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**Modification of
cardiovascular and renal risk
factors using antagonists of the
endothelin system**

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Declaration

I declare that all the work presented in this thesis is my own except where stated below, and it has been entirely composed by myself.

1. Studies

Chapter 3: I carried out study 1 by myself.

Chapter 4: I carried out study 2 by myself.

Chapter 5: I carried out this study with the help of Mrs V Melville.

Chapter 6: Dr N Dhaun and I carried out this study with the help of Mrs V Melville and Miss D Kerr.

Chapter 7: Dr N Dhaun and I carried out this study with the help of Mrs V Melville and Miss D Kerr.

2. Assays

Renal clearance studies and ET-1 concentrations: As these studies produce a very large number of samples that require analysis, these were performed by the laboratory staff of the Clinical Pharmacology Unit (Mr NR Johnston, Miss E Cole, Miss L Bruce). I and others, as outlined above, undertook all immediate processing of samples.

24 hour urinary protein and creatinine (Chapter 6): These were processed in the main hospital laboratory.

3. Data analysis

Chapter 3: I analysed the data for study 1 with the help of Dr J Goddard

Chapter 4: I analysed the data for study 2 with the help of Dr J Goddard

Chapter 5: I analysed the data for study 3 with the help of Dr J Goddard

Chapter 6: Dr N Dhaun, Dr J Goddard and I analysed the data for study 4

Chapter 7: Dr N Dhaun, Dr J Goddard and I analysed the data for study 5

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Abbreviations

ACE	Angiotensin Converting Enzyme
ACE-I	Angiotensin Converting Enzyme Inhibitor
ACR	Albumin:Creatinine Ratio
ADMA	Asymmetric Dimethylarginine
ANOVA	Analysis of Variance
ARB	Angiotensin Receptor Blocker
AS	Arterial Stiffness
BP	Blood Pressure
C&G	Cockcroft & Gault
cAIx	Central Augmentation Index
CI	Cardiac Index
CKD	Chronic Kidney Disease
CO	Cardiac Output
CRP	C-Reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
ECG	Electrocardiogram
ED	Endothelial Dysfunction
EFF	Effective Filtration Fraction
eGFR	Estimated Glomerular Filtration Rate
ERBF	Effective Renal Blood Flow
ERPF	Effective Renal Plasma Flow
ERVR	Effective Renal Vascular Resistance
ESRD	End-Stage Renal Disease
ET	Endothelin
ET _A	Endothelin-A
ET _B	Endothelin-B
ETRA	Endothelin Receptor Antagonist

FBF	Forearm Blood Flow
GFR	Glomerular Filtration Rate
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
hsCRP	High Sensitivity C-Reactive Protein
IL-6	Interleukin-6
In	Inutest
MAP	Mean Arterial Pressure
MDRD	Modification of Diet in Renal Disease
NO	Nitric Oxide
PAH	Pulmonary Artery Hypertension
PCR	Protein:Creatinine Ratio
PP	Pulse Pressure
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
RA	Rheumatoid Arthritis
RAAS	Renin Angiotensin Aldosterone System
RBF	Renal Blood Flow
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SVRI	Systemic Vascular Resistance Index
SVV	Small Vessel Vasculitis
SWG	Standard Wire Gauge
VSMC	Vascular Smooth Muscle Cells

Abstract

Chronic kidney disease (CKD) is an important independent risk factor in the development of cardiovascular disease (CVD). Indeed, patients with CKD are far more likely to die from CVD than reach end stage renal disease. Conventional cardiovascular risk factors and co-morbidity contribute to this increased risk of CVD. However, emerging evidence suggests other novel factors including inflammation, oxidative stress, and a shift in the balance of the vasodilator nitric oxide and vasoconstrictor endothelin system, are also important contributors. Despite increasing evidence that the endothelin system plays an important role in the development of CKD and CVD, there has been little research examining possible therapeutic benefits of its modification in patients with CKD. The overall aims of the work presented within this thesis were to examine CVD risk in patients with renal impairment and then to see what impact chronic inhibition of the endothelin system would have on risk factors for CVD and CKD progression.

In the first two studies I examined markers of arterial stiffness (AS) and endothelial function in a cohort of patients with immune-mediated renal disease. I was able to show in the acute setting that improvement in renal function following treatment for these conditions leads to significant improvements in AS. Interestingly, in patients who were in remission from their renal disease, only classical cardiovascular risk factors appear to be linked to AS. In the next study I was able to prove that sitaxsentan, a selective oral ETA antagonist, did not cause functional blockade of the ETB receptor in man. This was the first study of its kind to confirm that a “selective” endothelin antagonist truly is selective in vivo: a finding that will allow more accurate mechanistic investigation of the ET system. In the final studies, I showed that in subjects with stable non-diabetic proteinuric CKD, chronic selective ETA receptor antagonism reduces blood pressure and AS, and that these systemic benefits are associated with an increase in renal blood flow and reduction in proteinuria. The reduction in proteinuria is most likely haemodynamic and linked to a fall in GFR and filtration fraction, similar to what is seen with ACE inhibitors. Importantly, these benefits were seen in patients already taking maximally tolerated renin-angiotensin-

aldosterone system blockade, suggesting that chronic endothelin antagonism could be an important future therapy in the management of CKD.

In summary, I have shown that renal impairment can directly affect markers of arterial function and by inference increase the risk of CVD. Chronic antagonism of the endothelin system with ETA receptor blockers would appear to improve many of these biomarkers, including reductions in BP, AS and proteinuria. There were no adverse effects reported in these studies, suggesting that selective ETA antagonism may be safe enough for clinical development in CKD patients. Further larger clinical trials are warranted.

Chapter 1: Introduction

1.1 Chronic Kidney Disease

Chronic kidney disease (CKD) is a long-term condition caused by damage to the kidneys and is increasingly being recognised as a major public health problem. It is categorised, according to levels of renal function, by internationally accepted definitions requiring structural or functional abnormalities of the kidneys which persist for greater than 3 months (Table 1.1)^{1, 2}. It is common, with an average prevalence of approximately 11% within the populations of the United States of America (USA) and Western Europe³. Within the UK, prevalence of stage 3-5 CKD is 6%, with this rising to 13-14% when including stages 1 & 2⁴. Furthermore, as CKD is seen more frequently in the elderly it is likely that its prevalence will continue to increase as the population ages⁵.

Table 1.1 Stages of chronic kidney disease²

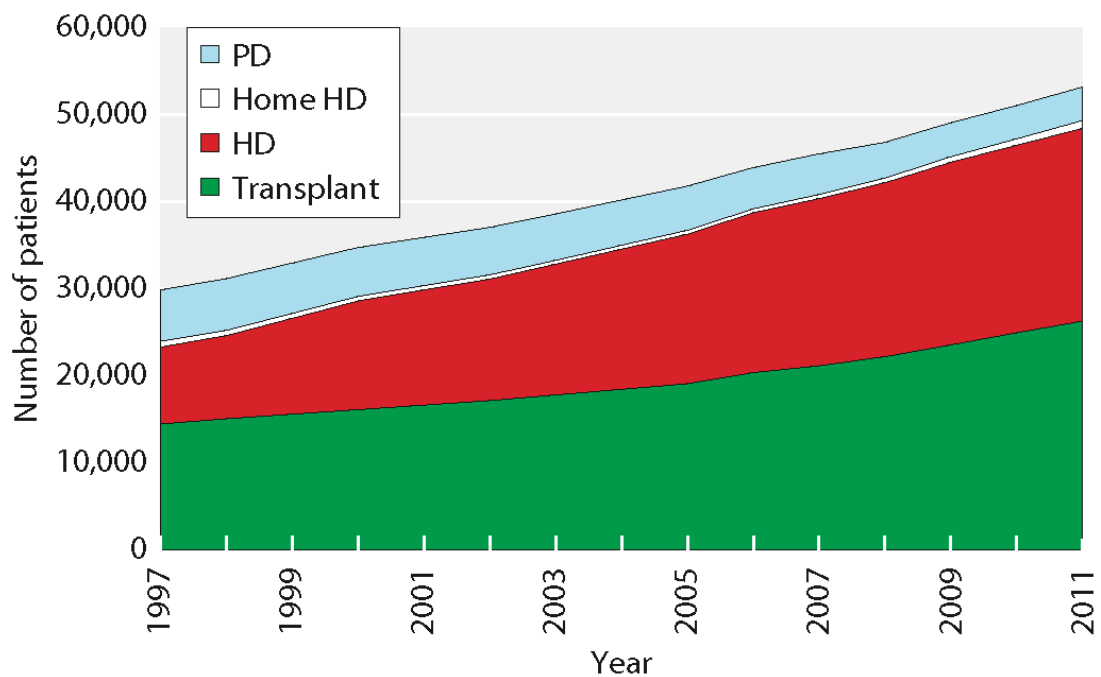
Stage	eGFR (ml/min/1.73 m ²)	Description
1	90 or more	Normal or increased eGFR, with other evidence of kidney damage
2	60 – 89	Slight decrease in eGFR, with other evidence of kidney damage
3A	45 – 59	Moderate decrease in eGFR, with or without other evidence of kidney damage
3B	30 – 44	Moderate decrease in eGFR, with or without evidence of kidney damage
4	15 – 29	Severe decrease in eGFR, with or without evidence of kidney damage
5	< 15	Established renal failure

CKD represents a major clinical and financial burden for two main reasons. Firstly, despite current best treatments, many patients with CKD continue to have declining function, with a small but significant number of patients progressing to end-stage renal disease (ESRD) requiring dialysis or transplantation. Estimates suggest the current world dialysis population is greater than 2 million patients with a treatment

cost of over \$100 billion per annum⁶. Within NHS England, annual spending on CKD was estimated at £445 million in 2002. However, by 2010 this had more than trebled to £1.64 billion⁷. Moreover, using data from the Renal Registry, the number of patients receiving renal replacement therapy in the UK has increased steadily over the last 15 years (~4% a year) and currently stands at 53,207 adult patients (Figure 1.1)⁸. Secondly it is now well recognised that CKD is strongly associated with cardiovascular disease⁹. Indeed, dialysis patients have mortality rates 10-100 times greater in than the general population^{10, 11}. Patients with less severe kidney dysfunction (chronic CKD stages 3-5) are also at increased risk of cardiovascular death^{12, 13}. Indeed those with stages 4-5 CKD are up to 10 times more likely to die from CVD than to reach ESRD¹⁴. While many patients with CKD have traditional risk factors for CVD such as diabetes mellitus, smoking and hypertension, part of the increased risk is attributable to CKD itself or more novel markers associated with CKD^{15, 16}. Such findings have led the US National Kidney Foundation Task Force on CVD in Chronic Renal Disease to recognise that patients with CKD should be considered in the highest risk group for subsequent cardiovascular events¹⁷.

Figure 1.1 Growth in end-stage CKD 1997-2011

(UK Renal Registry 15th Annual Report⁸)



It is clear from these figures that patients with CKD represent a large group within the population who suffer high morbidity and mortality. There is, therefore, a need to further investigate the cardiovascular burden associated with CKD and study possible treatment strategies that will not only slow renal decline, but also reduce cardiovascular risk in these patients.

1.2 The link between CKD and CVD

Evidence for the relationship between CKD and CVD was first recognised in the 1970s within the dialysis population¹⁸. In the UK approximately 20% of patients with ESRD will die from a cardiovascular (CV) cause¹⁹. Studies within the general population have shown that the relationship between CKD and CVD also extends to those with only mild to moderate renal impairment. The Cardiovascular Health Study was a prospective population-based study of 5,808 subjects aged ≥ 65 yr, with an average follow-up of 7.3 yr. Renal insufficiency, defined as a serum creatinine value $>130\mu\text{mol/L}$ in men and $115\mu\text{mol/L}$ in women, was present in 11.2% of participants²⁰. Subjects with renal insufficiency were more likely to develop cardiovascular disease, congestive heart failure, and symptomatic peripheral vascular disease, as well as to die: these associations were not eliminated by adjusting for traditional cardiovascular risk factors. More recently Go *et al.*¹² analysed the database of a large healthcare provider in Northern California, stratifying over a million subjects according to estimated glomerular filtration (eGFR) as calculated by the Modification of Diet in Renal Disease (MDRD) formula²¹. Mean follow up was 2.8 years. After adjustment for age, sex, race, coexisting illness and socioeconomic status, a stepwise increase in death, CV events and hospitalisation was seen with decreasing eGFR. This large study, along with others, clearly demonstrates an independent and inverse graded relationship between glomerular filtration and CVD.

1.2.1 Proteinuria

CKD may not only be identified by impaired glomerular filtration but also by the presence of proteinuria²². Indeed proteinuria often precedes reductions in eGFR and is a powerful predictor of renal disease progression²³. It is likely that proteinuria also has a direct deleterious effect on renal tubular cells, causing tubulointerstitial

inflammation with subsequent fibrosis and thereby contributing to progressive renal failure²⁴⁻²⁶. Furthermore, just like reductions in eGFR, proteinuria has been shown to be a strong independent determinant of CVD in both the diabetic and non-diabetic population. The association between proteinuria and CVD was first noted in those patients with overt macroalbuminuria (albumin:creatinine ratio [ACR] >30mg/mmol) and was found to be independent of traditional CV risk factors^{27, 28}. More recently it has become increasingly recognised that the risk of CVD is increased with microalbuminuria and even within the currently defined normal levels of albuminuria (ACR <3mg/mmol)²⁹⁻³¹. For example, Hillege *et al.* followed 40,000 members of the general public from the city of Groningen²⁹. Subjects were asked to fill in a medical questionnaire and provide an early morning urine specimen and were then followed up for an average of almost 3 years. After adjusting for known CVD risk factors, the investigators found that a doubling of the urinary albumin excretion rate, even when still within the 'normal' range was associated with a relative risk of 1.29 (95% confidence interval 1.18 to 1.40) for CV mortality. Furthermore, microalbuminuria outranked the predictive power of other classic CV risk factors.

Analysis of the urinary data from the Heart Outcomes Prevention Evaluation (HOPE) study also appears to support the view that microalbuminuria is an important independent risk factor for CVD. In short, the HOPE study recruited 9297 high-risk patients aged ≥ 55 years of age who had evidence of vascular disease or diabetes plus one other cardiovascular risk factor (hypertension, elevated total cholesterol levels, low high-density lipoprotein cholesterol levels, cigarette smoking, or documented microalbuminuria), and randomly assigned them to receive ramipril (10 mg once per day) or matching placebo for a mean of five years³². *Post hoc* analysis of the urinary data revealed that in the overall study population, a baseline ACR of >2.0 mg/mmol increased the adjusted relative risk of CV events to 1.83³³. Further analysis of the HOPE data suggests that albuminuria is a continuous risk factor for CV events with the adjusted hazard of major CV events increasing by 5.9% for every 0.4 mg/mmol increase in ACR³⁰.

It is well established that proteinuria is associated with renal disease progression^{34, 35} and reduction of proteinuria appears to reduce the rate of GFR decline^{23, 36-39}. Importantly, there is also evidence that proteinuria is a modifiable risk factor for CVD. The Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study involved around 8200 subjects with hypertension who were randomised to receive losartan or atenolol⁴⁰. Baseline albuminuria was measured in all patients and as expected was found to be a powerful predictor of CVD mortality. Despite losartan and atenolol achieving similar blood pressure reductions (30.2/16.6 mmHg vs. 29.1/16.8 mmHg), patients in the losartan group had significantly greater reductions in albuminuria and CVD outcomes suggesting that reductions in urine ACR over time translate into reduced cardiovascular risk. Similarly, in the Reduction in Endpoints in Non-insulin dependent diabetes mellitus with the Angiotensin II Antagonist Losartan (RENAAL) study there was an 18% reduction in cardiovascular risk for every 50% reduction in albuminuria⁴¹. All of this suggests that as well as being a marker of renal damage, proteinuria is a direct target for the treatment of CVD in patients with CKD.

1.2.2 Hypertension

Hypertension, defined as a systolic BP (SBP) of ≥ 140 mmHg or a diastolic BP (DBP) ≥ 90 mmHg, is accepted as one of the strongest prognostic markers for the development of cardiovascular disease and death^{42, 43} with BP values bearing a continuous linear relation with the incidence of cardiac events. Importantly, a 5 mmHg reduction in a given SBP results in a 22% reduction in cardiovascular events⁴⁴.

Hypertension can be either a consequence or cause of CKD and is associated with adverse outcomes including worsening renal function and CVD. Its prevalence increases as GFR falls, hypertension being found in around 20% of patients with stage 1 CKD compared to more than 80% of patients with stage 4 CKD⁴⁵. Moreover, treatment resistant hypertension, defined as a BP above target despite adherence to at least 3 different antihypertensive agents, including a diuretic, is 2.5-3 times more common in patients with CKD compared to those without⁴⁶.

Progression of renal damage secondary to hypertension can be split into 3 categories: the systemic BP load, the degree to which such a load is transmitted to the renal vascular bed and the local tissue susceptibility to any given degree of barotrauma⁴⁷. Under normal conditions, renal blood flow (RBF) varies little within a broad range of BPs. As BP increases vasoconstriction of the afferent glomerular arteriole occurs maintaining RBF and glomerular pressures. However, this autoregulatory process can be blunted as a result of renal disease or diabetes, leading to increased pressure load to the kidney, resulting in barotrauma to the glomerulus and, over time, glomerulosclerosis results. Furthermore, damage to the glomerular capillaries and mesangium also promotes increased proteinuria, which in turn promotes further glomerular and tubulointerstitial injury. Importantly, renal impairment can also drive systemic hypertension through numerous mechanisms including the expansion of the extracellular volume via sodium retention, increased sympathetic activity, an inappropriately increased activation of the renin-angiotensin-aldosterone system (RAAS), impaired endothelial function and high parathyroid hormone levels⁴⁸. As a consequence a vicious cycle of hypertension and renal damage can result.

Numerous studies have confirmed that BP is an important independent risk factor for ESRD. In the Kaiser Permanente cohort, a graded association between BP and risk of ESRD among subjects, without clinical evidence of CKD at baseline, was shown over a 20-year period. This held true even at BPs that would be described as normal (systolic of 120-129 mmHg) and suggests that non-malignant hypertension is an independent risk factor for CKD progression⁴⁹. Furthermore, the RENAAL study, which followed patients with diabetic nephropathy for an average of 3.4 years, found that for every 10 mmHg rise in baseline SBP there was a 7% increase the risk of death or ESRD⁵⁰. Importantly, reductions in BP have also been shown to reduce the rate of renal functional decline. In one meta-analysis of 11 studies involving 1860 patients with CKD, results showed that the lowest risk for kidney disease progression seemed to be at a SBP of 110 to 129 mmHg and urine protein excretion less than 2.0 g/d. Interestingly, at levels of urine protein excretion less than 1.0 g/d, there was little relationship between risk for kidney disease progression and current SBP from 110

to 159 mmHg²³. Based on these results and those of other studies, current guidelines suggest aggressive management of BP in patients with proteinuric CKD, aiming for ≤ 130 mmHg systolic and ≤ 80 mmHg diastolic⁵¹.

1.2.3 Endothelial dysfunction

The endothelium is a single layer of cells that line the entirety of the vascular system. It plays a pivotal role in a number of vascular functions including vascular tone, thrombosis, inflammation and permeability⁵². Endothelial dysfunction (ED), characterised by impaired endothelium-dependent vasodilatation, enhanced endothelium-dependent vasoconstriction and a proinflammatory and prothrombotic state⁵², is recognised as one of the initial mechanisms in the development of atherosclerosis and is associated with increased risk of subsequent CV events⁵³. Its development is linked to hypertension, reduced nitric oxide (NO) generation, oxidative stress and inflammation⁵².

ED has been demonstrated in patients with CKD when compared to controls, and undoubtedly plays an important role in the development of CVD in this group^{52, 54, 55}. Interestingly, there appears to be an association between ED and microalbuminuria that may explain, at least in part, why microalbuminuria strongly predicts CV events⁵⁶. Furthermore, animal models of CKD suggest that ED promotes further reductions in renal function and promotes proteinuria^{57, 58}. This in turn, may further exacerbate ED and promote further atherogenesis, in effect creating a vicious cycle. The Hoorn Study has shown that even mild impairment of renal impairment is independently associated with ED and this in turn with associated with increased cardiovascular mortality⁵⁵. In more severe renal disease reduced bioavailability of NO appears to be an important factor^{59, 60}, in large part due to increased oxidative stress and high concentrations of plasma asymmetric dimethylarginine (ADMA)^{59, 61, 62}.

ADMA, an endogenous competitive inhibitor of NO synthase, is formed during the catabolism of proteins containing methylated arginine residues⁶². Within the cardiovascular system it is synthesised within the heart, endothelium and smooth

muscle cells. Exogenous ADMA inhibits NO generation in vitro and in healthy humans reduces forearm blood flow, cardiac output and renal blood flow, as well as, increasing BP, systemic vascular resistance and sodium retention^{62, 63}. Plasma concentrations of ADMA are increased in association with ED, particularly in patients with renal disease^{62, 64}. The increased concentrations of ADMA in renal impairment may result from both increased activity of protein arginine methyltransferase and reduced metabolism of ADMA via dimethylarginine dimethylaminohydrolase (DDHA)⁶⁵. As well as being a marker for ED, ADMA levels strongly correlate to atherosclerosis and cardiovascular mortality⁶⁶⁻⁶⁸. With regard to renal disease, plasma ADMA is inversely related to GFR⁶⁹ and is an independent risk factor for progression to ESRD and mortality^{65, 70}.

1.2.4 Arterial Stiffness

One of the major functions of the large elastic arteries of the body (most notably the aorta) is to convert intermittent pulsatile blood flow to a more steady flow. This is achieved by the artery expanding during systole and then recoiling during diastole, promoting forward flow. AS is the reduced capability of an artery to expand and contract in response to pressure changes and leads to increasing systolic and lower diastolic pressures (See Figure 1.2)⁷¹. Increasing AS is a consequence of arterial damage and develops from a complex interaction between stable and dynamic changes involving structural and function of the vessel wall (Fig 1.3)⁷².

AS leads to increased SBP, which in turn, leads to increased left ventricular workload and subsequent gradual development of left ventricular hypertrophy (which is associated with a 2-5 fold increase in cardiovascular events⁷³). There is also an associated fall in diastolic pressures, potentially impairing coronary blood flow. Pulse pressure is increased and may have direct effects on end organs including on the kidney where it causes afferent arteriolar constriction and subsequent reduction in GFR⁷⁴. AS is also linked to ED⁷⁵. Evidence from animal^{76, 77} and human^{78, 79} studies has shown that the endothelium, via NO, is an important regulator of AS.

Figure 1.2 Pulse pressure - compliant vs. noncompliant aorta

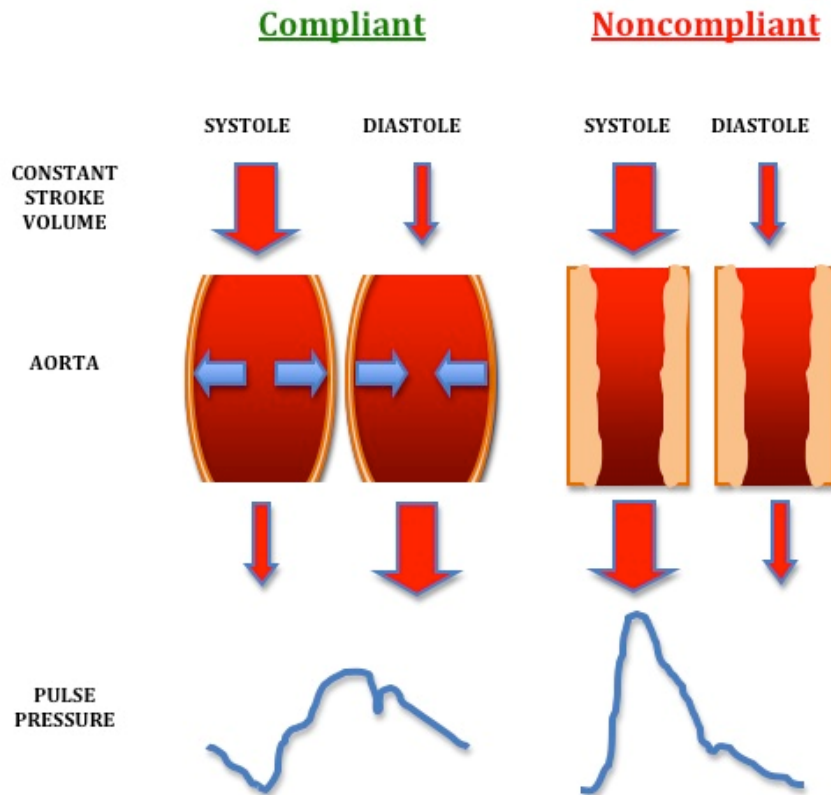
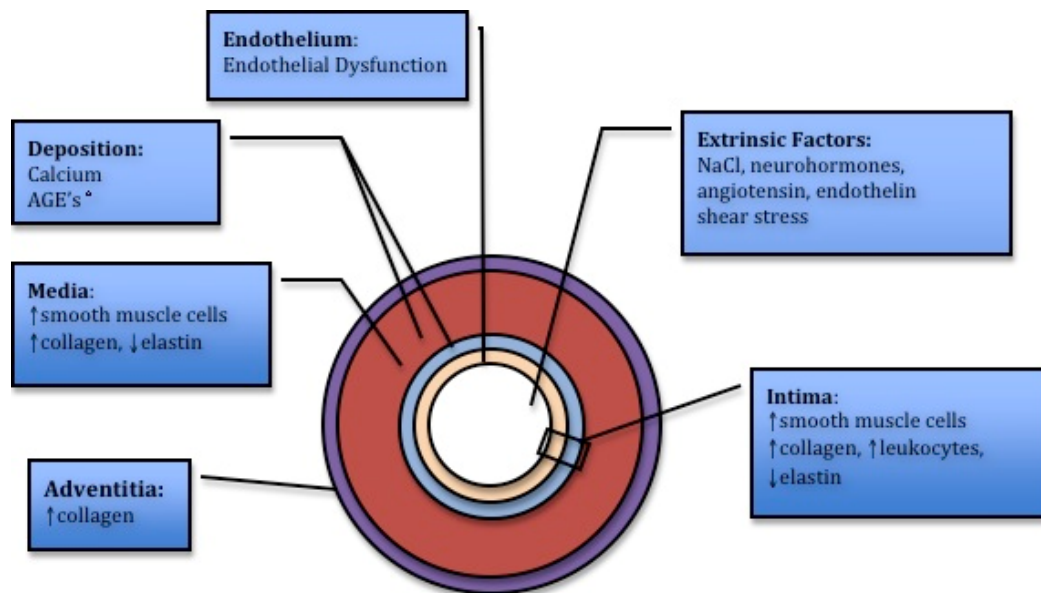


Figure 1.3 Factors involved in arterial stiffness.

Modified from Zieman *et al.*⁷²



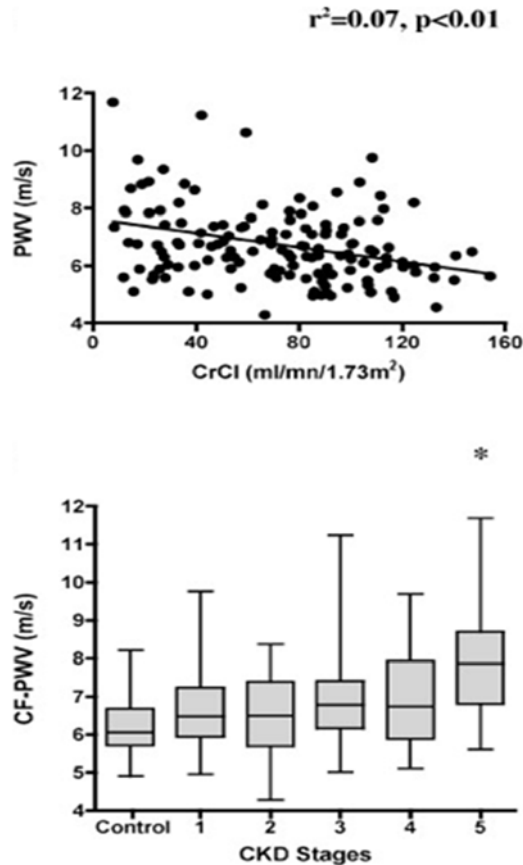
* AGE - advanced glycation end products

Aortic stiffness has predictive value for all-cause and cardiovascular mortality, as well as total cardiovascular events. A 2010 meta-analysis of 17 longitudinal studies that evaluated aortic pulse wave velocity (PWV; a marker of aortic stiffness) found that an increase in aortic PWV by 1 m/s corresponded to an age-, sex-, and risk factor-adjusted risk increase of 14%, 15%, and 15% in total CV events, CV mortality, and all-cause mortality, respectively, implying that AS is a strong predictor of future CV events and all-cause mortality⁸⁰. The additional value of AS above and beyond traditional cardiovascular risk factors has been quantified by 3 separate studies⁸¹⁻⁸³. Boutouyrie *et al.* assessed the predictive value of AS on coronary heart disease in 1045 patients with essential hypertension and without known clinical cardiovascular disease. In univariate analysis, the relative risk of follow-up coronary event or any cardiovascular event increased with increasing level of PWV; for 1 SD (3.5 m/s) relative risks were 1.42 (1.10 to 1.82; $P < 0.01$) and 1.41 (1.17 to 1.70; $P < 0.001$), respectively. With multivariate analysis, PWV remained significantly associated with the occurrence of coronary event after adjustment for Framingham scoring⁸¹. This improved ability of aortic stiffness to predict CV mortality was confirmed by Mattace-Raso *et al.*⁸² in a study of 2835 subjects participating in the third examination phase of the Rotterdam Study and by Sehestedt *et al.* in middle-aged subjects from a general population⁸³. These studies suggest that aortic stiffness in many ways could be considered as a surrogate end-point for CV events⁸⁴.

