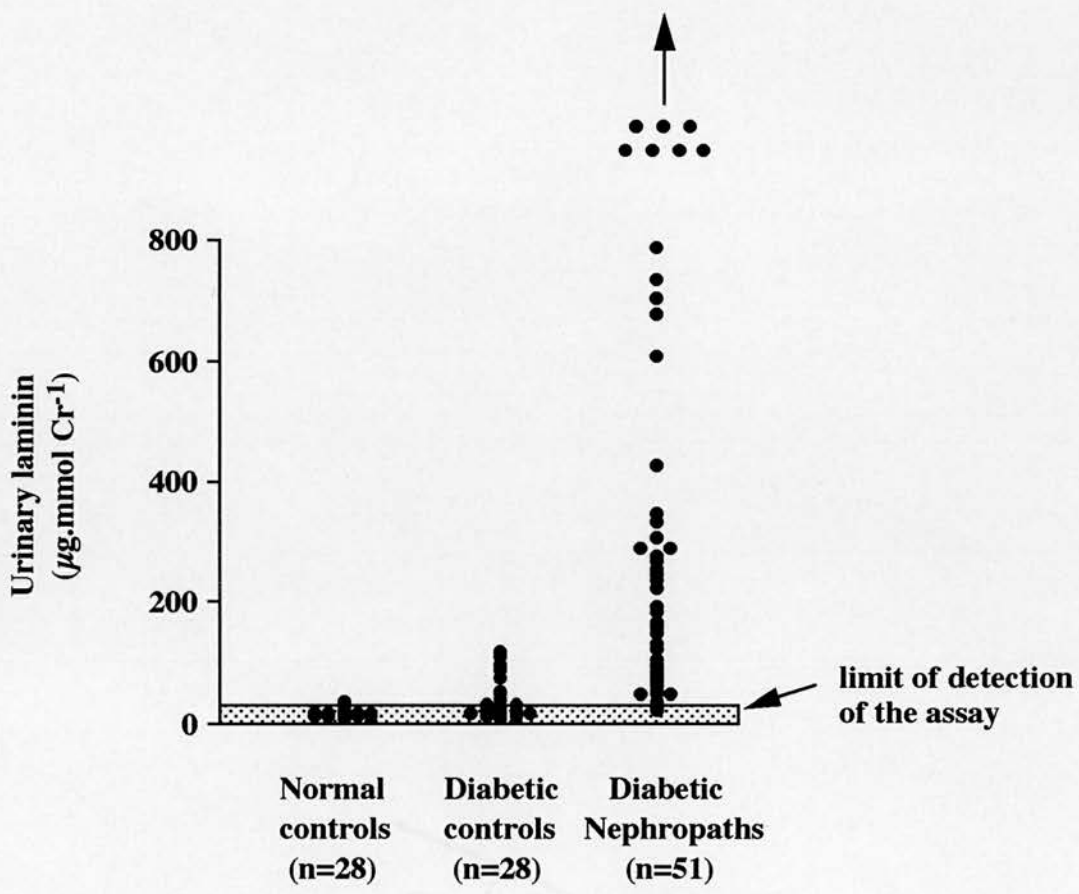
Figure 6.12 : Level of mixed laminin fragments in the urine of patients with diabetic nephropathy, together with diabetic and normal controls.
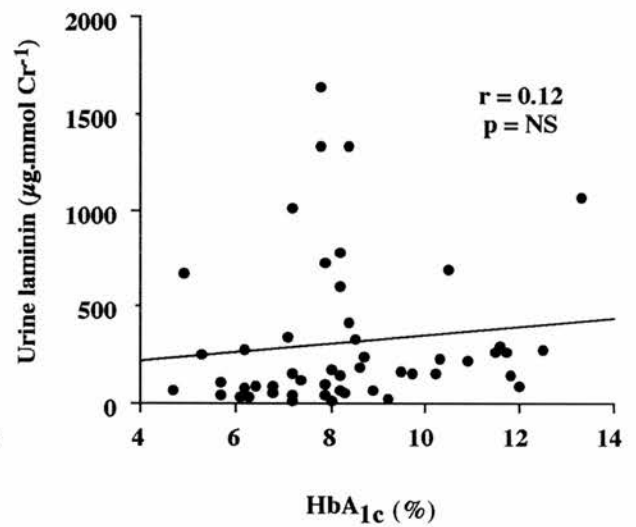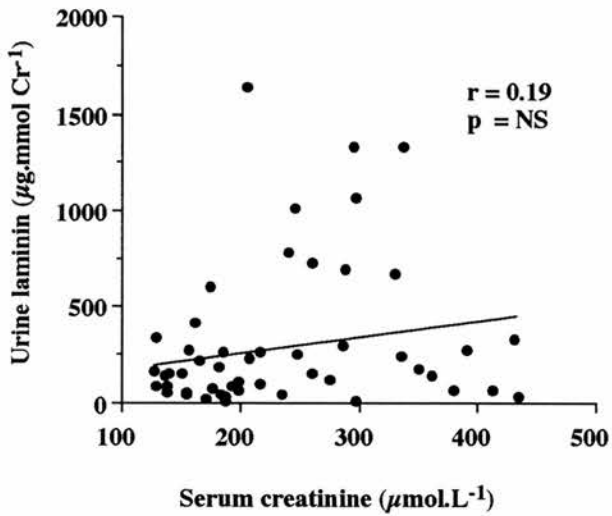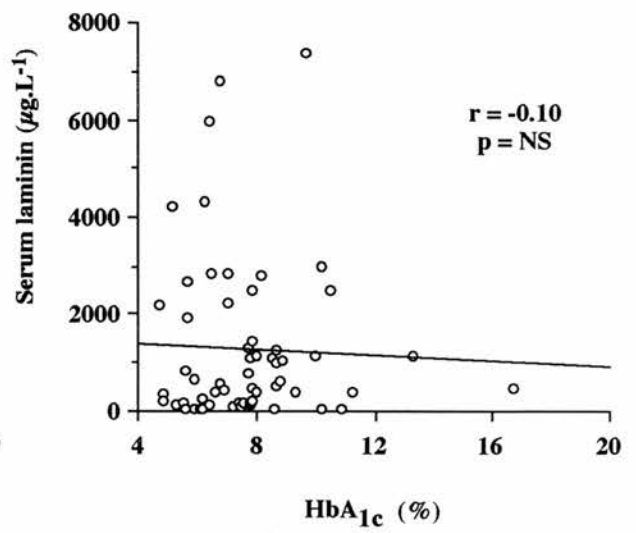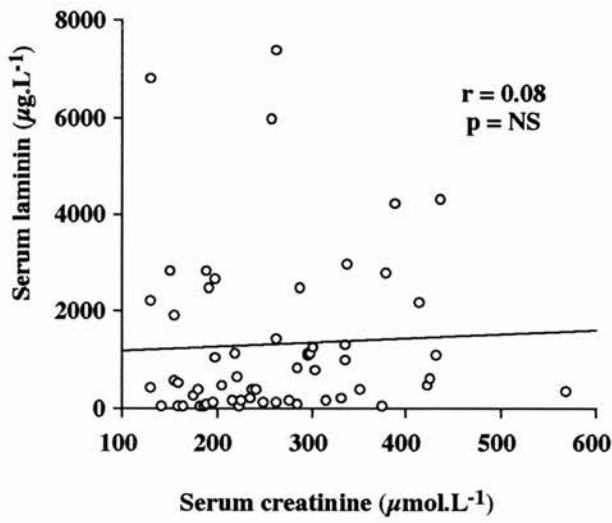
Figure 6.13 : Relationship between serum creatinine, glycosylated haemoglobin and serum and urine mixed laminin fragments. Univariate analysis. Regression coefficients as shown.
