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The Endothelin System & Its Antagonism In Chronic Kidney Disease

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Abstract

Since its discovery in 1988 the powerful vasoconstrictor endothelin-1 (ET-1) has been widely implicated in the pathophysiology of chronic kidney disease (CKD) as well as the cardiovascular disease with which it is associated. ET receptor antagonists have favourable effects in experimental models of these conditions and orally acting antagonists are now licensed for the treatment of pulmonary arterial hypertension. However, there is a paucity of human data regarding the role of ET-1 in CKD. In this thesis, I have therefore explored the utility of ET-1 as a biomarker in CKD, and, using selective ET receptor antagonists, the beneficial renal and cardiovascular effects of ET receptor antagonism in CKD.

I have shown that as glomerular filtration rate (GFR) declines plasma ET-1 increases linearly whereas urinary ET-1 shows an exponential increase. Furthermore, urinary ET-1 may be a useful marker of disease activity in patients with lupus nephritis. Its levels are high in those with biopsy-proven active renal inflammation and these fall with treatment.

I have shown that in subjects with stable non-diabetic proteinuric CKD, acute selective ET_A receptor antagonism reduces blood pressure and arterial stiffness and that these systemic benefits are associated with an increase in renal blood flow and reduction in proteinuria. Importantly, these effects are seen on top of those achieved with maximal therapy with angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers.

Following a study confirming unchanged pharmacokinetics in CKD, I have used an oral selective ET_A receptor antagonist to show that the reductions in BP, arterial stiffness and proteinuria seen in my acute studies are maintained longer term. This results of this study also suggest that the mechanism for the reduction in proteinuria is haemodynamic and relates to a reduction in GFR and filtration fraction.

In summary, these studies suggest that ET-1 may act as a potential biomarker of renal inflammation, and confirm its role in the pathophysiology of the systemic and renal vasoconstriction seen in CKD. They also suggest that selective ET_A receptor antagonism may provide a novel therapeutic approach in proteinuric CKD on top of standard therapies. Larger and longer term studies are now warranted to confirm this potential.

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 : study 1 was carried out by Dr P Lilitkarntakul.

Chapter 4 : this study was carried out by myself with the help of Mrs V Melville

Chapter 5 : this study was carried out by myself with the help of Mrs V Melville and Miss D Kerr.

Chapter 6 : this study was carried out by myself, Dr IM MacIntyre, Mrs V Melville and Miss D Kerr

2. Assays

Renal clearance studies : as these 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). All immediate processing of samples was undertaken by myself and others as outlined above.

Plasma sitaxsentan (Chapter 5): these were performed by Encysive Pharmaceticals (now Pfizer).

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

3. Data analysis

Chapter 3 : the data for study 1 were analysed by Dr P Lilitkarntakul

Chapter 5 : the pharmacokinetic analyses were carried out by Encysive Pharmaceuticals (now Pfizer).

Chapter 6 : the analysis was carried out by myself, Dr IM MacIntyre and Dr J Goddard.

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Finally, Professor David Webb who afforded me the opportunity to undertake this research in the first place and who has been unstinting in his backing, advice and support.

Abbreviations

ACE	Angiotensin converting enzyme
AIx	Augmentation index
ANG II	Angiotension II
ARB	Angiotensin receptor blocker
AS	Arterial stiffness
AVP	Arginine vasopressin
BP	Blood pressure
CI	Cardiac index
CO	Cardiac output
CKD	Chronic kidney disease
CTF	C-terminal fragment
CVD	Cardiovascular disease
ECE	Endothelin converting enzyme
ED	Endothelial dysfunction
EFF	Effective filtration fraction
ERBF	Effective renal blood flow
ERPF	Effective renal plasma flow
ERVR	Effective renal vascular resistance
ESRF	End-stage renal failure
ET	Endothelin
FeNa	Fractional excretion of sodium
GFR	Glomerular filtration rate
Hct	Haematocrit
IMCD	Inner medullary collecting duct
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
NO	Nitric oxide
PAH	Para-aminohippurate
PWV	Pulse wave velocity
RBF	Renal blood flow
SVRI	Systemic vascular resistance index
UFR	Urinary flow rate
UNaV	Urinary sodium excretion

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Chapter 1

Introduction

Chronic kidney disease (CKD) is common. A study in general practice has demonstrated that an elevated creatinine is present in 6% of hypertensives, 13% of diabetics and 17% of patients with both conditions[1]. A United States (US) population study suggests that more than 10% of the general adult population have an indicator of kidney damage – proteinuria, haematuria, and/or reduced glomerular filtration rate (GFR)[2] and there is no reason to think that these figures will be substantially different in the United Kingdom. Despite our best current treatments, progression to end-stage renal disease (ESRD) remains a major clinical and financial problem and there are currently over a million patients worldwide on dialysis, with the number continuing to increase yearly.

It is now well-recognised that cardiovascular disease (CVD) is strongly associated with CKD[3, 4] and constitutes one of its major causes of morbidity and mortality[4]. Indeed, CKD has emerged as an important and powerful independent risk factor for CVD[4]. As GFR declines the risk of CVD increases and patients with non-dialysis-requiring CKD are more likely to die from CVD than develop ESRD[4]. Furthermore, not only are individuals with CKD at increased risk of cardiovascular events but their outcome is worse than in those without CKD[5]. Although the prevalence of traditional risk factors (such as diabetes, hypertension, dyslipidaemia) in the CKD population is high, CVD events remain disproportionate to the underlying risk factor profile[4]. Thus, 'non-traditional' risk factors, such as endothelial dysfunction, arterial stiffness, oxidative stress and inflammation, which may contribute to this excessive uraemic cardiovascular risk, have become a major focus of interest.

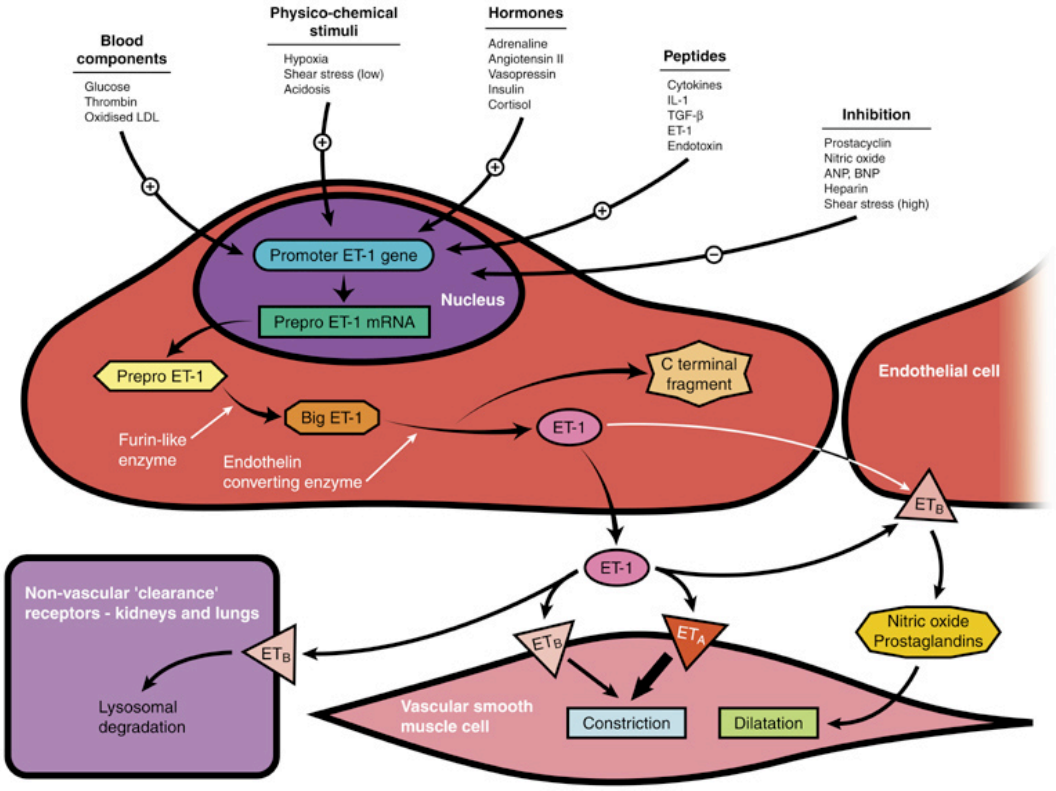
Thus, there is an important unmet need for treatments that not only slow the rate of progression of renal impairment, delaying the onset of dialysis in CKD, but also that might improve the cardiovascular risk profile in these patients. Blockade of the endothelin (ET) system has emerged as one potential strategy. The ET system has been widely implicated in renal disease, including acute renal failure[6] and emerging data suggest that inhibition of its actions might slow the progression of CKD and reduce the burden of CVD with which it is associated.

Biology of the endothelin system

First described by Yanagisawa in 1988[7], ETs are a family of 21 amino acid peptides with powerful vasoconstrictor and pressor properties. Three different isopeptides, ET-1, ET-2 and ET-3, are known, each with distinct genes and tissue distributions[7-9]. ET-1 is the major endothelial isoform and, in the human kidney, the only one so far shown to be expressed at the protein level[10]. Its main site of vascular production is the endothelial cell but it is also produced by other cell types including vascular smooth muscle cells and epicardial cells[11]. Within the kidney, it is produced by glomerular epithelial and mesangial cells, and renal tubular and medullary collecting duct cells[6]. Furthermore, the renal medulla is not only an important site of ET-1 generation but also contains among the highest concentrations of immunoreactive ET-1 of any organ[12].

The gene product is the 212 amino acid pre-pro-ET-1, and regulation of ET synthesis occurs at the level of gene transcription. Enhanced generation occurs with a wide range of stimuli[13, 14] (Figure 1.1). Those pertinent to CKD include other vasoactive hormones, such as angiotensin (ANG II) and vasopressin (AVP), the cytokine interleukin (IL)-1, oxidised LDL, reduced extracellular pH, and cyclosporin A (CyA). In contrast, prostacyclin, nitric oxide (NO), and the natriuretic peptides all inhibit gene transcription. Pre-pro-ET-1 is cleaved to big ET-1 (38 amino acids) which is largely biologically inactive[15]. Endothelin converting enzyme (ECE) then catalyses generation of the biologically active ET-1 and C-terminal fragment from big ET-1. Once synthesised, the secretion of mature ET-1 from endothelial cells is largely abluminal[16], towards the adjacent vascular smooth muscle, suggesting an autocrine or paracrine action.

Figure 1.1 *(overleaf)*: Pathways of endothelin-1 (ET-1) synthesis and sites of action.
ANP: atrial natriuretic peptide, BNP: brain natriuretic peptide, IL-1: interleukin-1, TGF-β: transforming growth factor β.



ET-1 acting in the vascular system

ET-1 acts by binding to two receptors, the ET_A and ET_B receptor[17, 18] (Table 1.1). Within blood vessels, ET_A receptors are found on smooth muscle cells and their activation results in vasoconstriction. ET_B receptors are also found on vascular smooth muscle cells[19], where they can mediate vasoconstriction, but are predominantly found on the vascular endothelium where their activation results in vasodilatation via prostacyclin and NO[20].

In addition, the ET_B receptor also acts as a clearance receptor for circulating ET-1. The half-life of ET-1 in the healthy circulation is ~1 min[21] with removal through receptor and non-receptor mediated mechanisms. ET-1 binds to ET_B receptors, with subsequent ligand-receptor complex internalisation and intracellular degradation accounting for the majority of clearance, particularly in the pulmonary circulation[22], though the splanchnic

and renal circulations also contribute[13]. Therefore, reductions in ET_B numbers, or ET_B receptor blockade, may reduce ET-1 clearance, increasing plasma concentrations without altering production. Importantly, because most ET-1 is released abluminally, plasma concentrations of ET-1 do not accurately reflect ET-1 production.

ET-1 acting in the renal system

ET receptors are widely distributed within the human kidney, with the ET_A subtype localised to vascular smooth muscle, 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 more widespread, with a high concentration in the collecting system[10, 23]. With respect to the renal system, ET-1 has a role in the paracrine/autocrine regulation of renal and intrarenal blood flow, glomerular hemodynamics, sodium and water homeostasis[24], and acid-base balance[25] (Table 1.1). It is also clear that ET-1 has many other functions within the kidney[26]. Evidence exists for renal and vascular ET-1 acting as two independent systems[27]. After systemic infusion of radiolabelled ET-1, labeled compound makes up less than 1% of total urinary ET-1[28]. Therefore, neither glomerular filtration nor tubular secretion of plasma ET-1 accounts for urinary ET-1, which is therefore assumed to be primarily of renal origin. Urinary excretion of ET-1 is thus thought to reflect renal ET-1 production.

Table 1.1: Actions of endothelin-1. The table shows the receptor responsible for each action but, particularly in the case of the ET_A receptor-mediated actions, does not exclude a small contribution from the ET_B receptor.

Actions of ET-1 in the systemic vasculature - animal studies	
ET_A receptor	**ET_B receptor**
Vasoconstriction Increased arterial stiffness Endothelial dysfunction Inflammation Atherosclerosis Cardiac hypertrophy	Vasoconstriction Endothelium-dependent vasodilation ET-1 clearance
Actions of ET-1 in the kidney - animal studies	
ET_A receptor	**ET_B receptor**
Renal vasoconstriction Cortical vasoconstriction Afferent arteriolar constriction Efferent arteriolar dilation Mesangial cell contraction Mesangial cell proliferation Extracellular matrix accumulation Interstitial fibrosis	Renal vasodilation Medullary vasodilation Afferent arteriolar constriction Efferent arteriolar dilation Natriuresis
Receptor uncertain	
Podocyte de-differentiation Diuresis Acid-base balance	

Defining the role of endothelin in physiology

ET-1 is a potent vasoconstrictor *in vitro*, and pressor in whole animals[29]. With respect to the kidney, exogenous ET-1 causes renal vasoconstriction[30]. Indeed, the renal vasculature is more sensitive to the vasoconstricting effects of ET-1 than other vascular beds[31]. Although exogenous ET-1 reduces total renal blood flow (RBF), a regional difference has been observed, with cortical vasoconstriction[32-34] and NO dependent medullary vasodilatation[32]. Exogenous ET-1 has also been shown to cause constriction of afferent and efferent arterioles, with a greater effect on the former[35], and reduce filtration coefficient by mesangial cell contraction[26]. In man, a similar vasoconstrictor[15] and pressor response has been demonstrated[36], as well as renal vasoconstriction, a fall in

total RBF and a consequent reduction in GFR[37]. As yet, there are no studies of the effects of ET-1 on intra-renal distribution of blood flow in man.

With respect to renal tubular functions, there is now a substantial body of evidence supporting a role for ET-1 in the regulation of volume homeostasis. ET-1 inhibits the vasopressin (AVP) stimulated retention of water in inner medullary collecting duct cells (IMCD) *in vitro*[38], and extra-cellular sodium concentrations may regulate IMCD ET-1 production[24, 39]. Additionally, ET-1 appears to have a natriuretic role, at least in animals. ET-1, acting via ET_B and NO, can inhibit chloride transport in the medullary thick ascending limb of Henlé, thus promoting natriuresis[40, 41]. Picomolar ET-1, binding to ET_B receptors, activates amiloride-sensitive sodium channels in distal tubular cells *in vitro*, though higher, nanomolar doses inhibit this channel by a non-ET_B receptor dependent mechanism[42]. This has been supported by *in vivo* experiments in rats demonstrating natriuresis due to reduced sodium transport in the proximal and distal nephron segments in response to low dose exogenous ET-1 with higher doses resulting in sodium retention due to glomerular vasoconstriction[43].

Agonist studies may not adequately represent the effects of a hormone the actions of which are largely autocrine/paracrine, and inhibition of the production or actions of ET-1 may better define its physiological and pathological effects. In this respect, ET receptor antagonists have proved to be useful tools in defining the role of ET in health and disease. ET receptor antagonists are classified as ET_A selective (such as the iv antagonist BQ-123), or ET_B selective (such as the iv antagonist BQ-788), depending on their relative affinity for a receptor subtype, or non-selective (such as the oral drug, bosentan)[13]. It should be noted, however, that the distinction between selective and non-selective antagonists is not pharmacologically well-defined. The so-called 'non-selective' antagonists are still selective for the ET_A receptor, but the ratio of ET_A to ET_B affinity is generally 10-100-fold selective for ET_A over $ET_{B,}$ compared to 1000-fold or more for recent ET_A selective agents[13].

With respect to systemic haemodynamics in healthy man, selective ET_A receptor antagonism is associated with vasodilatation[44] and a reduction in blood pressure (BP)[45], and selective ET_B receptor antagonism with vasoconstriction[44] and a pressor response[46]. This suggests that endogenous ET-1 contributes to the maintenance of vascular tone and BP via the ET_A receptor, and the balance of ET_B receptor function favors activation of the endothelial over the vascular smooth muscle ET_B receptor.

In the kidney, animal studies have suggested that both exogenous and endogenous ET-1 mediated reductions in total RBF are mediated via the ET_A receptor[47, 48]. Antagonist studies describe cortical vasoconstriction as ET_A receptor mediated and medullary vasodilatation as ET_B receptor mediated[32, 33, 47]. Furthermore, i*n vitro* studies have shown that combined $ET_{A/B}$ receptor antagonism is required to fully abrogate the vasoconstricting effects of exogenous ET-1 on the afferent arteriole suggesting that both ET_A and vascular smooth muscle cell ET_B receptors are involved. At the efferent arteriole the effect of ET-1 is blocked by ET_A receptor antagonism alone, and enhanced by ET_B receptor blockade, suggesting that ET-1 can modulate efferent arteriolar tone via the ET_A receptor and that the balance of ET_B receptor effects here is to produce vasodilatation[35]. The situation is less clear in healthy humans where there are few studies. One study has demonstrated an increase in RBF after non-selective ET receptor blockade[49]. Most, however, do not demonstrate an effect of selective ET_A receptor blockade, or combined $ET_{A/B}$ receptor blockade, on basal renal haemodynamics[50-53], suggesting that ET-1 acting via the ET_A receptor does not contribute to the maintenance of renal vascular tone in health. Selective and unopposed ET_B receptor antagonism can, however, produce profound renal vasoconstriction, suggesting that ET-1 mediated tonic renal vasodilatation via the ET_B receptor is important.