Studies have demonstrated that patients with renal dysfunction have stiffer arteries than healthy subjects and this stiffness increases with declining GFR⁸⁵⁻⁸⁸. Wang *et al.*⁸⁵ demonstrated a step wise increase in PWV with increasing CKD stage. Further work in a group of 113 patients with CKD but no history of cardiovascular disease or diabetes confirmed a step-wise increase in AS as renal function declines⁸⁸ (Fig 1.4). This suggests that arterial damage occurs long before ESRD is reached and would be in keeping with data showing that patients with non-dialysis-requiring CKD are more likely to die from CVD than develop ESRD¹⁴.

Figure 1.4 Aortic stiffness vs. GFR.

Scatter plots show a significant negative correlation between carotid–femoral pulse wave velocity (CF-PWV) and eGFR. Box plots show CF-PWV by CKD stage including a control group. *Stage 5 vs. control, $p < 0.01$. Figure taken with permission from Lilitkarntakul *et al.* 2011⁸⁸



AS has been shown to be an independent predictor of all-cause and CVD mortality in patients with ESRD^{89, 90}. It has also been shown by Guerin *et al.*⁹¹ that reduction in CVD mortality is only achieved when BP reduction and control is associated with a reduction in AS in ESRD patients. Those patients with adequate BP control but high PWV do not see as great a reduction in mortality. It would therefore appear that AS as well as being a marker of CVD is also a valuable target for early treatment.

1.2.5 Inflammation and oxidative stress

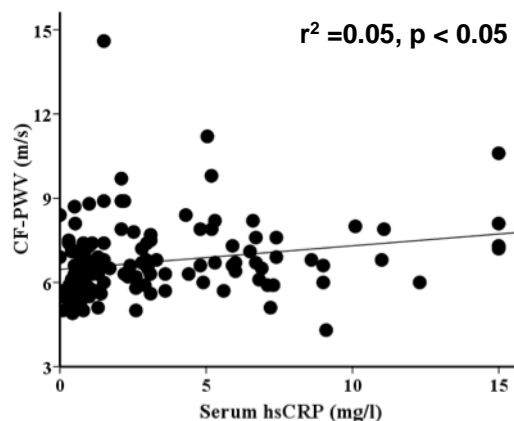
Chronic inflammation contributes to the development of atherosclerosis⁹². As CVD burden increases, established markers of inflammation such as high sensitivity C-reactive protein (hsCRP), the cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF α), and soluble intercellular adhesion molecule (sICAM-1) increase. This relationship holds true even after adjustment for traditional CVD risk factors⁹³.

Systemic low-grade inflammation has been linked to impaired endothelial function⁹⁴,⁹⁵. C-reactive protein (CRP) is an acute-phase protein, the main physiological role of which is to activate the complement system via the C1Q complex and clinically is widely used as a marker of inflammation. Fichtlscherer *et al.*⁹⁴ demonstrated an inverse correlation between CRP and forearm blood flow responses to acetylcholine in males with documented CVD. Further study has shown that CRP is an independent determinant of endothelium-dependent vascular function, even in apparently healthy subjects, and that there is a relationship between low-grade chronic inflammation and basal endothelial NO synthesis⁹⁵. CRP has also been shown to correlate to AS as measured by PWV⁹⁶. Interestingly, a consistently elevated CRP was found to have a far greater correlation with AS than many traditional CVD risk factors in a general population of middle-aged men⁹⁶. Even acute low-level inflammation in otherwise healthy individuals causes a rise in PWV. Vlachopoulos *et al.*⁹⁷ showed that vaccination (*Salmonella typhi*) produced a significant increase in PWV (at 8 hours by 0.43 m/s). There were associated significant increases in inflammatory markers (hsCRP and hsIL-6).

Irrespective of the cause of renal disease there is firm evidence that a chronic pro-inflammatory state exists in patients with CKD⁹⁸. While associated disease such as hypertension, diabetes and atherosclerosis may contribute, it is also likely that hypoalbuminaemia/malnutrition⁹⁹, dyslipidaemia¹⁰⁰, advanced glycation end products¹⁰¹, oxidative stress¹⁰² and upregulation of certain hormonal systems such as the RAAS¹⁰³ and the endothelin system¹⁰⁴ all play a role in causing inflammation in CKD. Unpublished data from our department examining traditional and emerging

cardiovascular risk factors in patients with CKD has also confirmed that hsCRP is significantly, but weakly, associated with PWV (Fig 1.5).

Figure 1.5 Relationships between CF-PWV and hsCRP



Oxidative stress is closely related to inflammation and is well documented in CKD. It appears to be present even in mild CKD and increases as glomerular filtration rate falls¹⁰⁵. It is characterised by an imbalance between free radical exposure and antioxidant defence and is thought to play an important role in CVD development. With regard to the endothelium, increased production of oxidants inactivates NO, which in turn impairs endothelial function¹⁰².

1.3 The endothelin system

One major area of interest is that of the endothelin (ET) system, discovered in 1988. ET has been widely implicated in the development of both renal and cardiovascular disease and may well be an important marker of disease activity as well as a possible site for disease modification through its inhibition¹⁰⁶. First described by Yanagisawa over two decades ago, the ET system comprises a family of 21-amino acid peptides with powerful vasoconstrictor and pressor properties¹⁰⁷. Additionally, the predominant vascular isoform, ET-1, has pro-inflammatory, proliferative, pro-fibrotic and hypertrophic effects¹⁰⁴. Within the kidney, ET-1 has a role in salt and water homeostasis¹⁰⁴. The ET system has been implicated in the pathogenesis of a number of diseases including hypertension, atherosclerosis, congestive heart failure, CKD, connective tissue disease and some forms of cancer. Thus the potentially wide

clinical potential of ET blockade has led to intense research in this field, with a number of ET receptor antagonists (ETRAs) either approved for clinical use or under development.

1.3.1 ET-1 synthesis and secretion

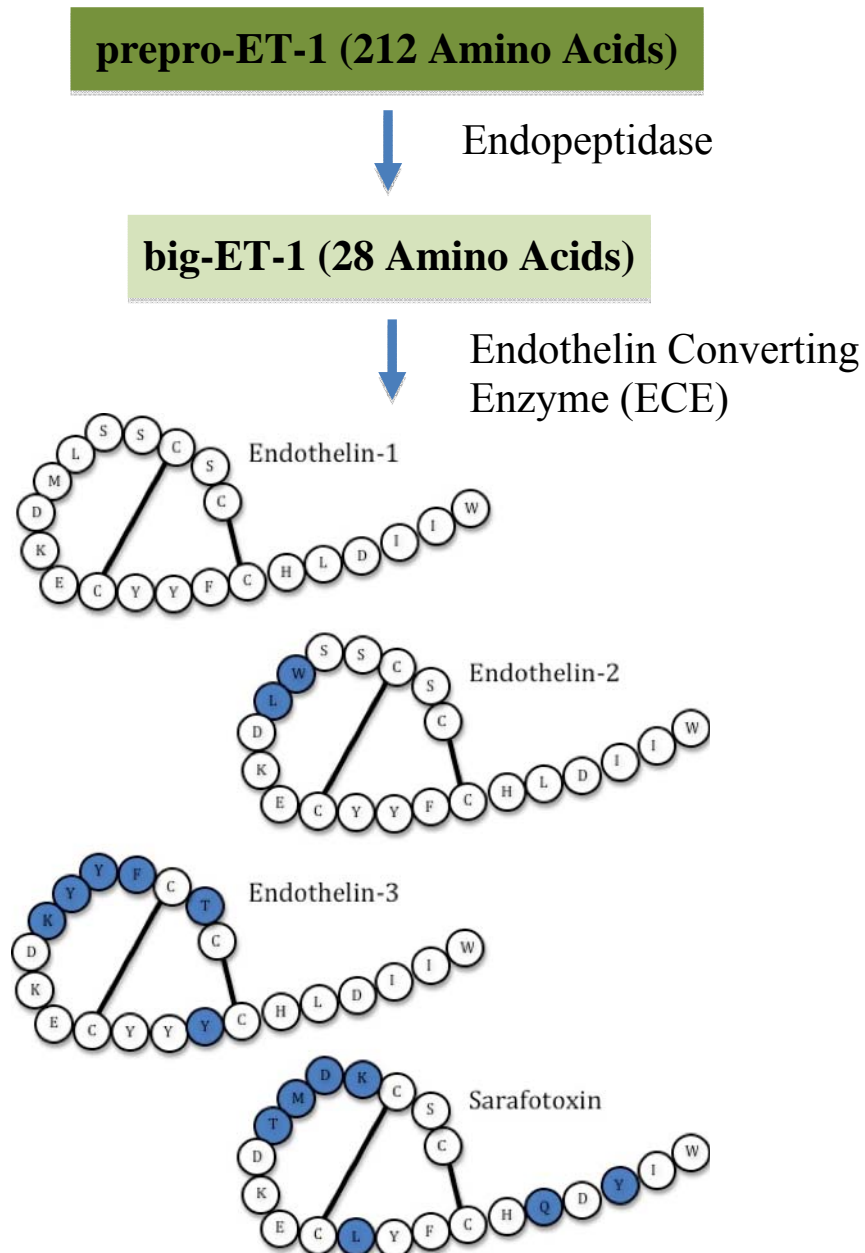
Three human isoforms of ET have been isolated: ET-1, ET-2 and ET-3. These are encoded by distinct genes located, in man, on chromosomes 6, 1 and 20 respectively¹⁰⁸. In the case of ET-1, the dominant vascular isoform, the gene product is the 212-amino acid prepro-ET-1. This is cleaved intracellularly by a furin-like endopeptidase, to yield an inactive 38-amino acid precursor, big-ET-1¹⁰⁸, which is then further cleaved by an endothelin-converting enzyme to generate the biologically active mature ET-1 peptide¹⁰⁹ (Figure 1.6). Within the vasculature, the endothelial cell is the major site of ET-1 production though other cells are also capable of its production in health and disease, including vascular smooth muscle cells (VSMC), macrophages and fibroblasts¹¹⁰. Within the kidney, ET-1 is produced in relatively high amounts particularly in the inner medulla, which has been found to have the highest concentration of ET-1 of any body tissue¹¹¹. It is synthesised by all glomerular cell types and by the tubules¹¹².

ET-1 synthesis, which is the key regulatory step in ET-1 generation, is modulated by a number of stimuli¹¹³. Synthesis is enhanced in response to low shear stress, turbulent blood flow, hypoxia, acidosis, cytokines, angiotensin II, adrenaline, insulin, cortisol and low-density lipoprotein. In contrast, high shear stress, NO, vasodilating prostaglandins and natriuretic peptides suppress ET-1 production¹¹³ (Figure 1.7).

ET-1 acts primarily in an autocrine and paracrine manner. Up to 80% is released abluminally from endothelial cells towards VSMC¹¹⁴. Importantly, because of the polarised abluminal secretion of ET-1, its short half-life (~1 minute)¹¹⁵ in blood, and the sensitivity of blood levels to changes in clearance, plasma ET-1 concentrations do not accurately reflect ET-1 production.

Figure 1.6 Synthesis and structure of the ET group and related sarafotoxin

ET-2 and ET-3 differ from ET-1 by two and five amino acids while sarafotoxin differs by 7.

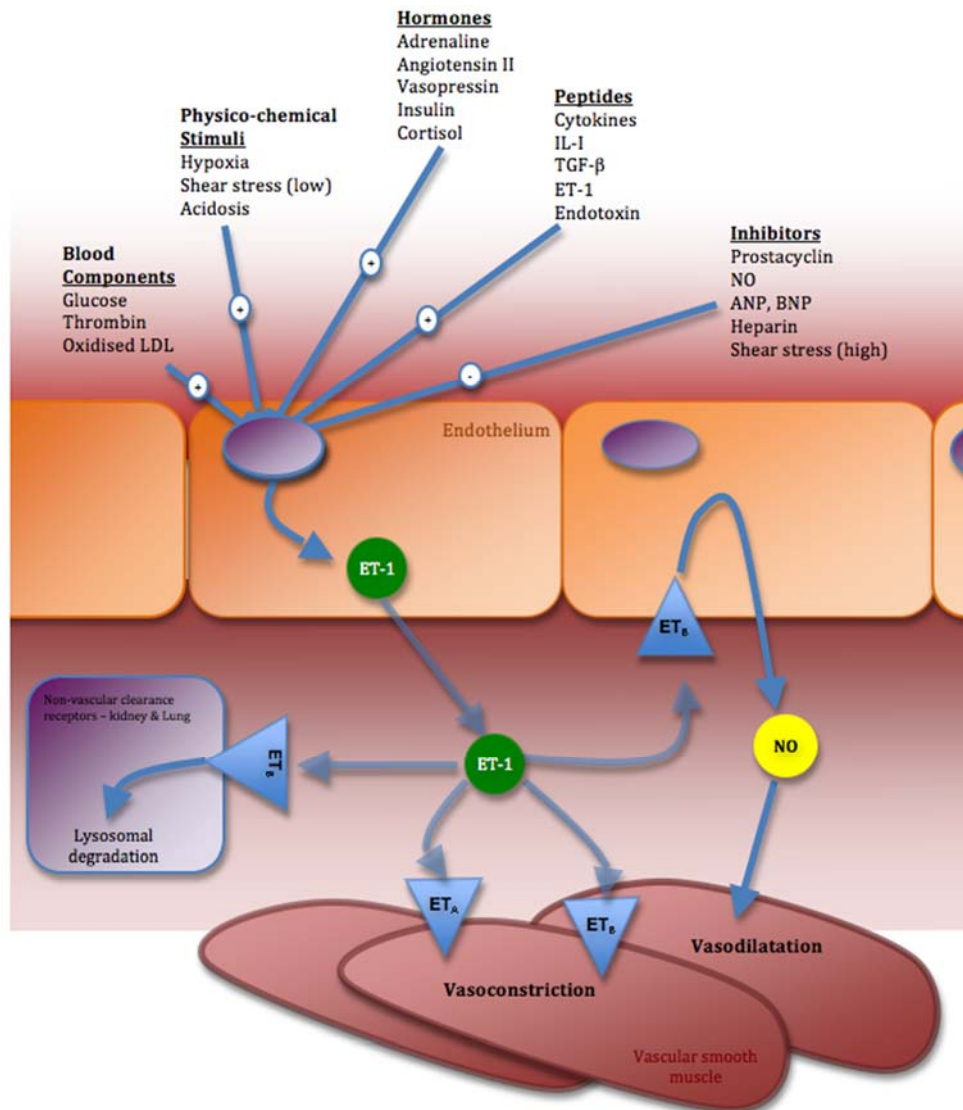


1.3.2 Endothelin Receptors

The effects of ET-1 are mediated by two receptors: endothelin-A (ET_A) and endothelin-B (ET_B), both of which belong to the rhodopsin-like G-protein-coupled superfamily^{116, 117}. Within the vasculature the ET_A receptor is expressed predominantly on VSMC, cardiomyocytes and fibroblasts¹¹⁰. Its activation results in sustained vasoconstriction, cell proliferation and fibroblast activation. In contrast, the ET_B receptor is predominantly expressed on endothelial cells. Its stimulation leads to vasodilatation through NO and prostacyclin release¹¹⁸. The ET_B receptor is also present on VSMC where its activation contributes to ET_A receptor-mediated vasoconstriction¹¹⁹. In addition, ET_B receptors, particularly in the pulmonary circulation, act as the primary clearance mechanism for circulating ET-1. This occurs through ligand-receptor complex internalisation and intracellular degradation¹²⁰. Up to 60% of circulating ET-1 is cleared following a single pass through the pulmonary vasculature¹²⁰. (Figure 1.7)

The human kidney has one of the highest concentrations of ET receptors in the body¹²¹. Renal ET-1 likely helps to control renal blood flow, glomerular haemodynamics, acid-base balance, and sodium and water homeostasis^{104, 122}. Within the kidney, ET receptors are located in the renal vasculature, glomeruli and tubules. The ET_A subtype is localised to VSMC, notably in the glomeruli, vasa recta and arcuate arteries, whereas ET_B receptors are more numerous (ET_B to ET_A ratio 2:1) and widespread, with a high concentration in the collecting system¹²³. Activation of ET_A receptors leads to increase glomerular filtration pressures through efferent arteriolar constriction¹²⁴. The ET_B receptor promotes vasodilatation and appears to play a role in sodium and water homeostasis, inhibiting chloride transport in the medullary thick ascending limb of Henlé, thereby promoting natriuresis¹⁰⁴. The vascular and renal ET-1 systems appear to act independently¹²⁵.

Figure 1.7 ET-1 secretion and site of action within the vasculature.



ANP: atrial natriuretic peptide, BNP: brain natriuretic peptide, IL-1: interleukin-1, TGF- β : transforming growth factor β

1.3.3 Endothelin-1 in normal physiology

1.3.3.1 Vascular tone

Up to 80% of ET-1 produced by endothelial cells diffuses through the basal wall of the vessel where it binds to both ET_A and ET_B receptors of VSMC which mediate the potent vasoconstrictor effects that are characteristic of ET-1¹¹⁴. The ET_B receptor,

however, exerts a dual effect on vascular tone, as activation of the ET_B receptors on endothelial cells stimulates the production of NO and vasodilatory cyclooxygenase metabolites, which in turn exert vasorelaxant effects on underlying VSMC¹²⁶ (Figure 1.7). In health, the predominant influence of ET-1 on vascular tone and basal BP is somewhat contentious. Systemic exogenous administration of ET-1 produces a biphasic response in BP, with transient hypotension followed by prolonged vasoconstriction and associated hypertension. The first phase corresponds to ET_B receptor activation on endothelial cells whereas the hypertensive phase corresponds to smooth muscle activation predominantly through the ET_A receptor though the ET_B receptor on smooth muscle also likely plays an important role¹¹⁰. Studies using ET receptor antagonist, however, suggest that, in health at least, the ET_B receptor may play a more important role in day-to-day vascular physiology. Acute administration of ET_A or ET_{A/B} antagonists causes a drop in peripheral resistance and arterial pressure. However, administration of selective ET_B antagonist causes progressive and sustained vasoconstriction¹²⁷. This suggests that the more important physiological role of ET-1 is through action on the ET_B receptors found on the endothelial cells¹²⁶.

1.3.3.2 Renal blood flow

Exogenous ET-1 causes significant renal vasoconstriction. Indeed the renal vasculature is more sensitive to the vasoconstricting effects of ET-1 than other vascular beds¹²⁸. Although total renal blood flow is reduced by exogenous ET-1 there are regional variations within the kidney with cortical vasoconstriction and medullary vasodilatation¹²⁶. Few studies have examined the effect of ET receptor antagonism on renal blood flow in health. Most studies to date have shown ET_A and ET_{A/B} antagonism to have no effect on renal blood flow suggesting that the ET_A receptor has little to no role in maintaining renal vascular tone in health¹²⁹⁻¹³². Unopposed ET_B receptor antagonism, however, leads to significant renal vasoconstriction suggesting that the ET_B receptor plays an important role in maintaining renal tone¹³².

1.3.3.3 Salt and water homeostasis

There is now a large body of evidence to suggest that ET-1 has a role in the regulation of salt and water homeostasis¹²⁶. Diuresis and natriuresis are explained by activation of the ET_B receptors at the level of the medullary collecting ducts. The collecting ducts produce and bind more ET-1 than any other cell type in the body¹³³. *In vitro* ET-1 and the ET_B agonist sarafotoxin 6c lowers water permeability in vasopressin-stimulated inner medullary collecting epithelial cells¹³⁴. *In vivo* this would lead to reduced water reabsorption in the collecting ducts and increased diuresis. Further work on the thick ascending loop of Henle has shown that ET-1, acting via the ET_B receptor, inhibits chloride and sodium reabsorption¹³⁵.

Animal studies also support a role for the ET_B receptor in sodium and salt handling within the kidney. Collecting duct ET-1 knockout mice have salt-sensitive hypertension, excessive weight gain and reduced urinary sodium excretion. All of these effects were reduced by amiloride and furosemide¹³⁶. Collecting duct-specific knockout of the ET_B receptor confirms the role of the ET_B receptor, with these mice having salt dependent hypertension and impaired sodium excretion¹³⁷.

1.3.4 Role of ET-1 in Hypertension

The potent effects of ET-1 on the vasculature have made it a plausible candidate mediator for the development of hypertension. The effects of ET-1 on BP appear to be at least partially dependent on sodium intake^{136, 137}. To date, no endothelin antagonists have been licensed for the treatment of hypertension. However, a recent study in patients with resistant hypertension showed darusentan (a marginally selective ET_A antagonist) had significant BP lowering effects that were maintained over the period of the study¹³⁸.

1.4 Endothelin Receptor Antagonists

Endothelin receptor antagonists (ETRAs) are classified according to their selectivity for the ET_A or ET_B receptors and molecular structure. Selectivity is calculated from

in vitro competitive receptor assays. Those antagonists with an affinity >100-fold for the ET_A receptor are said to be selective ET_A antagonists. Those with ≤100-fold affinity are said to be ‘non-selective’, ‘dual’ or ‘mixed’ receptor antagonists. Bosentan, for example, has a 20:1 affinity for the ET_A receptor and is therefore classified as a mixed ETRA¹³⁹. Sitaxsentan, on the other hand, has a >6500:1 affinity for the ET_A receptor and is therefore a selective ET_A receptor antagonist¹⁴⁰. Despite their differing receptor affinities, both bosentan and sitaxsentan belong to the sulphonamide class of ETRA. A list of selected ETRAs and their affinities can be seen in Table 1.2.

Table 1.2 Selected ETRAs approved or currently under evaluationModified from MacIntyre *et al.*¹⁴¹

Compound	Class	Relative ETA/ETB selectivity	Company	Conditions Studied
Ambrisentan LU-208075 BSF-208075	Selective ETA	77-4000x	Gilead Sciences	Pulmonary artery hypertension (licensed in Europe & USA)
Atrasentan ABT-627/ A-147627/ A-127722	Selective ETA	1860x	Abbott	Prostate cancer Diabetic Nephropathy
Avosentan SPP301	Selective ETA	50-600x	Speedel	Diabetic Nephropathy
Bosentan RO 47-0203	Mixed ETA/B	20x	Actelion	Pulmonary artery hypertension (licensed in Europe & USA) Scleroderma Hypertension Chronic Heart Failure
Clazosentan AXV-034343 VML-588 Ro 61-1790	Selective ETA	1000x	Actelion	Subarachnoid haemorrhage
Darusentan LU-125252 BSF-135252	Selective ETA	130x	Gilead Sciences	Hypertension Chronic Heart Failure
Edonentan BMS-207940	Selective ETA	80,000x	Bristol-Myers Squibb	Chronic Heart Failure
Enrasentan SB217242	Mixed ETA/B	110x	GlaxoSmithKline	Chronic Heart Failure
Sitaxsentan TBC11251	Selective ETA	6500x	Encysive (acquired by Pfizer)	PAH Chronic Heart Failure Chronic Renal Disease Hypertension
Tezosentan Ro 61-0612	Mixed ETA/B	30x	Actelion	Chronic Heart Failure Acute coronary syndrome Hepatorenal syndrome

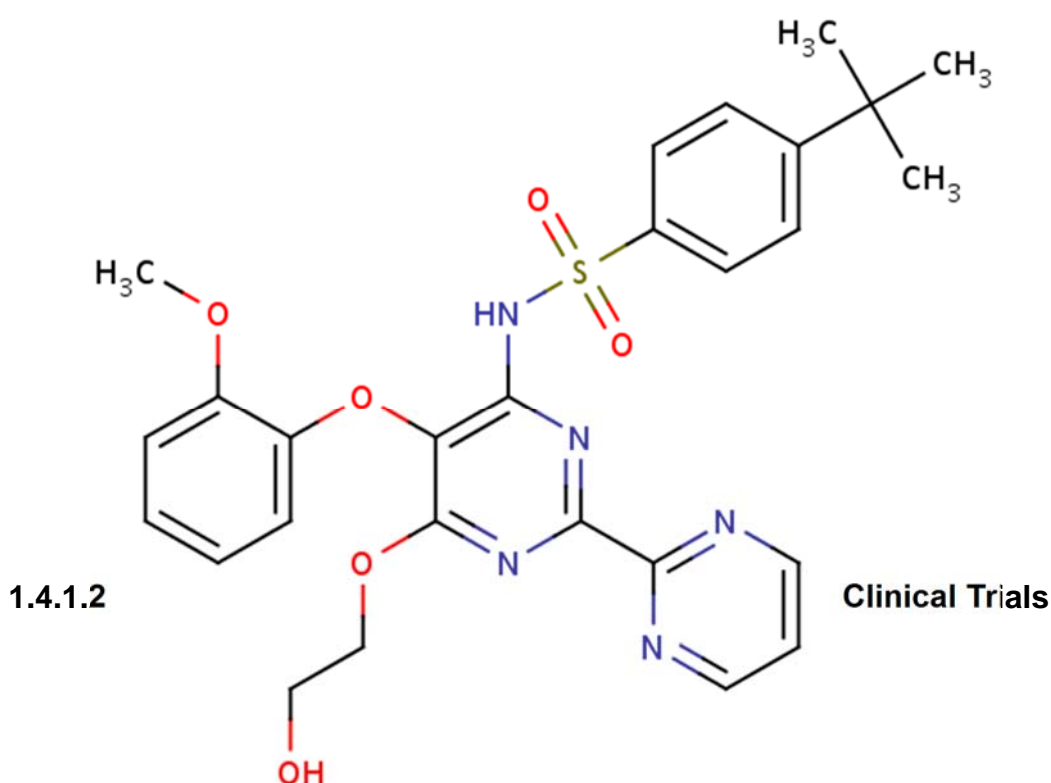
1.4.1 Bosentan

1.4.1.1 Pharmacokinetics and pharmacodynamics of bosentan

Bosentan is a mixed ETRA with a 20:1 affinity for the ET_A receptor¹³⁹. Its chemical structure can be seen in Figure 1.8. In healthy subjects, the absolute bioavailability of bosentan is around 50% with maximum plasma concentrations achieved at ~3 hours. It has a relatively short duration of action (terminal elimination half life ~5hours) requiring twice daily dosing. Steady state plasma concentrations are reached within 3-5 days¹⁴².

Bosentan is eliminated by biliary excretion following metabolism in the liver by the cytochrome P450 isoenzymes CYP2C9 and CYP3A4. Less than 3% of an administered oral dose is recovered in urine. Bosentan forms three metabolites and only one of these is pharmacologically active. This metabolite is mainly excreted unchanged via the bile. Bosentan is an inducer of CYP2C9 and CYP3A4 and possibly also of CYP2C19 and the P-glycoprotein¹⁴². In patients with significant renal impairment (creatinine clearance 15–30mL/min), plasma concentrations of bosentan decreased by ~10%. No dose adjustment is required in patients with renal impairment.

Figure 1.8 Chemical structure of bosentan



Bosentan has been extensively studied in the management of pulmonary artery hypertension (PAH) where it has been found to be both safe and effective¹⁴³. It has also been shown to be of benefit in reducing the number of new digital ulcers in patients with systemic sclerosis^{144, 145}. Furthermore, it is effective in reducing BP in patients with mild to moderate systemic hypertension, with BP reductions similar to those expected from ACE inhibition¹⁴⁶.

1.4.1.3 Bosentan tolerability and safety

Bosentan is well tolerated. Side effects most commonly attributed to treatment, after placebo correction, include flushing (4.9%), abnormal hepatic function (3.8%), leg oedema (3.3%), headache (3%) and anaemia (2.4%)¹⁴³. Of these side effects the most serious is elevation of liver enzymes and is the most common cause for discontinuation of the drug. The incidence of liver enzyme elevation greater than three times the upper limit of normal occurs in between 8-14% of patients, with drug discontinuation rates of 4% by 30 months¹⁴³. The development of fluid retention and peripheral oedema can also be problematic with bosentan and to an extent is dose dependent. It is likely at least in part due to functional ET_B blockade within the renal tubules leading to salt and fluid retention.

A class effect of ETRAs is their teratogenicity, an effect most apparent in the first trimester of pregnancy. As such, women of childbearing age are required to use reliable contraception and monthly pregnancy testing is advised.