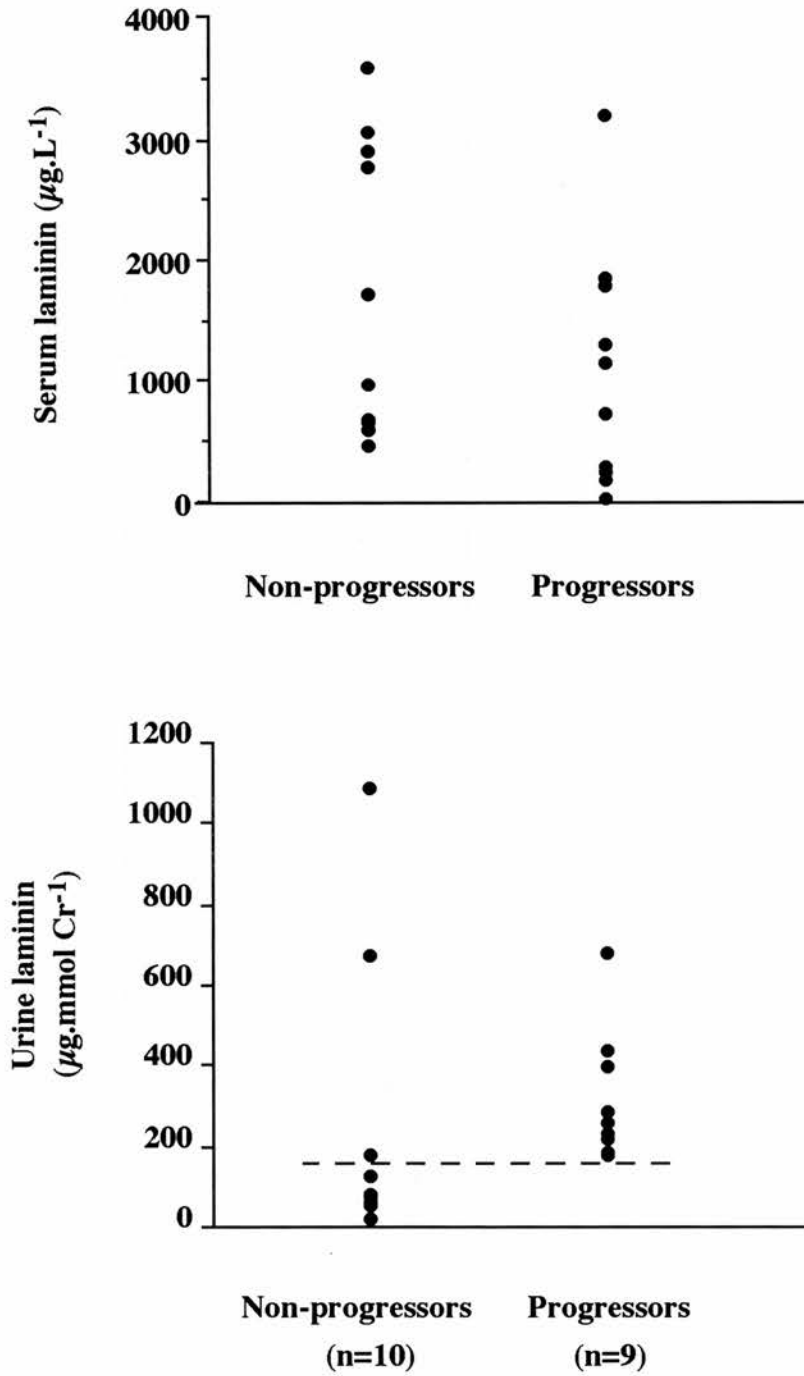
Figure 6.14 : Serum and urine laminin in subjects with NIDDM
nephropathy, subdivided into 'Non-progressors' and
'Progresors'. Each value plotted is the mean of three
samples. Dashed line represents lowest detected in
the 'Progressors'
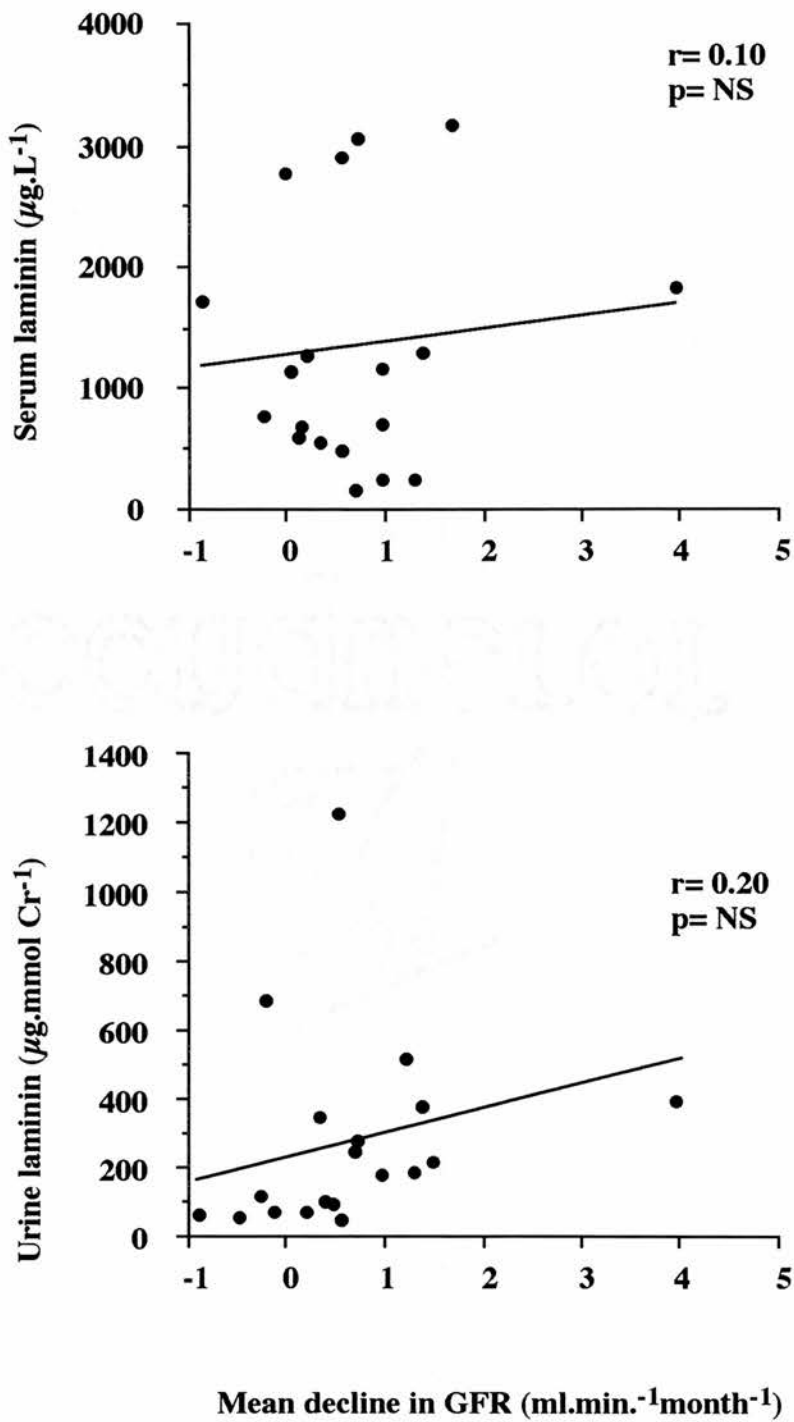
Figure 6.15 : Relationship between the decline in GFR and serum mixed laminin fragments in 19 nephropathic subjects. Each point represents the mean of three samples.