Studies have suggested a natriuretic role for the tubular ET_B receptor which is linked to NO generation. A potent inhibitory action of NO on tubular sodium reabsorption is well described[54]. A rat model deficient in renal ET_B receptors displays a salt-sensitive hypertension, with restoration of normal BP by amiloride, suggesting that the ET_B

receptor regulates sodium excretion at the epithelial sodium channel in collecting duct (CD) cells[55], and ET_B antagonist-treated rats develop a sodium-dependent hypertension[56]. Additionally, in the face of acute ET_B receptor blockade, pressure-natriuresis curves are shifted to the right such that a greater renal perfusion pressure is needed to excrete the same amount of sodium[57]. Finally, administration of exogenous low dose ET-1 to dogs in the presence of high grade selective ET_A receptor blockade results in renal vasodilatation and natriuresis, presumably by unmasking an ET_B receptor mediated effect[58]. Dissecting the different actions of the intrarenal ET system has however proved difficult, in part from an inability to discriminate between effects of ET-1 *in vivo* on the nephron and vasculature. To date, ET-1 associated natriuresis and diuresis have not been demonstrated in man.

Defining the role of endothelin in pathophysiology

Hypertension

Initial evidence of a pressor action of ET-1 led to the suggestion that ET-1 might be implicated in hypertension[7]. Production of vascular ET-1 is increased in some (such as the Dahl salt-sensitive and the stroke-prone spontaneously hypertensive rat (SHR)), but not all animal models of hypertension[59]. Those models where ET-1 production is increased (mostly, but not exclusively salt-dependent types) are associated with increased vascular growth and a response to both selective and non-selective ET receptor antagonism comprising not only a modest reduction in BP but also a marked regression of vascular growth[59].

In man, ET-1 message and protein are increased in the vascular smooth muscle cells of hypertensive patients[60]. Elevated plasma ET-1 concentrations, however, are not a consistent finding[59]. High concentrations would appear, mostly, to be a feature of severe hypertension or indicative of the presence of complications or co-existing disease. Some[61], but not all[62] local studies with ET receptor antagonists suggest increased vascular ET system activity in patients with hypertension compared to normotensive controls, and a greater forearm vascular response to non-selective receptor antagonism

compared to selective ET_A antagonism, consistent with an increased importance of vascular smooth muscle vasoconstrictor ET_B receptors in hypertension. In CKD, local administration of BQ-123 increases forearm blood flow[63]. Systemic administration of BQ-123 (+/- BQ-788) to hypertensive patients with CKD showed that selective ET_A receptor blockade produces a substantial reduction in BP (~10mmHg), whereas non-selective $ET_{A/B}$ receptor antagonism lowered BP to a lesser extent. In both cases, the reduction in BP was much greater than in healthy controls[64], supporting an upregulation of the ET-1 system in CKD-associated hypertension. Only one major study has examined the longer-term anti-hypertensive effects of ET receptor antagonism in man. Bosentan, an orally available, non-selective ET receptor antagonist reduced BP in essential hypertensives as much as did enalapril 20mg[65]. Importantly, this reduction was achieved without activation of the sympathetic nervous or renin-angiotensin system (RAS).

Altered intrarenal ET-1 production may contribute to hypertension[66, 67]. SHR have reduced medullary ET-1 levels after the development of hypertension[68]. More recently, Kohan *et al* have successfully created an elegant tissue-specific knockout of the renal ET system. Mice lacking CD expression of the ET-1 gene have reduced urinary ET-1. These animals are hypertensive and have an impaired ability to excrete a sodium load[67]. Interestingly, these knockout mice excrete acute water loads less well than wild-type mice, and have a heightened physiological response to AVP, consistent with an intra-renal role for ET-1 in blunting the response to AVP[69]. While plasma ET-1 concentrations are normal, urinary ET-1 excretion is reduced in hypertensive subjects compared to healthy controls suggesting that either renal ET-1 synthesis is reduced or breakdown is enhanced[70, 71]. Thus, renal ET-1 production or handling may be altered in hypertension, leading to inappropriate sodium and water retention, and aiding the development and/or maintenance of hypertension.

Renal function may also influence the relationship between ET-1 and hypertension. Firstly, as renal function declines plasma ET-1 levels increase[71, 72]. The effects of

exogenous ET-1 on the renal vasculature are to cause vasoconstriction, activating the RAS and causing salt and water retention, both of which have the potential to raise BP. It remains to be seen whether the rise in ET-1 concentrations seen in CKD is due to biologically-active or simply immunologically-competent peptide, but infusion of exogenous ET-1 to bilaterally nephrectomised rats results in an increased plasma half-life of ET-1 and a prolonged rise in BP compared to sham-operated rats[73], consistent with the idea that elevated plasma ET-1 concentrations in CKD may cause hypertension. Second, there is an upregulation of renal ET-1 in CKD[74], as reflected by increased urinary ET-1 excretion[71]. Third, there is a suggestion from an experimental model of nephritis associated with mesangial proliferation that the renal vasculature in this disease may be more sensitive to the vasoconstrictor effects of ET-1 than in normal kidneys[75]. Thus, an amplification of the renal vasoconstrictor effects of ET-1, promoting hypertension, could be envisaged in CKD.

Studies of ET_B receptor knockout animals suggest the ET_B receptors are important in protecting against hypertension. These animals exhibit a sodium-dependent hypertension attributed to an absence of tonic inhibition of the epithelial sodium channel in the distal nephron[55]. Interestingly, ET_B receptor deficient mice show renal injury, an impaired ability to excrete a sodium load and hypertension that persists when they are cross-transplanted with wild-type kidneys suggesting that it may not only be renal but also extra-renal ET_B receptors that play a protective role against hypertension[76].

Endothelial dysfunction and atherosclerosis

The endothelium is a crucial regulator of vascular tone[77] and its function is impaired, both in hypertension and in groups at risk of hypertension, with a shift towards reduced vasodilatation, associated with a pro-inflammatory and pro-thrombotic state. Endothelial dysfunction (ED) is also a well-recognised feature of CKD[77]. ED is recognised to be a key early determinant in the progression to atherosclerosis, and is now well established to be independently associated with increased cardiovascular risk[78]. Mechanisms that participate in ED include reduced NO generation, oxidative stress and upregulation of

inflammatory mediators[77]. Animal models of ED across a number of animal species have shown that antagonism of the ET system, predominantly with selective ET_A receptor antagonists, improves NO-mediated endothelial function[79-81], suggesting that ET-1, acting via ET_A receptors, is involved in the pathogenesis of ED. Treatment with selective ET_A receptor antagonism also improves endothelial function in the coronary vessels of patients with atherosclerosis, again suggesting that ET-1, acting via ET_A receptors, is involved in the pathogenesis of ED in these patients[82].

The ET system is also implicated in the development of atherosclerosis. In smooth muscle cells and foamy macrophages in atherosclerotic models both ET_A and ET_B receptors are highly expressed[83]. Increased expression of ET-1 and ECE is seen in human arteries at different stages of atherosclerosis[60, 84], and high levels of ET-1 have been found in human atherosclerotic lesions[60, 84-86]. Furthermore, plasma ET-1 concentrations correlate positively with the degree of atherosclerosis present[85]. Importantly, not only is restoration of the impaired activity of the NO system, and hence improvement in endothelial function, seen following ET receptor antagonism in a range of animal models of atherosclerosis[79, 80, 83], but so too is a reversal of atherosclerotic lesion development. Thus, ET antagonists reduce the activity of the ET system, increase NO bioavailability, improved endothelial function, and slow the progression of atherosclerosis. To date, there are no therapeutic studies on endothelial function or atheroma progression with ET antagonism in CKD patients.

Arterial stiffness

Arterial stiffness is an important independent predictor of all-cause and cardiovascular mortality in patients with ESRD[87]. Moreover, a therapeutic trial in ESRD patients by Guerin *et al*[88] has shown that after BP reduction, cardiovascular survival was observed mainly in those patients who also displayed a reduction in arterial stiffness[88]. Epidemiologic studies indicate that there is increased cardiovascular risk early on in CKD, but there are as yet few data that show how early arterial stiffness develops[89]. Increased arterial stiffness results in a selective elevation of pulse pressure causing

deleterious consequences for the heart. Through an elevation of central systolic BP, arterial stiffness enhances left ventricular load, favoring development of cardiac hypertrophy, and through reduction of central diastolic BP it decreases coronary perfusion pressure, contributing to myocardial ischaemia[90].

Arterial stiffness is linked to ED[90] and the two conditions commonly co-exist in patients at increased risk of CVD. A number of interventions that reduce arterial stiffness also improve endothelial function[90]. To date, there have been few studies addressing the relationship between these two markers of CVD after treatment, and none in patients with CKD. However, there is now evidence from both animal and human studies that the endothelium is an important regulator of arterial stiffness. Basal endogenous NO generation decreases arterial stiffness in animals[91] and humans[92-94]. By contrast, ET-1, at concentrations similar to those observed in the plasma of CKD patients, caused an increase in arterial stiffness that can be blocked by concomitant administration of an ET_A receptor antagonist[95]. Furthermore, endogenous ET-1 has recently been shown to increase arterial stiffness[96]. Thus, in ED, where NO is downregulated and ET-1 upregulated, the balance will likely shift in favor of increased arterial stiffness. Clinical studies of the effects of ET receptor antagonism on arterial stiffness in CKD will clearly be of great interest.

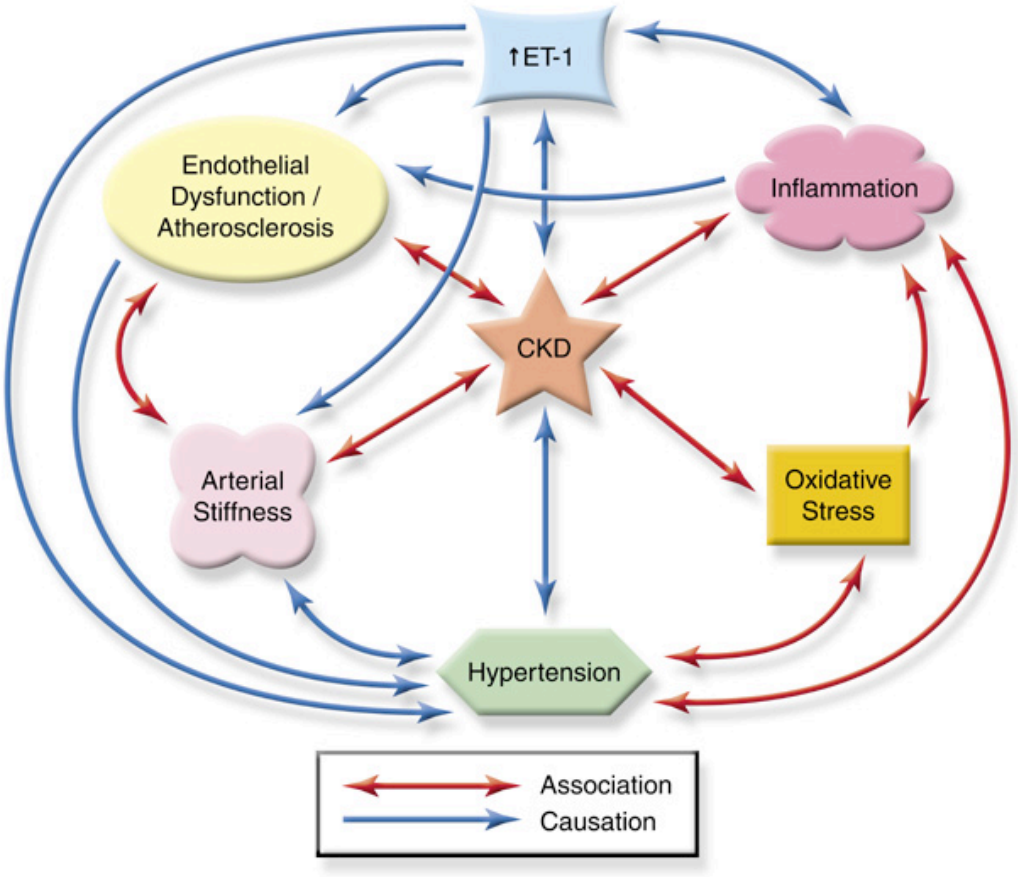
Oxidative stress and inflammation

Oxidative stress and inflammation are well documented in ESRD[97, 98]. Indeed they are very common even with mild renal insufficiency[99]. Oxidative stress is characterised by an imbalance between exposure to free radicals, principally derived from oxygen, and antioxidant defenses. As in ED, there is loss of NO availability in states of oxidative stress, and the close relationship between increased oxidative stress, reduced NO availability and subsequent cardiovascular events is well-established[100]. Additionally, there is now mounting evidence, although scarce human data, supporting the hypothesis that at least some of the injurious effects of ET-1 on the vasculature are mediated via an increase in oxidative stress, and that ET system blockade may be of use in reducing

this[101, 102]. Indeed, in DOCA-salt hypertension the ET-1 promoted production of reactive oxygen species (ROS), the principal mediators of oxidative stress, is ET_A receptor mediated, and selective ET_A receptor blockade normalises the ED found in this model[103], independent of changes in BP. Data in CKD are scarce at present and it remains unclear whether oxidative stress is a cause or consequence of renal insufficiency.

ROS likely promote the development of atherosclerosis through a number of mechanisms[104], including ED, increased production of pro-inflammatory cytokines, such as IL-6, and acute phase proteins, such as C-reactive protein (CRP)[105]. IL-6 and CRP are both independent predictors of cardiovascular events and mortality[106]. Recent evidence suggests that a reduction in kidney function per se may be associated with an inflammatory response both in mild[107] and advanced[108] kidney disease, and a number of studies have shown that CRP predicts all-cause and cardiovascular mortality in dialysis patients[109]. In addition to being an important prognostic marker for CVD, CRP may contribute to the atherosclerotic process mainly through the impairment of endothelial function[110, 111]. Furthermore, CRP has been shown to decrease the activity of the NO system[112] and to potentiate the release of ET-1[105]. Recently, ET receptor antagonism has been shown to attenuate the pro-atherogenic effects of CRP in *vitro*[105], consistent with anti-inflammatory and anti-atherogenic actions.

Figure 1.2: The role of ET-1 in CKD



Defining the role of endothelin in renal pathophysiology

CKD & Renal Haemodynamics

There are only few studies in animal models of CKD. Nevertheless, these show that ET receptor antagonism improves renal blood flow (RBF) and preserves GFR. Non-selective $ET_{A/B}$ receptor antagonism with bosentan can prevent the renal vasoconstriction seen in the early phases of streptozocin-induced diabetes mellitus[113] and selective ET_A receptor antagonism can preserve GFR and RBF during acute[114] and chronic CyA administration[115]. Additionally, in the Dahl salt-sensitive hypertensive rat, where a reduced RBF and GFR are observed after a high salt diet, systemic $ET_{A/B}$ receptor

antagonism tended to increase, and intra-renal interstitial infusion significantly increased, RBF and GFR[116].

Table 1.2: Animal models of CKD improved by ET receptor antagonism. CyA, cyclosporin.

Animal models of CKD improved by ET receptor antagonism
Streptozocin-induced diabetes mellitus
Renal mass reduction
Proliferative glomerulonephritis
Lupus nephritis
Hypertensive nephrosclerosis
Chronic CyA administration
Hypokalemic nephropathy

In patients with CKD, selective ET_A receptor antagonism produces an increase in RBF and decrease in renovascular resistance[64], suggesting that ET-1 acting via ET_A receptors is involved in the increased renovascular tone. These changes are accompanied by a fall in effective filtration fraction (EFF), suggesting that ET-1, acting via ET_A receptors, exerts a preferential efferent arteriolar vasoconstrictive effect, raising the possibility that ET-1 promotes hyperfiltration with its consequent potential for renal injury. The renal haemodynamic effects of selective ET_A receptor antagonism can be abolished by concomitant administration of an ET_B receptor antagonist, and, as in health, selective blockade of the ET_B receptor produced renal vasoconstriction[64]. Notably, in these studies, selective ET_B receptor antagonism increased renal vascular resistance by twice as much (~20-30%) as systemic vascular resistance (~10-15%), 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. The renal haemodynamic changes in these studies are consistent with other work in CKD patients where non-selective ET receptor blockade reduces EFF whilst maintaining GFR[56], and in diabetics with albuminuria, in whom selective ET_A receptor antagonism reduced both BP and urinary protein excretion[117, 118]

Proteinuria

Significant proteinuria has emerged as a powerful predictor of renal disease progression[119], and proteinuria reduction is an important strategy to retard or prevent renal functional loss[119, 120]. Additionally, proteinuria is no longer simply a renal risk factor. Alongside the concept of CKD as a global vascular disease state is emerging the global cardiovascular risk associated with proteinuria. Albuminuria is incrementally associated with increased cardiovascular risk in both individuals with pre-existing risk (such as hypertensive patients)[121], and in individuals with no known risk factor[122]. This is true even in the presence of normal renal function[123]. Furthermore, at least in hypertension, reduction of albuminuria confers cardiovascular protection[121].

Upregulation of the renal ET system exacerbates proteinuria. Through its haemodynamic effects ET-1 causes glomerular capillary hypertension, an increase in glomerular permeability and excessive protein filtration[124]. A reduction in microalbuminuria in patients with diabetes mellitus has recently been shown following chronic selective ET_A receptor blockade[117, 118]. Furthermore, the reductions in EFF observed following acute selective ET_A receptor antagonism in patients with CKD were accompanied by a reduction in proteinuria[64] suggesting that one mechanism for the anti-proteinuric effect of ET_A receptor antagonism in CKD may relate to alterations in glomerular haemodynamics with, potentially, a fall in glomerular capillary perfusion pressure. This would produce a situation analogous to that seen with angiotensin converting enzyme (ACE) inhibitors[119], in which case ET antagonists might be expected to be renoprotective and improve long term renal outcome.

The development of proteinuria is also associated with damage to the renal podocyte[125], the highly specialised glomerular epithelial cell that helps maintain an intact filtration barrier under normal conditions. Recent *in vitro* studies suggest that podocytes undergo phenotypic changes that resemble de-differentiation as a result of exposure to exuberant amounts of protein[126]. In parallel with these changes there was increased ET-1 production by the podocyte, which was, at least partly, dependent on the cytoskeletal

rearrangements brought about by excess protein exposure. In the same model, administration of exogenous ET-1 brought about similar podocyte cytoskeletal changes. Thus, the authors conclude that podocyte-derived ET-1 acting in an autocrine and paracrine manner promotes further podocyte ultrastructural degeneration and hence its own production, both of these contributing to a further breakdown in the glomerular filtration barrier. These data are in line with *in vivo* evidence in a murine model of protein overload that displays increased renal ET-1 production alongside the development of podocyte structural damage[127].Whether damage to the podocyte is the primary event leading to subsequent proteinuria or vice versa remains unclear. Nevertheless, it is not unreasonable to envisage a series of events, with initial ET-1 mediated glomerular hypertension exposing podocytes to unusually large amounts of protein and so leading to their de-differentiation and production of ET-1. This podocyte-derived ET-1 could then exacerbate glomerular hypertension and lead to further podocyte de-differentiation so setting up a vicious cycle.