1.4.1.4 Drug interactions

Bosentan is an inducer of the cytochrome P450 isoenzymes CYP2C9 and CYP3A4. As such, other medications which use this pathway for metabolism, such as warfarin will be metabolised more quickly and doses may need adjustment¹⁴².

1.4.1.5 Licensing

Bosentan is licensed both by the European Medicines Agency and Food and Drug Administration for the treatment of idiopathic PAH. In Europe it is also licensed for the treatment of digital ulceration in scleroderma.

1.4.2 Sitaxsentan

1.4.2.1 Pharmacokinetics and pharmacodynamics of sitaxsentan

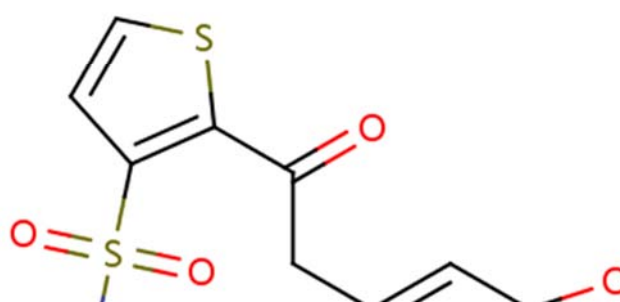
Sitaxsentan (also known as sitaxentan) is a highly selective ET_A receptor antagonist with ~6500 times greater affinity for the ET_A than the ET_B receptor¹⁴⁰. Its chemical structure can be seen in Figure 1.9. It has high oral bioavailability (>90%) and rapid absorption, reaching maximum plasma concentrations at 1-4 hours. It has a long duration of action (terminal elimination half life ~10 hours) allowing it to be given as a once-daily treatment. At the standard 100mg dose, sitaxsentan displays linear elimination in humans, with no increase in mean maximum plasma concentrations over a 12-week dosing period¹⁴⁷. Steady state plasma concentrations are achieved at ~6 days. At 300mg, however, non-linear elimination is seen, with disproportionately higher plasma concentrations (12-fold increase compared to 100mg dosing)¹⁴⁷, which may ultimately lead to higher rates of adverse effects.

Sitaxsentan is extensively metabolized (only 1% of the oral drug is excreted unchanged) primarily via cytochrome CYP2C9 and CYP3A4. Its primary metabolites have less than one-tenth the activity of the parent drug. In humans ~55% of the metabolites of sitaxsentan are excreted in the urine with the rest being eliminated in faeces¹⁴⁸. However, there is no evidence that renal impairment has any effect on the pharmacokinetics of the drug¹⁴⁹.

1.4.2.2 Clinical Trials

Sitaxsentan underwent a number of phase III clinical studies examining its role in the management of PAH and are listed in Table 1.3. They showed sitaxsentan to be effective in the management of PAH with significant improvements in exercise capacity, functional class and pulmonary vascular haemodynamics while increasing time to clinical worsening. Its effects appeared long lasting, with studies suggesting continued benefit at 2 years of treatment. Early data also suggested that selective ET_A receptor blockade is suitable substitute for patients who poorly tolerate, or gain no benefit from, bosentan therapy.

Figure 1.9 Chemical structure of sitaxsentan



1.4.2.3 Sitaxsentan tolerability and safety

Treatment with sitaxsentan is generally well tolerated. Side effects most commonly attributed to treatment, after placebo correction, include headache (5.5%), nasal congestion (5.5%), nausea (3%) and peripheral oedema (1%)^{147, 150}.

Liver function abnormalities appear to be a class effect of the sulphonamide ETRAs and, to a degree, dose related. During the initial open label study of sitaxsentan that used large doses of drug (up to 1g daily), 7 out of 20 patients experienced asymptomatic transaminase elevations¹⁵¹. During the extension arm of this study, one of these patients subsequently developed severe acute hepatitis, which resolved on stopping sitaxsentan. A further patient in this arm of the study developed acute fulminant hepatitis at 16 weeks of treatment, and despite discontinuation of sitaxsentan, died. More recent studies however (STRIDE-1 and STRIDE-2) demonstrated a far lower incidence of elevated transaminases with lower doses of sitaxsentan^{147, 150}. Combining the data from both studies, elevated transaminases (classed as 3 times the upper limit of normal) occurred in ~2% of patients taking 100mg per day compared to 5% in the placebo arm over an 18-week period. Furthermore, an interim analysis from the STRIDE-2X study suggested, that over a 1-year period, sitaxsentan was associated with a significantly lower incidence of abnormal liver function tests compared to bosentan (4% and 14% respectively)¹⁵².

Early data from STRIDE-3 and STRIDE-6, studies enrolling patients who have had to discontinue bosentan therapy due to raised transaminases, suggested that recurrence of altered liver function tests on sitaxsentan was low (13% at 18 weeks)¹⁵³.

Mild reductions in haemoglobin and haematocrit are seen with ETRAs and likely represent fluid retention. A fall in haemoglobin was reported in both sitaxsentan treatment arms in the STRIDE-1 study (1.0g/dL in the 100mg group vs. 1.6g/dL in the 300mg group)¹⁴⁷. Similar findings were reported in STRIDE-2 with 0.4g/dL reduction in the 50mg sitaxsentan arm and a 0.7g/dl reduction in both 100mg sitaxsentan and open-label bosentan arms¹⁵⁰.

Like all ETRAs sitaxsentan is teratogenic and as such, women of childbearing age are required to use reliable contraception and advised to undergo monthly pregnancy tests.

1.4.2.4 Drug interactions

Sitaxsentan has been shown to exert an inhibitory response on hepatic cytochrome CYP2C9 and to a lesser extent on CYP3A4/5. As such, metabolism of warfarin may be reduced, and its actions prolonged. This is an important interaction as many patients with a diagnosis of PAH will be on thromboprophylaxis. Results, however, from STRIDE-2 suggest that after an 80% initial dose reduction of warfarin upon commencement of sitaxsentan, further warfarin changes were equally frequent for patients treated with sitaxsentan, bosentan or placebo, suggesting no greater complexity in management^{150, 154}. Although the pharmacokinetics of cyclosporine A (metabolised by CYP3A4/5) are not altered by sitaxsentan, cyclosporine has been shown to alter the pharmacokinetics of sitaxsentan, with pre-dose levels of sitaxsentan increasing 6 fold. As such, concomitant use is contraindicated on the current license. There have been no significant interactions seen with nifedipine, digoxin, sildenafil or the oral contraceptive pill.

Table 1.3 Phase III clinical trials of sitaxsentan in patients with PAH.

Abbreviations: IPAH : idiopathic PAH, CHD : congenital heart disease, CTD : connective tissue disease, FC : functional class, TCW : time to clinical worsening, 6MWD: 6-minute walking distance

STRIDE-6 ¹⁵⁸	STRIDE-4 ¹⁵⁹	STRIDE-3	STRIDE-2X ⁴³	STRIDE-2 ¹⁵⁸	STRIDE-IXC ¹⁵⁶	STRIDE-IX ¹⁵⁵	STRIDE-1 ¹⁵⁵
48 50mg (24) 100mg (14)	98 50mg (32) 100mg (32) Placebo (34)	1450 as of May 2006	229 100mg (145) Bosentan (84)	245 50mg (62) 100mg (61) Bosentan (60) Placebo (62)	11	168 100mg (77) 300mg (91)	178 100mg (56) 300mg (63) Placebo (59)
PAH NYHA I-IV IPAH or secondary to CHD or CTD	PAH NYHA II-IV IPAH or secondary to CHD or CTD	PAH NYHA II-IV IPAH or secondary to CHD or CTD	PAH WHO II-IV IPAH or secondary to CHD or CTD	PAH WHO II-IV IPAH or secondary to CHD or CTD	PAH NYHA II-III IPAH or secondary to CHD or CTD	PAH NYHA II-IV IPAH or secondary to CHD or CTD	PAH NYHA II-IV IPAH or secondary to CHD or CTD
Randomised, double-blind study	Randomised, double-blind placebo-controlled study	Long term open label study	Extension study of STRIDE-2	Randomised double-blind placebo-controlled with additional open-label bosentan arm	Canadian open label extension study of STRIDE- 1	Extension study of STRIDE-1	Randomised double-blind placebo-controlled
Sitaxsentan 50mg or 100mg	Sitaxsentan 50mg or 100mg or placebo	Sitaxsentan 100mg	Sitaxsentan 100mg od or bosentan 125mg bd	Sitaxsentan 50mg or 100mg od or bosentan 125mg bd or placebo	Sitaxsentan 100mg	Sitaxsentan 100mg or 300mg od	Sitaxsentan 100mg or 300mg od or placebo
12 weeks	18 weeks	Ongoing in Europe, Canada and Australia	Mean duration 43 weeks sitaxsentan/35 weeks bosentan	18 weeks	2 years	Max 58 weeks (mean 26 weeks)	12 weeks
Safety, tolerability and efficacy (6MWD, FC, TCW, borg duration, cctasa)	Dose efficacy (6MWD, FC, TCW) safety	Long term safety and efficacy (6- MWD, FC, TCW)	Safety and efficacy (6- MWD, FC, TCW)	Safety and dose efficacy (6- MWD, FC, TCW)	Long term safety, tolerability and efficacy (6- MWD, FC, TCW)	Long term safety, tolerability and efficacy (6- MWD, FC, TCW)	Cardiopulmonary exercise testing 6- MWD, FC, TCW Tolerability and
Sitaxsentan well tolerated in patients who previously discontinued bosentan. Trend towards improvement with sitaxsentan 100mg.	No significant change between groups. Trend to suggest 100mg sitaxsentan beneficial. 50mg sitaxsentan sub- therapeutic	Not yet reported	No significant differences between groups in 6MWD or FC. 30% of bosentan group suffered clinical worsening compared to 20% with sitaxsentan (p=0.03). Higher rate of liver enzyme abnormalities with bosentan (14% vs 4% p=0.01)	50mg sitaxsentan dose ineffective. Treatment with 100mg improves exercise capacity and FC.	Sustained improvement in 6MWD and FC	53% of patients on 100 mg and 44% of patients on 300 mg sitaxsentan improved at least one FC. 100mg sitaxsentan better tolerated	Improvement in 6MWD, FC and TCW in both treatment arms. Increased rates of adverse events in 300mg sitaxsentan group

		Number of Patients	Patient Group	Study Type	Dosing	Duration of treatment	Endpoints	Outcome
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1.4.2.5 Licensing

Sitaxsentan was initially classified in September 2004 as an orphan medicinal product by the European Agency for the Evaluation of Medicinal Products (EMA) for the treatment of PAH. In June 2006, however, the Committee for Medicinal Products for Human Use of the European Medicines Agency recommended its approval at a dose of 100mg for the improvement of exercise capacity in patients with PAH functional class III. At this time sitaxsentan was still classed as an orphan drug in the USA with the FDA requesting further phase III data prior to any licence being granted.

In December 2010, Pfizer's voluntarily withdraw sitaxsentan from the market worldwide following two new cases of fatal liver injury.

1.5 Aims and hypotheses

In a series of acute and chronic studies, this thesis explores CVD risk in patients with CKD and examines the impact of ET-1 and its antagonism.

Study 1: Cardiovascular risk in patients in remission from immune-mediated inflammatory renal disease (Chapter 3) This study examined AS in patients with SVV and renal involvement, comparing these to a cohort of patients with non-inflammatory renal disease.

Study 2: Investigation of arterial stiffness in newly diagnosed immune-mediated renal disease (Chapter 4) This study examined AS in a cohort of 10 patients with immune-mediated inflammatory renal disease following them through the first 1-year of treatment.

Study 3: Investigation of functional ET_B receptor antagonism after bosentan and sitaxsentan in healthy men (Chapter 5) This study sought to clarify the functional ET_B receptor blockade produced by standard clinical doses of the ET receptor antagonists bosentan and sitaxsentan. The effect of these drugs on plasma ET-1 concentrations and their ability to block ET-3 mediated vasodilatation

as functional markers of ET_B receptor blockade was studied. The information from this study was used to verify the ET_A selectivity of sitaxsentan

Study 4: The effects of oral acute and chronic selective ET_A receptor antagonism on systemic and renal haemodynamics in CKD (Chapter 6)

This study sought to examine the effect of acute and chronic ET_A receptor blockade in a cohort of patients with CKD, examining its effect on systemic and renal haemodynamics.

Study 5: The effects of selective chronic ET_A receptor antagonism on selected markers of renal and cardiovascular disease progression (Chapter 7)

This study examined the effects of chronic dosing of a selective ET_A antagonist in a cohort of patients with CKD looking at its effects on risk factors of CKD and CVD progression, namely proteinuria, systemic BP and AS.

Chapter 2: Materials and Methods

Methods

All studies were performed in the University of Edinburgh's Clinical Research Centre with the approval of the local research ethics committees and the written informed consent of each subject. The investigations conformed to the principles outlined in the Declaration of Helsinki.

All subjects abstained from alcohol, nicotine and caffeine-containing products for 24 hours, and had a light breakfast, before attending on each study day. All studies were carried out in a quiet, temperature-controlled room, at 22-24C, with the subject recumbent throughout, except when voiding urine, during which they were allowed to stand.

Healthy subjects taking any medications in the previous 2 weeks were excluded from the study. Patients continued taking their normal medications up to and including each study day with the exception of diuretics, which they omitted that morning.

2.1 Drug administration

2.1.1 Locally active doses - intra-arterial administration

The brachial artery of the non-dominant arm was cannulated under local anaesthesia (1% lignocaine; Astra Pharmaceuticals, Stockholm, Sweden) with a 27 SWG steel needle (Cooper's Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK), and patency was maintained by infusion of physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) at 1 ml/min. Saline was infused for 30 min prior to the infusion of ET-3.

2.1.2 Systemically active doses - intravenous administration

For systemic intravenous administration, study drugs were infused via an 18 SWG cannula sited in an antecubital vein. Para-aminohippurate acid and Inutest were diluted

in dextrose (5%; Baxter Healthcare Ltd, Thetford, UK) and infused intravenously at a constant rate of 2 ml/min.

2.2 Drugs

2.2.1 Endothelin-3

Compared to ET-1, which has equal affinity for the ET_A and ET_B receptor (K_i 0.6nmol/L and 0.12nmol/L respectively), ET-3 has a greater affinity for the ET_B than the ET_A receptor (K_i 0.06nmol/L vs. 140nmol/L) and as such can be used as a relatively selective ET_B agonist¹⁶⁰. ET-3 (Merck Chemicals Ltd), dissolved in physiological saline, was administered intra-arterially at a dose of 60pmol/min for 5 minutes. This dose was based on previous work showing, *in vivo*, that 60pmol/min caused significant early forearm vasodilatation, suggesting functional stimulation of the endothelial ET_B receptor¹⁶¹.

2.2.2 Bosentan

Bosentan (Actelion Pharmaceuticals Ltd, Basel, Switzerland) is used in these studies at a dose of 125mg twice daily, the recommended maintenance dose in pulmonary arterial hypertension¹⁶². Bosentan is a dual ET-1 receptor antagonist with affinity for both ET_A and ET_B receptors¹³⁹. Its oral bioavailability is ~50% and is largely unaffected by food. It reaches maximum plasma concentrations approximately 3 hours after ingestion and has a relatively short duration of action (terminal half-life ~ 5 hours) requiring twice daily dosing¹⁶³. Steady state plasma concentrations are achieved in 3-5 days¹⁶³. Bosentan is metabolized by cytochrome P450 (CYP) 2C9 and CYP3A4 into three metabolites, Ro 48-5033(major metabolite), Ro 47-8634, and Ro 64-1056. Only Ro 48-5033 is metabolically active, accounting for up to 20% of drug activity. Oral bosentan is eliminated largely via faeces in healthy adults, mainly as Ro 48-5033; less than 3% of a dose is excreted in urine¹⁶³.

2.2.3 Sitaxsentan

Sitaxsentan (Encysive Pharmaceuticals Inc, Houston, USA) is used in these studies at a dose of 100mg once daily. Sitaxsentan is a highly selective, orally active ET_A receptor antagonist with around 6500 times greater affinity for the ET_A than the ET_B receptor¹⁴⁰. It has high oral bioavailability (>90%) and rapid absorption, reaching maximum plasma concentrations at 1-4 hours. It has a long duration of action (terminal elimination half life ~10 hours) allowing it to be given as a once daily treatment. At the standard 100mg dose, sitaxsentan displays linear elimination in humans, with no increase in mean maximum plasma concentrations over a 12-week dosing period¹⁴⁷. Steady state plasma concentrations are achieved at ~6 days. At 300mg, however, non-linear elimination is seen, with disproportionately higher plasma concentrations (12-fold increase compared to 100mg dosing)¹⁴⁷ that may ultimately lead to higher rates of adverse effects. Sitaxsentan is extensively metabolized (only 1% of the oral drug is excreted unchanged) primarily via cytochrome CYP2C9 and CYP3A4. Its primary metabolites are less than one-tenth as active as the parent drug. In humans ~55% of the sitaxsentan's metabolites are excreted in the urine with the rest being eliminated in faeces¹⁴⁸. There is no evidence that renal impairment has any effect on the pharmacokinetics of the drug¹⁴⁹.

2.2.4 Nifedipine

Nifedipine 30mg LA (Adalat, Bayer) was used as active BP control in Chapters 6 and 7, and was administered orally. Nifedipine is a calcium antagonist of the 1,4-dihydropyridine type. It reduces the transmembranal influx of calcium through the slow calcium channels into the cell, acting particularly on the cells of the myocardium and smooth muscle cells of the peripheral resistance vessels. The resultant action is of arterial vasodilatation and subsequent reduction in BP¹⁶⁴.

Adalat LA is formulated to provide nifedipine at a constant rate over a 24-hour period. This occurs via a membrane-controlled, osmotic push-pull process. Nifedipine is almost completely absorbed in the gastro-intestinal tract and after oral administration it is

metabolised in the gut wall and the liver by oxidative processes. The kidneys predominantly excrete its metabolites, with 5-15% being excreted in bile and faeces. There is no difference in the pharmacokinetics of nifedipine between healthy subjects and those with renal impairment and as such no dose adjustment is required¹⁶⁴.

2.2.5 Para-aminohippurate acid

Para-aminohippurate acid (Clinalfa AG) was used for the measurement of renal blood flow using standard clearance techniques¹⁶⁵ (Chapter 4). It is an amide derivative of the amino acid glycine and para-aminobenzoic acid that is not naturally found in man. Para-aminohippurate acid is an inert and non-toxic compound that only reaches the kidney via the blood stream and is filtered by the glomerulus and excreted by the proximal tubules. The extraction by the kidneys in a single transit is not complete (the full criteria for a marker of renal blood flow (RBF) by clearance) but about 80-90%, thus measurements are quoted as "effective" renal plasma flow (ERPF).

Para-aminohippurate acid was administered as a bolus loading dose of 0.4 g in 100 ml dextrose 5 over 15 min, and a maintenance infusion of 6.6 g/L at a rate of 2 ml/min. For subjects with a calculated GFR < 50 ml/min, the maintenance dose was reduced by one-third, and by two-thirds for those with a GFR < 30 ml/min. This regimen was based on previous work by Goddard *et al.*¹³².

2.2.6 Sinistrin

Sinistrin (Inutest®, Fresenius Pharma, Austria GmbH) was used for the measurement of glomerular filtration rate (GFR) by standard clearance techniques¹⁶⁵ (Chapter 6). Sinistrin, like inulin, is a naturally occurring sugar polymer of the fructan group. It has a molecular weight of ~3,500 daltons and is inert and non-toxic. It is not protein-bound, is freely filtered at the glomerulus, is neither secreted nor reabsorbed within the tubules, nor metabolised within the kidney and hence fulfils the criteria for the measurement of GFR by clearance measurement. Sinistrin differs from inulin due to its high solubility in water (also in cold water) and improved alkaline stability. It has identical renal clearance

to that of inulin. The quantitative determination of sinistrin in urine and blood plasma is identical to that of inulin¹⁶⁶.

Sinistrin was administered as a bolus loading dose of 3.5 g in 100 ml dextrose 5 over 15 min, and a maintenance infusion of 10 g/L at a rate of 2 ml/min. For subjects with a calculated GFR <40 ml/min, doses were reduced by a third. A steady-state concentration of sinistrin in the extracellular compartment is reached within approximately 70 minutes¹⁶⁶.

2.3 Haemodynamic measurement

2.3.1 Blood pressure

BP was recorded in duplicate at each time-point using a well-validated semi-automated non-invasive oscillometric sphygmomanometer (Omron HEM-705CP)¹⁶⁷. Recordings were required to be within 10 mmHg of each other (systolic and diastolic). If not, BP was repeated until two consecutive readings did fulfil these criteria. During forearm studies (Chapter 5), BP was recorded in the dominant arm (i.e. not in the arm with intra-arterial cannulation.)

2.3.2 Arterial stiffness

Pulse wave velocity (PWV) is a widely accepted indicator of AS.¹⁶⁸ PWV was measured using the SphygmoCor® system (SphygmoCor® Mx, AtCor Medical, Sydney, Australia, version 6.31) with the use of a high-fidelity micromanometer (SPC-301, Millar Instruments, Texas, USA) and electrocardiogram (ECG) gating to attain the pulse waves from both proximal (carotid artery) and distal (femoral artery) sites. The PWV is calculated from the transit time between the two sites relative to the R-wave within the ECG complex using the ‘foot-to-foot method’ and the intersecting tangent algorithm. The distance travelled was calculated using the following: the distance from the level of the sternal notch to the femoral location of the micromanometer minus the distance from

the level of the sternal notch to the carotid pulse. PWV was calculated using the following:

$$PWV (m/s) = \text{Distance travelled} / \text{wave transit time}$$

PWV was recorded in duplicate with values required to be 0.5 m/s of each other. If not, further recordings were made until two consecutive readings did fulfil the criterion.

The SphygmoCor apparatus was also used to measure the radial augmentation index. This was derived from averaged radial artery waveforms. Central augmentation index (cAIx), used as an additional measure of AS, was calculated from central aortic waveforms, which were derived by applying a generalised transfer function to the directly measured radial waveforms. All pulse wave analysis (PWA) measurements were made in duplicate with cAIx values within 5% of each other. When this criterion was not met, further recordings were made until two consecutive readings did fulfil the criterion. The quality control for PWA including average pulse height >100, pulse height variation <5%, and diastolic variation <5% were followed. As cAIx is partly dependent on heart rate all recordings were corrected to a heart rate of 75bpm.

2.3.3 Flow mediated Dilatation (FMD)

FMD is an endothelium-dependent process by which arterial dilatation occurs in response to increased shear stress¹⁶⁹. This dilatation is almost entirely due to NO release and is abolished with L-NMMA infusion¹⁷⁰. In chapter 3 of this thesis FMD of the brachial artery was measured. With individuals in a supine position and their arms outstretched perpendicular to the body, the brachial artery was imaged longitudinally with B-mode ultrasound (Acuson XP 128, Siemen plc, Bracknell, UK) 5 cm above the antecubital fossa using a linear array transducer with an imaging frequency of 11 MHz. The ultrasound probe was held in place with a stereotactic clamp throughout the study (see figure 2.1). A segment with clear anterior and posterior intimal interfaces between

the lumen and vessel wall was selected as the area to be analysed for the change of the arterial diameter. Every 3 seconds, end-diastolic frames (ECG R-wave triggered) were acquired on a computer equipped with DT-3152 progressive scan frame grabber (Data Translation Ltd, Basingstoke, UK) and image acquisition software (CVI Acquisition version 1.5, Information Integrity Inc, USA).

Baseline diameter was recorded for 1 minute. To create a flow stimulus in the brachial artery, a BP cuff, which was placed around the upper forearm, was inflated to 50 mmHg above SBP in order to occlude blood flow into the forearm for 5 minutes. Following deflation of the cuff the artery was scanned for a further 5 minutes. All the ultrasound recordings were stored on videotape. Brachial artery diameter was calculated off-line from the stored images using semi-automated wall tracking software (Brachial Analyzer, Medical Imaging Application, Iowa, USA). FMD was reported as a percentage change of the brachial artery diameter from baseline (FMD%).

Figure 2.1 Experimental setup for flow mediated dilatation



2.3.4 Forearm blood flow

FBF was measured by venous occlusion plethysmography with mercury-in-silastic strain gauges applied to the widest aspect of each forearm^{161, 171}. The hand was excluded during periods of blood flow study by inflation of wrist cuffs to 220 mmHg. An upper arm cuff was intermittently inflated to 40 mmHg for 10s in every 15s to temporarily prevent forearm venous outflow and obtain plethysmographic recordings. Voltage output from a dual-channel Vasculab SPG 16 strain gauge plethysmograph (Medasonics Inc) was transferred to a Macintosh personal computer (Macbook Pro, Apple Computer Inc, Cupertino, CA, USA) using a MacLab analogue digital converter and Chart software (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia). Calibration was achieved using the internal standard of the Vasculab plethysmography units. The experimental setup can be seen in Figure 2.2

Figure 2.2 Experimental setup of forearm blood flow



2.3.5 Clearance Studies

Effective renal blood flow (ERBF) and GFR were measured by standard clearance techniques¹⁶⁵ (Chapter 6). On each study day, an 18 standard wire gauge (SWG) cannula was sited in an antecubital vein in each arm. Diuresis was induced by 500 ml 5% dextrose over 30 min through the left arm cannula. After 15 min, the loading doses of para-aminohippurate acid & sinistrin were administered through the same cannula. Thereafter, maintenance infusions of para-aminohippurate acid and sinistrin, and 5% dextrose at 180 ml/hr continued throughout the study. Urine was collected by spontaneous voiding every 60 min. A 2.5-hour period was allowed for water, sinistrin and para-aminohippurate acid equilibration before baseline measurements, made over a 1 hour period. BP, cardiac output (CO) and heart rate (HR) were recorded every 15 min. Each collection period lasted 1 hour with urine being collected (spontaneous voiding) at

the beginning and end of each period with blood samples for para-aminohippurate acid, sinistrin and haematocrit being collected 30 min into each period. Each study day had 4 clearance periods.

2.3.6 Cardiac Output and Heart Rate

Cardiac output (CO, L/min) and heart rate (HR, bpm) were recorded using a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA)^{172, 173}. A constant sinusoidal current is applied through dual electrodes situated at the root of the neck bilaterally and to the lateral aspect of the trunk at the level of the lower chest. The changes in bioimpedance to this current are related to cardiac events and to blood flow. CO is estimated from the measures of bioimpedance by the Sramek-Bernstein formula, adapted from the original formula of Kubicek¹⁷³. HR is counted directly from detection of the cardiac electrical cycle. Each reading is the average of 15 consecutive beats. Four such readings were recorded for each measurement of CO and HR.

2.3.7 Ambulatory blood pressure monitoring

Ambulatory BP was measured at the brachial artery using a validated SpaceLabs 90217 ambulatory BP monitor¹⁷⁴. Measurements were taken every 30 minutes for 24 hours.

2.4 Measurements of Renal Function

Glomerular filtration is widely considered the best overall marker of kidney function in both health and disease¹⁷⁵. It cannot be measured directly in humans, but can be determined by plasma clearance of a filtered marker in to the urine. Ideally, this marker should be inert, not protein bound, be freely filtered by the glomerulus and not actively secreted, reabsorbed or metabolised by the kidneys. A number of exogenous and endogenous have been proposed. Within this thesis I used both sinistrin (exogenous marker) and creatinine (endogenous marker) to measure glomerular filtration.

2.4.1 Sinistrin

Urinary clearance of inulin (or sinistrin) is considered the gold standard measurement of GFR. Inulin is not protein bound, is freely filtered at the glomerulus, is neither secreted nor reabsorbed within the tubules, nor metabolised within the kidney and hence fulfils the criteria for the measurement of GFR by clearance measurement. Once steady state is reached, after around 70 minutes¹⁶⁶, repeated timed urine and blood samples are collected and GFR derived from the concentration of inulin and plasma and urine and the urinary flow rate (see section 2.5.2.2).

2.4.1 Creatinine

Creatinine is the most commonly used endogenous marker of glomerular filtration. It is primarily formed within skeletal muscle with a small contribution from dietary ingestion¹⁷⁶. With a mass of 113 daltons it is freely filtered by the glomerulus and is not reabsorbed by the tubules. It is, however, limited by the fact that its production varies with person and time dependent on muscle mass and diet. It is also actively secreted by the tubules; hence, creatinine clearance exceeds GFR¹⁷⁷.

Numerous equations using creatinine have been created to try and estimate GFR more accurately. Of these the Cockcroft & Gault and MDRD equations have been the most evaluated and studied, and have been used within this study.