6.5   DISCUSSION

This study fails to substantiate claims that the measurement of type IV collagen provides an effective index of disease progression in diabetic nephropathy (Matsumoto et al. 1990). In any case the current assay is probably too expensive ($\approx$£2 per sample) for routine clinical use. Likewise serum MLF does distinguish between diabetic control and nephropathic subjects. However these preliminary studies suggest that the measurement of urinary laminin fragments may prove useful in the identification and monitoring of disease progression, given that higher levels are observed in those who progress more rapidly.

The antibody used to measure type IV collagen in this study is thought to recognise the intact molecule and furthermore it is hypothesised that such collagen is excess to that incorporated into the matrix (Obata et al. 1989; Matsumoto et al. 1990). If this is the case then the levels measured should reflect the rate of deposition of collagen at a given time. Our study has demonstrated that, though type IV collagen is measurable in the serum, the overlap of individuals with nephropathy, normal and diabetic controls precludes its use as a marker of disease let alone progression. Only 11 samples from nephropathic individuals had collagen level in excess of the upper limit of the normal range. These results contrast markedly with those of Matsumoto and colleagues (1990) who observed significant differences between normal and diabetic subjects, with and without complications. Using the same assay we have found consistently higher levels in each group with greater variability within each group. Both studies were closely matched for age and the groups had comparable renal function. The nephropathic group in both show the greatest variability in the data.

Whilst it appears from this study that type IV collagen has no role as a serum marker of disease progression in diabetic nephropathy, is this the case for urine collagen? Initial examination of Figure 6.7 would suggest it may have a role. Intact type IV collagen was undetectable in the urine of the 10 normal and in all but 3 samples from

169

diabetic control subjects. Since intact type IV collagen has a molecular weight of 500000 and would not pass through the intact glomerulus, such findings are not surprising. Collagen was detectable in approximately 50% samples from nephropathic subjects. Only a few urine samples from patients with advanced nephropathy analysed by Matsumoto and colleagues (1990) yielded a positive result. The authors concluded that their assay was not sufficiently sensitive to detect the low concentration of collagen present in urine.

Banu and colleagues (1994) describe a novel method of concentrating urinary type IV collagen using precipitation with polyethylene glycol (PEG), based on the method of Ramshaw and colleagues (1984). Concentration of urine enabled the authors to detect type IV collagen in diabetic individuals with nephropathy and, more surprisingly, normal subjects. It is most unlikely that the latter represented filtered protein and presumably the origin was tubular secretion or else the assay was not measuring intact collagen. This raises the question as the relative contribution of the glomerulus and tubule to urinary collagen in the nephropath group. Falk and colleagues (1983) would argue the latter, observing that type IV collagen was increased in both the mesangium and the GBM in the early stages of nephropathy in renal biopsy tissue from IDDM subjects but decreased in the latter stages. In contrast the amount of type IV collagen was increased in the renal tubular basement membrane in more advanced disease.

There are no comparable studies to the above in NIDDM though one of three subjects described by Woodrow and colleagues (1992) had NIDDM. However their immunohistochemical studies, using immunogold on renal biopsies reported only on the glomeruli and did not mention the tubular basement membrane. Hayashi and colleagues (1992) filtered and concentrated urine by dialysing against PEG, prior to analysis. They too found measurable quantities in normal subjects. Given these findings it is perhaps not surprising that so many individuals had detectable collagen.

Were it possible to repeat the study with PEG precipitation then it might have been detected in many more. Donovan and colleagues (1994) have recently described increased urinary levels of type IV collagen in the urine of a small group of diabetic subjects, using an 'in-house' assay. Although highly sensitive (measuring down to 1 $\mu g.L^{-1}$) the assay unfortunately measures both 'intact' and 'degraded' collagen.

Torffvit and colleagues (1989, 1991) in their studies on NC1 levels in the urine found elevated levels in the patients with microalbuminuria but not in those with proteinuria. The authors interpret their findings in the light of the changing pattern of type IV collagen deposition in progressive disease described in the immuno-histochemical studies described above. The small number of subjects studied precludes definitive conclusions being drawn.