Clinical studies are supportive of a role for the ET system in proteinuric nephropathies. Patients with chronic glomerulonephritis and proteinuria displayed a rise in renal ET-1 and tubular ET_B receptor expression that increased with higher degrees of proteinuria[128], and patients exposed to selective ET_A receptor antagonism both in the acute[64] and chronic[118] setting showed a significant reduction in proteinuria.

Table 1.3: ET receptor antagonist studies in CKD patients. RVR, renal vascular resistance.

ET receptor antagonist studies in CKD patients		
Effect	***ET receptor blockade***	***Dosing***
↓forearm blood flow	ET_A	Acute
↓BP	ET_A, $ET_{A/B}$	Acute
↓RVR, ↓proteinuria	ET_A	Acute
↓proteinuria	ET_A	Chronic

CKD progression

Excess protein filtration at the glomerulus leads to increased tubular reabsorption. This can activate tubular-dependent pathways of interstitial inflammation and fibrosis, with progressive renal scarring[129]. Studies suggest a link between upregulation of the renal ET system and tubular protein reabsorption. Exposure of proximal tubular cells *in vitro* to a protein load leads to a dose-dependent increase in ET-1 production[130]. This phenomenon is not exclusively associated with albumin but may be seen with other proteins such as IgG and transferrin[130]. Within the interstitium, ET-1 has the capacity to bind to interstitial fibroblasts and promote their proliferation, and the generation of extracellular matrix (ECM)[131]. Furthermore, ET-1 is chemotactic for blood monocytes[132] leading to secretion of pro-inflammatory cytokines and growth factors, events that could contribute to interstitial remodeling and scarring. Hence, a potential pathway may be envisaged whereby ET-1 could link proteinuria to interstitial fibrosis.

In the remnant-kidney model, renal ET-1 gene expression and urinary ET-1 excretion correlate with degree of proteinuria and extent of renal damage[74]. Also, transgenic animal studies in which renal ET pathways are upregulated display renal tubulo-interstitial lesions independent of changes in BP[133]. These BP-independent effects of ET are supported by antagonist studies where ET receptor antagonists lead to a slowing of progressive renal damage, even in the absence of BP modification[134]. Non-selective $ET_{A/B}$ receptor antagonism can reduce the increase in collagen and ECM deposition seen in rats treated with L-NAME, an inhibitor of NO synthesis, independent of BP changes, and can also reduce collagen I gene activity to normal levels, suggesting that ET-1 promotes renal fibrosis via activation of this gene[135]. ET receptor antagonists have also been shown to attenuate the progression of renal insufficiency in a rat remnant-kidney model[136]. While non-selective $ET_{A/B}$ receptor antagonists have produced positive results[137], the effect is greater with selective ET_A receptor antagonism[138]. Indeed, concomitant administration of an ET_B receptor antagonist can abolish the beneficial effects of ET_A receptor antagonism[139]. In patients with nephrotic syndrome due to focal and segmental glomerulosclerosis, plasma and urinary ET-1 concentrations are

significantly higher than in healthy controls[140], and nephrotic patients who show a reduction in proteinuria with immunosuppressive therapy also show reductions in urinary ET-1 excretion[141].

Blockade of the endothelin & renin-angiotensin systems: a potential synergism

ET-1 and ANG II are powerful vasoconstrictors involved in the regulation of vascular tone, and there is considerable evidence for an interaction between the ET and RAS[142]. ANG II increases ET-1 transcription and secretion *in vitro* in a variety of cell types, including endothelial and vascular smooth muscle cells[143, 144]. ACE inhibitors also reduce renal ET-1 formation. Rats with reduced renal mass show a parallel fall in proteinuria, vascular and glomerular pre-pro-ET-1 mRNA, and ET-1 peptide, following RAS blockade with losartan and captopril[145]. Chronic ACE inhibitor treatment in animal models of glomerulosclerosis[146] and immune-mediated glomerulonephritis[147] leads to a reduction in proteinuria as well as a fall in renal ET-1 mRNA and protein expression.

Interestingly, animal data have suggested that concomitant acute blockade of the RAS and ET system produces greater haemodynamic changes than those seen with blockade of either system alone[148-152]. Also, many clinical studies using ET receptor antagonists, in patients with heart failure, demonstrate major additional haemodynamic effects[153, 154] in patients already receiving ACE inhibitors. Synergism in respect of acute systemic haemodynamic effects between ET_A receptor antagonists and angiotensin receptor type 1 antagonists (ARBs)[52], or ACE inhibitors[155] has been demonstrated in man. More recently, the combination of ET receptor antagonism and ACE inhibition has also been shown to improve endothelial function[156, 157].

In respect of the kidneys, when ET_A receptor antagonism is given in the presence of ACE inhibition in healthy subjects, contrary to a lack of effect of ET_A receptor antagonism alone, an increase in renal blood flow and natriuresis is observed, an effect which appears to be both ET_B dependent and NO mediated[155]. While it is tempting to attribute the increase in sodium excretion to the activity of an unblocked tubular ET_B

receptor, it is possible that the natriuresis is entirely a consequence of the renal vasodilatation and so essentially a haemodynamic effect. This would likely be the case if the intrarenal changes in RBF, of an ET_B mediated increase in medullary blood flow, seen in animal models also occur in man. Further human studies are needed to characterise the role of the renal ET_B receptor and the interaction between ET and ANG II in CKD, in which there is increased activity in the RAS, and in a setting where many patients are already treated with ACE inhibitors.

Endothelin antagonism as a treatment strategy in CKD

ET-1 plays a role in the maintenance of BP and arterial stiffness. It also contributes to endothelial dysfunction, oxidative stress and vascular inflammation. In animals, ET receptor antagonists have been shown to reduce BP, improve arterial stiffness and endothelial dysfunction, and retard the progression of atherosclerosis. Some of these observations are confirmed by clinical studies. However, studies in CKD are fairly limited but suggest that, in addition to having a beneficial effect on systemic haemodynamics, ET receptor antagonists improve renal function and may potentially reduce renal disease progression.

The question of whether selective or non-selective receptor blockade should be used is probably disease-specific, and in CKD remains to be clarified. However, preliminary evidence in patients with CKD suggests that both selective ET_A and non-selective $ET_{A/B}$ receptor blockade reduce BP but that selective ET_A blockade has additional desirable effects on renal haemodynamics[64]. From the current evidence base, concomitant ET_B receptor blockade seems at best to offer no advantage over selective ET_A antagonism and may potentially reduce the benefits. Further studies are needed to discern the theoretical beneficial effects of an unblocked ET_B receptor in terms of natriuresis, diuresis and glomerular haemodynamics. Additionally, more clinical data are essential to advancing our broader understanding of the role of ET receptor antagonism, not only as a potential renoprotective therapy in CKD, but also as a treatment for the CVD with which it is associated.

Aims and hypotheses

In a series of acute and chronic studies this thesis explores the role of ET-1 and its antagonism in CKD.

Study 1 (Chapter 3): This study investigated the impact of progressive renal dysfunction and renal inflammation on plasma and urinary ET-1 concentrations. We hypothesised that plasma and urinary ET-1 concentrations would increase as GFR declined, and that in subjects with varying degrees of inflammatory CKD, but normal renal function, urinary ET-1 would act as a surrogate measure of the underlying renal inflammation.

Study 2 (Chapter 4): Here we studied the effects of acute selective ET_A receptor antagonism on BP, proteinuria and renal haemodynamics, arterial stiffness and endothelial function in patients with proteinuric CKD. We hypothesised that selective ET_A receptor antagonism would reduce BP and proteinuria, and additionally reduce arterial stiffness and improve endothelial dysfunction. We expected these effects to be evident on top of standard treatment with blockers of the renin-angiotensin system.

Study 3 (Chapter 5): To inform our studies in Chapter 6, here we investigated the pharmacokinetic profile of a single 100 mg oral dose of sitaxsentan, a selective ET_A receptor antagonist, in subjects with normal and impaired renal function. Since over 50% of the administered dose of sitaxsentan is excreted via the kidneys, impaired renal function could potentially affect the pharmacokinetics of sitaxsentan.

Study 4 (Chapter 6): Our acute studies (Chapter 4) showed that acute selective ET_A receptor antagonism reduces proteinuria, BP and arterial stiffness - key independent, surrogate markers of CKD progression and CVD risk, in patients with proteinuric CKD. These studies therefore examined if these effects are maintained longer term using the selective ET_A receptor antagonist sitaxsentan.

Chapter 2

Materials & 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 22-24^{0}C, with the subject recumbent throughout, except when voiding urine.

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.

Estimation of glomerular filtration rate

GFR was calculated using the Cockcroft & Gault (C&G) equation as an estimate of creatinine clearance (Chapter 3): GFR = [140 - age (years)] x weight (kg) x (0.85 if female) / serum creatinine.[158] GFR was further corrected by body surface area (BSA): BSA = [71.84 x weight (kg)$^{0.425}$ x height (cm)$^{0.725}$] / 10,000 as defined by Du Bois, *et al* [159]. The C&G equation was selected to assess renal function in this study because it is more accurate than the Modification of Diet in Renal Disease (MDRD) equation when used to assess mild renal insufficiency.[160, 161]

Drug administration

For systemic intravenous administration, study drugs were infused via an 18 standard wire gauge (SWG) cannula sited in an antecubital vein. PAH and Inulin were diluted in 5% dextrose (Baxter Healthcare Ltd, Thetford, UK) and infused intravenously at a constant rate of 2 ml/min. All other drugs were dissolved in physiological saline and infused intravenously at a constant rate of 1 ml/min. Saline was administered as placebo.

Drugs

BQ-123

BQ-123 (Clinalfa AG, Laufelfingen, Switzerland) was used as a selective ET_A receptor antagonist (Chapter 4). It is a synthetic derivative of BE 18257B, a product of *Streptomyces misakiensisis* and is a cyclic pentapeptide that is highly selective for the ET_A receptor (IC_{50}: ET_A = 7.3 nM, ET_B = 18 μM[162]). Studies with radiolabelled BQ-123 demonstrate that it binds competitively to the ET_A receptor, achieving steady state within 7 minutes of injection and dissociates with a half-time of 1.4 min[163]. It is extracted from the circulation by the hepatic anion transport system[164].

On the basis of previous work from our department[53] a dose of 1000 nmol/min BQ-123 over 15 min was used as that achieving maximal haemodynamic effects. The haemodynamic effects of 1000 nmol/min for 15 min are demonstrable for ~2 hours after injection and BQ-123 is undetectable in the plasma 150 min following injection.

Sitaxsentan

Sitaxsentan is an orally active ET_A receptor antagonist licensed for the treatment of pulmonary arterial hypertension[165]. It is approximately 6,500-fold more selective as an antagonist for the ET_A receptor than for the ET_B receptor[166]. In healthy subjects sitaxsentan displays linear steady state pharmacokinetics at the 100 mg therapeutic dose (with non-linear kinetics at higher doses). It is rapidly absorbed, highly bound to plasma proteins (>99.5%), predominantly albumin, and extensively metabolised (CYP2C9 pathway). However, data suggest that the metabolites of sitaxsentan are unlikely to contribute significantly to its therapeutic efficacy. Following oral dosing with radiolabelled sitaxsentan at the maximum clinically recommended dose of 100 mg, ~50-60% of the radioactivity is eliminated via the urine, with only ~1.2% of this due to unchanged parent drug. The balance is excreted via the faeces, in which there is no detectable parent compound[167].

Nifedipine

Nifedipine 10 mg and nifedipine 30mg LA (Adalat, Bayer) were used as active controls in Chapters 4 and 6, respectively, and administered orally.

PAH

Para-aminohippurate sodium (PAH, Clinalfa AG) was used for the measurement of renal plasma flow by standard clearance techniques[168] (Chapters 4 and 6). It is an inert and non-toxic compound that is both filtered at the glomerulus and actively secreted by the proximal tubules, reaching the kidney only via the blood stream. 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). This extraction rate is not affected by BQ-123 in man[169].

PAH 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.

Inulin

Inutest (Fresenius Pharma, Austria GmbH) was used for the measurement of glomerular filtration rate (GFR) by standard clearance techniques[168] (Chapters 4 and 6). Inulin is an inert and non-toxic complex polyfructose with a molecular weight of 5,200 daltons. It is not protein bound, is freely filtered at the glomerulus, is neither secreted not reabsorbed within the tubules, nor metabolised within the kidney and hence fulfils the criteria for the measurement of GFR by clearance measurements. Its problems with solubility have been overcome by the introduction of Sinistrin (Inutest) a related polysaccharide with identical clearance.

Inutest 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 < 50 ml/min, the maintenance dose was reduced by one-third, and by two-thirds for those with a GFR < 30 ml/min.

Blood pressure

Blood pressure (BP) was recorded in duplicate at each time-point using a validated oscillometric sphygmomanometer, the Omron HEM-705CP[170]. 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. For studies in chapter 7 ambulatory BP was recorded at the brachial artery using a validated Spacelabs 90217 ambulatory BP monitor[171]. Measurements were taken every 30min for 24h and mean systolic (SBP), mean arterial pressures (MAP) and diastolic (DBP) calculated.

Arterial stiffness

Pulse wave velocity (PWV), the gold standard for measurement of arterial stiffness[90], was measured by the foot-to-foot wave velocity method using the SphygmoCor® system (SphygmoCor® Mx, AtCor Medical, Sydney, Australia, version 6.31), in which a high-fidelity micromanometer (SPC-301, Millar Instruments, Texas, USA) was used to determine carotid-femoral PWV.

Pulse wave recordings were made at two sites- the carotid and femoral arteries. At each site the foot of the flow wave was identified as the beginning of the sharp systolic upstroke. The time delay was measured between the feet of the flow waves at each site and designated as the pulse transit time. A tape measure applied to the surface of the body was used to find the distance travelled by the pulse wave. ECG gating allows the time lapse between the pulse waves at both sites to be calculated from sequential rather than simultaneous measurements. PWV was then calculated as the distance: transit time ratio and expressed as metres per second.

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 a additional measure of arterial stiffness, was calculated from central aortic waveforms, which were derived by applying a generalised transfer function to the directly measured radial waveforms.

Endothelial function

Brachial artery flow-mediated dilatation (FMD) was used to assess endothelium-dependent vasomotor function[172]. With individuals in a supine position and their arms out-stretched perpendicular to the body, the brachial artery was imaged longitudinally with 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. 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. We did not use glyceryl trinitrate as a measure of endothelium-independent vasomotor function to avoid interference with responses to study drugs. FMD was quantified both as the peak change from baseline and as the area under the curve of the change from baseline in brachial artery diameter after 5 min of forearm ischaemia.

Cardiac output and heart rate

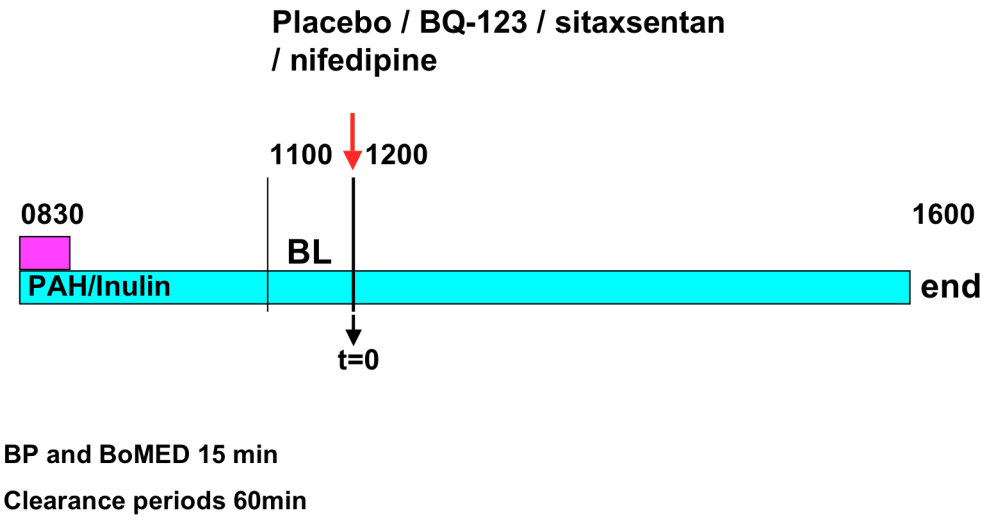
Cardiac output (CO, L/min) and heart rate (HR, bpm) were recorded using a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA)[173, 174]. This non-invasive technique measures CO by transthoracic bioimpedance. A constant sinusoidal current 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 xiphisternum**.** These electrodes then detect changes in bioimpedance related to the cardiac cycle and blood flow. CO is estimated from the measures of bioimpedance by the Sramek-Bernstein formula, adapted from the original formula of Kubicek[174]. 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 CI and HR.

Clearance Studies

ERPF and GFR were measured by standard clearance techniques[168] (Chapters 4 and 6). On each study day, an 18 SWG cannulae 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 PAH & inutest were administered through the same cannula. Thereafter, maintenance infusions of PAH and inutest, and 5% dextrose at 180 ml/hr continued throughout the study. Urine was collected by spontaneous voiding every 60 min. A two hour period was allowed for water, inutest and PAH equilibration before baseline measurements were made over one 60 min urine collection period. Blood pressure, CO and HR were recorded every 15 min. At the mid-point of each 60 min collection period, blood was sampled from the right antecubital cannula for PAH inulin, and haematocrit measurements. Following baseline recordings measurements were made for a further 4 hours.

Figure 2.1 : A standard clearance study. BL : baseline



Assays

At pre-specified time points, venous blood was collected via an 18 SWG cannula for plasma measurements. Blood was collected into plain tubes (Sarstedt) for the measurement of serum sodium, and into EDTA tubes (Sarstedt) for all other plasma measurements. 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.

Haematocrit (Hct) was measured on whole blood. All other blood samples were centrifuged immediately at 1000 *g* at 4^0C for 20 min, and plasma and urine stored in plain tubes at -80^0C until assay.