2.4.3 Cockcroft & Gault equation

The Cockcroft & Gault (C&G) formula was developed in 1973 based on the data from 249 men with a wide range of age and renal function and measures creatinine clearance and is based on 4 variables: age, sex, weight and serum creatinine level¹⁷⁸. Creatinine clearance = $[140 - \text{age (years)}] \times \text{weight (kg)} \times (0.85 \text{ if female}) / \text{serum creatinine}$.¹⁷⁸ Creatinine clearance can then be further corrected by body surface area (BSA): $\text{BSA} = [71.84 \times \text{weight (kg)}^{0.425} \times \text{height (cm)}^{0.725}] / 10,000$ as defined by Du Bois, *et al.*¹⁷⁹. As

previously mentioned creatinine clearance always overestimates GFR due to creatinine secretion by the tubules.

2.4.4 MDRD equation

The MDRD (Modification of Diet in Renal Disease study) equation was developed in 1999 based on creatinine from 1628 patients with CKD²¹. The initial equation was based on 6 variables: age; sex; ethnicity; and serum levels of creatinine, urea, and albumin²¹. It has since been simplified to a 4-variable equation consisting of age, sex, ethnicity, and serum creatinine concentration¹⁸⁰. $GFR (mL/min/1.73 m^2) = 175 \times (\text{serum creatinine})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if Black})$.

2.4.5 Limitations of Cockcroft & Gault and MDRD equations

The MDRD equation and C&G equations appear to be accurate in non-hospitalised patients with CKD, though C&G is less accurate in the elderly and obese¹⁷⁷. Both MDRD and C&G equations are less accurate in those without kidney disease though C&G may be slightly more accurate than the MDRD equation when used to assess mild renal insufficiency.^{181, 182}

2.5 Assay

At pre-specified time points, venous blood was collected via an 18 SWG cannula for plasma measurements. Blood was collected into serum-gel tubes (Sarstedt) for the measurement of serum sodium, and into ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt) for all other plasma measurements. Blood was centrifuged immediately at 2,500 g for 20 min at 4°C except for haematocrit which was measured on whole blood. Samples were stored at -80°C until analysis. 20 ml aliquots of urine from each voiding were collected into plain tubes for the urinary measurements. At pre-specified time points, 20ml aliquots of urine were also collected into plain tubes containing 2.5ml of 50% acetic acid for the measurement of urinary ET-1.

2.5.1 Plasma and urinary ET-1

After extraction¹⁸³, ET-1 was determined by radioimmunoassay using rabbit anti-human ET-1 (Bachem UK Ltd, St Helens, UK)¹³². The mean recovery of ET-1, from extraction to assay, was >90%. The intra- and inter-assay variations were 6.3 and 7.2%, respectively. The cross-reactivity of the antibody was 100% with ET-1, 7% for both ET-2 and ET-3, and 10% with big ET-1.

2.5.2 Plasma & urinary para-aminohippurate acid

Para-aminohippurate acid was determined by high performance liquid chromatography (HPLC) with fluorescence detection. Plasma samples were deproteinised with equal volumes of 6% perchloric acid and, after centrifugation at 1000 g for 10 min, supernatant was diluted by 1/40 with deionised water. Urine samples were diluted 1/4000. Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard. Samples were injected into the HPLC column. The HPLC system consisted of a Waters 510 HPLC pump, WISP (Waters Intelligent Sample Processor) and Spherisorb S5 ODS column (Waters Ltd, Watford, Herts. UK) with detection by an LS-5 fluorometric detector (Perkin-Elmer Ltd, Beaconsfield, Bucks, UK), with excitation and emission wavelengths of 280 nm and 360 nm, respectively. The mobile phase consisted of 0.1 molar citrate acetate buffer containing 100 mg/L octane sulphonic acid.

2.5.3 Plasma & urinary sinistrin

Sinistrin was determined by spectrophotometry after hydrolysis to fructose. Plasma samples were deproteinised with equal volumes of 6% perchloric acid and after centrifugation at 1000 g for 10 min supernatant was decanted. Urine was diluted 1/20 with 3% perchloric acid. Resorcinol (1.5 g dissolved in 1 l of ethanol) and HCl/FeCl₃ solution (7.5 mg FeCl₃ dissolved in 1 l of molar hydrochloric acid) were added in a 6:6:1 ratio to the plasma/urine. The samples were then vortexed and incubated at 80°C for 40 min. Sinistrin concentrations were then determined against standard curves by absorption spectrophotometry at 480 nm.

2.5.4 Haematocrit

Haematocrit was measured using a Coulter counter.

2.5.5 Plasma and urinary sodium

Urinary and plasma sodium concentrations were measured using an ion-selective electrode.

2.5.6 Urinary protein

Urine protein was measured using a colorimetric method with pyrogallol red¹⁸⁴.

2.6 Data analysis

Descriptive statistics are presented as mean \pm SEM unless otherwise indicated. Microsoft Excel 2004 for Windows and SPSS Statistics (Version 19, IBM New York, USA) were used for statistical analyses. For categorical data, means were compared by one-way analysis of variance (ANOVA), Kruskal-Wallis test, unpaired Student's *t*-test, and Mann-Whitney test where appropriate. Correlation coefficients were calculated using the Pearson method. Stepwise linear regression was used for multivariate analysis. A *p* value of < 0.5 was considered significant.

2.6.1 Forearm blood flow

Plethymographic data were extracted from Chart (v5.0.2 PowerLab 2003) data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (EXCEL 2004 for Macintosh; Microsoft Corporation). The last five measurements from each 3-minute recording period were averaged for the infused and non-infused arm. However to detect early, transient, changes in blood flow, every recording during a 13-minute period of continuous FBF measurement was analyzed. To reduce variability of blood flow data, the ratio of flows in the two arms was calculated for each time point. FBF results were calculated as the ratio of flow

between the infused and non-infused arms and shown as percentage change from baseline¹⁸⁵. The percentage change in forearm blood flow following drug administration was calculated as follows:

$$100\% \times \frac{F(i)_d/F(ni)_d - F(i)_v/F(ni)_v}{F(i)_v/F(ni)_v}$$

where $F(i)$ and $F(ni)$ represent measured blood flows in the infused and non-infused arms respectively during periods of drug (d) and vehicle (v) administration¹⁸⁶.

2.6.2 Systemic & Renal data

Data were stored and analysed using the Microsoft Excel data analysis package (EXCEL 2004 for Macintosh; Microsoft Corporation).

2.6.2.1 Blood Pressure

BP at each time point was calculated as the mean of 2 recordings and represented as mean arterial pressure (MAP). MAP was calculated as follows:

$$Diastolic\ BP + \frac{(Systolic\ BP - Diastolic\ BP)}{3}$$

2.6.2.2 Renal blood flow, glomerular filtration and sodium excretion

ERBF and GFR were calculated from para-aminohippurate acid and inulin clearance, respectively. Urinary sodium excretion and fractional excretion were calculated from plasma and urinary sodium and inulin concentration and urinary flow rates (Table 2.1)

Table 2.1 Calculations used for renal data

Measurement	Calculation	Units
Glomerular Filtration Rate (GFR)	$\frac{uIn \times UFR}{pIn}$	ml/min

Effective Renal Plasma Flow (ERPF)	$\frac{uPAH \times UFR}{pPAH}$	ml/min
Effective Renal Blood Flow (ERBF)	$\frac{ERPF}{1-Haematocrit}$	ml/min
Effective Renal Vascular Resistance (ERVR)	$\frac{MAP}{ERBF}$	mmHg.min/L
Effective Filtration Fraction (EFF)	$\frac{GFR \times 100}{ERPF}$	%
Urinary Flow Rate (UFR)	$\frac{Urinary\ volume}{Time\ of\ collection}$	ml/min
Urinary Sodium Excretion (UNaV)	$uNa \times UFR$	$\mu\text{mol/min}$
Fractional Excretion of Sodium (FeNa)	$\frac{uNa \times pIn}{pNa \times uIn}$	

PAH: Para-aminohippurate acid

2.6.2.3 Bioimpedance

Cardiac output was recorded using a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA)^{172, 173}. Cardiac output is calculated by detecting changes in the body's impedance to a small electrical current. Blood and tissues both impede electrical current, however, within the time frame of the cardiac cycle, only the volume of blood within the chest changes. This resulting change in thoracic blood volume causes changes in impedance, which can then be used to calculate cardiac output with the use of computer algorithms. Bioimpedance data at each time point were calculated as the mean of four recordings, each the average of 15 consecutive heartbeats. Data were corrected using body surface area to give cardiac index (CI) for direct comparison of subjects. Systemic Vascular Resistance Index (SVRI) was derived from BP and CI data using the following equation:

$$\frac{MAP \times 80}{CI}$$

Chapter 3: Cardiovascular risk in patients in remission from immune-mediated inflammatory renal disease

Abstract

Background

Systemic connective tissue diseases are associated with an increased risk of cardiovascular disease (CVD). This may in part be driven by chronic inflammation. Patients with small vessel vasculitides which effect the kidney, such as Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis and systemic lupus erythematosus (SLE), may be expected to confer greater CVD risk through a combination of classic CVD risk factors, renal impairment and chronic inflammation than in those with renal impairment alone. The aim of this study was to determine whether inflammation and renal impairment may impact on arterial stiffness (AS) and endothelial dysfunction in a cohort of patients in remission from small vessel vasculitis.

Methods

Aortic pulse wave velocity (PWV), central augmentation index (cAI) and flow-mediated dilatation (FMD) were assessed in 22 patients with a diagnosis of small vessel vasculitis who were currently in remission.

Results

PWV was significantly positively related to age ($r^2 = 0.40$, $p < 0.01$) systolic BP ($r^2 = 0.31$, $p < 0.01$), C-reactive protein (CRP) ($r^2 = 0.32$, $p < 0.01$), triglycerides ($r^2 = 0.51$, $p < 0.01$), and urate ($r^2 = 0.01$, $p < 0.01$), and inversely related to eGFR ($r^2 = 0.21$, $p < 0.05$). Following multiple regression analysis only age, systolic BP, and triglycerides remained independently associated with PWV. AIx was positively related to age ($r^2 = 0.42$, $p < 0.001$) and this remained the case following multiple regression analysis. FMD was inversely related to age ($r^2 = 0.27$, $p < 0.05$) and waist:hip ratio (WHR) ($r^2 = 0.29$, $p < 0.05$) and positively related to protein:creatinine ratio (PCR) ($r^2 = 0.48$, $p < 0.001$). Following multiple regression both WHR and PCR remained independent predictors of FMD.

Conclusion

These results suggest that in this cohort of patients classic risk factors for CVD namely age, blood pressure, dyslipidaemia and obesity appear to play a role in AS and ED. There was little evidence to suggest inflammation or renal impairment played a significant independent role in AS and ED in this cohort.

3.1 Introduction

Small vessel vasculitis (SVV) encompasses a group of inflammatory disorders characterised by leucocyte infiltration and necrosis of the small arteries. SVV includes the ANCA-associated systemic vasculitides (Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome), vasculitis secondary to connective tissue disorders (such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)), Henoch-Schönlein purpura and cryoglobulinaemia. Symptomatic involvement may either affect a single organ or multiple organs; the kidney is affected in the majority of cases. Patients with SVV and renal involvement may present with kidney function ranging from normal through to renal failure requiring dialysis. The presence of blood and/or protein in the urine (an active urinary sediment) is highly suggestive of renal involvement, and this is confirmed by characteristic inflammatory changes on renal biopsy. SVV is often serious and sometimes fatal, and requires prompt recognition and treatment. When the kidney is involved, and if untreated, these patients may suffer an unremitting and aggressive renal attack that, if not fatal, results in ESRD and the necessity for long-term dialysis¹⁸⁷. Fortunately, available treatments have proven to be successful in controlling SVV, increasing patient survival and turning this disease, in many cases, into a chronic relapsing condition.

Cardiovascular disease (CVD) burden is significantly increased in patients with chronic inflammatory conditions. For example, the risk of a woman under the age of 45 with SLE (in the absence of renal involvement) developing atherosclerosis is 50 fold greater than that of an age-matched control¹⁸⁸ and the mortality rate from cardiovascular disease is doubled in patients with RA¹⁸⁹.

In patients with SVV with renal involvement there are currently few data that relate to cardiovascular risk, and none that relate to cardiovascular mortality. However, in addition to traditional cardiovascular risk factors and the presence of CKD as a

cardiovascular risk factor, systemic inflammation may influence vascular function and thus increase cardiovascular risk in these patients.

Vascular endothelial injury is the primary event in atherosclerosis⁹². In SVV, pro-inflammatory cytokines depress endothelial function¹⁹⁰, antineutrophil cytoplasmic antibody/neutrophil interact close to the vessel wall triggering endothelial damage¹⁹¹, and LDL oxidation promoted by the inflammatory microenvironment, leads to direct endothelial cell toxicity. Any or all of these factors may contribute to vascular endothelial damage¹⁹².

There are few data that relate AS and ED to chronic inflammatory conditions. RA is associated with increased AS and this falls following treatment¹⁹³. ED is a feature of vasculitis *per se* and occurs in both active and quiescent disease¹⁹⁴. This latter study included patients with both larger vessel vasculitis and SVV, but the findings suggest that although SVV primarily affects small arteries, the impact of disease may actually be more widespread.

Studying patients with active disease and those in remission, Booth *et al.* have demonstrated that ANCA-associated SVV is associated with increased arterial stiffness measured by PWV, and that stiffness correlates with the degree of active inflammation, assessed by CRP¹⁹⁵. Additionally disease activity in ANCA-associated vasculitis has been associated with endothelial dysfunction, studied by forearm plethysmography, with an improvement in ED seen after successful induction of remission by immunosuppressive agents^{194, 196}.

It is likely, therefore, that as patient survival increases with SVV, late cardiovascular mortality will increase due to ongoing low-grade inflammation. It follows that, if inflammation is playing a significant role in long-term vascular disease in these patients, then early suppression of disease activity in chronic inflammatory disorders may reduce long-term vascular damage. Furthermore, those patients with SVV and renal

involvement are likely to have an even greater cardiovascular risk as a consequence of their superimposed renal impairment.

3.1.1 Aims and hypotheses

The aims of this study was to investigate the relationship between AS and ED to markers of renal impairment, metabolic dysfunction and inflammation in a cohort of patients with treated immune-mediated inflammatory renal disease who were classified as in 'remission'. Remission was defined as being on stable immunosuppression for a minimum of 1 year with no evidence of ongoing disease activity.

Hypotheses:

1. AS will worsen as renal function declines
2. Renal function will predict AS independent of conventional risk factors such as age and BP
3. This cohort of patients will have greater AS when compared to a matched group of patients with non-inflammatory CKD

3.2 Study design

3.2.1 Subjects

Subjects were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh. All patients had a diagnosis of immune-mediated inflammatory renal disease based on renal biopsy and were all classed as in remission and on maintenance therapy for ≥ 1 year. Remission was defined as the absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy.¹⁹⁷

Dr Pajaree Lilitkarntakul, working in our department, obtained the data for the comparison group of CKD patients. These patients were classified as low-comorbidity

patients with no history of CVD, diabetes or inflammatory renal disease⁸⁸. They were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh and categorised into 5 stages of CKD on the basis of the Kidney Disease Outcome Quality Initiative (K/DOQI) classification.

3.2.2 Study protocol

This was a prospective, cross-sectional study. Subjects refrained from alcohol for a minimum of 24 hours and caffeinated drinks, food and smoking for at least 12 hours prior to the study. Subjects were asked to not take their medications on the morning of the study. All studies were conducted in a quiet temperature-controlled room. Blood and urine samples as well as demographic data were taken and then subjects were rested in the supine position for 30 minutes. Following this, BP measurements, cAIx, PWV and FMD were performed as described in Chapter 2.

3.2.3 Statistical Analysis

Descriptive data are given as mean \pm SD. Correlation coefficients were calculated using the Pearson method. Stepwise linear regression was used for multivariate analysis using computer software package SPSS Statistics. The variables used in the analysis can be seen in table 3.1. Significance was taken at a p value of < 0.05 .

3.3 Results

3.3.1 Subject characteristics

In total 22 subjects were enrolled into the study, 9 males 13 females. Of these, 14 had a diagnosis of ANCA-positive vasculitis, 7 had type IV lupus and 1 had crescentic IgA nephropathy. All had undergone immune modulation therapy and all were classified as in remission (no evidence of disease activity of at least 1 year). The subject characteristics can be seen in table 3.1

3.3.2 Pulse wave velocity (PWV)

PWV increased significantly with increasing age, SBP, triglycerides, urate and CRP. (Figure 3.1). Although there was no significant relation with creatinine or eGFR using the C&G equation, there was, a significant relation with eGFR when using the MDRD equation.

Following multivariate analysis only age, triglycerides, and systolic BP predicted PWV. This remained constant whether C&G or MDRD equations were used to estimate GFR.

3.3.3 Central Augmentation index (cAIx)

cAIx increased significantly with increasing age (Figure 3.2) . This remained the case following multivariate analysis.

3.3.4 Flow Mediated Dilatation (FMD)

Only 19 of the 22 patients underwent FMD due to a breakdown of the ultrasound equipment. FMD fell significantly with increasing age and WHR and rose with increasing PCR. There was also a significant difference in FMD between male and female subjects with significantly great FMD seen in females. Following multivariate analysis both WHR and PCR remained independent predictors of FMD.

Table 3.1 Subject characteristics

N=22 unless otherwise stated	Mean (\pm SD)	Min	Max
Diagnosis			
<ul style="list-style-type: none"> • ANCA vasculitis N = 14 • Type IV lupus N = 7 • Crescentic IgA N=1 			
Age, years	47 \pm 16	20	70
Gender (Male:Female)	9:13		
Creatinine, μ mol/L	106 \pm 58	43	261
Creatinine clearance, mL/min/BSA	76 \pm 40	24	179
MDRD, mL/min/1.73 m ²	76 \pm 38	19	173
Systolic blood pressure, mmHg	124 \pm 18	101	163
Diastolic blood pressure, mmHg	68 \pm 7	57	84
Mean arterial pressure, mmHg	87 \pm 9	72	105
Waist-hip ratio	0.93 \pm 0.09	0.81	1.09
Body mass index, kg/m ²	29.5 \pm 6.9	20.6	43.2
Protein:creatinine ratio, mg/mmol	40 \pm 77	4	315
C-reactive protein, mg/L	5.8 \pm 4.5	3	18
Cholesterol, mmol/L	4.6 \pm 1.0	2.7	6.6
HDL, mmol/L	1.6 \pm 0.4	0.9	2.7
LDL, mmol/L	2.3 \pm 0.9	1.1	4.5
Triglycerides, mmol/L	1.6 \pm 0.8	0.6	4.1
Urate, μ mol/L	0.38 \pm 0.13	0.23	0.69
Pulse wave velocity, m/sec	6.5 \pm 2.2	4.2	14.4
Augmentation index corrected to heart rate of 75bpm, %	16.9 \pm 10.6	1.0	32.5
FMD (n = 19), %	6.7 \pm 5.6	0.7	20.2

Figure 3.1 Pulse wave velocity univariate analysis

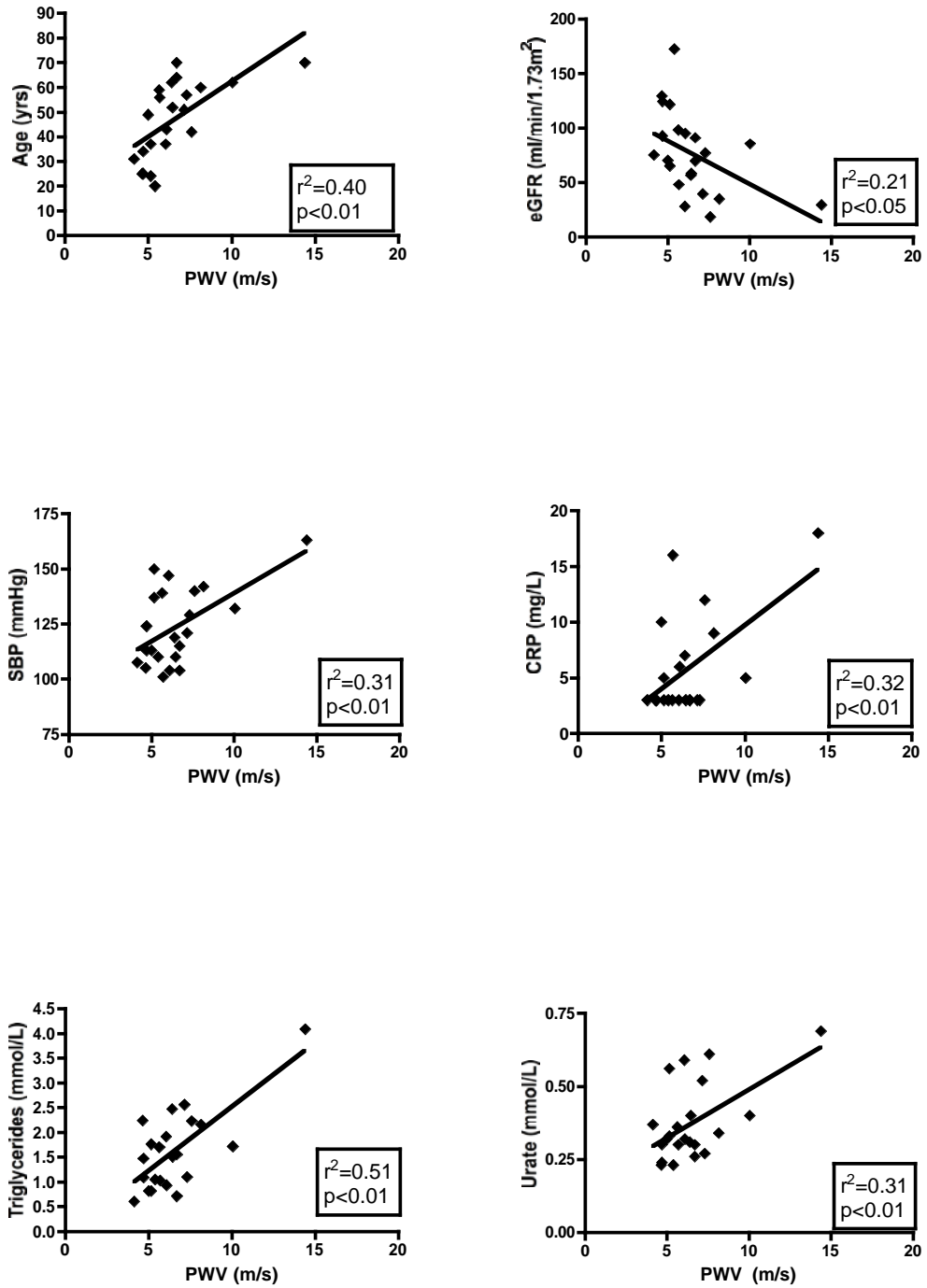


Figure 3.2 Augmentation index univariate analysis

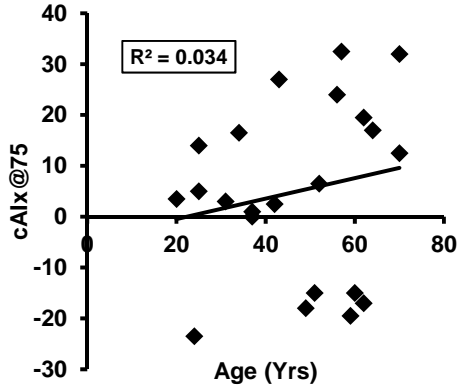
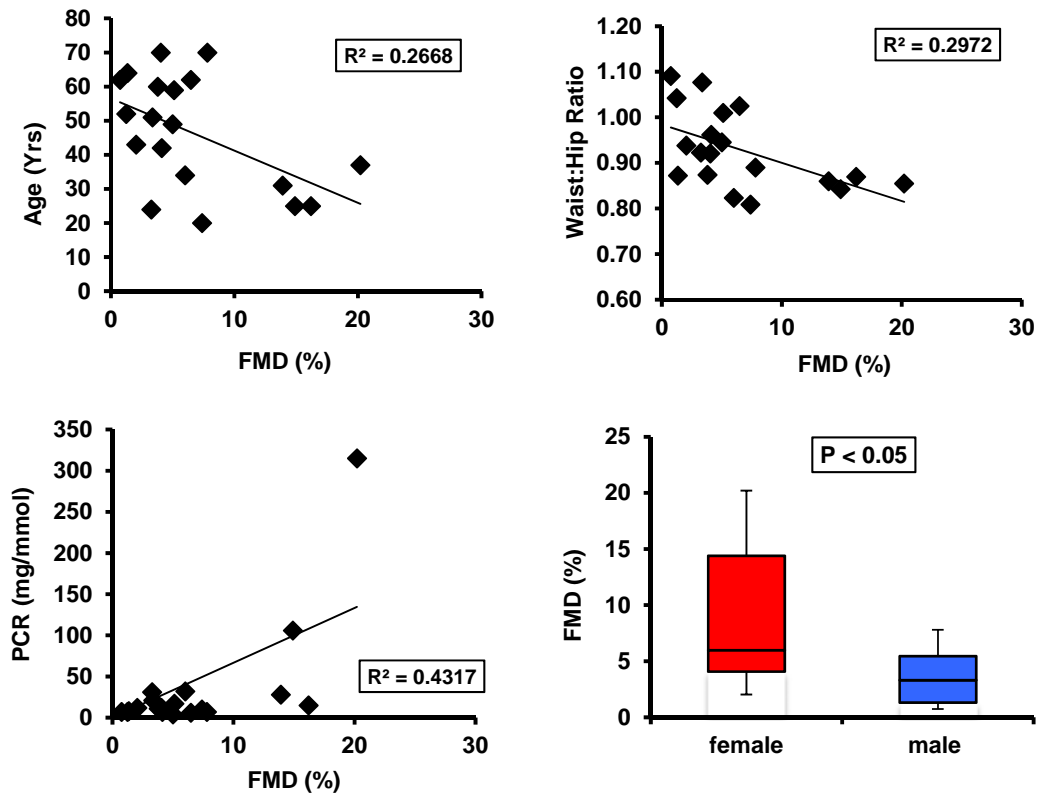


Figure 3.3 FMD univariate analysis



3.3.5 Comparison with low co-morbidity CKD patients

The current cohort of patients with inflammatory renal disease had the same age and sex distribution as the previous cohort of patients with non-inflammatory, low co-morbidity renal disease. Renal function in the current group was greater than that of the CKD patients. The current cohort also had a improved lipid profile, with higher HDL and lower LDL cholesterol (Table 3.2).

There was a non-significant difference in PWV between the inflammatory renal disease cohort and CKD cohort 6.5 m/s vs. 6.9 m/s. In the multivariate analysis combining both cohorts, age, MAP, urate, CRP were independent predictors of PWV. Multivariate analysis of cAix found only age and sex were independent predictors cAix. Multivariate analysis for FMD found age, creatinine, cholesterol and inflammatory renal disease to be independent predictors of FMD.

Table 3.2 Inflammatory renal disease vs. low co-morbidity CKD

* denotes significance $p < 0.05$

	Study 1 (n=22) Mean (STD)	Study 2 (n=113) Mean (STD)
Age (years)	47 (16)	47 (10)
Creatinine ($\mu\text{mol/L}$)	106 (58)	194 (165) *
MDRD ($\text{mls/min}/1.73\text{m}^2$)	76 (38)	53 (31) *
Corrected GFR (ml/min)	76 (40)	63 (35) *
CKD Stage	2.1 (1.0)	2.6 (1.2)
Sex (male)	0.59 (0.5)	0.65 (0.5)
SBP (mmHg)	124 (17)	118 (14)
DBP (mmHg)	68 (7)	74 (9) *
MAP (mmHg)	87 (9)	89 (10)

BMI (kg/m ²)	30 (7)	28 (5) *
Waist (cm)	96 (19)	96 (15)
Hip (cm)	103 (14)	107 (9)
WHR	0.93 (0.09)	0.89 (0.09)
HbA1c (%)	5.5 (0.6)	5.6 (0.4)
Glucose (mmol/L)	5.0 (0.8)	5.0 (0.5)
PCR (mg/mmol)	40 (77)	-
ACR (mg/mmol)	-	59 (86)
CRP (mg/L)	5.7 (4.5)	3.5 (3.8) *
ESR (mm/Hr)	14.7 (9.4)	15.2 (13.3)
Cholesterol (mmol/L)	4.58 (1.02)	4.62 (0.85)
HDL (mmol/L)	1.56 (0.43)	1.24 (0.34) *
LDL (mmol/L)	2.27 (0.87)	4.29 (0.82) *
Triglycerides (mmol/L)	1.62 (0.81)	1.47 (0.92)
Urate (mmol/L)	0.36 (0.13)	0.42 (0.12)
FMD (%)	6.7 (5.6)	4.2 (2.9) *
cAix (%)	16.9 (10.6)	15.8 (11.6)
PWV (m/s)	6.5 (2.2)	6.9 (1.3)

3.4 Discussion

I evaluated measurements of AS in a cohort of patients with known inflammatory renal disease who were classified as being in remission for at least 1 year. These inflammatory diseases are thought to confer a higher cardiovascular mortality. In SLE, for example, the risk of developing atherosclerosis is 50-fold greater than that of an age-matched control¹⁸⁸. Previous work has shown that patients with chronic inflammatory rheumatological diseases such as SLE, rheumatoid and Behçet's have increased PWV when compared to healthy controls^{193, 198, 199}. To date, however, there has been little study of the impact of CVD in patients with chronic inflammatory renal disease, particularly the ANCA positive vasculitides.