It is hard to envisage a role for the measurement of type IV collagen in diabetic nephropathy. Serum measurement appears to be of no clinical benefit, although urine measurement may merit further investigation. It has no obvious advantage over protein excretion, though may identify those individuals who are likely to fare badly. Only a greater understanding of the individual and temporal variation in urine levels together with an assessment of outcome would determine if it had any diagnostic or prognostic worth.

Although established as markers of hepatic fibrosis (Matsumoto et al. 1989) and proposed for metastatic disease (Yamamoto et al. 1992), laminin fragments have not yet been shown to be of clinical benefit in diabetic nephropathy. This new assay has revealed significantly elevated levels of MLF in sera from individuals with diabetes compared to normal controls; though in those with established nephropathy the level is not significantly different from those without. Pietschmann and colleagues (1988) have previously demonstrated increased laminin P1 in IDDM and NIDDM subjects with advanced nephropathy, though not in those with micro-albuminuria. A similar

finding was made by Hayashi and colleagues (1992), though only in eight subjects with renal impairment.

This assay has detected MLF in virtually all the urine samples from the nephropathic subjects, compared to only one of 42 normal control and <30% of diabetic controls. One individual in the nephropathic group had values in excess of 1000 μg.mmol Cr^{-1}. Price and colleagues (1994) reported a similar elevation of MLF in a pilot study. Mixed laminin fragments was significantly raised in those with clinical proteinuria though the levels in the 'micro-albuminuric' group did not differ from normal. Hayashi and colleagues (1992) found LP1 be elevated in the urine of a small group of NIDDM subjects, with and without nephropathy. The level of LP1 did not segregate with stage of nephropathy.

It is probable that the assay used in this present study measures the degradation products of laminin. The antibody raised to the LN_3U fragment did not cross-react with whole laminin, but cross-reaction did occur to other fragments. As yet it is not known what the relative contribution of each of these antigens is to the total measured. It remains to be seen whether any or all will prove helpful in monitoring the progression of nephropathy. Figure 6.14 suggests that MLF may be important in the identification of those individuals in whom nephropathy progresses more rapidly. No such separation is observed for serum. Unfortunately when the levels of MLF in the sequential urine samples were examined in these 19 subjects no consistent trend emerged, i.e. the 'Progressors' did not show increasing levels, nor did the 'Non-progressors' decline or even remain stable. Marked variation in urinary MLF occurred in sequentially obtained samples from several individuals, ranging from 180 to as much as 2500 μg.L^{-1}, yet in others the level of MLF remained remarkably constant over the two years.

MLF and, to a lesser extent, type IV collagen merit further study to determine whether they are of value in identifying those individuals with nephropathy who are destined to fare badly. Some support for this is provided here though more subjects need to be examined.

6.6 CONCLUDING REMARKS

- Serum type IV collagen from subjects with diabetic nephropathy is higher than found in diabetic control and normal subjects (both $p<0.0001$)

- Serum MLF was significantly higher in diabetic subjects with nephropathy ($p<0.005$), but was no different from diabetic control subjects.

- The levels of type IV collagen and serum MLF are not closely related to the degree of renal impairment.

- MLF was identified in the urine of almost all cases of diabetic nephropathy, and type IV collagen in approximately 50%.

- MLF and type IV collagen were not detected in urine from normal subjects and rarely in diabetic controls without proteinuria.

- Mean levels of urinary MLF and type IV collagen segregated with the rate of progression of nephropathy in the majority of cases, particularly the former.

- It is doubtful whether serum laminin or type IV collagen has a role in the clinical assessment of diabetic nephropathy,

- Further studies are warranted to determine whether urinary MLF have a use as non-invasive markers of progression of established nephropathy.

Chapter 7

Conclusions

Without treatment someone with NIDDM, proteinuria and near normal renal function can anticipate starting dialysis within 6 to 7 years. Even with anti-hypertensive therapy the interval may be as short as 40 months (Biesenbach et al. 1994). This study has demonstrated a decline of isotopically measured GFR of 0.48 ml.min.$^{-1}$ month^{-1} over a two year period in 19 subjects with a mean baseline GFR of ≈ 50 ml.min^{-1} and agrees closely with the only directly comparable study by Gall and colleagues, published in 1993. Translated, this suggests a doubling of the interval between the onset of proteinuria and dialysis to 12 to 15 years with current therapeutic regimens.

The observational study presented in Chapter 2 suggest an even more optimistic outlook for IDDM subjects. Although retrospective and based upon estimated GFR the data suggest a similar rate of decline of GFR in the most recent two years of observation, 0.20 ml.min.$^{-1}$month^{-1}, to that seen in closely monitored intervention studies (Parving et al. 1987). The retrospective study shows a more rapid rate in the decline of GFR in the NIDDM compared to the IDDM group and contrasts with the findings of Friedman and Gross (1991) and Humphrey and colleagues (1989), where it was slower in NIDDM and Biesenbach and colleagues (1994), where the rate was similar. Part of the reason for the difference seen here may relate to the findings of Parving and colleagues (1987), who observed that the rate of decline of GFR fell in successive time periods after initiation of anti-hypertensive therapy. It will be interesting to see whether this improvement will be maintained in IDDM (as might be suggested by our most recent one year data) and whether a similar pattern will emerge in NIDDM.

Certain individuals show no decline at all of estimated GFR over a five year period, despite a baseline GFR of <50 ml.min^{-1}. Of these eight individuals, three had NIDDM. Although these data are most encouraging, the variation in the rate of decline of GFR is wide and certain subjects progress rapidly to end-stage renal

disease, with loss of function up to 2 ml.min.$^{-1}$month^{-1}. Indeed in the prospective study the rate of decline of GFR in one individual was nearly double this over a 20 month period. Investigation of this subject revealed no additional renal disease, with a renal biopsy showing only glomerulosclerosis and no evidence of renal artery stenosis on arteriography. Because of the small numbers studied this case skewed the data and strengthened certain of the associations, but did not alter the overall conclusions. What then accounts for the variability in the rate of decline of GFR seen in this and other studies ?

Several differences were observed between the retrospective and prospective studies regarding the factors associated with the decline of GFR. Blood pressure, principally systolic, and serum cholesterol, emerged as significant variables in the prospective study, whereas protein excretion was a less important factor (p=0.07). By contrast the latter was the main factor accounting for the variability in the data in both NIDDM and IDDM in the retrospective analysis. Why this factor should assume less importance in the prospective study is uncertain, though protein excretion was 1g.24hr^{-1} less.