Plasma and urinary inulin

Inulin 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/$FeCl_3$ solution (7.5 mg $FeCl_3$ 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. Inulin concentrations were then determined against standard curves by absorption spectrophotometry at 480 nm.

Plasma and urinary PAH

PAH 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.

Haematocrit

Hct was measured using a Coulter counter.

Plasma and urine ET-1

After extraction[175], ET-1 was determined by radioimmunoassay[176]. The mean recovery of ET-1, from extraction to assay, was >90% for both plasma and urine. 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.

Plasma and urinary sodium

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

Urinary protein

Urine protein was measured using a colorimetric method with pyrogallol red[177].

Sitaxsentan

Samples for assessment of plasma sitaxsentan were collected into EDTA tubes and stored in wet ice. They were centrifuged for 20 minutes at 2,200 g at 4ºC within 30 minutes of collection. Plasma concentrations of sitaxsentan were measured using a validated LC/MS/MS method (inter- and intra-day assay variability +/- 15%) by MDS Pharma Services. In brief, this involved spiking plasma samples with a $^{13}C4^{15}N$ isotopically labeled internal standard and precipitating the protein with acetonitrile. The supernatant was transferred to clean culture tubes and the samples evaporated to dryness and reconstituted in acetonitrile/formic acid. The reconstituted extract was then injected into a HPLC system coupled to a MS/MS detector and the signal from the detector then back calculated to a

calibration curve to achieve a concentration value. The lower limit of quantitation for sitaxsentan was 0.005 μg/mL. The percent unbound sitaxsentan (F_U) in each sample was calculated according to $F_U = 100\% \times (C_U/C_T)$ where C_U and C_T represent the unbound and total concentrations, respectively.

Systemic and Renal Data

Data were stored and analysed using the Microsoft Excel data analysis package (Excel 5.0, Microsoft Ltd). BP at each time point was calculated as the mean of 2 recordings and represented as mean arterial pressure (MAP). Biompedance data at each time point were calculated as the mean of four recordings, each the average of 15 consecutive heart beats. Data were corrected using body surface area to give cardiac index (CI) for direct comparison of subjects, and systemic vascular resistance index (SVRI) derived from BP and CI data (Table 2.1).

GFR and ERPF were calculated from inulin and PAH clearances, respectively. Effective renal blood flow (ERBF), effective renal vascular resistance (ERVR) and effective filtration fraction (EFF) were derived from these indices (Table 2.1). Urinary sodium excretion and fractional excretion were calculated from plasma and urinary sodium and inulin concentration and urinary flow rates.

Table 2.1 : Calculations

Measurement	Calculation	Units
Mean Arterial Pressure (MAP)	$DBP + \frac{(SBP-DBP)}{3}$	mmHg
Systemic Vascular Resistance Index (SVRI)	$\frac{MAP}{CI} \times 80$	dyne.s /m^2/cm^5.
Glomerular Filtration Rate (GFR)	$\frac{uIn}{pIn} \times UFR$	ml/min
Effective Renal Plasma Flow (ERPF)	$\frac{uPAH}{pPAH} \times UFR$	ml/min
Effective Renal Blood Flow (ERBF)	$\frac{ERPF}{1-Hct}$	ml/min
Effective Renal Vascular Resistance (ERVR)	$\frac{MAP}{ERBF}$	mmHg.min/L
Effective Filtration Fraction (EFF)	$\frac{GFR}{ERPF} \times 100$	%
Urinary Flow Rate (UFR)	$\frac{\text{Urinary volume}}{\text{Time of collection}}$	ml/min
Urinary Sodium Excretion (UNaV)	uNa x UFR	µmol/min
Fractional Excretion of Sodium (FeNa)	$\frac{uNa}{pNa} \times \frac{pIn}{uin}$	

DBP - diastolic blood pressure, SBP - systolic blood pressure, u - urine, p - plasma, In - Inulin, PAH - para-amino hippuric acid, Hct - haematocrit, Na - sodium,

Chapter 3

Urinary endothelin-1 as a marker of chronic kidney disease

Abstract

Background: Chronic inflammation contributes to the development and progression of CKD. Identifying renal inflammation early is important. There are currently no specific markers of renal inflammation. ET-1 is pro-inflammatory and implicated in the pathogenesis of CKD. Thus, we investigated the impact of progressive renal dysfunction and renal inflammation on plasma and urinary ET-1 concentrations.

Methods: In a prospective study, plasma and urinary ET-1 were measured in 115 subjects with CKD stages 1 to 5 and 27 age- and blood pressure-matched non-CKD controls, and fractional excretion of ET-1 (FeET-1) calculated. FeET-1, serum C-reactive protein (CRP), urinary ET-1:creatinine ratio, and urinary albumin:creatinine ratio were also measured in 29 healthy volunteers (HV), 85 subjects with different degrees of inflammatory renal disease but normal renal function, and in 10 subjects with rheumatoid arthritis without renal involvement (RA). In subjects with nephritis associated with systemic lupus erythematosus (SLE) measurements were before and after 6 months of treatment.

Results: In subjects with CKD, plasma ET-1 increased linearly as renal function declined, whereas FeET-1 rose exponentially. In subjects with normal renal function, FeET-1 and urinary ET-1:creatinine ratio were higher in SLE subjects than in other groups (7.7 ± 2.7%, 4.7 ± 2.1 pg/μmol, both $p < 0.001$), and correlated with CRP. Notably, they were also significantly higher than in RA subjects (both $p < 0.01$) with similar CRP concentrations. In SLE patients, following treatment, FeET-1 fell to 3.6 ± 1.4% ($p < 0.01$).

Conclusions: Renal ET-1 production increases as renal function declines. In subjects with SLE, urinary ET-1 may be a useful measure of renal inflammatory disease activity whilst measured renal function is still normal.

Introduction

Chronic inflammation is a major contributor to the development and progression of CKD[178]. Current treatments for inflammatory CKD include immunosuppressive therapy, which is often associated with significant side effects[179]. Despite this, however, some patients develop progressive renal injury resulting in end-stage renal disease. Also, those who respond to treatment remain at risk of further disease relapses. Identifying renal inflammatory disease early and assessing its response to treatment remain important clinical challenges. Measurement of renal function, using serum creatinine for example, is often inadequate because substantial renal tissue damage can occur before function is impaired to a detectable extent[180]. However, serial renal biopsies are not appropriate in clinical practice. Current disease markers include serum C-reactive protein (CRP) and proteinuria. However, these lack both sensitivity and specificity for renal inflammation. There are currently no easily assessable clinical biomarkers specific to renal inflammation. Such markers would not only allow early implementation of appropriate treatments, with the hope of preventing disease progression, but also help identify future disease relapses.

Endothelin-1 (ET-1) is implicated in the development and progression of CKD[181], and is produced both within the vasculature and the kidney[181]. Although plasma ET-1 levels are not a reliable measure of vascular ET-1 production, owing to its predominantly abluminal release[16], urinary ET-1 excretion is independent of plasma ET-1 concentrations [182, 183] and is well-correlated with renal ET-1 production[25, 28].

A few small studies have shown a rise in plasma[72] and urine[184] ET-1 in severe CKD, and our group has previously demonstrated increases in plasma and urinary ET-1 concentrations in 8 subjects with non-inflammatory renal disease, across a range of glomerular filtration rates (GFR)[183]. However, there are no data on how renal inflammation may alter these profiles and hence on the utility of urinary ET-1 as a potential biomarker of renal inflammation.

Thus, we hypothesised that, as a result of reduced clearance and increased renal production respectively, plasma and urinary ET-1 concentrations would increase as GFR declined, and that in subjects with varying degrees of inflammatory CKD, but normal renal function, urinary ET-1 would act as a surrogate measure of the underlying renal inflammation. Our main groups of interest were thin basement membrane disease (TBM), immunoglobulin A nephropathy (IgAN) and systemic lupus erythematosus (SLE) with nephritis as examples of non-inflammatory, mild and more florid inflammatory renal diseases respectively.

Methods

This was a prospective and cross-sectional study.

Subjects

For Study 1, the inclusion criteria were: male or female CKD patients, 18 – 65 years old with a BP ≤ 160/100 mmHg. We excluded patients with a renal transplant, those requiring dialysis, and patients with a history of established cardiovascular disease, peripheral vascular disease, diabetes mellitus, respiratory disease, and neurological disease. Additionally, a systemic inflammatory disorder such as SLE or vasculitis was a specific exclusion criterion. Age- and BP-matched non-CKD subjects were recruited from the community.

For Study 2, we included male and female subjects aged 18-70, with haematuria and/or proteinuria of presumed glomerular origin. All subjects had a serum creatinine and GFR in the normal range and no history of hypertension. We excluded subjects with any significant co-morbidity. Rheumatoid arthritis (RA) subjects (as a control group for the SLE cohort) were recruited from the Rheumatology outpatient clinic at the Western General Hospital, Edinburgh.

Study protocol

Following a brief medical enquiry to confirm suitability for the study, body weight and height of the participants were recorded. After 30 min of supine rest, BP and heart rate

were recorded. Following this, blood was taken for analysis (serum creatinine, cholesterol, CRP and plasma ET-1), and urine collected (creatinine, albumin:creatinine ratio and ET-1), and tested for presence of blood and/or protein. Glomerular filtration rate was assessed using the Cockcroft & Gault equation (Chapter 2).

Data and statistical analysis

Data were stored and analysed in Microsoft Excel (version 11.3.7, Microsoft Ltd). Fractional excretion of ET-1 (FeET-1) was calculated by [(urine ET-1/plasma ET-1 x plasma creatinine/urine creatinine) x 100]%. Descriptive data are given as means ± SD. The D'Agostino and Pearson omnibus test was used to evaluate the distribution characteristic of the 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. To measure the sensitivity and specificity for FeET-1 at different values, a receiver-operator curve (ROC) curve was generated using subjects with IgAN and microhaematuria as controls. The area under curve was calculated to ascertain the quality of FeET-1 as a biomarker. An area of 0.5 is no better than expected by chance, whereas a value of 1.0 signifies a perfect biomarker. A significant level was p value ≤ 0.05.

Results

Study 1

142 subjects were enrolled into this study (115 CKD and 27 matched non-CKD subjects). CKD diagnoses were autosomal dominant polycystic kidney disease (n = 26), IgAN (n = 24), reflux nephropathy (n = 11), chronic glomerulonephritis (n = 10), non-inflammatory glomerular disease (n = 8), obstructive nephropathy (n = 5), TBM (n = 2), cystinuria (n = 2), Alport's disease (n = 1), and medullary cystic kidney disease (n = 1). 25 CKD subjects had no known cause for their renal disease. GFR ranged from 8 to 154 ml/min/1.73m^2. Baseline characteristics of all study subjects are shown in Table 3.1.

There was a negative linear correlation between GFR and plasma ET-1 (Table 3.2 and Figure 3.1a, $r^2 = 0.22$, $p < 0.001$). FeET-1 increased exponentially as GFR declined (Table 3.2 and Figure 3.1b, $r^2 = 0.47$, $p < 0.001$). Whereas plasma ET-1 did not correlate with BP, there was a positive correlation between BP and FeET-1 ($r^2 = 0.04$, $p < 0.05$). There was no relationship between GFR and serum CRP, consistent with our subjects comprising of a non-inflammatory cohort of CKD patients.

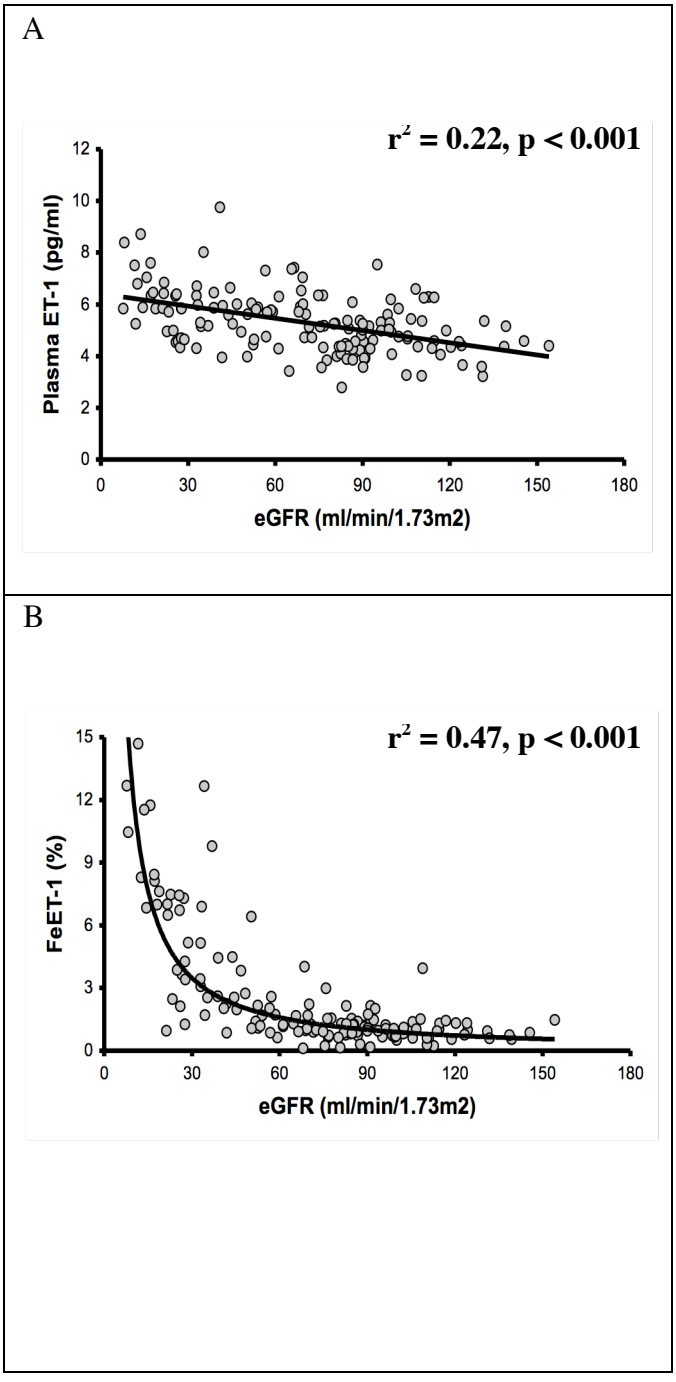
Table 3.1: Demographic data for non-CKD and CKD subjects in Study 1. BMI: body mass index, FeET-1: fractional excretion of ET-1, SBP: systolic blood pressure, DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein, ACR: albumin:creatinine ratio. Values are mean ± SD (range).

	Non-CKD subjects (n = 27)	**CKD subjects (n = 115)**	**p value**
Age, y	48 ± 9 (32 – 64)	47 ± 10 (23 – 65)	ns
Sex, M/F	13/14	77/38	-
BMI, kg/m^2	26 ± 5 (18 – 46)	28 ± 5 (19 – 41)	ns
SBP, mmHg	110 ± 17 (83 - 152)	119 ± 15 (85 – 159)	ns
DBP, mmHg	70 ± 10 (54 – 90)	74 ± 9 (52 – 96)	ns
Creatinine, μmol/l	78 ± 13 (55 – 98)	194 ± 164 (55 – 825)	$p < 0.001$
eGFR ml/min/$1.73m^2$	94 ± 18 (68 – 131)	63 ± 35 (8 – 154)	$p < 0.001$
Cholesterol , mg/dl	5.0 ± 0.8 (3.4 – 6.6)	4.6 ± 0.9 (3.0 – 8.2)	$p < 0.05$
CRP, mg/l	2 ± 3 (0 -12)	4 ± 4 (0 – 15)	$p < 0.05$
Plasma ET-1, pg/ml	4.6 ± 1.0 (2.8 – 7.5)	5.5 ± 1.1 (3.4 – 9.8)	$p < 0.001$
FeET-1, %	1.1 ± 0.7 (0 – 3.0)	3.0 ± 3.1 (0.2 – 14.7)	$p < 0.01$
ACR, mg/mmol	0.5 ± 0.8 (0 – 3.3)	48.6 ± (0 – 428)	$p < 0.001$

Table 3.2: Plasma ET-1 (pg/ml) and fractional urinary excretion of ET-1 (FeET-1, %) for subjects in Study 1 at different estimated glomerular filtration rates (eGFR). Values are mean ± SD (range).

	eGFR			
	< 30 ml/min/m^2	**30 – 60 ml/min/m^2**	**> 60 ml/min/m^2**	**p value**
Plasma ET-1 pg/ml	6.1 ± 1.2 (4.3 – 8.7)	5.8 ± 1.2 (4.0 – 9.8)	4.9 ± 1.0 (2.8 – 7.5)	< 0.001
FeET-1 %	6.8 ± 3.5 (1.0 – 14.7)	3.2 ± 2.7 (6.0 – 12.7)	1.1 ± 0.7 (0 – 4.0)	< 0.001

Figure 3.1: Scatter plots for estimated glomerular filtration rate (eGFR, ml/min/1.73m^2) and (A) plasma ET-1 (pg/ml), $r^2 = 0.22$, $p < 0.001$, and (B) fractional urinary excretion of ET-1 (FeET-1, %), $r^2 = 0.47$, $p < 0.001$.



Study 2

114 subjects took part in Study 2: healthy volunteers (HV, n = 29), TBM (n = 8), IgAN (n = 22), microscopic haematuria of presumed glomerular origin (MH, n = 35), SLE with nephritis (n = 10), and rheumatoid arthritis without renal involvement (RA, n = 10). All subjects with TBM, IgAN and lupus nephritis had biopsy-proven renal diagnoses. Of those with SLE, 4 had type IV lupus nephritis on renal biopsy, 2 type V, and 4 both types IV and V[185]. These subjects were studied before, and 6 months after, the start of treatment. This comprised of oral prednisolone for all 10 subjects, with 6 additionally receiving mycophenolate mofetil as a steroid-sparing agent, and the remaining 4 subjects receiving cyclophosphamide. All subjects in Study 2 had normal renal function with GFRs ranging from 61 to 153 ml/min/1.73m^2. A cohort of subjects with rheumatoid arthritis (RA) were included as a control group for those with lupus nephritis as having a similar degree of systemic inflammation, as measured by serum CRP, but no evidence of renal disease as shown by a clear urinalysis and GFR >60 ml/min/1.73m^2. Subject characteristics are shown in Table 3.3.