In the current study, following multivariate analysis, age predicted AS as measured by PWV and cAix. Triglycerides and systolic BP were also correlated with PWV. These findings broadly agree with previous work in other cohorts of patients. For example the cross-sectional analysis of the Framingham Heart Study by Mitchell *et al.* has previously shown that AS significantly increases with age. Following age adjustment, other important correlates included higher MAP, higher BMI, impaired glucose metabolism and abnormal lipids²⁰⁰. A further study by Aatola *et al.* examined PWV in 1691 white healthy adults in Finland. In this study sex, age, systolic BP, insulin and triglycerides were all independent predictors of PWV²⁰¹. Interestingly a recent study in patients with Behçet's disease (an SVV that can also affect large vessels and commonly affects the mucous membranes) also found that triglycerides were an important predictor of PWV²⁰². Almost all the patients within the current study were treated with chronic prednisolone therapy (N=20). As prednisolone is known to increase both LDL and triglyceride levels²⁰³, based on our results and those mentioned, lipid lowering therapy should be considered particularly when commencing patients on steroids.

In the current study there was no evidence of a relationship of PWV to renal function as measured by C&G or MDRD equations following multivariate analysis. This is in contrast to a previous larger low morbidity CKD cohort⁸⁸. eGFR as measured by the

MDRD equation, however, was significantly correlated with PWV in the univariate analysis. It is likely that, due to the wide distribution in renal function within the current study, there simply were not enough patients to show a significant correlation.

CRP has previously been shown in some, but not all, studies to be an independent predictor of arterial stiffness in health²⁰⁴⁻²⁰⁶ and disease¹⁹⁵. Wilkinson's team in Cambridge were one of the first to look at this in 2005. They found, in a cohort of over 400 healthy volunteers, that PWV was significantly related to age, BMI, MAP, LDL cholesterol, triglycerides and CRP. There was no link with CRP and AIx²⁰⁴. Interestingly, a very similar study performed by Kullo *et al.* in a cohort of just over 200 health subjects found CRP was not significantly associated with PWV but was with AIx²⁰⁶. Wilkinson's team went on to examine AS in a group of 32 patients with ANCA-associated vasculitis without renal involvement. They again found that CRP was positively correlated to PWV. Interestingly, this time a link between CRP and AIx was also found following multivariate analysis¹⁹⁵. On the other hand a recent large study involving over 800 individuals with type II diabetes found CRP had no association with AS²⁰⁷. In the current study there was a significant relationship between PWV and CRP in univariate analysis. However, this disappeared following multivariate analysis. There was no association between CRP and AIx. It may be that CRP is only very weakly, if at all, associated with AS, if at all. However, it may be that sample size was too small to detect a significant correlation, particularly as most of these subjects had a CRP within the normal range because they were in remission (only 9/22 had a CRP above the lower limit of detection in the laboratory).

In previous work from this department in 113 patients with CKD and low co-morbidity⁸⁸, CRP (again, largely in the normal range) was an independent predictor of PWV. When the results from these SVV patients in remission are added to this group, CRP remains an independent predictor of arterial stiffness (along with BP, age and urate) but having SVV in remission, as opposed to low co-morbidity CKD, was not an independent predictor of PWV in the combined cohort. When the two studies are

compared, the relationship between renal function and PWV is the same. It is likely, therefore, that low-grade inflammation is important but that the numbers in this study were insufficient to show a significant relationship.

FMD measures endothelium-dependent dilatation of the artery in response to shear stress and, as such, can be used as a surrogate marker of endothelial function²⁰⁸. It has been shown to be adversely affected by classical CVD risk factors, with age, dyslipidaemia, diabetes and smoking being particularly strong determinants²⁰⁹. In the current study FMD was adversely affected by increasing age and WHR, which, would be in keeping with previous studies²⁰⁹. The present study also found a significant difference in FMD between the sexes. This is in keeping with previous studies, which have shown that women have significantly greater FMD responses than men^{210, 211}. This may, in large part, be a consequence of females having smaller diameter brachial arteries; therefore % change is often greater. There is also some evidence that oestradiol may play a role in this sex difference²¹¹.

The positive relationship between FMD and proteinuria seen with this study was unexpected and is in contradiction to previous studies, which have shown a negative relationship. It is likely that small sample size and one major outlier may explain these current results

In conclusion, the major determinants of arterial stiffness and endothelial dysfunction in this cohort of patients with inflammatory renal disease (in remission) appear, in large part, to be based on known significant cardiovascular risk factors, namely; age, BP, dyslipidaemia and obesity. Therefore good BP control, lipid control eg. with statins and lifestyle advice should be the mainstay of primary and secondary CVD prevention in this group. It is not possible to determine the role of low-grade inflammation from this study because the majority of the patients had CRPs at the lower limit of detection once in remission. However, there is a case for further investigation in this area, given that it appears to play a role in CKD patients in general.

Chapter 4: Investigation of arterial stiffness in active immune-mediated renal disease

Abstract

Background

I have previously shown that, in patients with immune-mediated renal disease in remission, that markers of arterial stiffness and endothelial dysfunction are linked to classic cardiovascular risk factors: namely age, blood pressure, dyslipidaemia and obesity. This current study followed a cohort of patients with a new diagnosis of immune-mediated inflammatory renal disease, following them for 1 year to examine what effect if any treatment has on arterial stiffness, hypothesising that as patients entered remission arterial stiffness would improve.

Methods

Aortic pulse wave velocity PWV was assessed in 10 patients over a 1-year period. Patients were studied at before treatment, and 2-weeks, 1-month, 3-months, 6-months and 12 months after treatment. These results were then compared to markers of disease activity and classic cardiovascular risk factors.

Results

All 10 patients achieved clinical remission of their disease by 1 year. Overall renal function improved significantly from baseline to 1-year as measured by eGFR (67 ± 39 vs. 81 ± 39 ml/min $p < 0.01$) and PCR (367 ± 333 vs. 48 ± 63 mg/mmol $p < 0.05$). There was also a significant fall in PWV over this period (6.1 ± 1.1 m/s vs 5.7 ± 1.3 m/s, $p < 0.01$). This fall in PWV was correlated to improvement in renal function on both univariate and multivariate analysis. PWV did not correlated with any other measures.

Conclusion

PWV falls following treatment of acute immune-mediated renal disease and its improvement seems largely related to improvements in renal function during the course of treatment.

4.1 Introduction

In chapter 3 I looked at cardiovascular risk factors in a cohort of patients with immune-mediated renal disease who were currently in remission from the disease. In this study I further examined that risk in a cohort of newly diagnosed patients, following them for 1 year to examine what impact, if any, treatment and subsequent disease remission had on markers of AS.

4.1.1 Aims and hypotheses

The aims of this study was to investigate the relationship between AS and markers of renal impairment, metabolic dysfunction and inflammation in a cohort of patients with newly diagnosed ‘active’ immune-mediated inflammatory renal disease

Hypotheses:

1. AS would improve as renal function improves.
2. AS would improve as markers of inflammation fall.

4.2 Study design

4.2.1 Subjects

Subjects were recruited from the renal ward of the Royal Infirmary of Edinburgh. All patients had a diagnosis of an inflammatory autoimmune disease on renal biopsy and all were commenced on immunomodulatory therapy.

4.2.2 Study protocol

This was a longitudinal study following patients for 1 year. Subjects were studied at before treatment, and after 2 weeks, 1 month, 3 months, 6 months and 12 months of treatment. Subjects refrained from alcohol for a minimum of at least 24 hours and caffeinated drinks, food and smoking for at least 12 hours prior to the study. Subjects were asked to not take their medications on the morning of the study. All studies were

conducted in a quiet temperature-controlled room. Blood and urine samples as well as demographic data were taken and then subjects were rested in the supine position for 30 minutes. Following this, measurements of BP and AS were performed as described in Chapter 2.

4.2.3 Statistical Analysis

Descriptive data are given as mean \pm SD. Correlation coefficients were calculated using the Pearson method. Stepwise linear regression was used for multivariate analysis. Significance was taken at a p value of < 0.05 .

4.3 Results

4.3.1 Subject characteristics

10 subjects, 5 males and 5 females, were enrolled and followed up over a 1-year period. Of these 5 had a diagnosis of type III or IV lupus nephritis, 4 ANCA + vasculitis and 1 crescentic IgA nephropathy. A summary of individual diagnoses and treatment regimens can be reviewed in Table 4.1 with baseline characteristics in Table 4.2

Table 4.1 Diagnosis and treatment regimens of study patients

Patient	Diagnosis	Involvement	Induction therapy	Maintenance Therapy	Antihypertensive Therapy	Statin Therapy
1	Type IV LN	Kidney Joint Skin	Prednisolone MMF Hydroxychloroquine	Prednisolone MMF Hydroxychloroquine	Nil	Nil
2	Type III LN	Kidney Joint	Rituximab MMF Hydroxychloroquine	MMF Hydroxychloroquine	Ramipril	Nil
3	PR3 +	Kidney Lung Skin	Prednisolone PEX Pulse CYC	Prednisolone Azathioprine	Ramipril	Atorvastatin
4	PR3 +	Kidney GI Eyes Joint	Prednisolone PEX Pulse CYC	Prednisolone Azathioprine	Bisoprolol	Atorvastatin
5	Type IV LN	Kidney	Prednisolone MMF	Prednisolone MMF Hydroxychloroquine	Doxazosin	Atorvastatin
6	MPO +	Kidney Lung Nerve Joint	Prednisolone Pulse CYC	Prednisolone MMF	Candesartan	Atorvastatin
7	Type IV/V LN	Kidney Pericarditis GI	Prednisolone MMF	Prednisolone MMF	Ramipril	Simvastatin
8	MPO+	Kidney	Prednisolone PEX MMF	Prednisolone Azathioprine	Nil	Nil
9	Crescentic IgA	Kidney	Prednisolone Pulse CYC	Prednisolone MMF	Amlodipine Doxazosin Ramipril Candesartan	Atorvastatin
10	Type IV LN	Kidney Joint Skin	Rituximab MMF	MMF	Irbesartan Lisinopril	Atorvastatin

LN: Lupus Nephritis, PEX: Plasma Exchange, CYC: Cyclophosphamide, MMF: Mycophenolate Mofetil

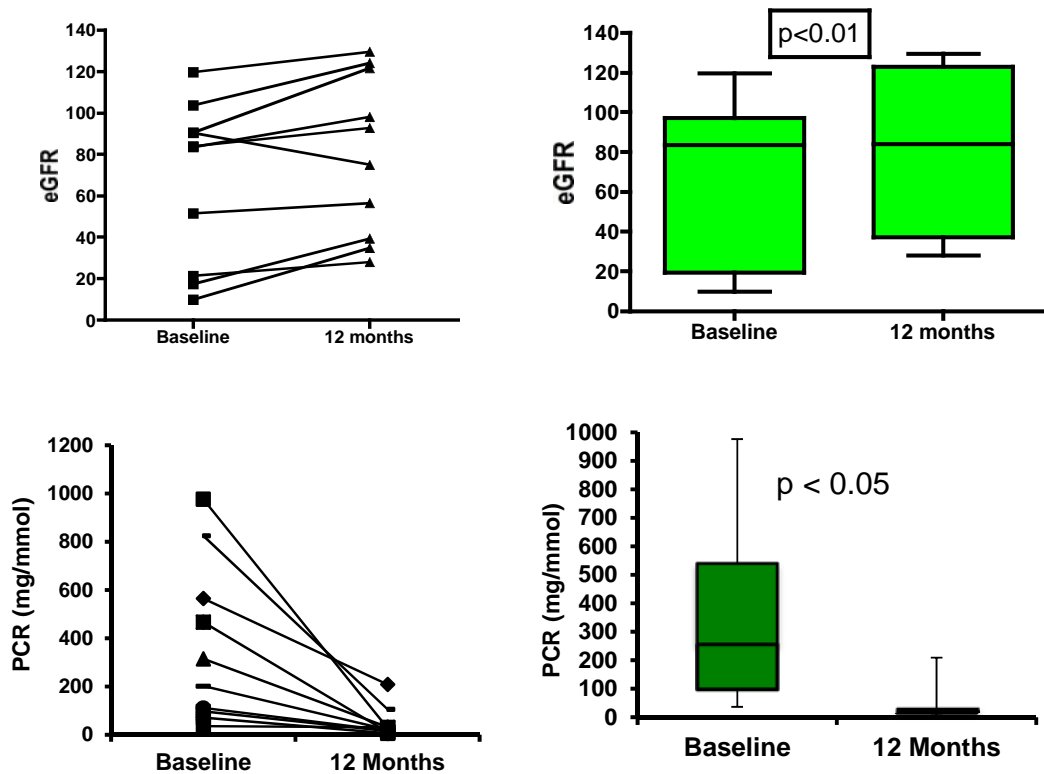
Table 4.2 Baseline characteristics

N= 10	Mean (\pm SD)	Min	Max
Age, years	40 \pm 16	23	61
Gender (Male:Female)	5:5		
Creatinine, μ mol/L	147 \pm 189	60	389
Creatinine clearance, mL/min/BSA	67 \pm 39	10	120
MDRD, mL/min/1.73 m ²	72 \pm 40	11	113
Systolic blood pressure, mmHg	140 \pm 20	102	167
Diastolic blood pressure, mmHg	77 \pm 13	56	102
Mean arterial pressure, mmHg	98 \pm 14	71	120
Waist-hip ratio	0.95 \pm 0.09	0.79	1.11
Body mass index, kg/m ²	27.9 \pm 4.4	21.3	34.2
Protein:creatinine ratio, mg/mmol	367 \pm 333	36	976
C-reactive protein, mg/L	24 \pm 18	3	54
Cholesterol, mmol/L	5.5 \pm 1.7	2.6	7.9
HDL, mmol/L	1.1 \pm 0.3	0.7	1.5
LDL, mmol/L	3.4 \pm 1.5	1.3	5.6
Triglycerides, mmol/L	2.1 \pm 0.9	0.9	3.7
Urate, μ mol/L	0.36 \pm 0.12	0.22	0.59
Pulse wave velocity, m/sec	6.1 \pm 2.2	4.2	7.5
Augmentation index corrected to heart rate of 75bpm, %	20 \pm 17	-15	45

4.3.2 Renal function

Over the 12-month period 9 out of the 10 patients had improvements in renal function. Overall, there was a significant increase in renal function as measure by the C&G equation (67 \pm 39 vs. 81 \pm 39 ml/min p < 0.01). There was a trend to increasing eGFR after the 2-week time point but this did not reach significance level. Proteinuria as measured by PCR also fell significantly by 12 months (367 \pm 333 vs. 48 \pm 63 mg/mmol p <0.05) (Fig 4.1).

Figure 4.1 Effect of treatment on renal function



4.3.3 Pulse wave velocity (PWV)

PWV was significantly lower at 12 months compared to baseline (6.1 ± 1.1 m/s vs. 5.7 ± 1.3 m/s, $p < 0.01$) (Fig 4.2A). There was a trend to reducing PWV after the 2-week time point but this did not reach significance (Fig 4.2B).

Age and CRP were independent predictors of PWV at baseline, before induction treatment. At 12 months, no one variable independently predicted the absolute PWV value. Absolute PWV at baseline and at 12 months was significantly correlated with renal function in univariate analysis but this was not an independent predictor of PWV at either time point. However, in multivariate analysis, change in PWV over 12 months correlates only with change in creatinine ($R^2=0.40$, $p < 0.05$), and is not significantly

correlated with change in blood pressure, CRP, ESR, lipids, PCR or weight. There is an identical relationship when PWV is plotted against eGFR at both baseline and 12 months, suggesting that the effect of treatment on PWV is largely due to the effect on eGFR, with 9/10 patients having an improvement in GFR (67.2 ± 39.2 vs 80.6 ± 38.9 ml/min) (Fig 4.3)

Figure 4.2 Change in PWV over time

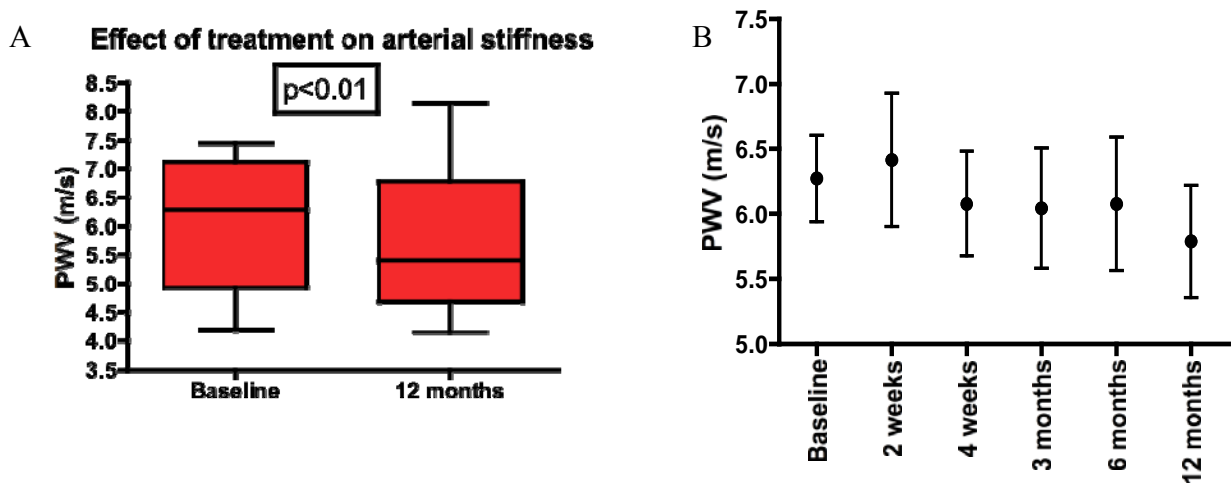
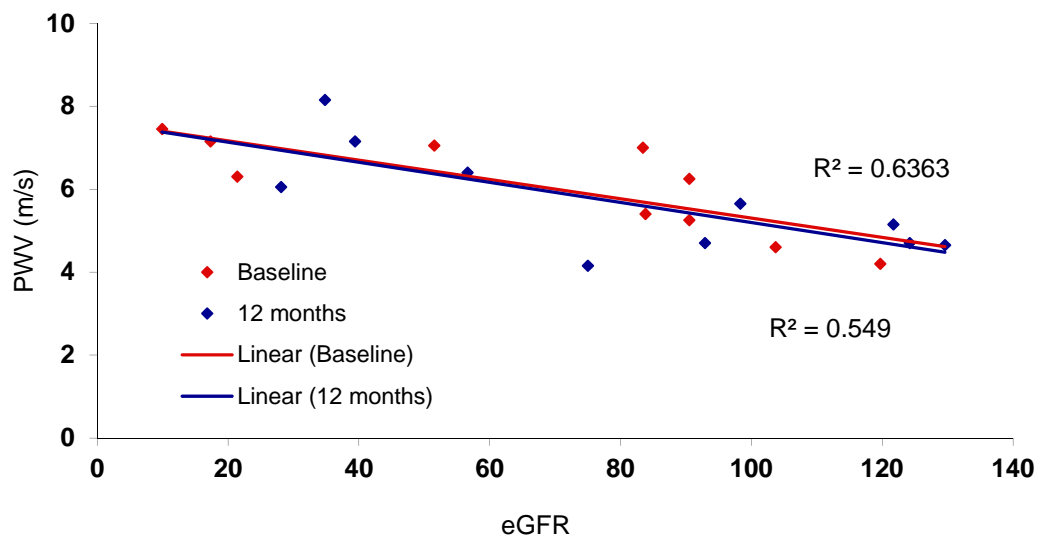


Figure 4.3 PWV vs. eGFR at baseline and 12 months



4.4 Discussion

In the current study, renal function improved notably following treatment with a significant increase in eGFR and fall in proteinuria. All patients were classed as in remission by 12 months. PWV also significantly fell from baseline to 12 months with a trend to reducing PWV after the 2-week time point suggesting that the effect of treatment on arterial stiffness begins relatively rapidly though, with such a small sample size, this was not significant.

In comparison to the previous chapter, where classical cardiovascular risk factors such as age, BP and lipids were correlated to PWV, no such correlations were found when the patients were in remission, though the numbers studied were small. Inflammation, measured by CRP, did independently predict AS as measured by PWV at diagnosis (before treatment), as did age but by 12 months, CRP had normalised and no significant relationship between inflammation and arterial stiffness was found. Strikingly, the only correlation to PWV in this current cohort of patients was renal function in that the change in PWV from diagnosis (baseline) to remission (12 months) was only predicted by change in creatinine. Indeed the overlapping trend lines seen in figure 4.3 suggest that the improvement in PWV is linked to improvement in renal function, though it is not clear if this is a direct consequence of improved renal function.

This is the first study to examine PWV in a cohort of adult patients with immune-mediated inflammatory renal disease and follow them through the first year of treatment. A previous study by Booth *et al.*, the only other study to examine PWV in a cohort of patients with ANCA-associated vasculitis, showed that PWV was increased in active disease compared to a matched group of patients in disease remission, which would be in keeping with my current results. They also showed that PWV was linked to CRP and BP. This study, however, excluded subjects with renal impairment as defined as a creatinine clearance of <50 ml/min and as such found no link with renal function¹⁹⁵.

Only one other study has examined PWV in AKI, a cohort with acute post-streptococcal glomerulonephritis (APSGN). APSGN is an immune-mediated disease occurring after streptococcal infection. Its cause is unclear, but 2 possibilities have been postulated; one that circulating immune complexes form with streptococcal antigenic components and these subsequently become deposited within the glomerulus with associated complement activation; the other is that of molecular mimicry²¹². It is in effect similar to the conditions studied within the current study, though its management is normally only supportive and complete recovery is seen in most patients. Yu *et al.* examined PWV in a group of 16 children with APSGN comparing them to a similar cohort of children with acute pyelonephritis (APN) and healthy controls. Subjects were followed up for an average of 2 years. They found that PWV was significantly elevated in the APSGN group compared to the APN and healthy control groups. As renal function improved PWV in the ASPGN group fell to normal levels²¹³. Unfortunately, this study did not examine other markers of disease so it is not clear if inflammation or other cardiovascular markers had any impact on improvement in PWV.

In conclusion I have shown in a small cohort of patients with acute immune-mediated renal disease that improvements in renal function confer significant improvements in PWV. Failure to achieve significant improvements in renal function may lead to increased risk of CVD. Further work is required in this field particularly examining the effect that AKI has on arterial stiffness and endothelial function.

Chapter 5: Investigation of functional ET_B receptor antagonism after bosentan and sitaxsentan in healthy men

Abstract

Background

ET-1 is implicated in the development of hypertension and a role for ETAs in the management of hypertension is emerging. ETAs are classified as selective or mixed depending on their degree of ET_A:ET_B receptor blockade. As yet, there are no comparative studies in humans that measure biochemical and functional ET_B blockade achieved by currently licensed ETAs. I therefore investigated the effects of bosentan, a mixed ETA, and sitaxsentan, an ET_A selective ETA, on plasma ET-1 concentrations and ET_B-mediated vasodilatation to ET-3.

Methods

In a randomized, double-blind, 3-way cross-over study, 10 healthy subjects received 7 days of placebo, bosentan 250mg and sitaxsentan 100mg daily. Plasma ET-1 concentrations were measured at baseline and 3h on day 1 and predose on day 7. Subjects also underwent forearm blood flow (FBF) measurements on day 7 of each period with brachial artery infusion of ET-3 (60pmol/min for 5min).

Results

Bosentan, but not placebo or sitaxsentan, significantly increased plasma ET-1 concentrations at day 7 ($+0.70\pm 0.20\text{pg/ml}$; $P<0.005$). Maximal ET-3 mediated vasodilatation was seen at 2min following placebo ($30\pm 6\%$) and sitaxsentan ($21\pm 11\%$), however this was abolished by bosentan, with a reduction in FBF of $8\pm 3\%$ ($P<0.01$ vs. placebo and sitaxsentan)

Bosentan but not sitaxsentan increases circulating plasma ET-1 levels and abolishes acute ET-3 mediated vasodilatation, confirming that the mixed ET_{A/B} antagonist bosentan, but not the selective ET_A antagonist, sitaxsentan, causes functional ET_B blockade, at clinically relevant doses, in healthy human subjects.

5.1 Introduction

ET-1 acts via two specific receptors, the ET_A and the ET_B receptors²¹⁴. Within the vasculature ET_A receptors are expressed predominantly on vascular smooth muscle cells (VSMC), cardiomyocytes and fibroblasts¹¹⁰. Their activation results in sustained vasoconstriction, cell proliferation and fibroblast activation¹¹⁰. In contrast, ET_B receptors are predominantly expressed on endothelial cells and mediate vasodilatation primarily through NO²¹⁵. The role of the ET_B receptor however is more complex, as they are also present on VSMC and fibroblasts where they too contribute to vasoconstriction, proliferation and fibrosis^{119, 216, 217}. In addition, ET_B receptors, particularly in the pulmonary circulation, act as the primary clearance mechanism for circulating ET-1²¹⁸.

Despite the hypothesized benefits of the unblocked ET_B receptor, which include preserved endothelial dependent NO mediated vasodilatation, ET-1 clearance and natriuresis and diuresis, to date no clinically relevant differences have been demonstrated *in vivo* between selective ET_A and mixed ET_{A/B} ET_A antagonists.

I therefore examined biochemical and functional markers of ET_B receptor antagonism *in vivo* in healthy subjects following 7-day oral dosing with either the selective ET_A antagonist sitaxsentan or the mixed ET_{A/B} antagonist bosentan at clinically licensed doses. I hypothesized that bosentan, but not sitaxsentan, would affect biomarkers for ET_B receptor function, specifically to increase plasma ET-1 and reduce ET-3 mediated vasodilatation through functional ET_B receptor blockade.

5.2 Study Design

5.2.1 Subjects

The study was undertaken in 10 healthy male volunteers recruited from the local community and had the approval of the local research ethics committee. Written informed consent was obtained from each subject before entry into the study. All

subjects were allocated to a randomized treatment sequence of placebo, sitaxsentan and bosentan.

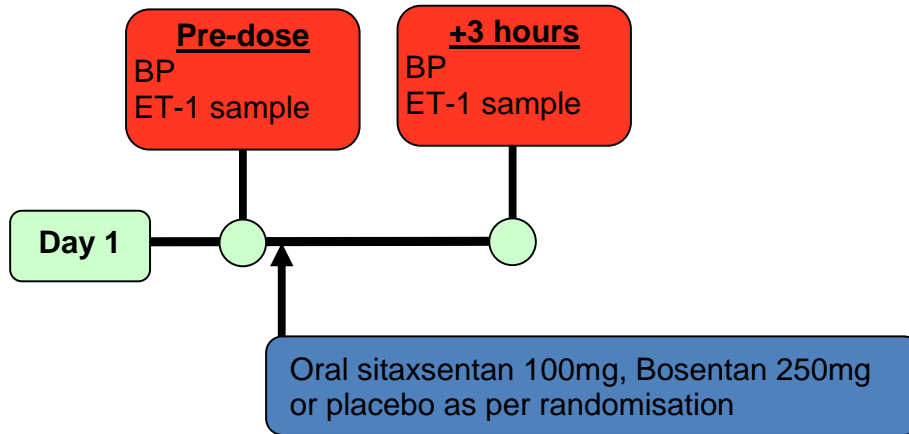
5.2.2 Study protocol

This was a three-way, randomized, double-blind placebo-controlled cross-over trial. The study consisted of three 7-day treatment periods with placebo, sitaxsentan 100mg or bosentan 125mg twice daily in a randomized order (see methods for justification of dose). As the bosentan arm had twice daily dosing, to maintain blinding, placebo was given as a twice-daily tablet and during the sitaxsentan phase a matched placebo was given in the evening. There was a minimum 14-day washout between periods. On day 1 of each study period subjects were required to attend the research centre fasted at 0900h. Following baseline blood sampling the study drug was administered. A further blood sample was then taken at 3 hours after dosing. Subjects were then allowed home and continued to take the study drug for 7 days. (Figure 5.1a)

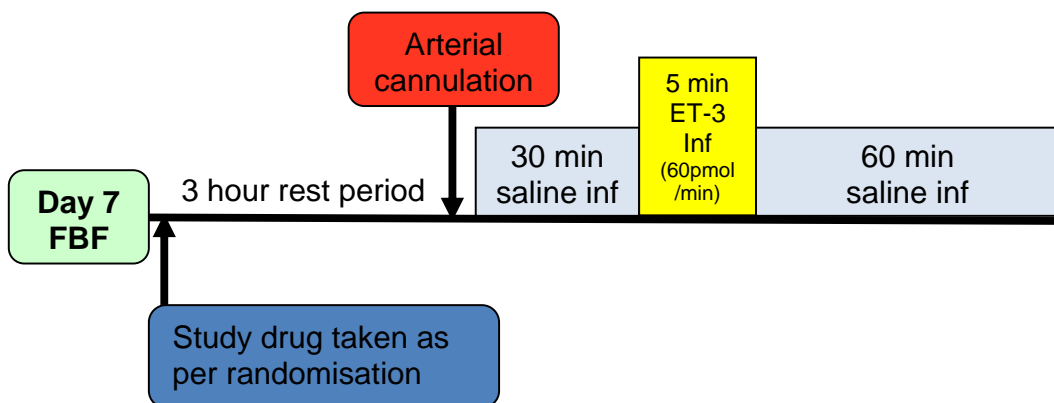
On day 7 patients attended the research centre at 0900h. Following baseline BP and plasma ET-1 sampling, the last dose of study drug was taken. Patients then rested for 3 hours prior to forearm blood flow measurements at peak plasma concentrations of the study drugs^{162, 219}. Following left brachial arterial cannulation saline was infused for 30 minutes during which two measurements of FBF were made (at -20 and -10 minutes). ET-3 was then infused via the brachial artery at 60 pmol/min for 5 minutes, followed by physiological saline for 60 minutes. Forearm blood flow was recorded from 3 minutes before to 10 minutes after the ET-3 infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes¹⁶¹. (Figure 5.1b)

Figure 5.1 Study design day 1 & 7

a.



b.