What should the therapeutic targets be for NIDDM subjects with renal failure? The mean blood pressure in 'Non-progressors' in the prospective study was 142/82 mmHg giving a MAP of 102 mmHg, and is comparable to the target of 100 to 105 mmHg set by Mogensen and colleagues (1991). Those achieving such a level in our study had a mean decline of GFR of 0.44 ml.min.$^{-1}$month^{-1}. More stringent criteria have been set by the American Diabetes Association and the National Kidney Foundation (Consensus Development Conference 1994) where a MAP of 92 mmHg is suggested for those diabetic subjects with chronic renal insufficiency and a protein excretion exceeding 1g.24hr^{-1}. This study has also demonstrated that serum cholesterol below 6 mmol.L^{-1} is associated with a much reduced rate of decline of GFR, 0.04 ml.min.$^{-1}$ month^{-1} compared to 1.14 ml.min.$^{-1}$month^{-1} in those over 6

mmol.L^{-1}. These data suggest that the targets for NIDDM subjects with renal impairment should be a MAP of 100 mmHg and a serum cholesterol of ≤ 6 mmol.L^{-1}.

As in many other studies neither protein intake (as determined here by urinary urea excretion) nor glycaemic control influenced the rate of decline of GFR. The small numbers studied make it difficult to draw conclusions on ethnic differences in the rate of decline of GFR. Afro-Caribbean NIDDM subjects did progress more slowly than their Caucasian counterparts, a finding which agrees with the work of Cowie (1993). In the prospective study the Asian subjects had slower rate of decline of GFR than the other groups, though there were only 4 individuals. No benefit was seen from renal artery angioplasty in any subject and raises the question as to whether angiography seeking a RAS is justified, where it is known that diabetic nephropathy already exists.

Proteinuria may be both a promoter and marker of disease progression in diabetic nephropathy. Anecdotally those individuals with the greatest protein excretion often show the most rapid rate of decline of GFR (e.g. Cases 5, 16 and 18 this study). Although the retrospective study lends weight to these claims, such a finding is not universal (Gall et al. 1993; Sawicki and Berger 1994). A more reliable marker of progression may derive from the components of the mesangial matrix and the basement membrane, both of which are increased in diabetic nephropathy. Both type IV collagen and mixed laminin fragments were examined in this study. The serum and urinary levels of the former were significantly greater in the nephropathic group compared to both the diabetic and normal control groups. Unfortunately there was considerable overlap in the serum levels between the groups and it was not detectable in the urine of many nephropathic subjects. Furthermore it did not discriminate between the 'Progressors' and the 'Non-progressors'. Although the level of MLF in the serum was very similar in both diabetic groups it may prove a more reliable urinary marker of nephropathy, given that it was detectable in all but 2% of the

nephropathic subjects, compared to 64% diabetic controls (all low levels) and 96% of normal subjects. There is also a suggestion that the levels are higher in those showing the most rapid decline of GFR.

The early identification of NIDDM subjects at risk of developing nephropathy is essential but beyond the scope of this discussion (see Introductory chapter). Once identified, all subjects should have an isotopically determined GFR, together with the simultaneous measurement of serum creatinine. Where a timed collection for urinary albumin (overnight or 24-hour) is routinely performed the creatinine clearance could be determined, although this may be logistically difficult in many clinics and compliance a problem for those with visual impairment or in the elderly. Ideally GFR should be measured annually with simultaneous creatinine until the GFR falls to 80 ml.min^{-1} (this study would suggest 50 ml.min^{-1}). For individual subjects EDTA clearance could be correlated directly with predicted GFR (from Cockcroft-Gault) and those subjects showing a close correlation could be followed by predicted GFR alone, unless GFR exhibited a sharp decline when confirmation using EDTA clearance may be desired. Those subjects showing a poor agreement between the methods should continue to be followed with EDTA clearance. The results of this study suggest that endogenous creatinine clearance may be a helpful indicator of progression in obese subjects. Until such time as the specific factors contributing to the decline of GFR are determined and corrected (and this may never be achievable) clinicians will continue to watch helplessly whilst renal function is lost. That the decline of renal function has been accurately charted will prove of little comfort to either the physician or the patient.

The study has its limitations. Principal amongst these are the relatively small numbers studied and the period of observation. That said, there is no study in the published literature which follows as many cases of NIDDM with this degree of renal impairment. Outwith a collaborative study, it is unlikely that any one centre has

significant numbers of NIDDM subjects where the GFR is <50 ml.min^{-1}. A longer period of follow-up with a greater number of GFR measurements would have been desirable to increase the accuracy of the determination of the rate of decline of GFR. Neither was feasible in this case.

Future studies in NIDDM will need to determine whether subjects do show a progressive reduction in the rate of decline of GFR as is seen in IDDM. Intervention studies with lipid-lowering therapy are required in more advanced nephropathy in the light of the very slow rate of decline seen here in those with low serum cholesterol. Given that the introduction of such therapy is becoming increasingly commonplace, efforts should be made to monitor the changes in GFR with an isotopic method, if randomised controlled studies are not to be carried out.

The value of MLF as urinary markers of diabetic nephropathy and possibly of disease progression merit further study. It may prove that specific laminin fragments, or other basement membrane components, such as fibronectin, have a role in identifying those likely to progress more rapidly.

The recent discovery that the ACE gene insertion/deletion polymorphism may be a marker of therapeutic responsiveness to ACEI in IDDM is potentially an exciting one. Although the DD genotype was associated a more rapid rate of decline of GFR the variation within each group was wide (Parving et al. 1996b). It remains to be seen what will be found in NIDDM, where the therapeutic efficacy of ACEI is much less well established than in IDDM.

Whilst aggressive anti-hypertensive therapy and perhaps lipid lowering drugs may slow the overall rate of decline of GFR in diabetic nephropathy, only a greater understanding of the genetic, physiological and biochemical events at the cellular level will is likely to provide an explanation as to why certain individuals progress rapidly and inexorably to renal replacement therapy.