Table 3.3 *(overleaf):* Study 2 subjects: healthy volunteers (HV), and subjects with thin basement membrane disease (TBM), immunoglobulin A nephropathy (IgAN), microhaematuria of presumed glomerular origin (MH), systemic lupus erythematosus with nephritis (SLE) and rheumatoid arthritis (RA). BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein, ACR: albumin:creatinine ratio. Values are mean ± SD (range). For CRP ***$p < 0.001$ for SLE vs. all renal groups. For ACR *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. SLE. $^{\dagger}p < 0.01$ for HV vs. IgAN.

Table 3.3

	HV (n = 29)	TBM (n = 8)	IgAN (n = 22)	MH (n = 35)	SLE (n = 10)	RA (n = 10)
Age, y	46 ± 10 (32 – 64)	42 ± 11 (26 – 60)	41 ± 11 (24 – 59)	45 ± 13 (22 – 64)	40 ± 14 (26 – 60)	44 ± 8 (29 – 56)
Sex M/F	12/17	3/5	17/5	14/21	4/6	3/7
BMI kg/m^2	25 ± 4 (18 – 34)	26 ± 5 (19 – 31)	27 ± 4 (22 – 36)	28 ± 5 (21 – 39)	26 ± 5 (19 – 29)	25 ± 4 (20 – 33)
SBP mmHg	119 ± 18 (84 - 152)	115 ± 10 (101 – 124)	117 ± 9 (106 – 132)	121 ± 14 (8 – 156)	124 ± 17 (96 – 151)	131 ± 13 (109 - 149)
DBP mmHg	73 ± 10 (59 – 93)	76 ± 5 (67 – 81)	74 ± 8 (62 – 96)	78 ± 11 (57 – 111)	77 ± 11 (63 – 87)	76 ± 8 (63 – 87)
Creatinine µmol/l	77 ± 12 (55 – 93)	73 ± 10 (60 – 87)	92 ± 23 (49 – 115)	81 ± 16 (49 – 118)	83 – 21 (59 – 126)	81 ± 9 (72 – 101)
eGFR $ml/min/1.73m^2$	94 ± 16 (62 – 130)	102 ± 13 (74 – 115)	97 ± 26 (61 – 153)	97 ± 25 (68 – 130)	97 ± 27 (61 – 142)	89 ± 19 (61 – 132)
Cholesterol mg/dl	5.0 ± 0.9 (3.8 – 7.3)	5.3 ± 1.1 (3.3 – 5.9)	4.6 ± 0.7 (3.6 – 5.9)	5.2 ± 1.1 (3.2 – 8.8)	4.9 ± 0.8 (3.7 – 6.1)	5.0 ± 1.1 (4.3 – 6.0)
CRP mg/l	1 ± 2 (0 – 10)	1 ± 2 (0 – 6)	2 ± 2 (0 – 10)	3 ± 4 (0 - 14)	63 ± 15*** (45 – 87)	61 ± 16 (38 – 91)
ACR mg/mmol	0.6 ± 1.0*** (0 – 3.6)	1.6 ± 2.3* (0 – 6.6)	26.9 ± 46.0† (0 – 173.5)	2.3 ± 3.5** (0 – 15.2)	27.8 ± 27.0 (4.3 – 89.3)	1.1 ± 0.8* (0 – 2.6)

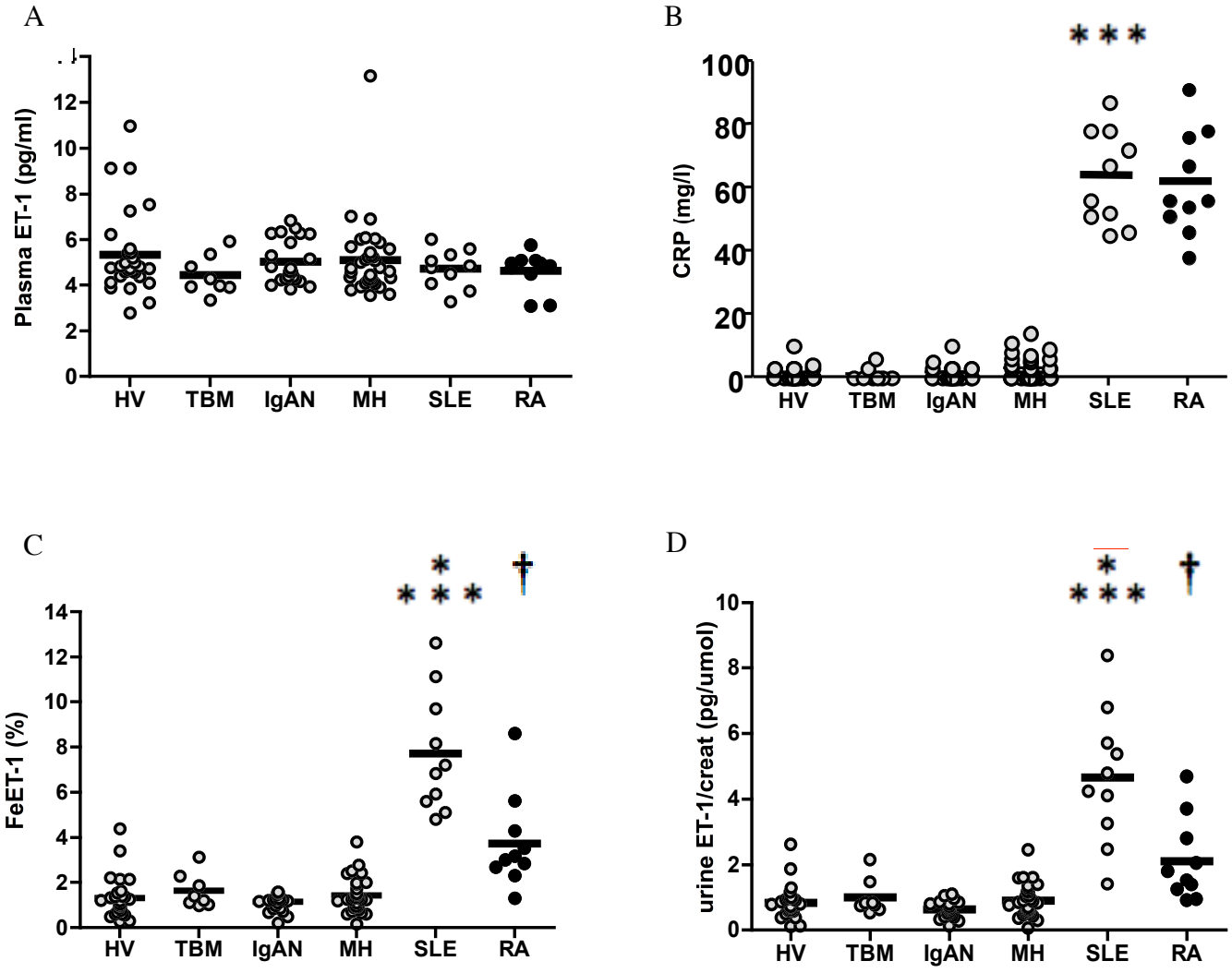
Table 3.4: Legend as for Table 3.3. FeET-1: fractional excretion of ET-1. *p < 0.05 for TBM vs. SLE, ***p < 0.001 for SLE vs. all other renal groups, †p < 0.01 fro SLE vs. RA.

	HV (n = 29)	TBM (n = 8)	IgAN (n = 22)	MH (n = 35)	SLE (n = 10)	RA (n = 10)
Plasma ET-1 pg/ml	5.3 ± 1.8 (2.8 – 11.0)	4.4 ± 0.8 (3.3 – 6.0)	5.1 ± 1.0 (4.0 – 6.9)	4.5 ± 0.7 (3.6 – 13.2)	4.7 ± 0.9 (3.3 – 6.0)	4.6 ± 0.9 (3.1 – 5.8)
FeET-1 %	1.3 ± 0.9 (0.3 – 4.4)	1.6 ± 0.8* (1.0 – 3.1)	1.1 ± 0.4 (0.2 – 1.6)	1.4 ± 0.7 (0.2 – 3.8)	7.7 ± 2.7*** (4.8 – 12.6)	3.7 ± 2.1† (1.3 – 8.6)
Urinary ET-1:creatinine	0.8 ± 0.5 (0.1 – 2.6)	1.0 ± 0.5* (0.5 – 2.2)	0.9 ± 0.5 (1.4 – 8.4)	0.9 ± 0.5 (1.4 – 8.4)	4.7 ± 2.1*** (1.4 – 8.4)	2.1 ± 1.3† (0.9 – 4.7)

All groups of subjects had similar plasma ET-1 concentrations (Table 3.4 & Figure 3.2a). However, both FeET-1 and urinary ET-1:creatinine ratio were significantly higher in lupus nephritis subjects compared to HV and all other renal groups (Tables 3.3, 3.4 & Figure 3.2c & 3.2d). Both FeET-1 and urinary ET-1:creatinine ratio were similar between HV, TBM, IgAN and MH.

CRP concentrations followed a similar pattern (Table 3.3 & Figure 3.2b, SLE: 63 ± 15 mg/l, p < 0.001 vs. HV and other renal subjects). Despite similar CRP concentrations to subjects with RA (Table 3.3 & Figure 3.2b, RA: 61 ± 16 mg/l), subjects with lupus nephritis had a significantly higher FeET-1 and urinary ET-1:creatinine ratio (Table 3.4 & Figure 3.2c & 3.2d). RA subjects also had higher urinary ET-1:creatinine ratio (p < 0.05), and FeET-1 (p < 0.01) than HV and subjects with MH and IgAN. There was no relationship between degree of proteinuria and FeET-1, urinary ET-1:creatinine ratio, or CRP.

Figure 3.2: (A) Plasma ET-1 (pg/ml), (B) serum C-reactive protein (CRP, mg/l) (C) FeET-1 (%), and (D) urinary ET-1: creatinine (pg/µmol). For all panels horizontal black bars show mean value. Panel (B): ***$p < 0.001$ for SLE vs. all groups except RA, for which p = ns. Panels (C) and (D): *$p < 0.05$ for SLE vs. TBM, ***$p < 0.001$ for SLE vs. all other groups, and †$p < 0.01$ for SLE vs. RA.



For FeET-1, the area under the ROC was 1.0 (curve not shown). Table 3.5 lists the derived sensitivities and specificities at different cutoff values for FeET-1. A value above 2.7% in renal patients yielded good sensitivity and specificity for the detection of SLE.

Table 3.5: FeET-1 test characteristics for renal subjects at different cutoff values. CI: confidence interval.

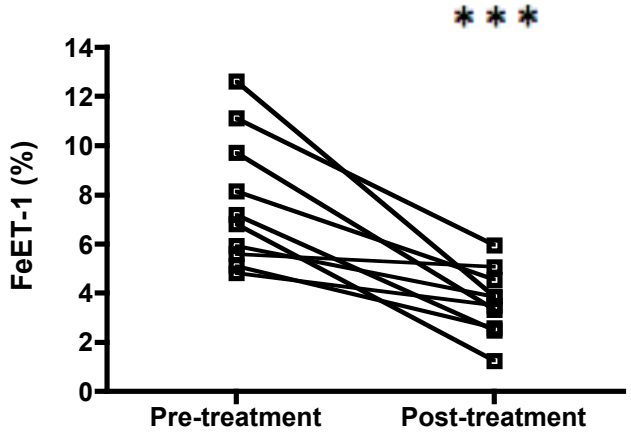
Cutoff for FeET-1 (%)	Sensitivity	95% CI	Specificity	95% CI
>2.08	1.00	0.69 – 1.00	0.96	0.85 – 0.99
>2.70	1.00	0.69 – 1.00	0.98	0.88 – 1.00
>3.96	1.00	0.69 – 1.00	1.00	0.92 – 1.00
>4.96	0.90	0.56 – 1.00	1.00	0.92 – 1.00

For subjects with SLE and nephritis, FeET-1 fell significantly following treatment (Figure 3, 7.7 ± 2.7 vs. 3.6 ± 1.4%, $p < 0.01$). Effects of treatment on other disease markers is shown in Table 3.6.

Figure 3.3 *(overleaf)*: (A) FeET-1 (%) and (B) albumin:creatinine ratio (ACR) in patients with systemic lupus erythematosus with nephritis pre- and post-treatment. *$p < 0.05$ and ***$p < 0.001$.

Figure 3.3

A.


***
FeET-1 (%)
14
12
10
8
6
4
2
0
Pre-treatment
Post-treatment

B.

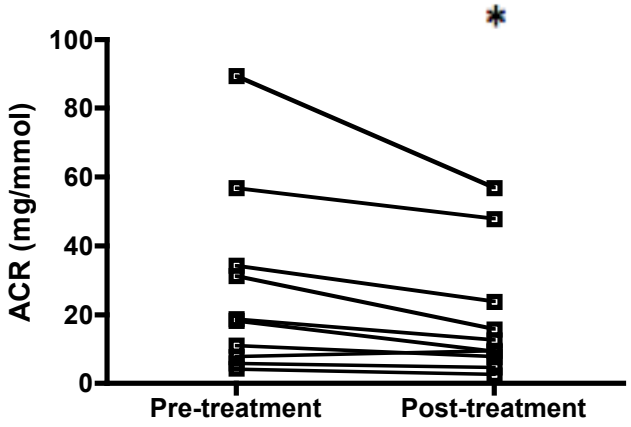

*
ACR (mg/mmol)
100
80
60
40
20
0
Pre-treatment
Post-treatment

Table 3.6: C-reactive protein (CRP), albumin:creatinine ratio (ACR), double-stranded DNA (dsDNA) and complement levels in subjects with lupus nephritis pre- and post-treatment. Values are mean ± SD. Normal ranges for dsDNA and complement are shown in brackets.

	Pre-treatment (n = 10)	**Post-treatment (n = 10)**	**p value**
CRP (mg/l)	63 ± 15	5 ± 3	$p < 0.001$
ACR (mg/mmol)	27.8 ± 27.0	19.1 ± 18.6	$p < 0.05$
dsDNA, (0 – 15, iu/ml)	102 ± 86	92 ± 93	$p < 0.05$
Complement (g/l)			
C3 (0.73 – 1.4)	0.73 ± 0.20	0.78 ± 0.11	p = ns
C4 (0.12 – 0.3)	0.13 ± 0.06	0.17 ± 0.03	$p < 0.05$

Discussion

Consistent with our previous findings in a limited number of subjects (8 CKD and 8 healthy controls)[183], we have now demonstrated in a large cohort of subjects that plasma ET-1 increases linearly as GFR declines, whereas FeET-1 shows an exponential rise. We have also shown for the first time that urinary ET-1 concentrations are raised in patients with systemic inflammatory disease and active renal involvement (but not systemic inflammatory disease *without* renal involvement), even when GFR is normal, whilst there is little impact of inflammation on plasma ET-1. Finally, in inflammatory renal disease urinary ET-1 concentrations fall following successful disease treatment. Thus, urinary ET-1 may be a useful marker of renal inflammation in the early stages of inflammatory renal disease, before renal function is affected, and may help direct treatment in these conditions.

Previous studies have shown increased plasma ET-1 concentrations in pre-dialysis[72, 186] and dialysis-requiring[72, 187] CKD patients. Our results are in keeping with these studies. However, we have also demonstrated that plasma ET-1 increases linearly as renal function declines, across a wide range of GFRs and in a relatively homogeneous, non-inflammatory CKD population. This is likely to be largely due to reduced renal filtration of ET-1 and thus renal clearance from the circulation. Importantly, we have also shown an exponential rise in FeET-1 as GFR declines. It is well established that a number of renal cell types are able to synthesise ET-1[6, 26, 181], and that ET-1 is an important regulator of renal function in CKD[53, 181]. It has been shown in animal models that plasma ET-1 does not account for urinary ET-1[28] and that renal cortical interstitial ET-1 levels correlate with urinary ET-1 excretion[25]. We have also previously demonstrated that plasma and urinary ET-1 change independently of one another in CKD[183]. These data support the view that urinary ET-1 concentrations reflect renal ET-1 production in CKD. Our finding of an exponential increase in FeET-1 as GFR declines is consistent with this evidence.

The results of our study suggest that urinary ET-1 is a useful marker of active renal inflammation in patients with lupus nephritis and normal renal function. Urinary ET-1 levels in subjects with TBM disease, a non-inflammatory condition, and in those with IgAN nephropathy, associated with mild renal inflammation, were no different to those in healthy volunteers. These findings are consistent with renal ET-1 production being driven by more florid inflammatory renal disease as is seen in lupus nephritis. In this study we used spot urine samples, as are collected routinely in the clinic, rather than timed urine collections making the data more widely clinically applicable. Whilst a urinary biomarker of disease activity would be ideal, being non-invasive and readily available, our results show an overlap in urinary ET:creatinine ratios between different renal groups. However, our sample size is small and it will be important to see if the same holds true in a larger cohort of subjects. Interestingly, the lack of overlap between FeET-1 levels in subjects with active lupus nephritis and levels in healthy volunteers and those with other renal diagnoses does support its use as a discriminatory test in the clinical management of lupus nephritis. Consistent with this is the area under the ROC of 1.0. Although calculation of FeET-1 requires both a blood and urine sample, it should still be feasible in most renal clinics. This would be of particular help in the assessment of renal disease activity because involvement of different organs in SLE is variable. Thus, in the group of patients who present to the renal clinic with an active urinary sediment but in the presence of normal renal function an elevated urinary ET-1 level may help identify those who have more active inflammatory renal disease, such as lupus nephritis.

FeET-1 and CRP were both significantly elevated in subjects with lupus nephritis compared to healthy volunteers and subjects with other renal diagnoses. Thus, one may argue that it is the systemic inflammation in lupus nephritis that is driving the increase in renal ET-1 production. However, when we compared urinary ET-1 concentrations in subjects with newly diagnosed and untreated rheumatoid arthritis, with a similar degree of systemic inflammation as reflected by a similar CRP, but with no evidence of renal involvement, FeET-1 was significantly higher in those with lupus nephritis. These data

suggest that the increase in urinary ET-1 in lupus nephritis is predominantly in response to the renal inflammation. As the current study had limited numbers we are unable to comment on the relationship between histological class of lupus nephritis and renal ET-1 production, but this is an area of ongoing study. Interestingly, although subjects with rheumatoid arthritis had a lower FeET-1 than those with lupus nephritis, they had a *greater* FeET-1 than healthy volunteers and those with other renal diagnoses suggesting that systemic inflammation may, in part, contribute to renal ET-1 production. Indeed, previous studies suggest that inflammatory mediators stimulate ET-1 production[188, 189].