5.2.3 Statistical Analysis

Based on previous data¹⁶¹, it was calculated that for 10 subjects there was an 80% probability that the study will detect a treatment difference at a two sided 5% significance level, if the true difference in the ratio of flow between the infused and non-infused arms between the treatments is 14%. This was based on the assumption that the standard deviation of the difference in the response variables was 10%. I expected to see a difference between the bosentan arm vs. placebo and sitaxsentan arms of approximately 20%¹⁸⁶.

The co-primary end points were change from baseline of plasma ET-1 and maximal forearm vasodilatation to ET-3. Three comparisons of interest were pre-identified: placebo versus sitaxsentan, placebo versus bosentan, and sitaxsentan versus bosentan.

5.3 Results

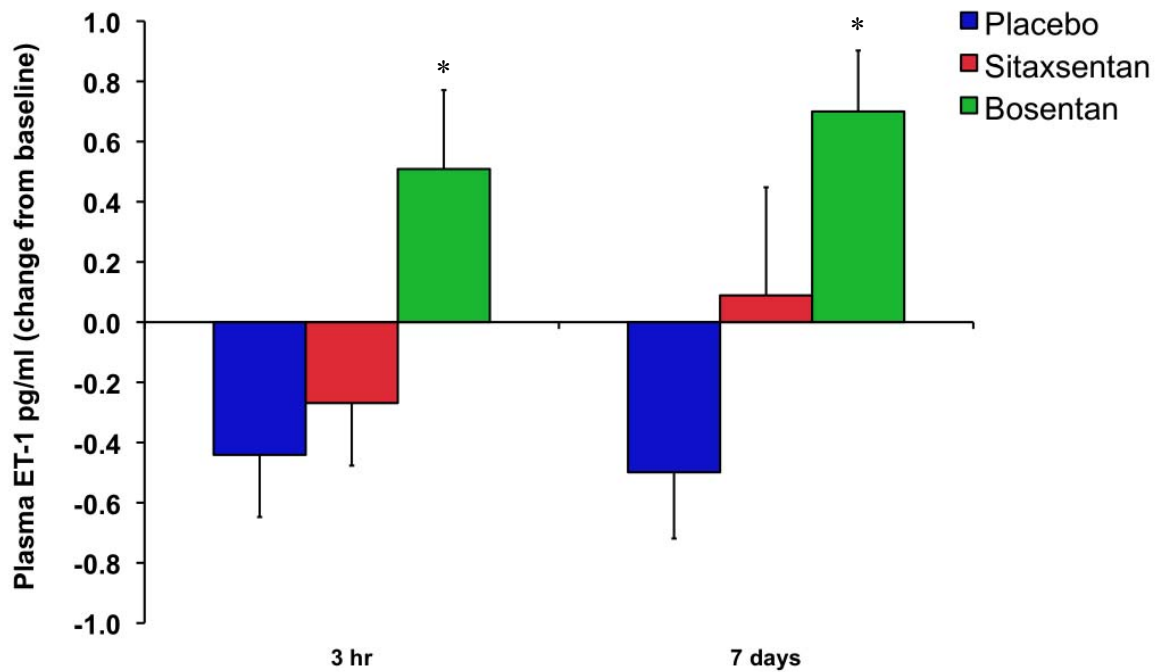
Ten healthy men with a mean age of 36 ± 16 years (range 20-66 years) were recruited and completed all 3 phases of the study. Three of the ten subjects were cigarette smokers. Overall subjects had a mean SBP of 121 ± 11 and diastolic BP of 68 ± 7 mmHg. Mean body mass index was 23 ± 2 kg/m². No adverse effects of treatment were reported.

5.3.1 Plasma ET-1

Baseline plasma ET-1 concentrations were not significantly different between study periods (placebo; 3.44 ± 0.27 pg/ml, sitaxsentan 3.42 ± 0.21 pg/ml, bosentan 2.92 ± 0.22 pg/ml). Following pre-treatment with either placebo or sitaxsentan there was no significant change in plasma ET-1 concentrations from baseline to 3 hours or on day 7. Following pre-treatment with bosentan, however, there was a trend to a rise in plasma ET-1 concentrations at 3 hours ($+0.51 \pm 0.26$ pg/ml $P=0.07$) and a significant increase at day 7 ($+0.70 \pm 0.20$ pg/ml; $P<0.005$). (Figure 5.2)

Figure 5.2 Plasma ET-1 concentrations

Values are given as absolute change from baseline \pm SEM. Bosentan vs. baseline * P <0.005.



5.3.2 FBF study

Baseline BP, heart rate and forearm blood flow were similar during the study days and there was no significant difference in basal FBF between infused and non-infused arms (Table 5.1). BP and heart rate, and FBF in the non-infused arm, did not significantly change after infusion of ET-3, confirming that the effects of ET-3 were confined to the infused arm (Table 5.1).

Table 5.1 Forearm blood flow haemodynamic dataValues are mean \pm SEM

Hemodynamic data	Placebo (n=10)	Sitaxsentan (n=10)	Bosentan (n=10)
MAP, mmHg			
Basal	83 \pm 2	83 \pm 3	81 \pm 2
60 min post ET-3	88 \pm 2	85 \pm 3	83 \pm 2
HR, BPM			
Basal	60 \pm 3	59 \pm 3	61 \pm 3
60 min post ET-3	60 \pm 3	65 \pm 3	62 \pm 4
Infused FBF, mL/100 mL/min			
Basal	2.3 \pm 0.3	2.7 \pm 0.4	2.4 \pm 0.2
3 min ET-3	3.2 \pm 0.4	3.3 \pm 0.5	2.6 \pm 0.3
60 min	2.9 \pm 0.4	3.1 \pm 0.5	2.6 \pm 0.4
Control FBF, mL/100 mL/min			
Basal	2.0 \pm 0.2	2.4 \pm 0.4	2.1 \pm 0.2
3 min ET-3	2.2 \pm 0.2	2.7 \pm 0.5	2.4 \pm 0.2
60 min	2.6 \pm 0.4	2.7 \pm 0.3	2.4 \pm 0.3

Following pre-treatment with placebo, intra-arterial infusion of ET-3 caused significant local vasodilatation with a maximal increase in FBF of $30\pm 6\%$ at 2 min ($P < 0.01$ vs. baseline). This dilatation persisted for 5 minutes. Similar results were seen following pre-treatment with sitaxsentan, with ET-3 causing a peak increase in FBF of $21\pm 11\%$ at 2 min ($P = 0.44$ vs. placebo). Pre-treatment with bosentan, however, abolished ET-3 induced vasodilatation, and was associated with a reduction of FBF at 2 min of $8\pm 3\%$ ($P < 0.01$ vs. placebo and sitaxsentan) (Figures 5.4 & 5.5).

Figure 5.3 Forearm blood flow (FBF) during the whole study (65min)

Shaded area indicates period of ET-3 infusion ($0.16\mu\text{g}/\text{min}$). Values are given as the ratio between infused and non-infused arm ($\% \pm \text{SEM}$)

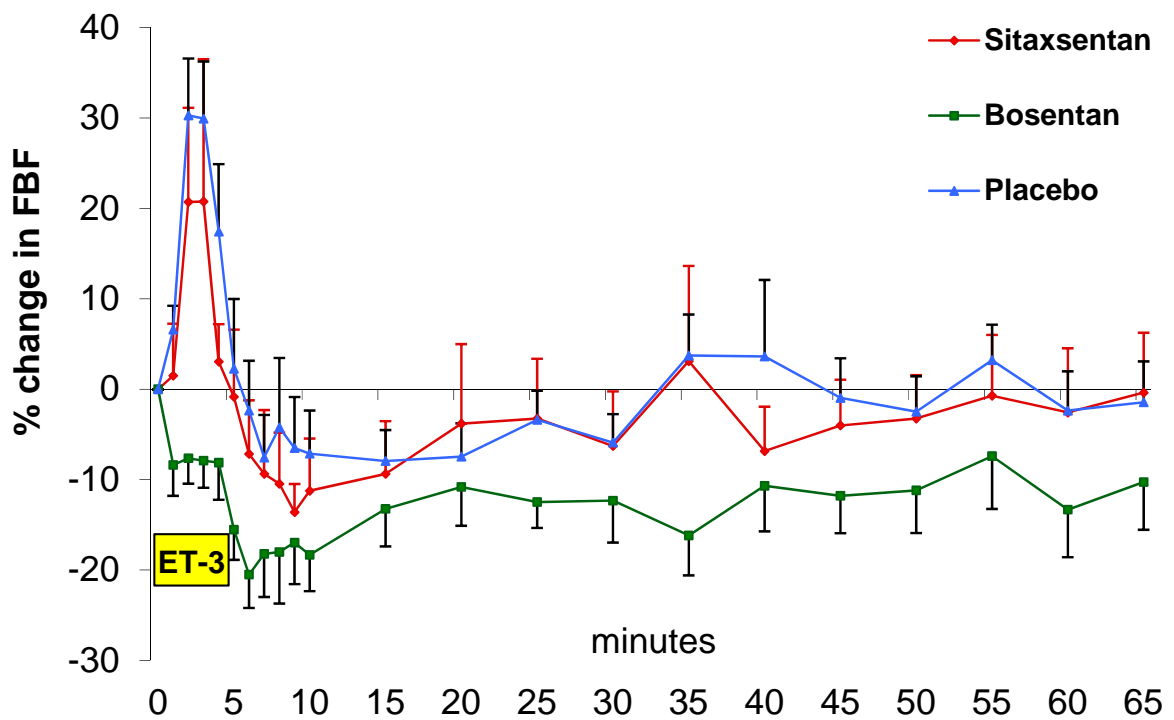
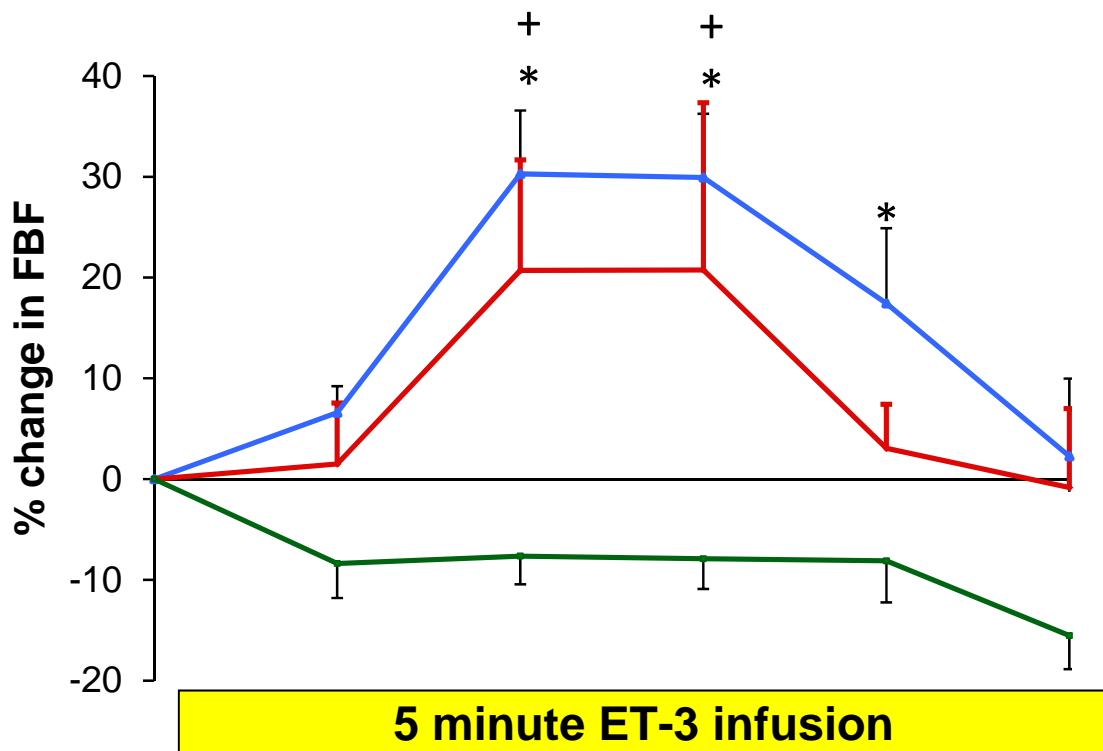


Figure 5.4 FBF expanded to show 5 min period of ET-3 infusion.

Values are given as the ratio between infused and non-infused arm (%) \pm SEM.

* P <0.005 bosentan vs. placebo; + P<0.05 bosentan vs. sitaxsentan (ANOVA plus Bonferroni correction for significance at specific time points).



5.4 Discussion

This study has demonstrated that 7-days treatment with bosentan, a mixed $ET_{A/B}$ receptor antagonist, increases plasma ET-1 concentrations and abolishes acute ET-3 mediated vasodilatation. Neither of these effects was seen with the selective ET_A receptor antagonist sitaxsentan, confirming that bosentan, but not sitaxsentan, causes functional ET_B blockade, at the standard clinically licensed doses, in healthy humans. This is the first study to show that selective and mixed antagonists differ in their

biochemical and functional effects in humans, which may be relevant to the benefits and harms of these drugs.

The ET_B receptor has an important role in clearing circulating ET-1. Thus, blockade of this receptor would be expected to cause a rise in plasma ET-1²¹⁸. Indeed ET-1 concentrations are elevated in animal ET_B receptor knockout models^{220, 221}. Furthermore, studies with both selective ET_B^{132, 222} and mixed ET_{A/B} receptor antagonists^{132, 223} in humans have shown that plasma ET-1 concentrations rise following their administration. In contrast, the highly selective ET_A receptor antagonists BQ-123, sitaxsentan and ZD4054 (ZD4054 has no measurable affinity for the ET_B receptor) do not increase ET-1 concentrations^{132, 224-226}. In the current study I have shown that plasma ET-1 concentrations increase significantly following bosentan, but not sitaxsentan, therapy in keeping with the important clearance role of the ET_B receptor. Accumulation of ET-1 may, theoretically, compete with the antagonist at the ET_A receptor making the agent less effective.

I use FBF response to ET-3 was used as a functional marker of ET_B activation following 7-day ETRA dosing. Activation of ET_B receptors with either ET-3¹⁶¹ or sarafotoxin S6c¹⁸⁶ (another selective ET_B agonist) cause brief vasodilatation which is likely to be, in large part related to NO generation in humans²¹⁵. Importantly the transient vasodilatation is abolished by BQ-788 (a highly selective ET_B receptor antagonist), confirming that this is likely to be an ET_B receptor-dependent effect¹⁸⁶, consistent with a multitude of studies in animals¹¹⁰. In keeping with previous studies¹⁶¹, I found that intra-arterial ET-3 infusion caused significant early forearm vasodilatation, during placebo treatment. A similar response was seen during treatment with sitaxsentan signifying that endothelial ET_B receptors were still functionally active in this group. The abolition of ET-3 induced vasodilatation during treatment with bosentan, however, demonstrates functional ET_B receptor blockade following this treatment. Interestingly, in the current study, following vasodilatation there was no significant vasoconstriction seen in any of the treatment arms. This is contrary to previous studies using ET-3¹⁶¹ and sarafotoxin S6c¹⁸⁶ and it is

not clear why this should be. Importantly, however, within the forearm it appears that vasoconstriction is likely to be primarily mediated via the ET_A receptor in health¹⁸⁶ and as such provides no further information on the function of the ET_B receptor.

To date, ETRA selectivity has been based solely on *in vitro* competitive receptor assays. However, different assays can produce greatly varying results depending on cell types and concentrations of drug used. For example the ET_A:ET_B ratios reported for ambrisentan vary widely, from 29:1 in rat aorta²²⁷ to 4000:1 in human myocardial membranes²²⁸. Furthermore, it is possible that “selective” ET_A receptor antagonists may cause functional ET_B receptor blockade if given at sufficiently high doses. Indeed, there are a number of “selective” ET_A antagonists that have been shown to increase circulating plasma ET-1 concentrations²²⁹⁻²³¹, suggesting that these compounds, at the doses used, may not be acting as functionally selective antagonists, with significant degrees of ET_B receptor blockade. These effects may be relevant to clinical outcomes. For example, I have shown that 7-day treatment with a mixed ETRA abolishes ET_B dependent vasodilatation, a pathway that may well have therapeutic benefit in a number of conditions including hypertension. It would also suggest that other beneficial actions of the ET_B receptor in hypertension such as natriuresis and diuresis may also be lost and would be in keeping with previous acute studies which shown that concomitant ET_B blockade impairs natriuresis and renal blood flow^{132, 232}. Conversely, mixed ET_{A/B} receptor antagonism may be required to block the secretagogue effect of ET-1 on aldosterone within the zona glomerulosa²³³. It is therefore important to be able to clearly classify ETRAs as selective or mixed based on *in vivo* rather than *in vitro* models prior to study.

Chapter 6: The effects of oral acute and chronic selective ET_A receptor antagonism on systemic and renal haemodynamics in CKD

Abstract

Background

It has previously been shown that acute intravenous ET_A but not acute ET_{A/B} antagonism lowers systemic BP and renal blood flow in patients with CKD. These effects are similar to those seen with blockade of the RAAS, and would suggest a degree of renal protection. I therefore investigated the effects of an oral ET_A antagonist on acute systemic and renal haemodynamics in a cohort of CKD patients.

Methods

In a randomized, double-blind, 3-way cross-over study, 13 patients were randomised to receive placebo, sitaxsentan 100mg or nifedipine LA 30mg given once daily for a period of 6 weeks. On day 1 and end of week 6 for each study block, patients underwent a renal clearance study where systemic and renal haemodynamics were measured.

Results

Sitaxsentan produced a significant drop in systemic vascular resistance but not BP. Sitaxsentan had no effect on renal blood flow or renal vascular resistance. However, EFF fell significantly by 6 weeks (20.8 ± 1.0 to 16.6 ± 0.7 ; $p < 0.01$). There was an associated fall in GFR over the same 6-week period (57 ± 8 to 48 ± 8 ; $p < 0.05$). Sodium handling by the kidney was not affected.

Conclusion

The significant drop in EFF and GFR following sitaxsentan therapy suggests that it cause preferential dilation of the afferent arterioles within the kidney. This suggests a similar effect to that of RAAS blockade.

6.1 Introduction

Goddard *et al.*¹³² have previously shown that acute ET_A but not acute ET_{A/B} antagonism lowers systemic BP and renal blood flow in patients with CKD. These effects were consistent with a renoprotective action. The aim of the current study was to evaluate whether Sitaxsentan, a selective ET_A antagonist that has been shown to have no functional ET_B blockade *in vivo* (see chapter 5), would have similar effects on systemic and renal haemodynamics in a CKD population.

6.2 Study design

6.2.1 Subjects

We enrolled 13 subjects with stable CKD stages I to IV²² and significant proteinuria (>300 mg/day). Subjects were already on maximally tolerated ACE inhibitor (ACE-I) and/or angiotensin receptor blocker (ARB) therapy to control BP and proteinuria. All medications were unchanged over the 3 months preceding the study.

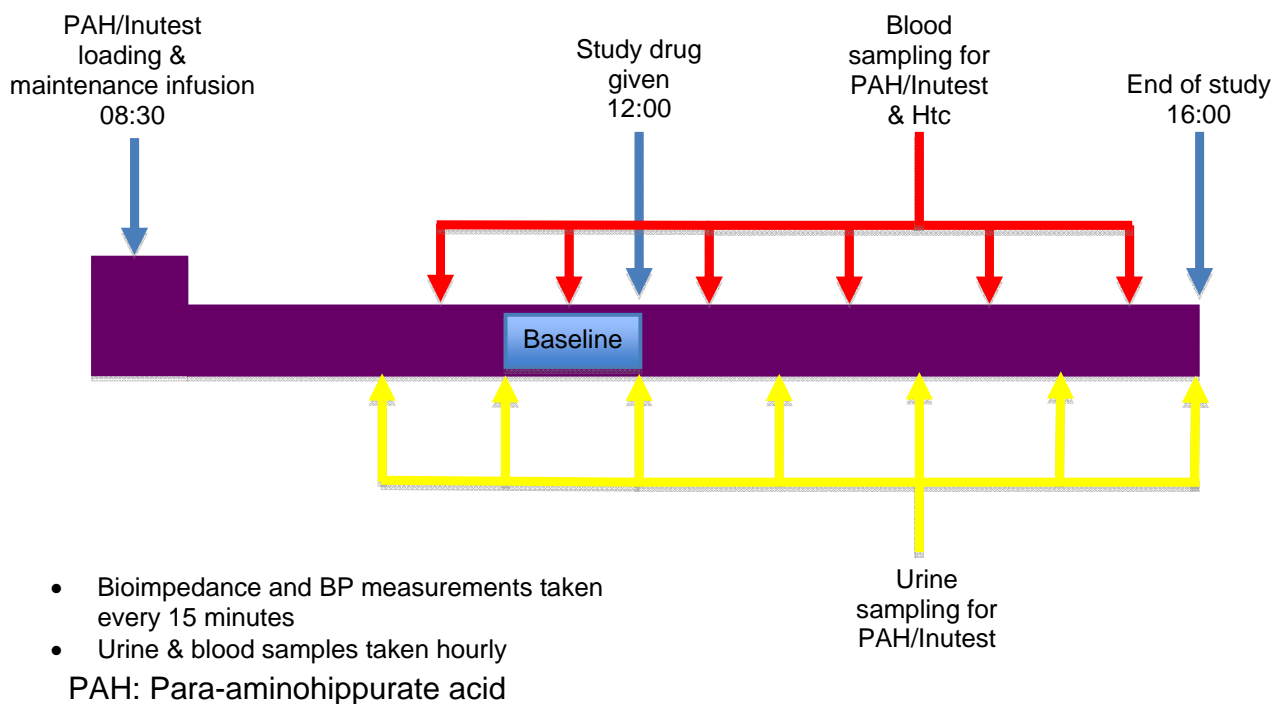
To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis or other systemic inflammatory renal disease, polycystic kidney disease or obstructive uropathy were excluded. Furthermore patients with significant co-morbidities, including diabetes mellitus, heart or lung disease, liver disease and peripheral vascular disease were excluded. As sitaxsentan is teratogenic women of childbearing potential were also excluded from taking part in the study.

6.2.2 Study protocol

This was a three-way, randomised placebo-controlled study. The study consisted of three 6-week treatment phases with placebo, sitaxsentan 100mg or nifedipine LA 30mg given once daily in addition to their regular medications. Each phase was separated by a minimum 14-day washout period.

Patients underwent standard clearance studies (see chapter 2) on day 1 and at the end of week 6 for each phase. Bioimpedance and BP measurements were taken every 15 minutes. Urine and blood samples were taken hourly. Study drug (sitaxsentan, nifedipine or placebo) was given following baseline measurements after a 2-hour period to allow equilibration of water, para-aminohippurate acid and Inutest. Following study drug ingestion measurements were taken for a total of 4-hours (Fig 6.1)

Figure 6.1 Clearance study day design



6.3 Results

Thirteen patients were enrolled in the study with all completing the 3 phases of the study. Baseline characteristics can be seen in table 6.1.

Table 6.1 Baseline characteristics

Values are given as mean of 3 baseline pre-treatment periods \pm SD. GFR: glomerular filtration rate; PCR: protein:creatinine ratio; ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker.

Study (n = 13)	
<i>Demographic</i>	
Age, y	46 \pm 13
Male sex (%)	12 (92)
Caucasian	13 (100)
<i>Clinical</i>	
Body mass index, kg/m ²	28.2 \pm 4.7
24h BP, mmHg	
Systolic	127 \pm 10
Diastolic	80 \pm 8
Mean	95 \pm 7
Creatinine, μ mol/l	152 \pm 67
Estimated GFR, ml/min/1.73m ²	55 \pm 26
Haemoglobin, g/l	132 \pm 16
Serum potassium, mmol/l	4.6 \pm 0.4
Cholesterol, mmol/l	4.4 \pm 1.0
<i>Urinary protein excretion</i>	
g/24h	2.01 \pm 1.6
PCR, mg/mmol	150 \pm 144
<i>Medications, n (%)</i>	
ACE inhibitor	10 (77)

ARB	3 (23)
ACE inhibitor + ARB	2 (15)
No ACE inhibitor or ARB	1 (8)
α blocker	1 (8)
β blocker	4 (31)
Calcium channel blocker	3 (23)
Diuretic	0 (0)
Statin	8 (62)

6.3.1 Systemic haemodynamics

After placebo correction there was a non-significant trend for BP reduction over the 4-hour clearance study period with both sitaxsentan and nifedipine (Figure 6.2). There was, however, a significant drop in SVRI of almost 20% in both nifedipine and sitaxsentan by the end of the study period (Figure 6.3)

6.3.2 Renal blood flow

There was no change in ERBF from day 0 to week 6 with placebo, sitaxsentan or nifedipine. GFR was comparable at day 0 and week 6 with placebo and nifedipine, however, sitaxsentan produced a substantial fall in GFR by week 6. Similarly EFF was unchanged between day 0 and week 6 with both placebo and nifedipine, however, EFF was lower with sitaxsentan. This was a consistent finding with 12 out of 13 subjects demonstrating a fall in EFF. 10 subjects had a EFF of >20% at baseline. These subjects showed a fall of >2% (range 2.1 – 8.9%) after 6 weeks' sitaxsentan treatment. The 3 subjects with a EFF <20% at baseline showed less impressive reductions in EFF following sitaxsentan dosing. All changes in renal haemodynamics had returned to baseline before starting the next phase of the study (minimum 2 weeks).

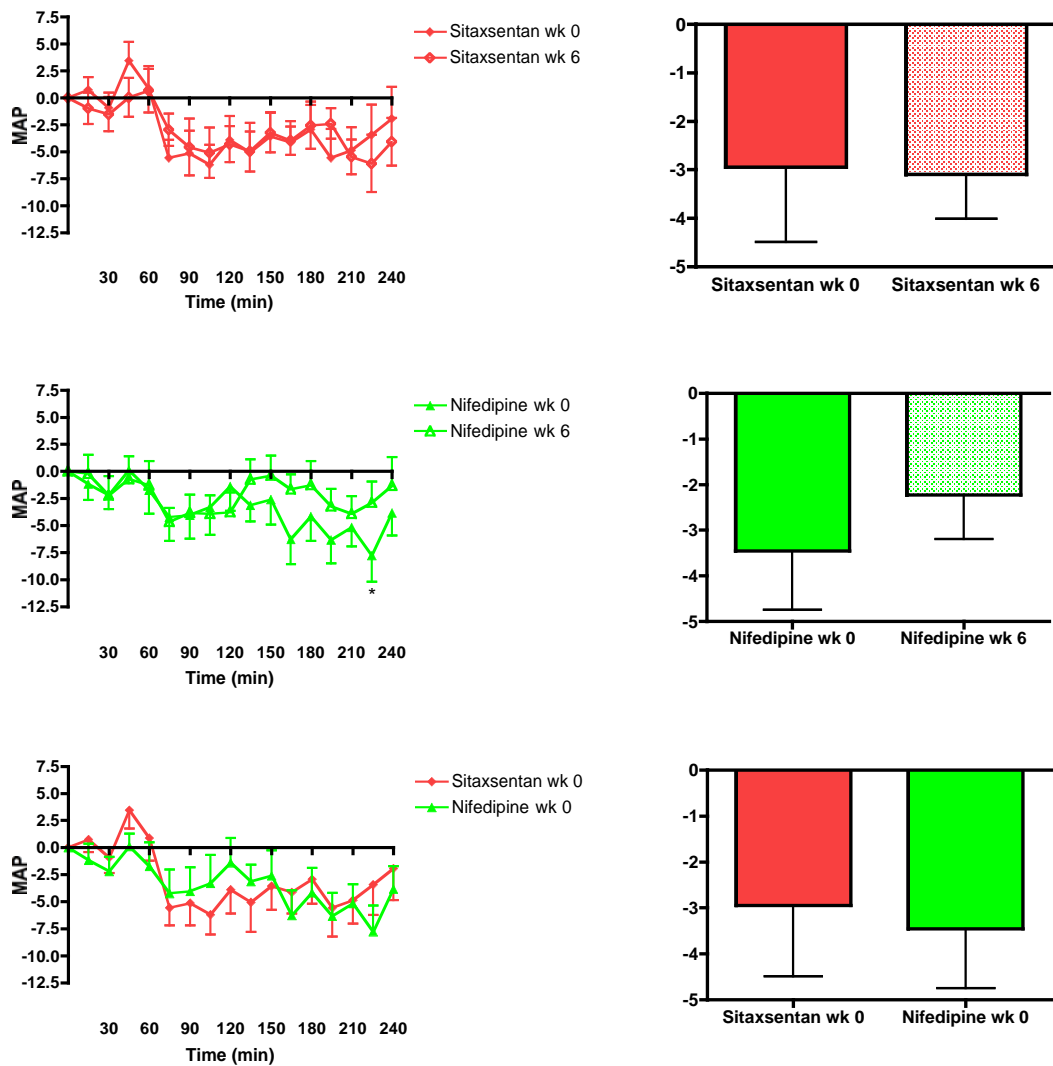
6.3.3 Natriuresis

There was no significant difference in sodium excretion between sitaxsentan and placebo over the 4-hour study period (Figure 6.6)

Figure 6.2 Mean Arterial Pressure during clearance studies

Data presented are placebo corrected % change from baseline and area under the curve.