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Appendices

Table A2.1 : Clinical and demographic data entered onto computer

Patient details   name, date of birth, height, gender, ethnic origin

      Visit date
      Weight
      Urinalysis for protein
      Blood pressure (systolic and diastolic)

Serum/Urine measurements

      Blood glucose
      Serum creatinine
      HbA_{1c}
      24-hour urinary protein
      Serum cholesterol
      Serum triglycerides
      Serum calcium
      Serum phosphate
      Serum albumin
      Haemoglobin

Complications

Retinopathy	-details
Neuropathy	-details
Peripheral vascular disease	-details
Ischaemic heart disease	-details
Visual acuity	-right/left

Therapy

Sulphonylurea	Yes/No
Biguanide	Yes/No
Insulin	Yes/No
Insulin dose	

Computed data

      $BMI = weight\ (kg)/height^2\ (m)$

      $GFR = \{140\text{-age(years)} \times body\ weight\ (kg) \times K/serum\ creatinine(\mu mol.L^{-1})$

            where K is 1.23 for men and 1.05 for women

MEASURED VARIABLE	METHOD
Serum creatinine	Alkaline picrate (kinetic Jaffe)
Serum total cholesterol	Cholesterol oxidase
HDL cholesterol	Heparin/manganese precipitations
LDL cholesterol	Friedwold formula
Serum triglyceride	Lipase/peroxidase
Plasma glucose	Glucose oxidase
Serum calcium	Cresolphthalein complexone
Serum phosphate	Molybdo phosphate (UV detection)
HbA1	Corning gel-electrophoresis
HbA1c	DCA 2000 Autoanalyser
Urine protein	Pyrogallol red
Urine urea	Urease
Urine creatinine	Jaffe (as for serum)
Urine phosphate	Molybdate (as for serum)
Urine sodium	Indirect ion selective electrode (ISE)

Table A4.1 : Methodologies for the variables measured in the study

All serum and urine samples, with the exception of glycosylated haemoglobin, were measured on a Hitachi BM Auto-analyser, using the methodologies shown.

Parameter	Correlation coefficient	Significance
Age	-0.006	NS
Duration of DM	-0.250	NS
BMI	0.144	NS
SBP	0.683	$p=0.01$
DBP	0.581	$p<0.05$
MAP	0.730	$p<0.001$
HbA_{1c}	-0.042	NS
Serum cholesterol	0.635	$p=0.01$
Serum triglyceride	0.270	NS
$CaPO_4$ product	0.300	NS
Uprotein	0.690	$p<0.05$
UPO_4	0.148	NS
UNa	-0.025	NS
UUrea	0.052	NS

Table A4.2: Univariate regression analysis. EDTA GFR is the dependent variable. The parameters are those measured throughout the study.

	cGFR vs EDTA	1/Sc vs EDTA	CrCl vs EDTA	cGFR vs CrCl
Afro-Caribbean	y = 0.98x + 10.5	y = 0.84x + 3.1	y = 0.63x + 15.2	y = 1.3x + 1.5
Asian	y = 1.04x + 1.4	y = 0.58x + 8.9	y = 1.2x - 8.7	y = 0.89x + 7.1
Caucasian	y = 0.85x + 6.9	y = 0.79x - 2.1	y = 1.26x - 8.4	y = 0.70x + 11.3
All subjects	y = 0.82x + 10.2	y = 0.78x + 1.35	y = 0.79x + 6.8	y = 0.93x + 7.6

Key : 1/Sc = reciprocal creatinine; EDTA = EDTA clearance; CrCl = creatinine clearance

Table A5.1 : Regression equations for measures of renal function in the different racial groups

	cGFR vs EDTA GFR	1/Sc vs EDTA GFR	CrCl vs EDTA GFR	cGFR vs CrCl
Gender				
Female (n = 13)	r = 0.06	r = 0.07	r = 0.14	r = 0.41
Male (n = 58)	r = 0.79	r = 0.73	r = 0.84	r = 0.78
Weight				
≤ 85 kg (n = 52)	r = 0.84	r = 0.75	r = 0.72	r = 0.83
> 85 kg (n = 19)	r = 0.49	r = 0.67	r = 0.89	r = 0.49
Serum creatinine				
≤ 300 µmol.L-1 (n = 52)	r = 0.72	r = 0.62	r = 0.80	r = 0.68
> 300 µmol.L-1 (n = 19)	r = 0.36	r = 0.69	r = 0.25	r = 0.37

Key : Sc = serum creatinine; cGFR = estimated GFR; CrCl = creatinine clearance

Table A5.2 : Correlation matrix between the different measures of renal function for gender, weight and serum creatinine

227

Table A6.1 : Materials and solutions used in type IV collagen and mixed laminin fragments assays.

TYPE IV COLLAGEN

1. Anti CL-IV antibody coated beads

Mouse anti-human CL-IV antibody coated polystyrene beads

2. Enzyme labelled anti CL-IV antibody solution

Horseradish peroxidase labelled monoclonal antibody ($0.8 \ \mu g.ml^{-1}$).

3. Colour reagent (TMBZ)

3,3',5,5' -tetramethyl benzidine ($14.72 \ mg.100ml^{-1}$.)

4. Solvent for colour reagent (DMF)

N,N-Dimethylformamide

5. Buffer for colour reagent

100 mM Acetate buffer (pH 5.5)

6. Substrate

10 mM Acetate buffer (pH 5.5) with 0.015% Hydrogen Peroxide

7. Stopping reagent

1.33 N Sulphuric acid

8. Dilution buffer for standard

10mM Phosphate buffer (pH 7) with 1% BSA and 10% horse serum

9. CL-IV standard

Human CL-IV ($2\mu g.ml^{-1}$)

10. Concentrated buffer

100mM Phosphate buffer (pH 7) containing 1M NaCl

Washing solution:

100 ml concentrated buffer was added to 900 ml of deionised water and mixed gently.

TMBZ-DMF

The tetramethyl benzadine was dissolved in 0.5 ml of N,N dimethyl formamide.

Substrate solution

0.4 ml of the TMBZ-DMF was added to 40 ml of 100 mM Acetate buffer (pH 5.5) and mixed gently.

MIXED LAMININ FRAGMENTS

1. Blocking agent   0.5% casein in PBS/0.04% Tween 20

2. Coating buffer   400 ng.ml^{-1} of laminin fragment N3 in 0.05 M carbonate buffer (pH 9.6). Carbonate buffer - 0.015 M anhydrous sodium carbonate/0.035 M anhydrous sodium hydrogen carbonate.