The current clinical study was designed to look at a pre-selected group of patients: those referred to the renal clinic on the basis of an abnormal urinalysis (haematuria +/- proteinuria) but in the presence of normal renal function. Our active control group ideally needed to comprise of subjects with a similar degree of systemic inflammation to those with lupus nephritis but with no evidence of renal disease. We chose patients with rheumatoid arthritis as they commonly present to the rheumatology clinic. They often have evidence of systemic inflammation but it is uncommon for the kidney to be involved[190], especially at the outset of disease. Although one may argue subjects with untreated SLE without nephritis may have been a better choice of control, these patients have variable CRP levels[191, 192] despite other evidence of systemic inflammation[193, 194], and their degree of inflammation is considerably less when not associated with nephritis. Clearly, comparing urinary ET-1:creatinine ratio and FeET-1 in those with SLE in the presence and absence of nephritis would be of some interest and an area of future study.

One problem in the management of lupus nephritis is assessing the response to immunosuppressive treatment as well as the early detection of relapse. A rising serum creatinine may be due to active disease or progressive renal scarring. However, serial renal biopsies are not without risk, making a simpler test desirable. The data from the current study show a significant fall in FeET-1 in subjects with lupus nephritis following successful treatment. At 6 months, all subjects were in clinical disease remission as defined by an improvement in symptoms and a fall in CRP. Importantly, *all* subjects

showed a fall in FeET-1. By contrast, some (6 of 10), but not all, showed a resolution of their microscopic haematuria, and/or reduction in proteinuria (7 of 10). Other immunological markers of disease activity (double-stranded DNA and complement levels) also showed variable changes. Thus, these data suggest that a fall in urinary ET-1 may be a useful additional marker of response to therapy. Whether monitoring urinary ET-1 levels may be useful in detecting patients who do not respond to therapy remains unclear. As a limitation, we recognise that the heterogeneity in treatment given may impact on the data and this should be studied further. Furthermore, none of the 10 patients relapsed within the 6-month period and it remains speculative whether urinary ET-1 levels would rise before clinical relapse and this should also be the focus of larger studies.

It is important to consider whether renal impairment or significant proteinuria would affect the utility of urinary ET-1 as a biomarker of renal inflammation. The results of Study 1 demonstrate that in patients with non-inflammatory CKD FeET-1 only begins to increase at a GFR of ~60 ml/min. All of the subjects with lupus nephritis in Study 2 had better renal function than this. It would be of great interest to see whether FeET-1 levels were higher in subjects with severe enough inflammatory renal disease to cause deterioration in GFR (<60 ml/min), such as in those with small vessel vasculitis, than in subjects with similar GFRs but non-inflammatory CKD, and whether levels remained higher in those without active inflammation. With regard to proteinuria, our data show no relationship between urinary ET-1 concentrations and degree of proteinuria. This is in contrast to others who have shown both microalbuminuria[195] and nephrotic range proteinuria[141] associated with urinary ET-1 albeit in different cohorts of patients. Certainly, both *in vitro*[126] and *in vivo*[127] experiments have shown that proteinuria may stimulate renal ET-1 production and this was associated with damage to the podocyte. All our subjects had low-grade proteinuria and whether higher degrees would relate to urinary ET-1 and whether alterations in podocyte function contribute to the development of lupus nephritis remains unclear and should be another area for further study.

Our data add to the existing and expanding literature on urinary biomarkers of inflammatory renal disease, in particular relating to lupus nephritis[196]. In a recent study by Pitashny *et al*[197], urinary lipocalin-2 levels were found to be higher in subjects with SLE in the presence of nephritis than in its absence. However, there was significant overlap in lipocalin-2 levels between the two groups, the population studied included a majority of Hispanics and African Americans, and the response to disease treatment was not assessed.

In conclusion, in the current study we have found that urinary ET-1 may act as a useful marker of active renal inflammation in lupus nephritis and provide additional clinically relevant information about disease activity to that given by established markers. Further study is needed to investigate whether rising urinary ET-1 concentrations are useful in identifying patients who do not respond to therapy or predicting a relapse, and whether different therapies may have variable effects on urinary ET-1. Furthermore, studying patients with other inflammatory renal diseases such as those with small vessel vasculitis, would be of great interest. ET-1 is pro-inflammatory and its upregulation in CKD may contribute to disease progression[181]. Thus, antagonising the effects of ET-1 may offer therapeutic benefits in patients with CKD, and this is supported by pre-clinical and clinical data[53, 198].

Chapter 4

Acute endothelin-A receptor antagonism reduces blood pressure, arterial stiffness and proteinuria in chronic kidney disease

Abstract

Background: ET-1 is implicated in the development and progression of CKD. We therefore studied the effects of selective ET_A receptor antagonism with BQ-123 on BP, proteinuria and renal haemodynamics, arterial stiffness and endothelial function in patients with CKD.

Methods: In a double-blind, randomised crossover study, 22 subjects with proteinuric CKD received, on two separate occasions, placebo or BQ-123. 10 of these subjects also received nifedipine 10mg as an active control for the antihypertensive effect of BQ-123. BP, pulse wave velocity (PWV), flow-mediated dilatation (FMD), renal blood flow (RBF) and glomerular filtration rate (GFR) were monitored following drug dosing.

Results: BQ-123 reduced BP (mean arterial pressure: -7±1%, $p < 0.001$ *vs* placebo), and increased RBF (17±4%, $p<0.01$ *vs* placebo). GFR did not change. Proteinuria (-26±4%, $p<0.01$ *vs* placebo) and PWV (-5±1%, $p<0.001$ *vs* placebo) fell after BQ-123, but it had no acute effect on FMD. Nifedipine matched the BP and RBF changes seen with BQ-123. Nevertheless, BQ-123 reduced both proteinuria (-38±3 *vs* 26±11%, $p<0.001$) and PWV (-9±1 *vs* -3±1%, $p<0.001$), to a greater extent than nifedipine.

Conclusions: Selective ET_A receptor antagonism reduces BP, proteinuria and arterial stiffness on top of standard treatment in CKD patients. Furthermore, these studies suggest the reduction in proteinuria and arterial stiffness is independent of BP. If maintained longer term, the effects of selective ET_A receptor antagonism would confer both cardiovascular and renal protection in patients with CKD.

Introduction

Chronic kidney disease (CKD) is common[199]. Hypertension is an independent risk factor for CKD progression[200], and is a frequent finding in patients with CKD. Its prevalence increases as CKD progresses, with over 75% of patients with a glomerular filtration rate (GFR) <30ml/min having a blood pressure (BP) >140/90mmHg[201]. Despite treatment with multiple antihypertensive agents the majority of CKD patients fail to reach target BP[202].

Proteinuria is a common feature of CKD and its presence is independently associated with an adverse renal outcome[203]. Current treatments for proteinuria focus on BP reduction[119], ideally using angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs)[204], both of which are inhibitors of the renin-angiotensin system and thought to reduce proteinuria to a greater extent than accounted for by BP-lowering alone[205]. Nevertheless, many CKD patients have significant residual proteinuria despite optimal treatment[206].

CKD is also strongly associated with incident cardiovascular disease (CVD)[207]. The US National Kidney Foundation Task Force on CVD in Chronic Renal Disease recognises that patients with CKD should be considered in the 'highest risk group' for subsequent cardiovascular events[208]. Hypertension[209] and proteinuria[210] make an important contribution to CVD risk in CKD, as do arterial stiffness[87, 89] and endothelial dysfunction[77]. Thus, there remains an unmet need for newer treatments in CKD that will not only lower BP and proteinuria beyond the levels achieved with standard therapies, but may also have favorable effects on arterial stiffness and endothelial dysfunction and so offer longer term cardiovascular and renal protection.

Endothelin-1 (ET-1) is is implicated in both the development and progression of CKD, and contributes to CVD[181]. Our group has previously shown that selective ET_A receptor antagonism, but not mixed $ET_{A/B}$ antagonism, reduces BP, increases renal blood flow and reduces effective filtration fraction in patients with CKD[53]. ET-1 also contributes to

arterial stiffness[22] and endothelial dysfunction[82, 95] in patients with vascular disease, however, there are no studies in renal patients.

Based on our previous work[53], we hypothesised that in patients with proteinuric CKD, acute selective ET_A receptor antagonism would reduce BP and proteinuria, and additionally reduce arterial stiffness and improve endothelial dysfunction. As we have previously demonstrated a synergism between ACE inhibitors and selective ET_A receptor antagonism in health[211], we expected these effects to be evident on top of standard treatment with ACE inhibitors and/or ARBs.

Methods

Subjects

We enrolled subjects 18-70 years of age with stable CKD stages 1 to 4[201] and proteinuria (>300 mg/day). Subjects were on treatment with ACE inhibitors and/or ARBs for their proteinuria (see Table 4.3). Explicitly, doses of one or both of drugs were titrated to the maximum tolerated, dependent on BP, renal function, serum potassium levels and side effects. All medications were unchanged over the 3 months preceding the studies.

Patients with significant co-morbidity, including diabetes mellitus, heart or lung disease, and peripheral vascular disease were excluded. To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis, other systemic inflammatory disease, polycystic kidney disease, nephrotic syndrome, or obstructive uropathy were excluded.

Study protocol

This was a randomised, double-blind, placebo-controlled study with a sub-study using an open-label active control arm. The main study looked at the effects of the selective ET_A receptor antagonist BQ-123 compared to saline placebo. As previous studies with BQ-123 have shown a reduction in BP in CKD patients, and as this may contribute to changes in arterial stiffness, protein excretion and natriuresis, nifedipine 10mg given

orally was used as an active control for this reduction in MAP in those subjects taking part in the sub-study.

All subjects attended for 2 visits, receiving placebo and BQ-123 in a randomised order, with those taking part in the sub-study (nifedipine 10mg) attending for three randomised visits. Since previous studies with the same dose of BQ-123 have demonstrated that haemodynamic changes return to baseline after 4 hours,[46, 53, 212], and the half-life of nifedipine is ~2 hours, each visit was separated by ≥7 days to ensure complete washout of the study drugs. On each study day, a clearance study was carried out as previously described (Chapter 2).

Statistical analysis

The number of subjects required to show a significant difference in BP and ERVR was based on previous data in CKD patients using the same dose of BQ123[53]. The co-primary endpoints were reductions in BP and proteinuria, with the secondary endpoints being a reduction in arterial stiffness and improvement in endothelial function. Baseline haemodynamic data were calculated as the mean of the 2 time points that immediately preceded administration of the study drug. For urine data, only one baseline measurement was used immediately prior to drug dosing. Haemodynamic and urine results are expressed as mean ± SEM change from baseline for drug and placebo, and placebo-corrected change from baseline for sub-study results. Statistical analysis was performed on untransformed data. Three comparisons of interest were pre-identified as placebo vs. BQ-123, placebo vs. nifedipine, and BQ-123 vs. nifedipine. Responses were examined by repeated measures ANOVA, and Bonferroni correction was used to assess significance at specific time points. Statistical significance was taken at the 5% level.

Results

22 patients with stable proteinuric CKD aged 46 ± 3 (29 – 69) years were recruited into the studies. All 22 CKD patients completed the placebo and BQ-123 phases of the study without adverse events. All subjects had similar baseline urinary protein leak on each

study day. Patient diagnoses were IgA nephropathy (n = 9), membranous glomerulopathy (n = 5), and focal segmental glomerulosclerosis (n = 8). Subject demographics are shown in Table 4.1 with baseline parameters, including renal function and proteinuria, shown in Table 4.2. Table 4.3 shows individual subject characteristics.

Table 4.1: Subject demographic data for main and sub- studies. Values are mean ± SEM (range).

	Main study (n = 22)	**Sub-study (n = 10)**
Age, y	46 ± 3 (29 – 69)	45 ± 11 (29 – 64)
Body mass index, kg/m^2	28 ± 1 (20 – 37)	30 ± 6 (21 – 37)
MAP, mmHg	93 ± 2 (75 - 118)	93 ± 2 (84 – 101)
Creatinine, μmol/l	191 ± 29 (78 – 487)	199 ± 88 (100 – 343)
Cholesterol, mmol/l	170 ± 8 (127 – 228)	166 ± 35 (127 – 228)
Urinary sodium excretion, mmol/24h	163 ± 18 (39 – 300)	174 ± 75 (39 - 300)
Urinary protein excretion, g/24h	2.7 ± 0.7 (0.4 - 8.7)	2.9 ± 2.8 (0.4 – 8.0)

Table 4.2*:* Baseline data for main study and sub-study. Values are given as mean of baseline pre-treatment periods over the 2 or 3 study days ± SEM (range).

	Main study (n = 22)	**Sub-study (n = 10)**
MAP, mmHg	92 ± 2 (80 – 103)	93 ± 2 (84 – 101)
SVRI, dyne/s/m^2/cm^5	3360 ± 230 (1800 – 5510)	3530 ± 230 (1980 – 5510)
CI, l/min^1/m^2	3.0 ± 0.2 (1.8 – 4.7)	2.9 ± 0.2 (1.8 – 4.4)
Heart rate, bpm	56 ± 2 (38 – 75)	57 ± 2 (42 – 66)
PWV, m/s	7.5 ± 0.4 (5.5 – 12.2)	7.4 ± 0.5 (5.7 – 10.5)
FMD, %	4.4 ± 0.6 (0.6 – 12.7)	4.4 ± 0.9 (0.1 – 8.2)
ERBF, ml/min	1810 ± 233 (106 – 4632)	1968 ± 390 (530 – 4632)
ERVR, mmHg/min^1/ml^1	11.5 ± 4.4 (2.0 – 107.8)	7.1 ± 1.5 (2.0 – 18.2)
GFR, ml/min/1.73m^2	43 ± 5 (12 – 99)	43 ± 7 (15 – 99)
UNaV, μmol/min	197 ± 21 (27 – 392)	193 ± 28 (95 – 392)
Urinary protein excretion, μg/min	1570 ± 371 (165 – 8616)	1520 ± 577 (109 – 8616)
Plasma ET-1 pg/ml	5.7 ± 0.3 (3.6 – 10.5)	6.7 ± 0.5 (3.8 – 8.7)

Table 4.3*:* Subject diagnoses and medications. Doses are total per day. IgAN: immunoglobulin A nephropathy; FSGS: focal & segmental glomerulosclerosis; Membranous: membranous glomerulopathy; EPO: erythropoietin.

Subject	Diagnosis	ACE inhibitor	ARB	Other drugs
1	IgAN	Ramipril 2.5mg		
2	Membranous	Ramipril 10mg	Candesartan 4mg	Atorvastatin 20mg Furosemide 40mg
3	FSGS	Lisinopril 40mg	Valsartan 80mg	Allopurinol 200mg Atorvastatin 20mg Fluoxetine 20mg Furosemide 40mg
4	FSGS	Ramipril 10mg	Candesartan 4mg	Simvastatin 80mg Zopiclone 12.5mg
5	IgAN	Lisinopril 40mg	Candesartan 4mg	Atorvastatin 20mg
6	IgAN	Ramipril 2.5mg		
7	IgAN	Ramipril 5mg		
8	FSGS	Lisinopril 20mg		Amlodipine 5mg Atenolol 50mg Atorvastatin 20mg Bendroflumethazide 2.5mg Omeprazole 20mg Sodium bicarbonate 3g
9	FSGS	Ramipril 10mg		Allopurinol 200mg Atorvastatin 40mg Bendroflumethazide 2.5mg Diltiazem 180mg Doxazosin 8mg EPO 12,000IU/week Ferrous sulphate 600mg Furosemide 80mg Omeprazole 20mg

10	Membranous	Enalapril 40mg	Losartan 50mg	Amlodipine 5mg
11	Membranous	Enalapril 40mg		Allopurinol 200mg Amlodipine 5mg Furosemide 40mg Metoprolol 100mg
12	IgAN	Ramipril 10mg	Candesartan 32mg	Aspirin 75mg Atorvastatin 40mg
13	FSGS	Lisinopril 40mg		Aspirin 75mg Atenolol 50mg Simvastatin 80mg Amlodipine 5mg
14	IgAN	Lisinopril 40mg	Candesartan 16mg	Aspirin 75mg Rosuvastatin 40mg
15	FSGS	Ramipril 10mg	Valsartan 160mg	Amlodipine 5mg Allopurinol 200mg
16	IgAN	Lisinopril 40mg	Candesartan 16mg	Amlodipine 5mg Atorvastatin 20mg Furosemide 40mg
17	IgAN	Ramipril 10mg		
18	Membranous	Ramipril 10mg		Amlodipine 5mg Atenolol 50mg
19	Membranous	Lisinopril 40mg		Metoprolol 100mg Omeprazole 20mg
20	FSGS	Lisinopril 40mg	Valsartan 80mg	Atorvastatin 20mg
21	IgAN	Lisinopril 40mg	Valsartan 160mg	Atenolol 50mg Allopurinol 200mg Atorvastatin 20mg
22	FSGS	Ramipril 10mg	Candesartan 8mg	

Main study

Systemic haemodynamics (Figure 4.1a & 4.1b)

Placebo was associated with increases in both MAP (93.1 ± 2.1 vs. 98.8 ± 2.4 mmHg, $p < 0.001$), and SVRI (3360 ± 230 vs. 3670 ± 290 dyne/s/m^2/cm^5, $p < 0.05$) from baseline to study end. BQ-123 led to reduction in both MAP (-9.2 ± 1.2 mmHg, $p < 0.001$ vs. placebo), and SVRI (-610 ± 100 dyne/s/m^2/cm^5, $p < 0.001$ vs. placebo), with the peak effects at 75 min following drug administration. There were no significant differences in heart rate between placebo and BQ-123 throughout the time course of the study.

Arterial stiffness and endothelial function (Figure 4.1c)

Whereas PWV increased following placebo (7.5 ± 0.4 vs. 7.8 ± 0.4 m/s, $p < 0.001$), BQ-123 was associated with a significant fall in PWV (-0.8 ± 0.1 m/s, $p < 0.001$ vs. placebo). With regards to endothelial function, there were no differences in FMD response between BQ-123 (4.4 ± 0.5 vs. 5.5 ± 0.8%, $p = 0.056$), and placebo (4.4 ± 0.6 vs. 5.1 ± 0.7%, $p = 0.082$).