* $p < 0.05$ vs. placebo



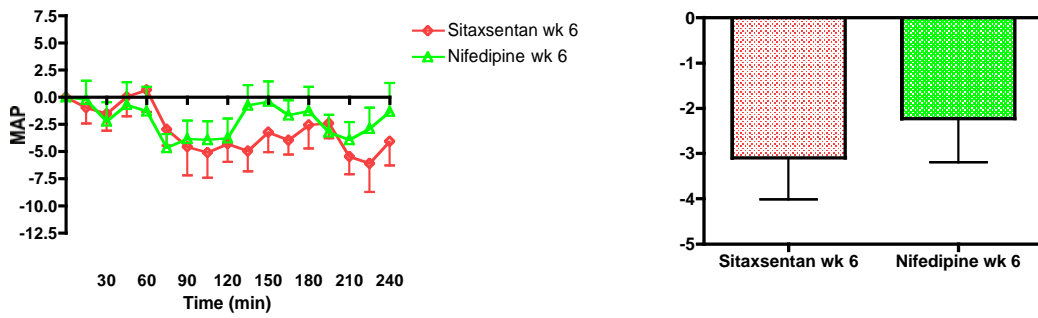
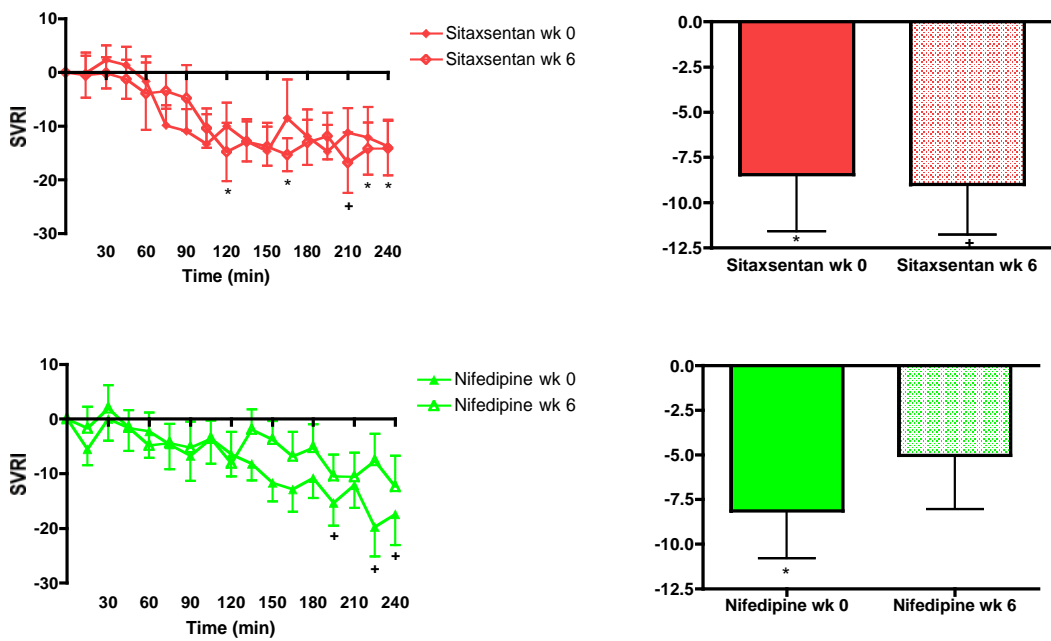


Figure 6.3 Systemic Vascular Resistance Index during clearance studies

Data presented are placebo corrected % change from baseline and area under the curve. * $p < 0.05$, + $p < 0.01$ vs. placebo



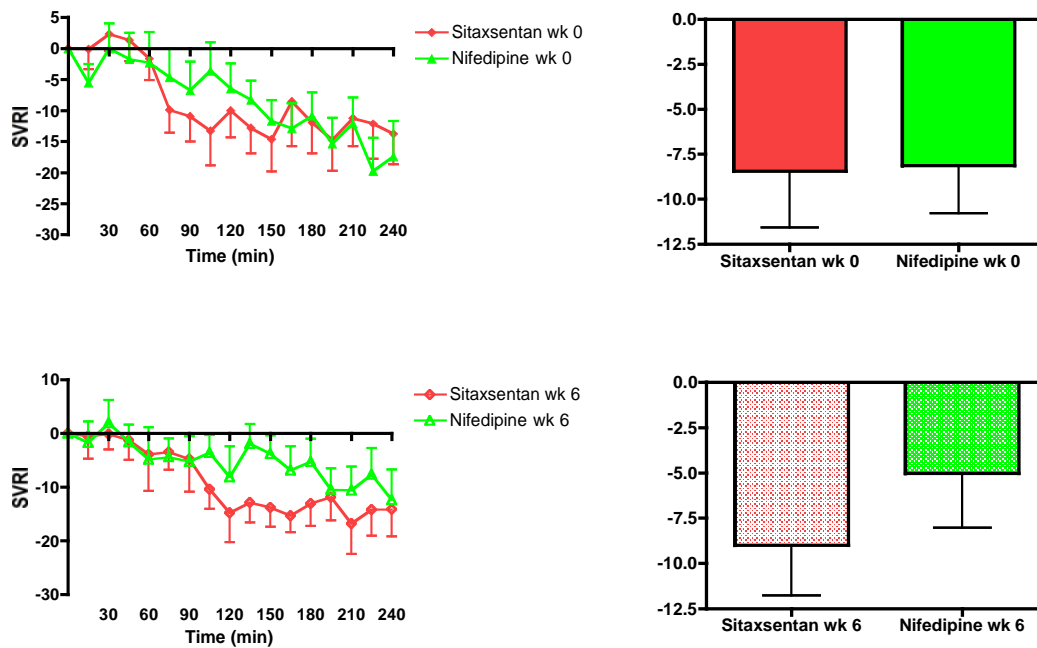


Table 6.2 Renal data from clearance studies at baseline and week 6

Values are given as pre-dosing baseline \pm SEM. GFR: glomerular filtration rate; ERBF: effective renal blood flow; ERVR: effective renal vascular resistance; EFF: effective filtration fraction. * $p < 0.01$ and + $p < 0.05$ for sitaxsentan at week 6 vs. sitaxsentan at baseline.

	Placebo Baseline	Week 6	Sitaxsentan Baseline	Week 6	Nifedipine Baseline	Week 6
GFR (ml/min)	56 \pm 7	54 \pm 8	57 \pm 8	48 \pm 8⁺	59 \pm 8	58 \pm 9
ERBF (ml/min)	533 \pm 66	552 \pm 65	511 \pm 63	543 \pm 73	562 \pm 82	530 \pm 72
ERVR (mmHg/min/l)	230 \pm 52	206 \pm 39	236 \pm 44	232 \pm 48	248 \pm 58	254 \pm 56
EFF (%)	19.1 \pm 1.1	17.9 \pm 1.3	20.8 \pm 1.0	16.6 \pm 0.7*	20.3 \pm 1.1	20.5 \pm 1.4

Figure 6.4 Effect of placebo, sitaxsentan and nifedipine LA 30mg on EFF.

Individual subject data are presented, as well as the mean \pm SEM at baseline and 6 weeks.

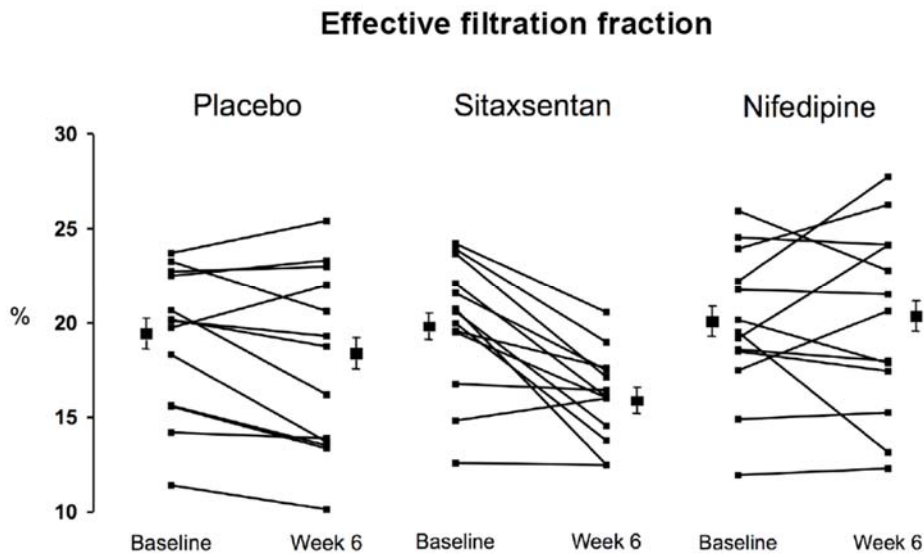
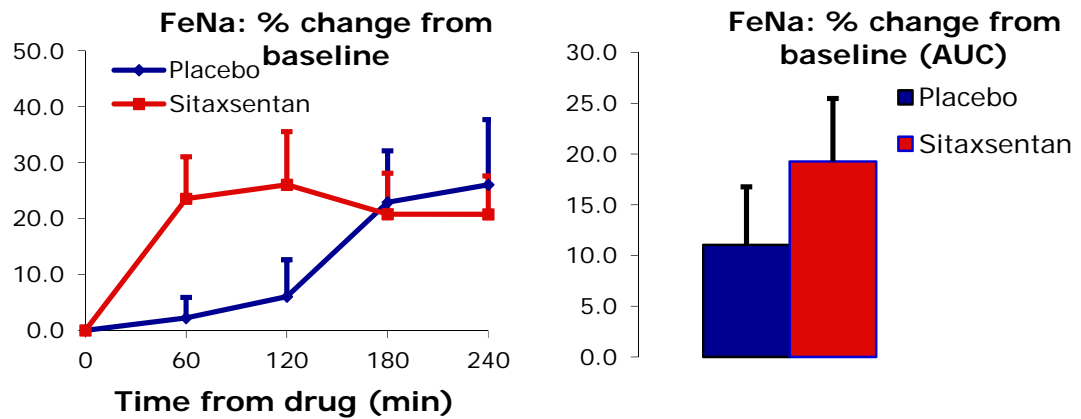


Figure 6.5 Effect of placebo, sitaxsentan on urinary sodium excretion

Data presented are % change from baseline and area under the curve



6.4 Discussion

This is the first study to examine the acute effects of an oral selective ET_A antagonist on systemic and renal haemodynamics in a cohort of patients with proteinuric CKD.

While there was no statistical change in BP over the 4-hour study period with sitaxsentan or nifedipine there was a clear trend towards a fall. There was however a significant drop in SVRI which would be in keeping with systemic vasodilatation and has been previously reported with both nifedipine²³⁴ and sitaxsentan¹⁵¹. These findings suggests that ET-1 contributes to the maintenance of vascular tone in patients with CKD via the ET_A receptor, and would be in keeping with previous studies^{132, 235, 236}. Interestingly, while the drop in SVRI was only observed acutely with nifedipine, sitaxsentan continued to have a significant effect on SVRI at 6 weeks.

In this study sitaxsentan had no effect on renal blood flow or renal vascular resistance. However, as in previous studies^{132, 235}, there was a very consistent fall in filtration fraction (−4%). This suggests that ET-1 induces an ET_A receptor-mediated preferential efferent arteriolar constriction. These effects are analogous to, and occur in addition to, those seen with RAAS blockade. This postulated reduction in efferent arteriolar tone with ET_A receptor antagonism should reduce glomerular perfusion pressure. This will result in a reduction in proteinuria with an associated short-term fall in GFR. Consistent with this proposed effect, we observed a significant fall in GFR (−9 mL/min) after 6 weeks of sitaxsentan treatment. In patients already prescribed blockers of the RAAS, these effects, despite an initial fall in GFR, should correlate with longer-term slowing of the rate of CKD progression.

Surprisingly, given the evidence for ET_B receptor-mediated natriuresis¹²⁶, no changes in sodium excretion or fractional excretion were observed in the present study and is in agreement with Goddard *et al.*¹³². Importantly, however, during ET_A receptor blockade, despite systemic vasodilatation and reduction in EFF, sodium retention did not occur, which is important if these drugs are to be prescribed safely to patients with CRF.

In conclusion, I have shown that the oral ET_A receptor antagonist sitaxsentan leads to system vasodilatation while reducing EFF patients with CKD. In addition, chronic dosing with sitaxsentan leads to a drop in GFR, which presumably relates to a fall in glomerular pressures. There was no evidence of acute sodium retention following oral dosing of this drug.

Chapter 7: The effects of selective chronic ET_A receptor antagonism on selected markers of renal and cardiovascular disease progression

Abstract

Background

Proteinuria, hypertension and AS are associated with progression of CKD and are also independent risk factors for CVD. Acute blockade of the ET_A receptor in patients with proteinuric renal disease has previously been shown to reduce proteinuria and improve BP and AS. This study was designed to investigate if these effects would be seen with chronic dosing of an oral ET_A antagonist

Methods

In a randomized, double-blind, 3-way cross-over study, 27 patients with proteinuric CKD on maximal tolerated RAAS blockade received 6 weeks of placebo, 100mg once daily sitaxsentan, and 30mg once daily of long acting nifedipine. Twenty-four-hour proteinuria, PCR, 24-hour ambulatory BP and PWV were measured at baseline and week 6 of each treatment.

Results

Sitaxsentan reduced 24-hour proteinuria (-0.61 ± 0.1 g/d; $P < 0.0001$), PCR (-42 ± 8 mg/mmol; $P < 0.0001$), MAP (-3.7 ± 1.0 mm Hg; $P = 0.004$), and PWV (-0.41 ± 0.16 m/s; $P = 0.003$). Although nifedipine matched BP and PWV when compared to sitaxsentan, it had no significant effect on proteinuria.

Conclusion

These results suggest that chronic dosing with a selective ET_A antagonist may provide additional renal and cardiovascular protection above that already offered by current therapy.

7.1 Introduction

Sitaxsentan a selective ET_A antagonist has previously been shown to have acute effects within the kidney that are similar to ACE-I, most notably reduction in EFF (See Chapter 6). The aim of this study was to evaluate what effect chronic oral dosing of sitaxsentan would have in a cohort of patients with non-diabetic proteinuric kidney disease, who were already on maximal tolerated ACE-I +/- ARB therapy. We hypothesised that chronic dosing of sitaxsentan would have favourable effects on proteinuria, systemic BP and AS.

7.2 Study design

7.2.1 Subjects

27 patients with stable CD stages I to IV²² and significant proteinuria (>300 mg/day) were enrolled. Subjects were already on maximally tolerated ACE inhibitor and/or ARB therapy to control BP and proteinuria. All medications were unchanged over the 3 months preceding the study.

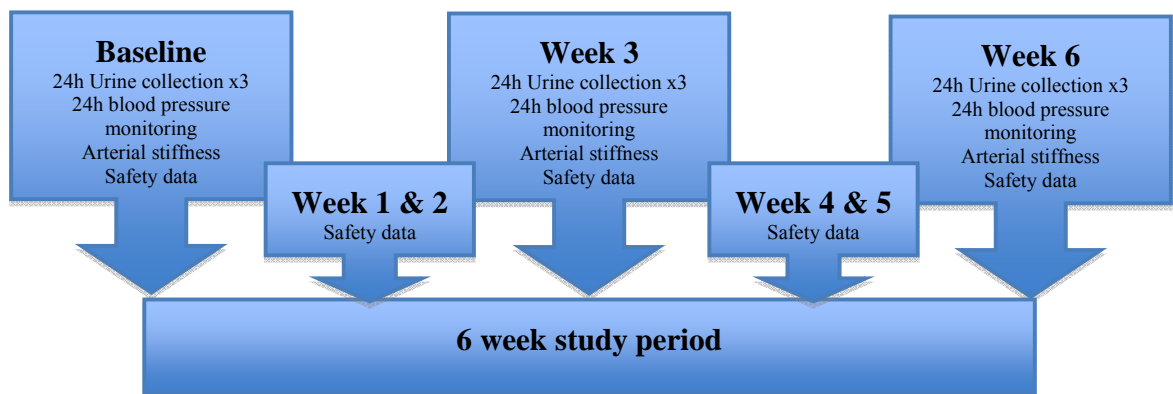
To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis or other systemic inflammatory renal disease, polycystic kidney disease or obstructive uropathy were excluded. Furthermore patients with significant comorbidities, including diabetes mellitus, heart or lung disease, liver disease and peripheral vascular disease were excluded. As sitaxsentan is teratogenic, women of childbearing potential were also excluded from taking part in the study.

7.2.2 Study protocol

This was a three-way, randomised placebo-controlled study. The study consisted of three 6-week treatment phases with placebo, sitaxsentan 100mg or nifedipine LA 30mg given once daily in addition to their regular medications. Each phase was separated by a minimum 14-day washout period.

Proteinuria, BP and AS were assessed at baseline, week 3 and week 6 of each study period (Fig 7.1). Proteinuria was assessed using both the mean 24-hour protein excretion and the mean PCR of 3 consecutive 24-hour urine collections. Ambulatory BP was measured at the brachial artery with measurements taken every 30 minutes for a 24-hour period. Measurements of AS (PWV and augmentation index) were measured as described in Chapter 2. Safety data: “office BP”, weight, haemoglobin, haematocrit, liver enzymes, serum potassium and adverse effects were recorded at baseline and at weeks 1, 2, 3, 4, 5 and 6.

Figure 7.1 Study period design



7.2.3 Statistical analysis

The primary end point of this study was change in proteinuria at 6 weeks. Secondary end points were change in systemic BP and AS. Using data from a previous study it was calculated that a minimum of 24 patients would be required for the study to have 80% power to detect a reduction in proteinuria of 0.7g/d²³⁷.

7.3 Results

Of the 27 patients recruited, all completed all the 3 phases of the study. Baseline characteristics can be seen in table 7.1

Table 7.1 Baseline characteristics

Values are given as mean of 3 baseline pre-treatment periods \pm SD. GFR: glomerular filtration rate; PCR: protein:creatinine ratio; ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker.

	Study n = 27 (%)
<i>Demographic</i>	
Age, y	48 \pm 12
Male sex (%)	23 (85)
Caucasian (%)	27 (100)
<i>Clinical</i>	
Body mass index, kg/m ²	29.3 \pm 4.6
24h BP, mmHg	
Systolic	125 \pm 12
Diastolic	78 \pm 7
Mean	94 \pm 78
Creatinine, μ mol/l	153 \pm 75
Estimated GFR, ml/min/1.73m ²	54 \pm 26
Haemoglobin, g/l	136 \pm 18
Serum potassium, mmol/l	4.6 \pm 0.4

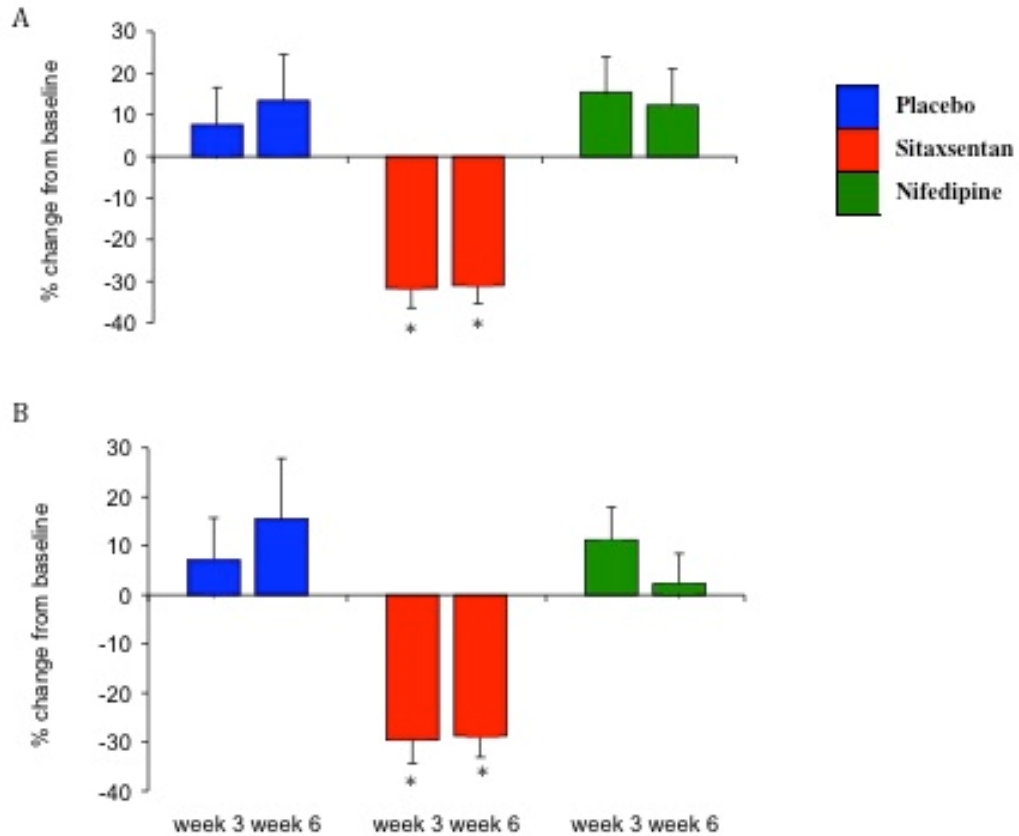
Cholesterol, mmol/l	4.6 ± 0.8
<i>Urinary protein excretion</i>	
g/24h	2.03 ± 1.7
PCR, mg/mmol	156 ± 143
<i>Arterial stiffness</i>	
PWV, m/s	8.3 ± 2.4
cAix, %	28 ± 12
<i>Medications, n (%)</i>	
ACE inhibitor	18 (67)
ARB	11 (41)
ACE inhibitor + ARB	5 (19)
No ACE inhibitor or ARB	3 (11)
α blocker	6 (22)
β blocker	8 (30)
Calcium channel blocker	3 (11)
Diuretic	2 (7)
Statin	18 (67)

7.3.1 Proteinuria

Following 6 weeks' dosing there was no change in proteinuria, as measured by 24-hour protein excretion or PCR, in the placebo or nifedipine arms. Sitaxsentan, however, significantly reduced proteinuria (Fig 7.2). The observed means (± SEM) for 24-hour proteinuria were 2.07 ± 0.34 g/d at baseline and 1.46 ± 0.26 g/d at 6 weeks ($P < 0.0001$). For PCR these were 156 ± 28 and 114 ± 23 mg/mmol ($P < 0.0001$).

Figure 7.2 Effects of placebo, sitaxsentan and nifedipine on proteinuria

(A) 24-hour proteinuria and (B) PCR. Values are given as mean % change from baseline \pm SEM at week 3 and week 6. * $p < 0.001$ for sitaxsentan vs. both placebo and nifedipine



7.3.2 Mean 24-hour ambulatory blood pressure

In the placebo phase there was no significant change in BP as measured by MAP, systolic and diastolic BP. Sitaxsentan significantly reduced all 3 parameters by ~ 4 mm Hg when compared to baseline, this effect was seen by week 3. After 6 weeks of dosing there were no differences in reduction from baseline BP between sitaxsentan and nifedipine LA. Systolic BP was reduced by -3.6 ± 1.5 versus -4.9 ± 1.6 mm Hg, diastolic BP by -3.6 ± 1.0 versus -3.2 ± 1.0 mm Hg and MAP by -3.7 ± 1.0 versus -3.8 ± 1.1 mm Hg (mean \pm SEM for sitaxsentan and nifedipine respectively).

7.3.3 Diurnal variation in systolic BP (SBP)

At baseline, for all 3 phases of the study there was a nocturnal dip in SBP that did not differ between treatment arms: placebo $-5.2 \pm 5.3\%$, sitaxsentan $-5.4 \pm 4.6\%$, nifedipine $-5.7 \pm 5.0\%$ (ANOVA $p = 0.88$). Following 6 weeks' treatment this nocturnal dip persisted in all 3 treatment arms: placebo $-4.8 \pm 7.3\%$, sitaxsentan $-8.8 \pm 5.9\%$, nifedipine $-4.6 \pm 4.9\%$. However, whereas placebo and nifedipine did not change the degree of nocturnal dip in SBP between baseline and week 6, the dip was greater following treatment with sitaxsentan (Figure 7.4), baseline *vs.* week 6: placebo -6.8 ± 7.5 *vs.* -6.5 ± 9.8 mmHg, $p = 0.88$; sitaxsentan -7.0 ± 6.2 *vs.* -11.0 ± 7.8 mmHg, $p < 0.05$; nifedipine -7.5 ± 6.9 *vs.* -6.0 ± 6.4 mmHg, $p = 0.27$.

7.3.4 Diurnal variation in diastolic BP (DBP)

As for SBP, there was a nocturnal dip in DBP at baseline that did not differ between treatment arms: placebo $-7.6 \pm 6.4\%$, sitaxsentan $-7.4 \pm 4.2\%$, nifedipine $-8.3 \pm 5.1\%$ (ANOVA $p = 0.73$). Following 6 weeks' treatment this nocturnal dip in DBP persisted in all 3 treatment arms: placebo $-7.2 \pm 5.7\%$, sitaxsentan $-10.4 \pm 5.9\%$, nifedipine $-7.0 \pm 5.1\%$. However, similar to SBP, whereas placebo and nifedipine did not change the degree of nocturnal dip in DBP between baseline and week 6, sitaxsentan increased the dip (Figure 7.4), baseline *vs.* week 6: placebo -6.3 ± 5.6 *vs.* -6.0 ± 4.9 mmHg, $p = 0.83$; sitaxsentan -6.0 ± 3.6 *vs.* -8.3 ± 5.1 mmHg, $p < 0.05$; nifedipine -6.9 ± 4.4 *vs.* -5.7 ± 4.1 mmHg, $p = 0.08$.

7.3.5 Diurnal variation in pulse pressure (PP)

There was no significant nocturnal dip in PP at baseline in the 3 phases of the study. Although this remained the case following 6 weeks of placebo and nifedipine, treatment with sitaxsentan was associated with the development of a nocturnal dip in PP, day *vs.* night: day *vs.* night: 46 ± 7 *vs.* 43 ± 7 mmHg, $p < 0.01$.

7.3.6 Arterial Stiffness

Placebo had no significant effect on PWV or cAIx over the 6-week study period, whereas, sitaxsentan reduced both by study end. PWV fell by 4.8% compared to baseline a difference of ~ 9% when compared to placebo ($P < 0.001$ Figure 7.5) and cAIx fell by 5.3% compared to baseline a difference of ~5% when compared to placebo ($P = 0.001$). Although nifedipine caused a similar fall in PWV when compared to sitaxsentan (-0.4 ± 0.2 versus -0.4 ± 0.2 m/s; $P > 0.05$), only sitaxsentan reduced cAIx after 6 weeks of dosing.

7.3.7 Plasma ET-1

Plasma ET-1 levels were unchanged throughout the study. In particular there was no change between baseline and 6 weeks of sitaxsentan therapy (3.6 ± 0.5 versus 3.7 ± 0.5 pg / ml)

7.3.8 Adverse events

There were no significant differences in adverse events between the 3 groups (see table 7.2). In particular, there was no evidence of significant fluid retention as measured by weight gain or fall in haemoglobin or haematocrit.

Figure 7.3 Effects of placebo, sitaxsentan and nifedipine LA 30mg on 24h BP

(A) mean arterial pressure, (B) systolic blood pressure, and (C) diastolic blood pressure. * $P < 0.01$ and + $P < 0.05$ for sitaxsentan and nifedipine vs. placebo.