3. PBS/0.04% Tween 20   0.04% Tween in 0.15 M phosphate buffered saline(PBS) pH 7.3.

4. Primary antibody   Rabbit IgG to purified laminin N3 fragments

5. Second antibody   Alkaline conjugated goat anti-rabbit IgG

6. Stopping buffer   0.2 M Dipotassium hydrogen phosphate containing 100 mM EDTA {pH 9.6}

7. Substrate   0.3 mM SLPr-phosphate in diethanolamine buffer containing 0.05 mM MgCl$_2$

8. Washing buffer   PBS/0.04% Tween 20
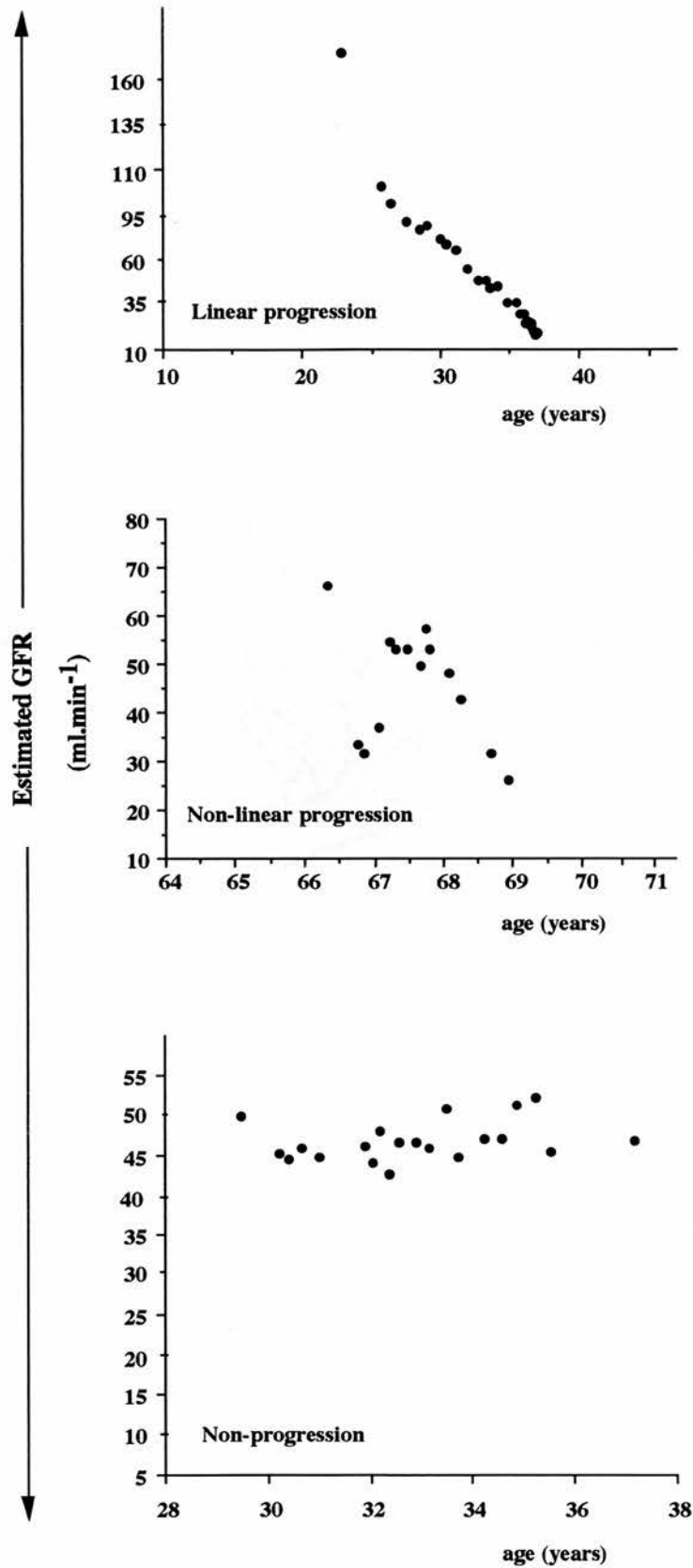
Figure A2.1 : Examples of linear progression, non-linear progression and non-progression of renal function as determined by estimated GFR
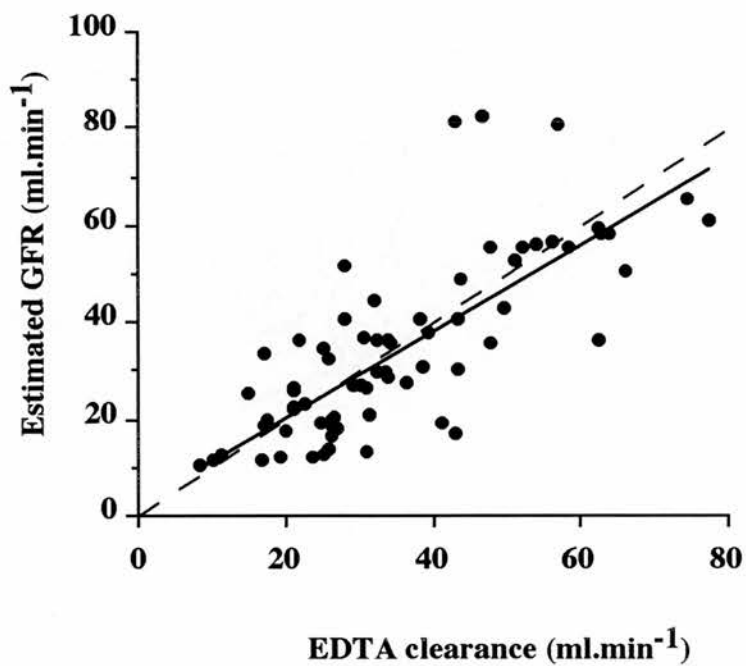
Figure A5.1 : Relationship between the simultaneously measured plasma clearance of ^{51}Cr EDTA and glomerular filtration rate predicted from Cockcroft-Gault and corrected for surface area for all subjects (n = 71 observations; r=0.76; p<0.0001; y = 0.86x + 8.4). The line of identity (dashed) is shown.