Renal haemodynamics (Figure 4.2a & 4.2b)

Administration of placebo was associated with a gradual reduction in ERBF (1810 ± 233 vs. 1454 ± 181 ml/min, $p < 0.001$), and increase in ERVR (11.5 ± 4.4 vs. 12.8 ± 3.8 mmHg/min/ml, $p < 0.05$) to study end. In contrast, BQ-123 produced a striking increase in ERBF (365.2 ± 103.6 ml/min, $p < 0.01$ vs placebo), and a reduction in ERVR (-3.0 ± 0.9 mmHg/min/ml, $p < 0.01$ vs placebo). There were no significant changes in GFR with either placebo or BQ-123.

Urinary sodium and protein excretion (Figure 4.2c & 4.3)

Placebo had little effect on sodium excretion, whereas BQ-123 produced a marked natriuresis with a maximum increase of 35.5 ± 15.4 μmol/min ($p < 0.05$ vs placebo).

Whereas placebo had little effect on urinary protein excretion, BQ-123 led to a sustained reduction in proteinuria throughout the time course of the study. This reduction at its maximum equated to -495.7 ± 141.0 μg/min ($p < 0.01$ vs. placebo), a fall in protein leak of about 30%. The size of this effect related to baseline urinary protein excretion with subjects with higher baseline proteinuria achieving a greater reduction ($r^2 = 0.78$, $p < 0.05$). This effect was seen across all levels of GFR.

Plasma ET-1

There were no changes in plasma ET-1 concentrations with either drug or placebo.

Sub-study: nifedipine 10mg

12 subjects were initially randomised to placebo or BQ-123 alone, after which 10 subjects took part in a randomised 3-way crossover sub-study, which included placebo, BQ-123 and nifedipine. All 10 subjects completed the 3 phases of the sub-study without adverse events. Subject demographics are shown in Table 4.1 with baseline parameters shown in Table 4.2.

BQ-123 and nifedipine produced a similar reduction in MAP (Figure 4.4a). Nifedipine caused an initial increase in heart rate (mean increase of 10 beats per minute from baseline) that returned to baseline by 60 min. Despite the consistent changes in systemic haemodynamics, BQ-123 reduced arterial stiffness to a greater extent than did nifedipine (PWV: -0.6 ± 0 vs. -0.3 ± 0.1 m/s, $p < 0.001$, Figure 4c). BQ-123 and nifedipine also increased ERBF to a similar degree (Figure 4.4b), and produced a similar natriuresis (at maximum, BQ-123: 64.4 ± 37.4 μmol/min, nifedipine: 37.2 ± 33.8 μmol/min). By contrast to BQ-123, nifedipine was associated with a gradual increase in urinary protein excretion (at maximum 190.4 ± 142.4 μg/min, $p < 0.01$ vs placebo, Figure 4.4d), whereas BQ123 produced a consistent reduction in proteinuria.

Figure 4.1: Systemic haemodynamics and arterial stiffness after ET_A receptor antagonism. MAP: mean arterial pressure; SVRI: systemic vascular resistance index; PWV: pulse wave velocity. Values are given as mean % change from baseline ± SEM (left), and mean area under curve (AUC) of % change from baseline ± SEM (right). Blue line/block, placebo; red line/block, BQ-123. **+**p < 0.05 *vs.* placebo, **†**p < 0.01 *vs.* placebo, *p < 0.001 *vs.* placebo (ANOVA plus Bonferroni correction for significance at specific time points).

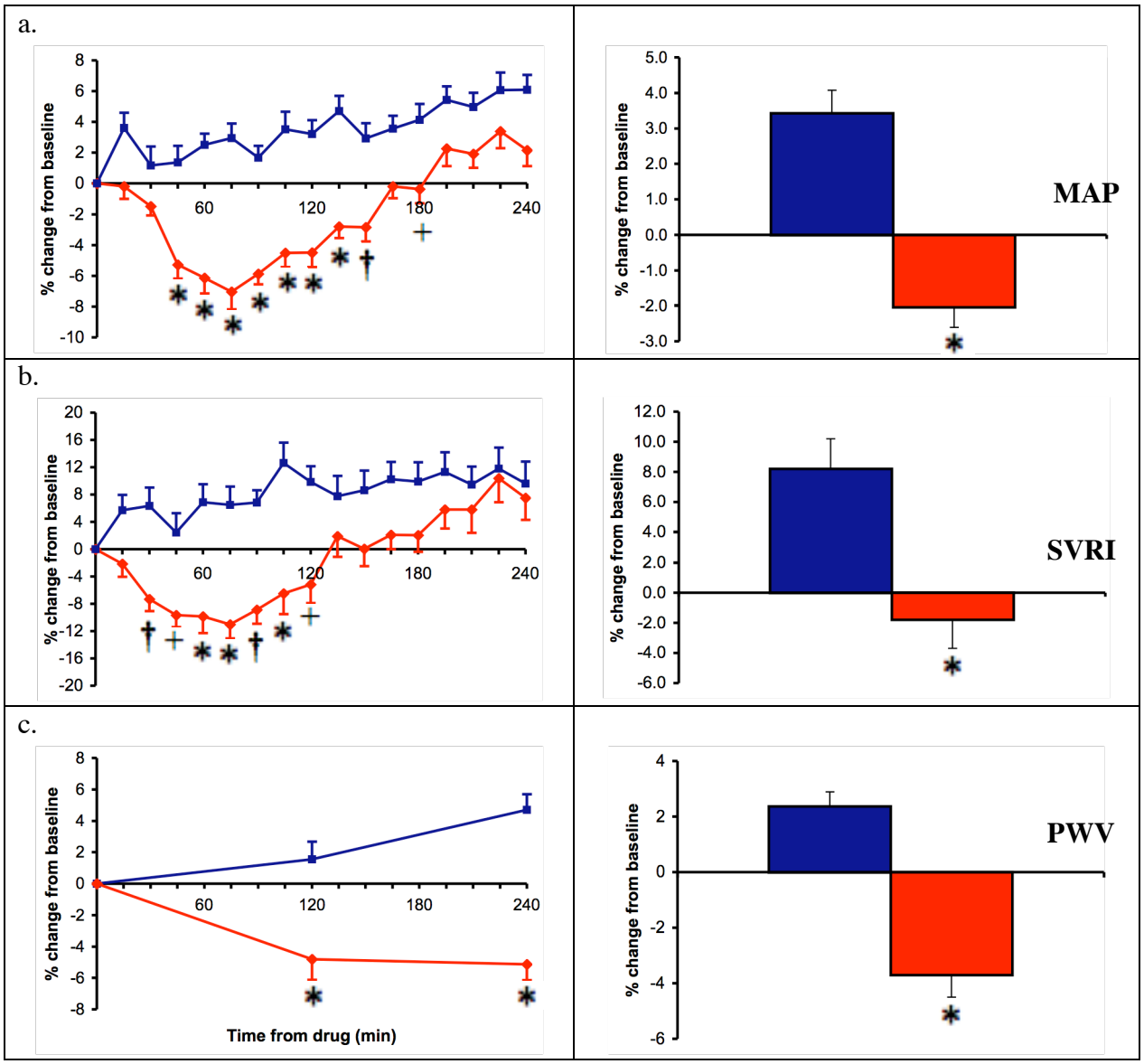


Figure 4.2: Renal haemodynamics and UNaV after ET_A receptor antagonism. RBF: renal blood flow; RVR: renal vascular resistance; UNaV: sodium clearance. Legend as for Figure 4.1.

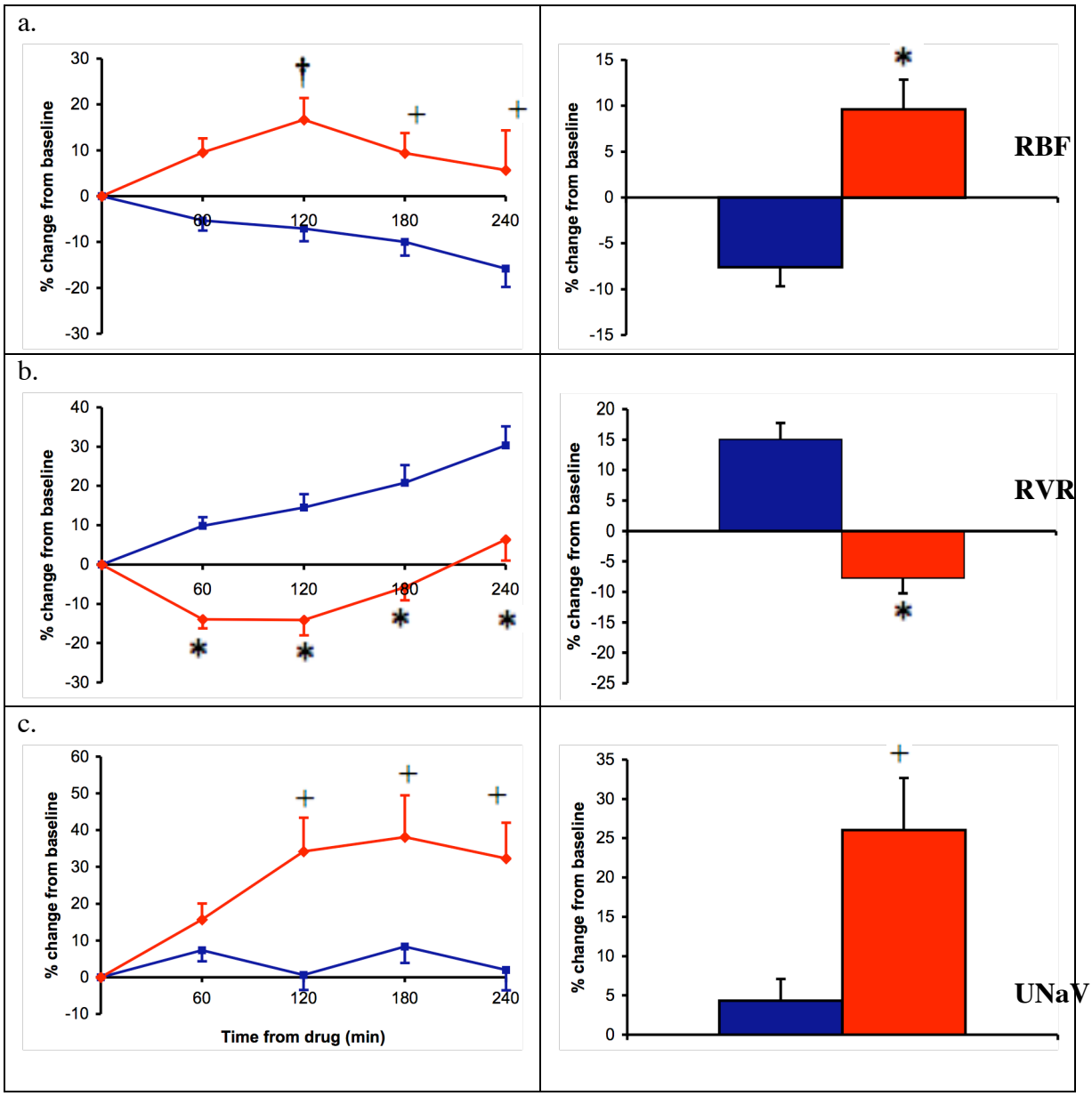


Figure 4.3: Protein excretion after ET_A receptor antagonism. Values are given as mean % change from baseline ± SEM (left), and mean area under curve (AUC) of % change from baseline ± SEM (right). Blue line/block, placebo; red line/block, BQ-123. +p < 0.05 vs. placebo, †p < 0.01 vs. placebo, *p < 0.001 vs. placebo (ANOVA plus Bonferroni correction for significance at specific time points). Figure 4.3c: effect of baseline protein excretion on maximal proteinuria reduction (mg/min) with BQ-123.

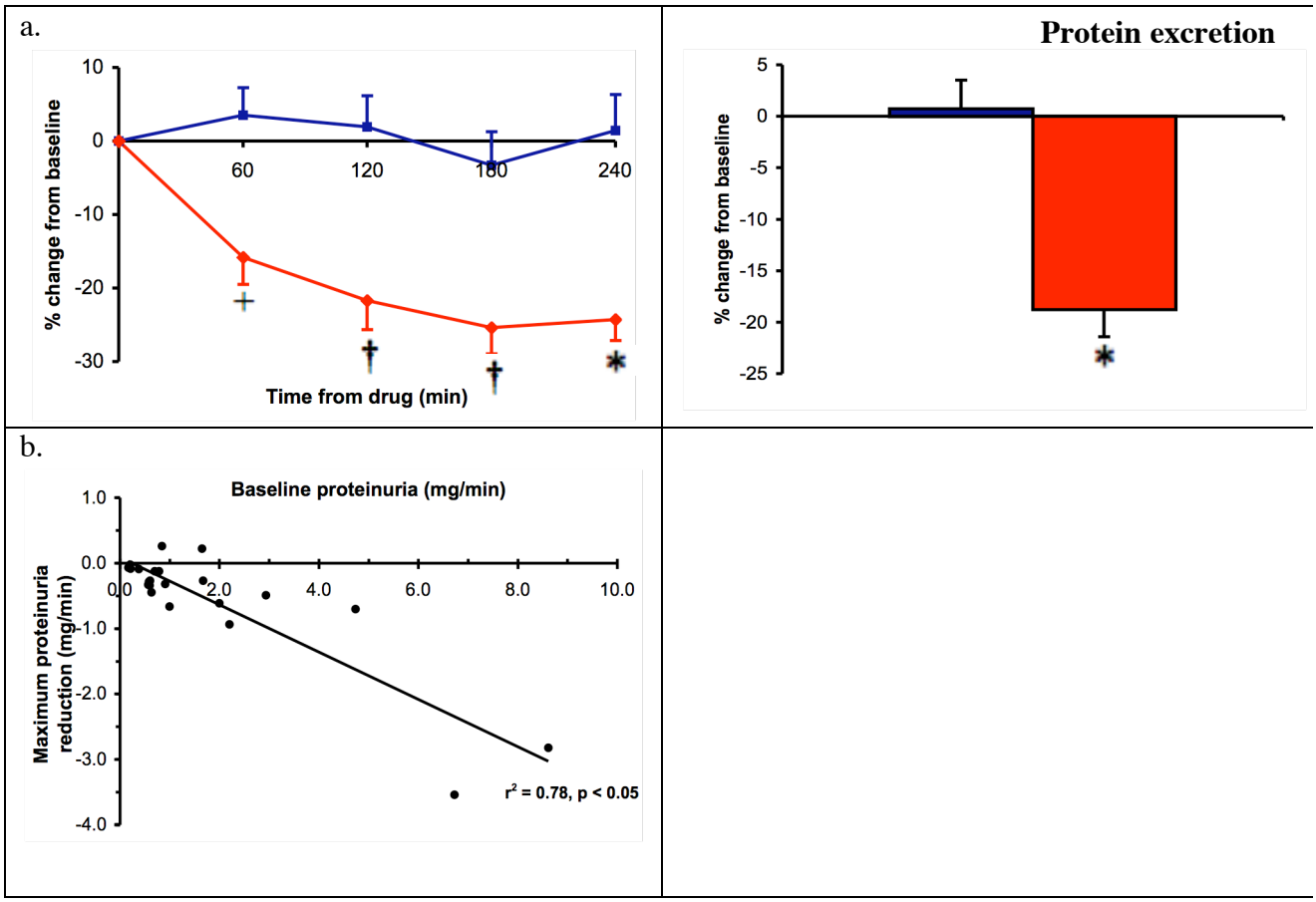
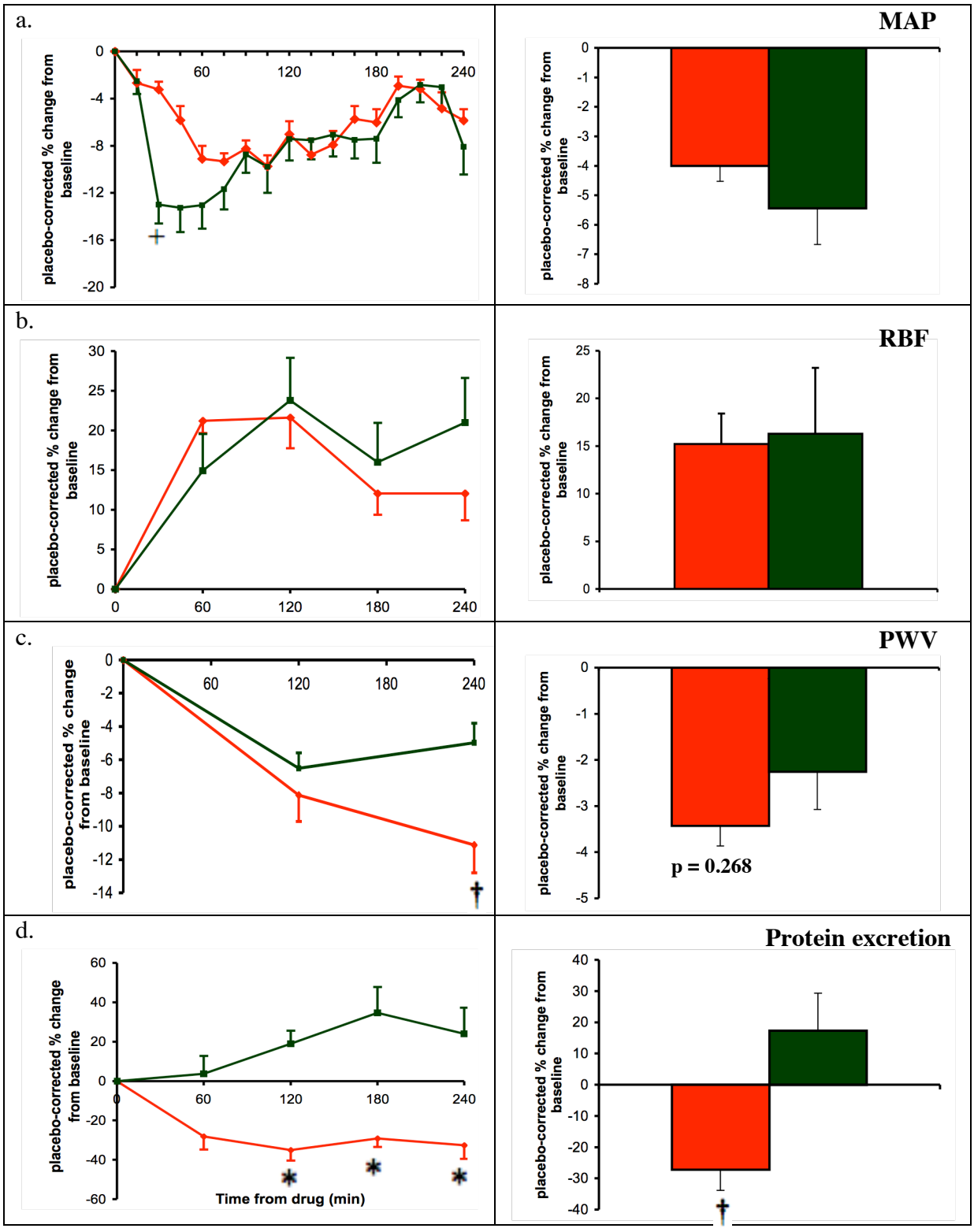


Figure 4.4 *(overleaf)*: Systemic and renal haemodynamics, arterial stiffness and protein excretion after ET_A receptor antagonism and nifedipine 10mg. Values are given as mean placebo-corrected % change from baseline ± SEM (left) and mean area under curve of placebo-corrected % change from baseline ± SEM (right). Green line/block, nifedipine; red line/block, BQ-123. +p < 0.05 vs. nifedipine, †p < 0.01 vs. nifedipine, *p < 0.001 vs. nifedipine (ANOVA plus Bonferroni correction for significance at specific time points).