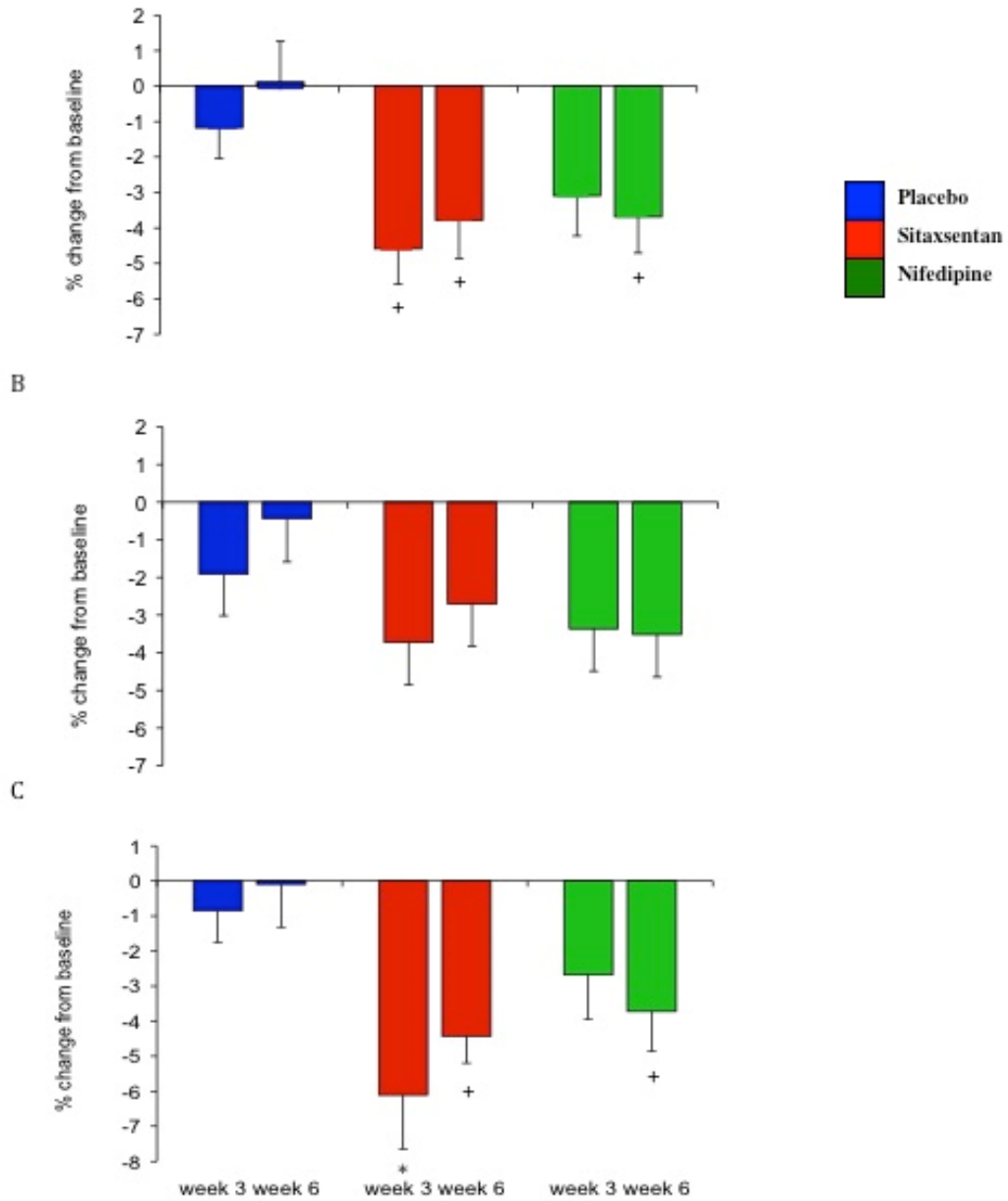


Figure 7.4 Nocturnal dip systolic and diastolic BP and pulse pressure

Baseline and following 6 weeks' treatment with placebo, sitaxsentan and nifedipine. Comparisons are for week 0 vs. week 6.

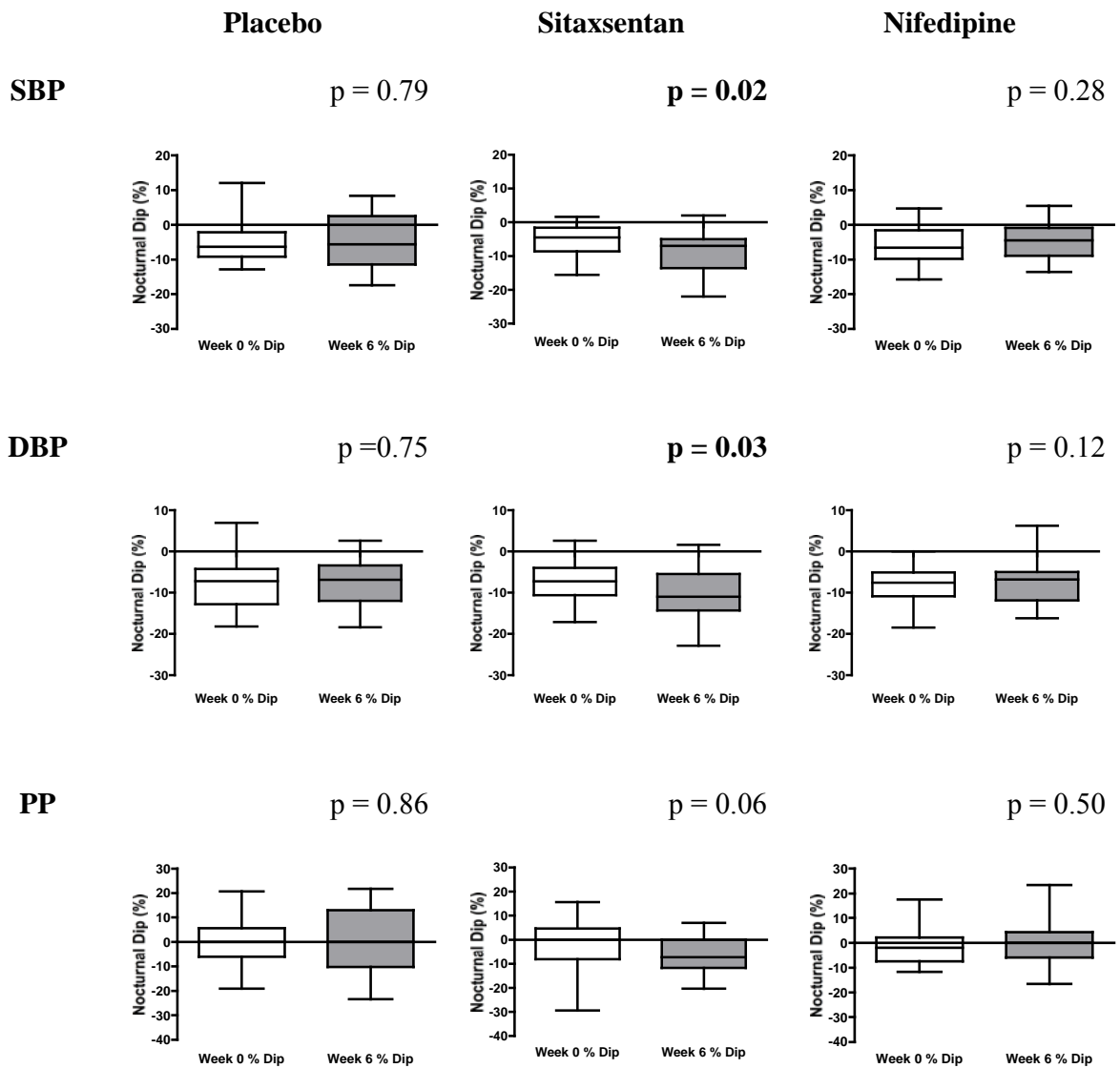


Figure 7.5 Effects of placebo, sitaxsentan and nifedipine LA 30mg on AS

(A) pulse wave velocity, and (B) central augmentation index.
 For (A), * $P < 0.01$ and + $P < 0.05$ for sitaxsentan and nifedipine vs. placebo.
 For (B), + $P < 0.05$ for sitaxsentan vs. both placebo and nifedipine.

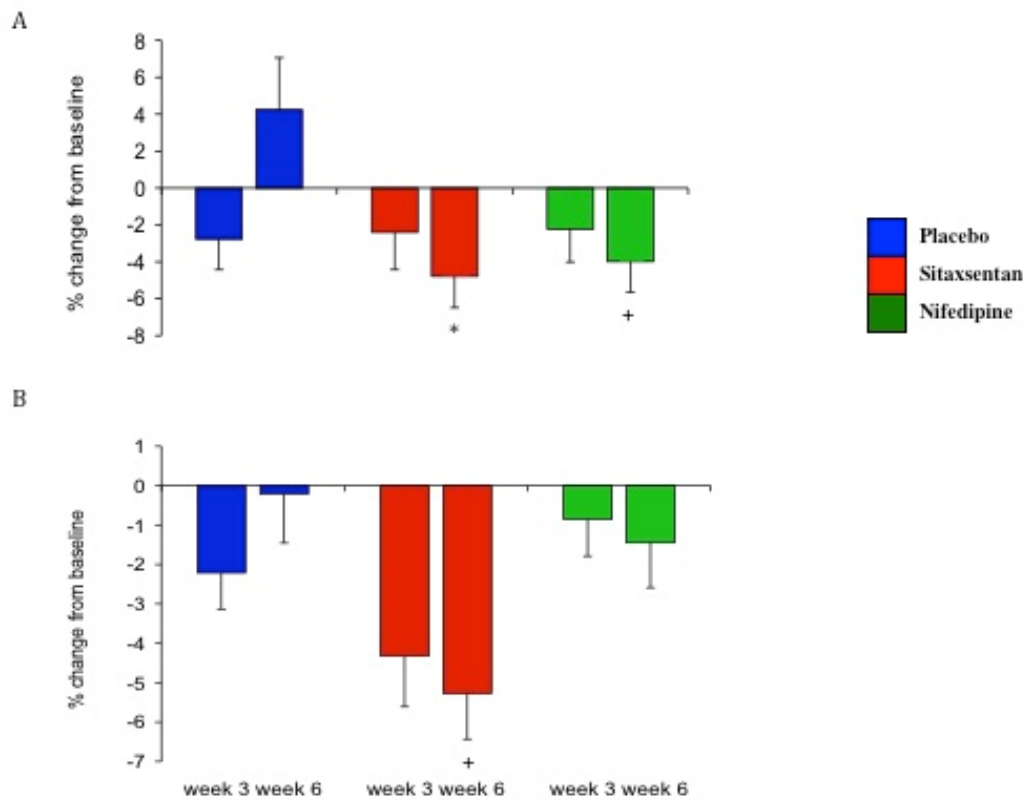


Table 7.2 Adverse events reported

	Placebo (n = 27)	Sitaxsentan (n = 27)	Nifedipine (n = 27)
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Adverse events, n	27	15	32
Subjects with adverse events, n (%)	21 (78)	13 (48)	18 (67)
Any serious adverse events, n (%)	0 (0)	0 (0)	0 (0)
Discontinuation due to adverse events, n (%)	0 (0)	0 (0)	0 (0)
<i>Adverse events reported >5%, n (%)</i>			
Headache	12 (48)	3 (11)	10 (37)
Nasal congestion	2 (7)	1 (4)	2 (7)
Flushing	0 (0)	1 (4)	2 (7)
Diarrhoea	2 (7)	1 (4)	0 (0)
Nausea & Vomiting	2 (7)	0 (0)	0 (0)
Back pain	2 (7)	0 (0)	2 (7)
Dizziness	2 (7)	1 (4)	1 (4)

7.4 Discussion

Previous studies have suggested that acute selective ET_A antagonism lowers BP and proteinuria and improves renal blood flow^{132, 237}. These beneficial changes in renal haemodynamics were abolished with concomitant ET_B antagonism¹³². Furthermore these effects appear to be synergistic with RAAS blockade suggesting that ET_A antagonism has a potential additional renoprotective role^{232, 238}. Indeed, our department has shown

that proteinuria can be reduced by an additional ~30% with acute ET_A antagonism in patients on maximal RAAS blockade²³⁸.

This study has demonstrated that the acute effects of ET_A antagonism on proteinuria BP and AS are sustained with chronic dosing of an oral ET_A antagonist. Again, these effects were seen in patients already receiving optimal ACE-I/ARB therapy. This would suggest a possible role for ET_A receptor antagonists in the treatment of proteinuric renal disease.

Reduction of proteinuria is seen as important in both reducing the risk of CKD and associated CVD²³⁹. Current management of proteinuria revolves around good BP control and blockade of the RAAS. However, despite maximal therapy, many patients continue to have ongoing significant proteinuria²⁴⁰. The cohort of patients from this study, despite being on maximal tolerated RAAS blockade, had ongoing significant proteinuria at ~2 g/d (range 0.3 to 7.8 g/d). The results of this study show that chronic ET_A antagonism is a novel therapeutic approach for further reducing proteinuria.

The effects of sitaxsentan on proteinuria in this study are, at least in part, independent of changes in systemic BP and are likely do to changes in renal haemodynamics (See chapter 6). Furthermore, despite nifedipine achieving similar BP effects to sitaxsentan, only sitaxsentan significantly reduced proteinuria. These findings are in keeping with a subsequent study using atrasentan (another selective ET_A antagonist), which showed that chronic selective ET_A antagonism in proteinuric kidney disease has additional beneficial effects on proteinuria when used in conjunction with RAAS blockade²⁴¹.

The current study confirms that ET_A antagonism reduces BP in CKD. In the current study sitaxsentan caused a modest reduction in MAP (~4 mm Hg). It may be that this effect would have been more impressive had the subjects not had such good BP control. Previous studies in hypertensive patients suggest that chronic ET_A antagonism is effective in reducing BP.^{138, 242} Furthermore the recent study by Andress *et al.* using atrasentan in diabetic nephropathy showed a mean fall in SBP of ~10 mmHg, though

these patients had much poorer BP control at baseline with a mean SBP of ~137 mm Hg²⁴¹.

In health, BP shows a diurnal variation with a nocturnal dip of ~10-20% (commonly known as a ‘dipping’ BP profile)^{243, 244}. Non-dipping BP (often defined as a fall in nocturnal BP of <10%²⁴⁴) is associated with an increased risk of CVD, so BP dipping is thought to confer benefit.²⁴⁵ A few studies have examined the diurnal variation of BP in CKD²⁴⁶⁻²⁴⁸. They suggest that not only is non-dipping a feature of CKD but also, as GFR declines, reverse dipping (nighttime BP readings that are higher than those during the day) becomes more apparent. Importantly, loss of nocturnal dipping is associated with CKD progression²⁴⁹.

The results of this study show that 6 weeks’ treatment with a selective ET_A receptor antagonist increased the nocturnal dip in both SBP and DBP in patients with CKD. Sitaxsentan also allowed the development of a nighttime dip in pulse pressure. A higher 24h ambulatory pulse pressure, along with a loss of nocturnal BP dip, has been shown to be independent predictors of nephropathy progression in patients with type 2 diabetes²⁵⁰. Furthermore, a high pulse pressure is associated with brain²⁵¹ and kidney²⁵² damage. AS is a major determinant of pulse pressure and it may be that the beneficial effects of sitaxsentan on PWV partly explain the effects on pulse pressure.

Sitaxsentan has a significant effect on AS, as measured by PWV and cAix, when compared to placebo in this study. These effects are most likely to be related to BP change rather than direct effects on endothelial function, supported by similar PWV results in the nifedipine arm of the study.

There have been observations in previous studies of significant and occasionally life-threatening fluid retention in subjects receiving ET antagonists²⁵³. The ASCEND study examining avosentan in diabetic nephropathy is the most notable, having to be terminated prematurely due to greater serious adverse cardiovascular events in the

avosentan groups, including a 3-fold increase in episodes of acute congestive heart failure²⁵⁴. It has been postulated that the cause of excess fluid retention could be secondary to ET_B blockade, altering sodium and water homeostasis¹²⁶. In the current study there was no observed weight gain, clinically significant oedema, or fall in haemoglobin or haematocrit. Interestingly, plasma ET-1 levels were unaltered throughout the study, which would suggest that there was no functional blockade of the ET_B receptor (see also chapter 4) affecting ET-1 clearance. I have previously shown that this dose of sitaxsentan does not block ET-3 mediated endothelium-dependent vasodilatation, whereas bosentan does.

In summary, the current study suggests that selective ET_A antagonism is a novel therapeutic target in patients with CKD already on maximally tolerated RAAS blockade. Reduction of BP may depend on the initial level of BP, whereas effects on proteinuria appear to be BP-independent. Further larger trials are warranted to assess the efficacy and safety of this class of drug.

Chapter 8: Conclusions

The studies presented in this thesis have examined cardiovascular risk in CKD and how this risk may be modified by antagonism of the ET_A receptor. These studies support a potential clinical role for ET antagonists, particularly in patients with non-diabetic proteinuric CKD.

Cardiovascular risk factors in a cohort with inflammatory renal disease

The studies presented in chapter 3 and 4 provide further information regarding cardiovascular risk in patients with renal disease. I have shown in a cohort of patients with acute inflammatory renal disease that renal impairment is likely to have a significant impact on AS and a far greater impact than inflammation or classic CVD risk factors. Interestingly, however, once remission is achieved it is the classic CVD risk factors which become the most important predictors of AS and endothelial dysfunction. These observations would suggest that every effort should be made to achieve remission quickly to protect and preserve renal function in this cohort, and that BP and lipid management should form the basis of CVD primary prevention.

Functional blockade of ET receptors

The study presented in chapter 4 supports the theory that bosentan, but not sitaxsentan, causes functionally important blockade of the ET_B receptor. Prior to this study, selectivity of ET_A antagonists was based on *in vitro* competitive receptor assays with little known of the functional effects of these drugs *in vivo*, and particularly in the target species, man. This study has, for the first time, provided evidence that a highly selective (6,500-fold ET_A/ET_B) ET_A antagonist, namely sitaxsentan, does not cause functional ET_B antagonism. This work is consistent with that of Maguire *et al.* showing, when moving from selectivity in cloned receptors to tissues, that selective inhibitors, like sitaxsentan, gain selectivity where as less selective agents, like bosentan, lose any selectivity they have shown²⁵⁵.

The method described here could be used to investigate the selectivity of other ETRAs. Indeed, the failure of previous trials such as the ASCEND²⁵⁴ trial may well in part be down to lack of ET_A receptor specificity at the doses used. A recent editorial in the *Journal of the American Society of Nephrology* also made the point that a good understanding of the pharmacodynamics of ETRAs remains elusive, particularly the mechanisms behind adverse events and suggest further research is required in this area²⁵⁶. Specific knowledge of the *in vivo* selectivity of these drugs would significantly help achieve this goal, and would be a valuable addition to the clinical investigation of new ET_A selective drugs.

Acute and chronic ET_A antagonism on renal and systemic haemodynamics

The study presented in chapter 6 is the first study to examine the systemic and renal haemodynamic effects of an oral ETRA. It confirmed that ET-1 acting through the ET_A receptor plays an important role in systemic and renal vascular tone in patients with CKD. Acute dosing of the oral ET_A antagonist, sitaxsentan, caused a sustained drop in vascular resistance over the 4-hour study period. Within the kidney there was a reduction in EFF (~4%), but no change in renal blood flow. After 6 weeks, sitaxsentan was still exerting its systemic and renal effects. The significant reduction in GFR (9/ml/min) over the 6-week study period would be in keeping with reduced efferent arteriolar tone with subsequent fall in glomerular pressures and filtration rate, an effect similar to RAAS blockade and one which is thought to be renoprotective²⁵⁷. Interestingly, as these patients were already receiving maximal tolerated RAAS blockade these effects on renal haemodynamics are of more interest and suggest that ETRAs could be used in conjunction with RAAS blockade to reduced glomerular pressure and in turn slow renal progression.

Importantly, despite a fall in BP, vascular resistance and GFR over the 6-week dosing period there was no evidence of impaired salt handling by the kidney, suggesting that

there was no significant ET_B receptor blockade and that, at least in this relatively healthy group of CKD patients, complications secondary to salt and water retention are unlikely.

Effect of chronic ET_A antagonism on markers of renal and cardiovascular disease progression

The study described in chapter 6 suggests that selective ET_A antagonism may have a potential therapeutic role in slowing renal disease progression and reducing cardiovascular risk, namely through a reduction in BP and proteinuria.

Hypertension is common in the CKD population and is associated with an increased risk in cardiovascular disease and renal disease progression. Conversely, reducing BP reduces cardiovascular mortality²⁵⁸ and slows the rate in decline of eGFR³⁵, particularly in patients with proteinuric CKD. Unfortunately, though hypertension can be difficult to treat in this population and despite treatment with multiple antihypertensive agents the majority of CKD patients fail to reach target BP²⁵⁹. Proteinuria is a common feature of CKD and its presence is independently associated with an adverse renal outcome³⁹. Current management revolves around BP reduction, primarily via blockade of the RAAS with either ACE-I or ARB²⁶⁰. Unfortunately, however, many CKD patients have significant residual proteinuria despite optimal treatment²⁶¹. The study in chapter 7 showed that ET_A antagonism can further lower BP and proteinuria in this population and that this effect is sustained over a 6-week period. These findings were in keeping with those of Chapter 6 and suggest that one of the major benefits of this treatment is its direct effect on glomerular pressures. More importantly, these effects were observed in a group of patients who were already receiving maximal therapy with an ACE-I and/or ARB and had reasonable BP control. This would suggest that ETRA therapy could offer a novel approach to improve BP and proteinuria in CKD patients, which, in theory, may slow renal disease progression and reduce cardiovascular risk.

Safety of chronic ET_A antagonism in the CKD population

The safety of ET_{RA}s remains of paramount concern, particularly the risk of salt and water retention, which has been observed with mixed and “selective” ET_{RA}s²⁶²⁻²⁶⁴. Indeed, fluid retention has led to the premature termination of clinical trials and has been blamed, in large part, for the neutral/negative results in heart failure studies²⁶⁵. In the studies described within this thesis, there was no evidence of fluid retention as measured by weight gain or drop in haematocrit, with the treatment being well tolerated by all participants. It may be that previous issues relating to fluid retention are due to blockade of the ET_B receptor and would explain why it was not observed in the studies described here. Importantly though, to date sitaxsentan is the only ET_{RA} that has been shown to have no functional effects on the ET_B receptor (see chapter 5). However, ours were small studies, not in diabetic nephropathy, and in patients selected to avoid associated CVD, so it may be unsafe to extrapolate too far.

In our cohort of patients we saw no abnormalities of liver function with sitaxsentan. However, shortly after the completion of these studies it became clear that there was a small but significant risk of liver failure. In total, 7 patients with severe liver toxicity have been described, of whom 2 died, 1 underwent heart/liver/lung transplant and the others fully recovered with drug withdrawal and steroids²⁶⁶. Sulphonamide ET_{RA}s are known to cause liver impairment, which appears to be direct, and dose-dependent toxicity. There is some evidence this may be due to drug-induced impairment of a bile salt transporter²⁶⁷. With bosentan, withdrawal of the drug leads, in most cases, to complete normalisation of liver function within a few weeks. Similar cases have been seen with sitaxsentan. However, the 7 severe cases appear different. Following withdrawal of the drug, liver function continued to decline. The histological pattern (presence of lymphocytes and eosinophils) and the clinical course suggested an idiosyncratic mechanism. Furthermore, no reports of death secondary to liver failure have been reported with bosentan (>80,000 patients treated) or ambrisentan (>10,000 patients treated) compared to two deaths in ~2000 patients treated with sitaxsentan. In view of these findings sitaxsentan was voluntarily withdrawn from the global market in

2010, just 3 years after it had been licensed for use in PAH by the European Medicines Agency

Future work

The observations in this thesis raise further questions to be answered and areas to explore. Some of these are discussed below:

1. Arterial stiffness and endothelial function in acute kidney injury

Based on the finding of chapter 3 and 4 it would be of great interest to further investigate markers of arterial stiffness and endothelial function in other cohorts of patients with acute kidney injury. In particular, comparing a cohort with a non-inflammatory cause, such as urinary obstruction, with the current cohort would help to confirm or refute the finding that renal function is the main determinant of AS in acute kidney injury.

2. Examination of functional ET_B blockade produced by other ETRAs

Following on from the study described in chapter 5 it would be interesting to further investigate the degree of ET_B blockade produced with other ETRAs particularly following the voluntary withdrawal of sitaxsentan. Theoretically, truly selective ET_A antagonism should confer benefits in terms of natriuresis, diuresis, and glomerular haemodynamics. However, to date there is no evidence to suggest the selective ET_A antagonists such as ambrisentan, atrosentan and zibotentan truly are functionally selective at their current doses, and good reasons to think they are not²⁶⁸. Important questions regarding efficacy and safety remain with this class of drug, and functional *in vivo* receptor studies would be of great benefit. The study described in chapter 4 is a simple and novel method for testing functional receptor antagonism in man using well described and tested techniques and could easily be applied to investigate other ETRAs.

3. Examination of the effects of ETRAs on sodium homeostasis

Peripheral oedema is one of the most common and troublesome adverse events seen with both mixed and selective ETRAs^{138, 146, 242}. This adverse effect has had significant clinical consequences leading to the failure of many trials^{262, 263}. Antagonism of the ET_B receptor with mixed antagonists may be contributing to fluid retention through inhibition of natriuresis and diuresis. The marginal selectivity of some “selective” ET_A antagonists may mean that at high doses they also cause functional ET_B blockade. However, there is also some evidence that ET_A receptors within the collecting ducts may also contribute to natriuresis and diuresis¹²⁶. Further studies are therefore required to define how these ETRAs exert their effects on sodium homeostasis.

4. Selective ET_A antagonism in proteinuric CKD

Based on the results of studies 6 and 7 and the more recently published data regarding atrasentan, a highly selective ET_A antagonist, in diabetic nephropathy, ET_A antagonists appear to confer additional renal and cardiovascular protection via reductions in proteinuria and BP in CKD patients^{264, 269}. Further research is therefore warranted in this area. A large phase III trial examining atrasentan in diabetic nephropathy is currently underway. This study, which started in May 2013, aims to enrol 4148 patients with diabetic nephropathy, randomising them to atrasentan or placebo. Treatment will continue for 48 months with the primary endpoints being time to doubling of serum creatinine or onset of end stage renal disease. The study is due to complete in March 2017 and will be the first large scale trial of endothelin antagonism in CKD (ClinicalTrials.gov NCT01858532).

5. Mixed vs. Selective ETRAs in proteinuric CKD

Both selective ET_A and mixed ET_{A/B} receptor antagonists are now available for clinical use. However, there remain few comparative studies of their effects in CKD. It would be of great interest to see if the results seen in Chapter 6 & 7 (proteinuria, BP and AS

reduction) are similar with a mixed ET_{A/B} blocking strategy. In particular, whether or not the side effect profile is as favourable given the theoretical risk of blocking ET_B receptor mediated natriuresis and so potentially risking fluid retention with a mixed approach.

In acute studies in subjects with CKD, the renal vasodilatation seen with selective ET_A receptor antagonism is attenuated with additional ET_B receptor blockade²⁷⁰, suggesting that tonic ET_B receptor-mediated renal vasodilatation plays a key role in opposing renal vasoconstriction. This is likely to be of particular importance in CKD, where baseline renal vascular resistance is high. Conversely, proteinuria reduction is seen with both approaches^{237, 271}. Although both approaches may be of benefit in CKD, there are no head-to-head studies with chronic dosing.

6. Management of scleroderma kidney

Scleroderma renal crisis (SRC) is an important complication of scleroderma associated with significant morbidity and mortality. Current treatment of patients with SRC focuses on RAAS blockade, ideally using ACE-I. ET-1 is implicated in the development of scleroderma with patients showing increased plasma and tissue concentrations of ET-1^{272, 273}. A trial of the mixed receptor antagonist bosentan in 122 patients with scleroderma showed a 48% decrease in mean number of new digital ulcers during the 16-week treatment period¹⁴⁴. Currently, data regarding the use of ETRAs in patients with scleroderma are limited, with the majority of studies focusing on pulmonary arterial hypertension¹¹³. I, in conjunction with other renal colleagues, have described a case study using sitaxsentan in the treatment of scleroderma kidney²⁷⁴. In this case the patient was already established on maximal tolerable RAAS-blocking treatment. Introduction of a selective endothelin-A receptor antagonist followed by a direct renin inhibitor provided excellent BP control and complete abrogation of heavy proteinuria. This was associated with a decrease in kidney function, with serum creatinine level increasing by around 30% before stabilizing. Whether this would protect residual renal function in the longer term remains unclear. Our observational case study suggests that ETRAs may offer therapeutic benefits in patients with SRC on top of standard treatment and warrants

further investigation²⁷⁴.

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