Figure A5.2 : Difference between EDTA GFR and calculated GFR vs mean of the two methods at baseline in the three racial groups. Mean difference shown as horizontal bar. Regression lines shown for Afro-Caribbean and Asian groups.
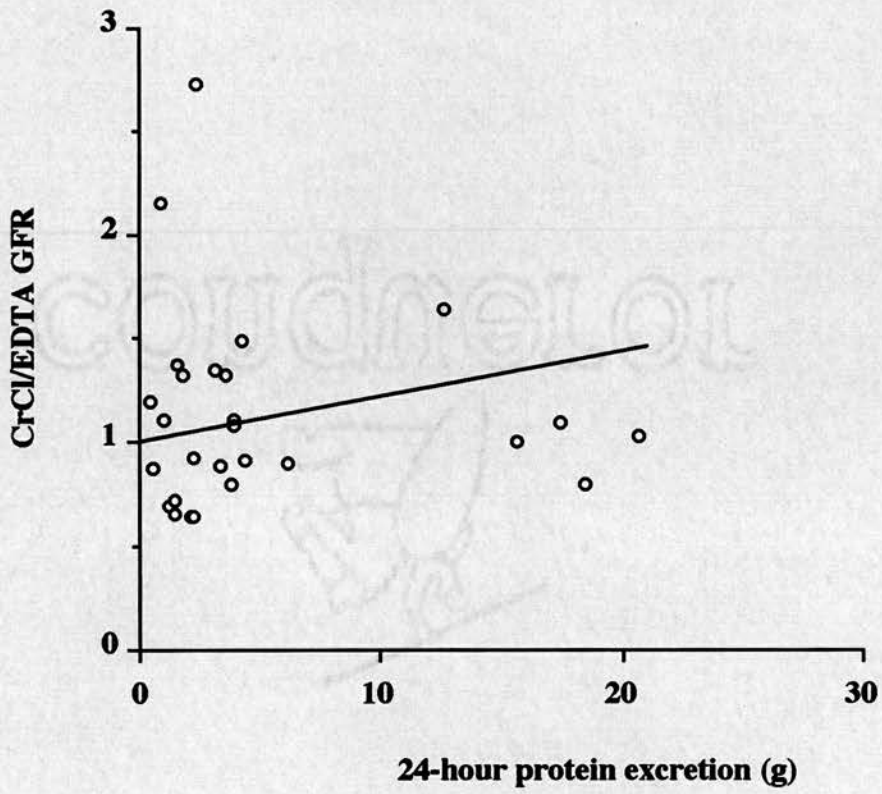
Figure A5.3 : Relationship between the ratio of endogenous creatinine clearance to EDTA GFR and 24 hour protein excretion. All subjects visit 1 (n=26; r=0.2, NS).
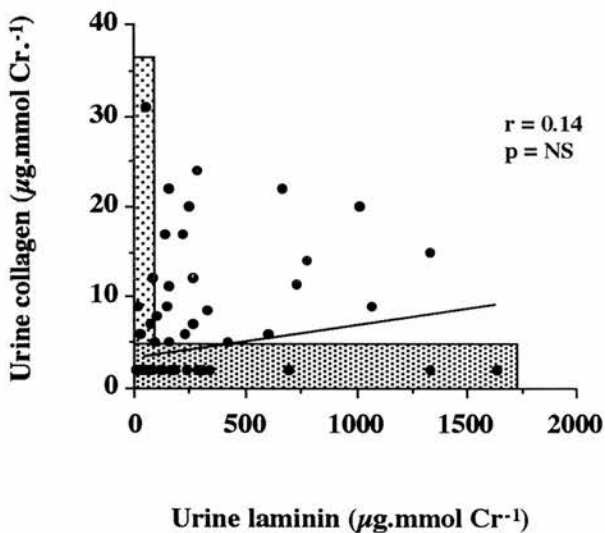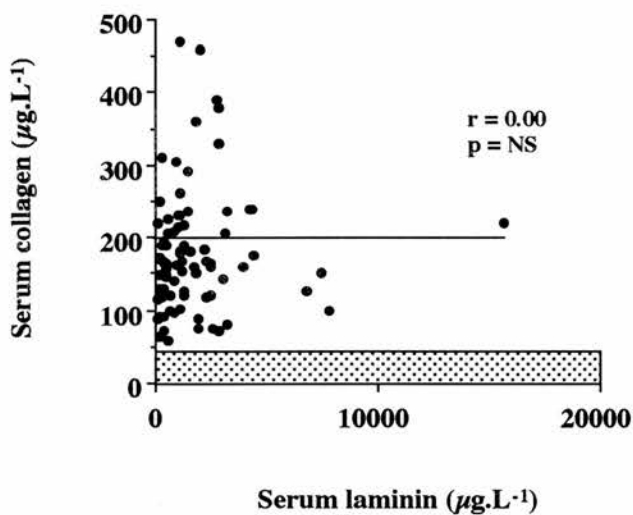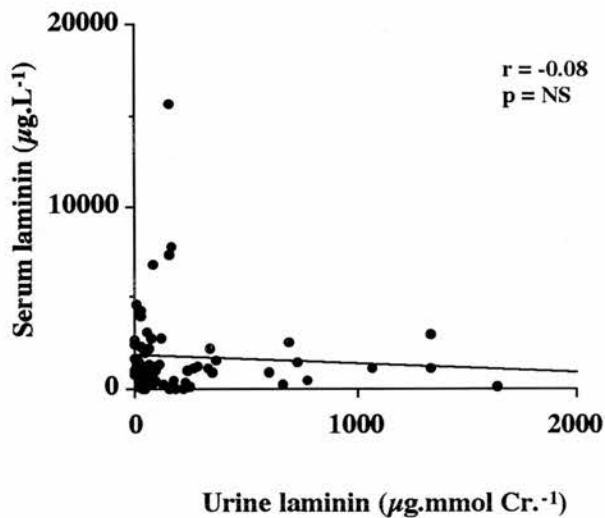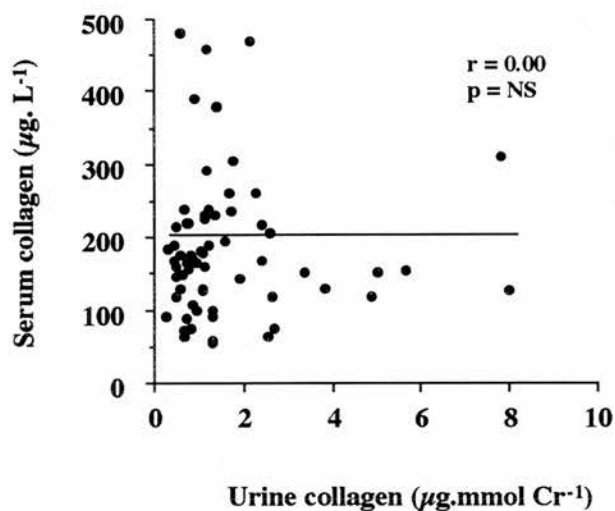
Figure A6.1 : Interrelationships of serum and urinary levels of Type IV collagen and mixed laminin fragments. The shaded areas are below the limit of detection of the assay.