Figure 4.4:


a.
placebo-corrected % change from baseline
0
-4
-8
-12
-16
-20
60
120
180
240
†
MAP
placebo-corrected % change from baseline
0
-1
-2
-3
-4
-5
-6
-7
-8
b.
placebo-corrected % change from baseline
30
25
20
15
10
5
0
RBF
placebo-corrected % change from baseline
25
20
15
10
5
0
c.
placebo-corrected % change from baseline
0
-2
-4
-6
-8
-10
-12
-14
†
PWV
placebo-corrected % change from baseline
0
-1
-2
-3
-4
-5
p = 0.268
d.
placebo-corrected % change from baseline
60
40
20
0
-20
-40
-60
*
*
*
Time from drug (min)
Protein excretion
placebo-corrected % change from baseline
40
30
20
10
0
-10
-20
-30
-40
†

Discussion

We have demonstrated for the first time that selective ET_A receptor antagonism reduces BP, proteinuria and arterial stiffness in patients with varying degrees of proteinuric nephropathy, and that these effects are seen on top of optimal treatment with ACE inhibitors and ARBs. Importantly, the reductions in proteinuria and arterial stiffness are greater than those expected with BP reduction alone. These findings suggest a potential role for ET_A receptor antagonism in conferring longer-term cardiovascular and renal protection in patients with CKD.

The current study confirms the importance of ET-1, acting through the ET_A receptor, in maintaining the increased vascular tone seen in CKD. BQ-123 produced a fall in BP that was associated with systemic vasodilatation, as reflected by the reduction in SVRI. However, we observed no change in heart rate suggesting no reflex activation of the sympathetic nervous system, in keeping with previous acute studies[213]. There are only two studies of the longer term antihypertensive effects of ET receptor antagonism. These suggest that both selective ET_A[214] and mixed $ET_{A/B}$ antagonists[65] are effective at reducing BP, but neither study included patients with CKD. Furthermore, both studied untreated hypertensive patients. Our current data suggest that, at least in patients with CKD, where BP control is often difficult, ET receptor antagonism may provide a novel strategy to lower BP to a greater extent than that achieved with existing treatments.

BQ-123 also significantly reduced PWV compared to placebo. This effect is likely to be due largely to the reduction in BP seen with BQ-123[90]. However, PWV continued to fall at the end of the study even when BP had returned to baseline. Furthermore, when the antihypertensive effect of BQ-123 was matched with nifedipine, the reduction in arterial stiffness was significantly greater with BQ-123. Although nifedipine did cause an expected increase in heart rate, which may have a minor impact on PWV[215, 216], this had returned to baseline within an hour of study start and before the measurement of PWV. These observations suggest that the effects of ET_A receptor antagonism on arterial stiffness seen here are not accounted for by changes in BP alone. There are few clinical

trials to date demonstrating that differential lowering of PWV with medical treatment results in different cardiovascular or renal outcomes[88, 217] but the importance of such studies is underscored by epidemiological data that suggest that PWV is an independent risk factor for CVD morbidity and mortality[87, 218, 219]. Karalliedde *et al* recently showed a BP-independent reduction in PWV with valsartan compared to amlodipine in patients with type 2 diabetes and proteinuria[220]. Our current data suggest that ET_A receptor antagonism may reduce arterial stiffness even further in patients established on blockers of the renin-angiotensin system and, similarly, in a BP-independent manner.

The mechanism for a BP-independent effect of ET_A receptor antagonism on arterial stiffness remains unclear. One plausible explanation relates to the balance between the ET and nitric oxide (NO) systems. Both ET-1 and NO are vasoactive mediators released by the endothelium. However, whereas ET-1 increases PWV[96], NO reduces it[91] and, in animal models, ET receptor antagonism is associated with an up-regulation of activity in the NO system[79, 80, 83]. Thus, in the current study, in addition to the effects of BP lowering on arterial stiffness, BQ-123 may have increased NO availability and so reduced arterial stiffness. However, the effects of ET_A receptor antagonism on endothelial function, as assessed by FMD, were less impressive, although there was a trend towards improvement.

ET_A receptor antagonism increased renal blood flow in association with a reduction in renal vascular resistance, suggesting that ET-1, acting through ET_A receptors, is involved in the increased renovascular tone seen in CKD. We observed no significant changes in GFR but we did, as in previous studies[53, 221], see a fall in filtration fraction (-7% at maximum; data not shown), suggesting that ET-1 induces an ET_A receptor mediated vasoconstriction, preferentially affecting the efferent arterioles, although not excluding an effect on mesangial cells and filtration coefficient. Our observations are consistent with animal data[35] and raise the possibility that ET-1 promotes hyperfiltration, with its consequent potential for renal injury. Consistent with a reduction in filtration fraction, BQ-123 produced a sustained reduction in urinary protein excretion that was only

beginning to slow at the end of the study. As with the reduction in arterial stiffness, the reduction in proteinuria continued even when the antihypertensive effect of BQ-123 had waned and BP had returned to baseline, suggesting of a BP-independent effect. Our control drug, nifedipine, closely matched both the decrease in BP and increase in renal blood flow seen with BQ-123. Nifedipine acts predominantly at the afferent arteriole, and so, as expected, produced steady increases in both GFR (7 ml/min at maximum; data not shown) and proteinuria throughout the study period. As BQ-123 had little effect on GFR and substantially reduced filtration fraction and proteinuria over the same time scale as the increase in renal blood flow, these findings are consistent with preferential dilation at the efferent arteriole, similar to, and on top of, that seen with ACE inhibitors.

The reduction in proteinuria seen here was related to baseline proteinuria, with subjects with a higher level of baseline proteinuria achieving greater reductions. This effect was seen across the range of GFRs. This is similar to the effects seen with ACE inhibitors[119, 120, 203]. Proteinuria reduction is important both for reducing risk of CKD progression[119, 120, 203] and consequent CVD[222]. Futhermore, the greater the proteinuria reduction, the lower these risks[120, 223]. Despite maximum achievable renin-angiotensin system blockade, many patients with proteinuric CKD have significant residual proteinuria[206]. Importantly, in this study, all subjects were established on treatment with ACE inhibitors, with the majority also taking ARBs. The ET and renin-angiotensin systems are known to interact[142], and a synergistic effect, in terms of systemic haemodynamics, has been demonstrated between ET_A receptor antagonism and both ACE inhibition[211] and angiotensin AT_1 receptor antagonism[52] in humans. Our data suggest ET_A receptor antagonism can produce a further reduction in proteinuria of ~30% on top of that achieved with optimal treatment with inhibitors of the renin-angiotensin system. If maintained longer term this should reduce both CKD progression and CVD morbidity and mortality.

BQ-123 produced a significant natriuresis that, if maintained, equated to about 50 mmol/24h. This is likely to be mainly due to the increase in renal blood flow seen with

ET_A receptor antagonism. Indeed, nifedipine which caused a similar change in renal haemodynamics, also caused natriuresis. In addition, all subjects showed a net diuresis, even with placebo, a likely consequence of the protocol used. These are important observations if ET receptor antagonists are to be used in trials involving CKD patients, in whom salt and water retention is an issue.

The current data support a role for ET_A receptor antagonism as a novel and worthwhile therapeutic target in CKD. As limitations, ours were acute studies and it is important to confirm that these effects are maintained longer term. Furthermore, we studied a relatively homogeneous CKD population and further work is needed in a broader population of patients with CKD, including those with diabetes, vasculitis and renal vascular disease. Finally, our choice of active control agent was based, most importantly, on the need for the drug to match the antihypertensive profile of BQ-123, to produce a similar change in renal haemodynamics, and to be a clinically tolerable agent that is also a standard treatment in CKD patients. Although we investigated other agents, nifedipine was the only drug to fulfill all these criteria. In summary, our current data support a role for selective ET_A receptor antagonism as a potential therapeutic approach in CKD and on this basis, larger and longer term studies in CKD are justified.

Chapter 5

The pharmacokinetic profile of sitaxsentan, a selective endothelin-A receptor antagonist, in varying degrees of chronic kidney disease

Abstract

Background

Sitaxsentan is an oral selective endothelin type A receptor antagonist. The aim of this study was to investigate the pharmacokinetic profile of a single 100 mg dose in subjects with normal and impaired renal function.

Methods

This was an open label, single oral dose study in subjects with normal and reduced glomerular filtration rate, as assessed by 24 hour creatinine clearance (CrCl). Normal GFR was defined as a CrCl ≥80 ml/min, mild chronic kidney disease (CKD) as a CrCl 51-80 ml/min, moderate CKD CrCl 31-50 ml/min, and severe CKD CrCl ≤30 ml/min). All subjects received a dose of 100 mg sitaxsentan.

Results

24 subjects were enrolled, 6 in each of the 4 groups. The mean plasma sitaxsentan concentrations were comparable across the groups as were the mean values for C_{max} (10.3-13.9 μg/ml), AUC_{∞} (18.7-22.5 h.μg/ml), oral clearance (CL/F, 82.3-94.9 ml/min), volume of distribution (Vz/F, 64.8-69.6 l), and elimination half-life ($t_{1/2}$, 8.6-9.6 h). There was substantial overlap among the four groups in the individual subject values for CL/F and Vz/F and no relationship between either of these parameters and CrCl.

Conclusion

After a single 100mg oral dose of sitaxsentan there were no differences in its pharmacokinetics among subjects with normal or impaired renal function.

Introduction

Since over 50% of the administered dose of sitaxsentan is excreted via the kidneys, impaired renal function could potentially affect the pharmacokinetics of sitaxsentan. We therefore evaluated the effect of impaired renal function on the pharmacokinetics of total sitaxsentan following a single oral dose of sitaxsentan at the recommended human therapeutic dose of 100 mg. This may be an important issue because ET_A receptor antagonism shows therapeutic promise in the treatment of CKD[181].

Methods

Subjects

Male or female subjects, 18 to 65 years old, who were willing and able to provide written informed consent, were eligible for inclusion in the study. All subjects with known or suspected ischaemic heart disease, elevated liver enzymes (aspartate transaminase and alanine transaminase) >3 times the upper end of the reference range, positive results for hepatitis B, C or the human immunodeficiency viruses, history of drug or alcohol abuse, significant blood loss or donation (>480 ml) within 30 days of the study, a history of organ transplantation, the presence of the nephrotic syndrome, or who had taken other investigational medication within 1 month of the study medication, were excluded from the study. Additionally, all subjects were required to be surgically sterile or using effective birth control. Pregnant or lactating women were excluded. Subjects taking any medications known to interact with sitaxsentan (those drugs metabolised by cytochrome P450, CYP2C9, for example, macrolide antibiotics, phenytoin were excluded).

Subjects were allocated to a group on the basis of creatinine clearance (CrCl) calculated from serum creatinine concentration during the screening period using the Cockcroft and Gault equation[158]. Subjects with CrCl ≥80 ml/min were classified as 'normal'. Subjects with 'mild', 'moderate' and 'severe' CKD had a CrCl of 51-80 ml/min, 31-50 ml/min, and ≤30 ml/min, respectively. These definitions were those suggested by the Food & Drugs Administration (FDA).

Study protocol

This was an open label, single oral dose study in subjects with normal and impaired renal function. All subjects received a dose of 100 mg sitaxsentan after an overnight fast.

The study was performed at two centres, the Clinical Research Centre, University of Edinburgh and DaVita Clinical Research, Minneapolis. The study was approved by the Lothian NHS board Scotland and the Human Subjects Research Committee, Hennepin County Medical Center, Minneapolis, USA.

Safety assessments included the incidence of adverse events, clinical laboratory test results, haemodynamic parameters, electrocardiogram, changes in physical examination assessments from baseline, and the monitoring of concomitant medications.

Sample collection and analysis

Blood samples for the measurement of plasma concentration of sitaxsentan were collected prior to and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, 16, 24 (day 2), 36 (day 2), 48 (day 3), and 72 (day 4) hours after dosing. As described in Chapter 2, plasma concentrations of sitaxsentan were measured using a validated LC/MS/MS method. The (LLOQ) for sitaxsentan using this method was 0.005 μg/mL. The percent unbound sitaxsentan (F_U) in each sample was calculated according to $F_U = 100\% \times (C_U/C_T)$ where C_U and C_T represent the unbound and total concentrations, respectively.

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters for sitaxsentan were calculated using non-compartmental analysis. Only plasma concentrations greater than or equal to the lower limit of quantitation (LLOQ) for the assays were used in the pharmacokinetic analyses. Actual blood sampling times were used in all pharmacokinetic analyses. Per protocol times were used to calculate mean plasma concentrations for graphical displays.

Maximum observed plasma drug concentration (C_{max}) and time to maximum observed plasma drug concentration (T_{max}) were taken directly from the data. The elimination rate constant (λz) was calculated as the negative of the slope of the terminal log-linear segment of the plasma concentration-time curve. The range of data to be used for each subject and treatment was determined by visual inspection of a semi-logarithmic plot of concentration vs. time. Elimination half-life was calculated according to the following equation, $t_{1/2} = 0.693/\lambda z$. Area under curve to the last time with a concentration equal to or greater than the validated LOQ of the bioanalytical method (AUC_{0-t}) was calculated using the linear trapezoidal method and extrapolated to infinity (AUC_{∞}) according to $AUC_{\infty} = AUC_{0-t} + (C_{tf}/\lambda z)$, where C_{tf} is the final concentration ≥ LOQ. Apparent total body clearance after oral dosing (CL/F) and volume of distribution (Vz/F) were calculated according to CL/F = dose / AUC_{∞}, and VZ/F = dose / (λz x AUC_{∞}), respectively. All pharmacokinetic calculations were done using SAS® for Windows® Version 9.1.

The pharmacokinetic parameters C_{max}, T_{max}, AUC_{∞}, $t_{1/2}$, CL/F, and Vz/F were compared among renal function groups using an analysis of variance (ANOVA) model with renal function group as the classification variable. C_{max} and AUC_{∞} were natural log-transformed prior to analysis; all other parameters were analysed on the original scale.

Relationships between CL/F and Vz/F and renal function as measured by CrCl were examined using linear regression.

Results

Demographics

A total of 24 subjects took part in the study, 15 males and 9 females. All subjects completed the study. There were 6 subjects in all four CrCl groups. Mean (range) of age, weight, serum creatinine and CrCl for the four groups are shown in Table 5.1.

Table 5.1: Subject demographics. Values are mean (range).

	Normal	**Mild CKD**	**Moderate CKD**	**Severe CKD**
Age	54 (47 – 59)	57 (41 – 66)	49 (38 – 58)	54 (45 – 65)
Weight (kg)	73 (48 – 88)	77 (69 – 92)	75 (68 – 80)	54 (45 – 65)
Creatinine (μmol/l)	73 (45 – 100)	112 (71 – 182)	219 (151 – 315)	423 (181 – 858)
CrCl (ml/min)	100 (91 – 116)	67 (61 – 78)	39 (31 – 46)	22 (16 – 30)

Pharmacokinetics

As illustrated in Figure 5.1 (semi-logarithmic axes), the mean plasma sitaxsentan concentrations were comparable across the four renal function groups as were the mean values for C_{max}, AUC_{∞}, CL/F, Vz/F, and $t_{1/2}$ (Table 5.2). There were no significant differences in these parameters amongst renal function groups ($p > 0.05$). There was little difference in F_U among the four renal function groups (Table 5.2). There was substantial overlap among the four renal function groups in the individual subject values for CL/F and Vz/F and no relationship between either parameter and CrCl, indicating no effect of renal impairment on the pharmacokinetics of sitaxsentan (Figures 5.2 and 5.3).

Table 5.2: Summary of sitaxsentan pharmacokinetic parameters after oral administration of a single 100mg dose to subjects with normal and impaired renal function. Results are presented as mean ± standard deviation except for T_{max} for which the median is reported.

Parameter	Normal	Mild CKD	Moderate CKD	Severe CKD	p value
C_{max} (μg/ml)	13.9 ± 3.12	11.6 ± 4.01	10.3 ± 4.45	13.0 ± 6.88	0.4834
T_{max} (h) (Range)	1.00 (1.0-1.5)	1.50 (1.0-2.5)	1.01 (1.0-3.5)	1.00 (0.5-1.5)	0.9072
AUC_{∞} (h.μg/ml)	21.8 ± 9.59	22.5 ± 8.64	18.7 ± 6.26	20.8 ± 10.2	0.3265
$T_{1/2}$ (h)	9.0 ± 1.2	9.6 ± 1.1	8.6 ± 1.1	8.8 ± 1.5	0.5656
CL/F (ml/min)	84.5 ± 34.7	82.3 ± 38.2	94.9 ± 37.3	88.0 ± 30.7	0.9310
Vz/F (l)	64.8 ± 24.0	65.4 ± 22.9	69.6 ± 28.2	68.3 ± 30.1	0.9869
F_U					
1h	0.0133 ± 0.0012	0.0123 ± 0.0070	0.0130 ± 0.0066	0.0176 ± 0.0045	-
1.5h	0.0140 ± 0.0013	0.0127 ± 0.0067	0.0127 ± 0.0066	0.0174 ± 0.0026	-
2h	0.0117 ± 0.0058	0.0152 ± 0.0023	0.0133 ± 0.0068	0.0166 ± 0.0039	-

Figure 5.1: Mean plasma concentrations of sitaxsentan after oral administration of single 100mg doses to subjects with normal and impaired renal function- semi-logarithmic axes.

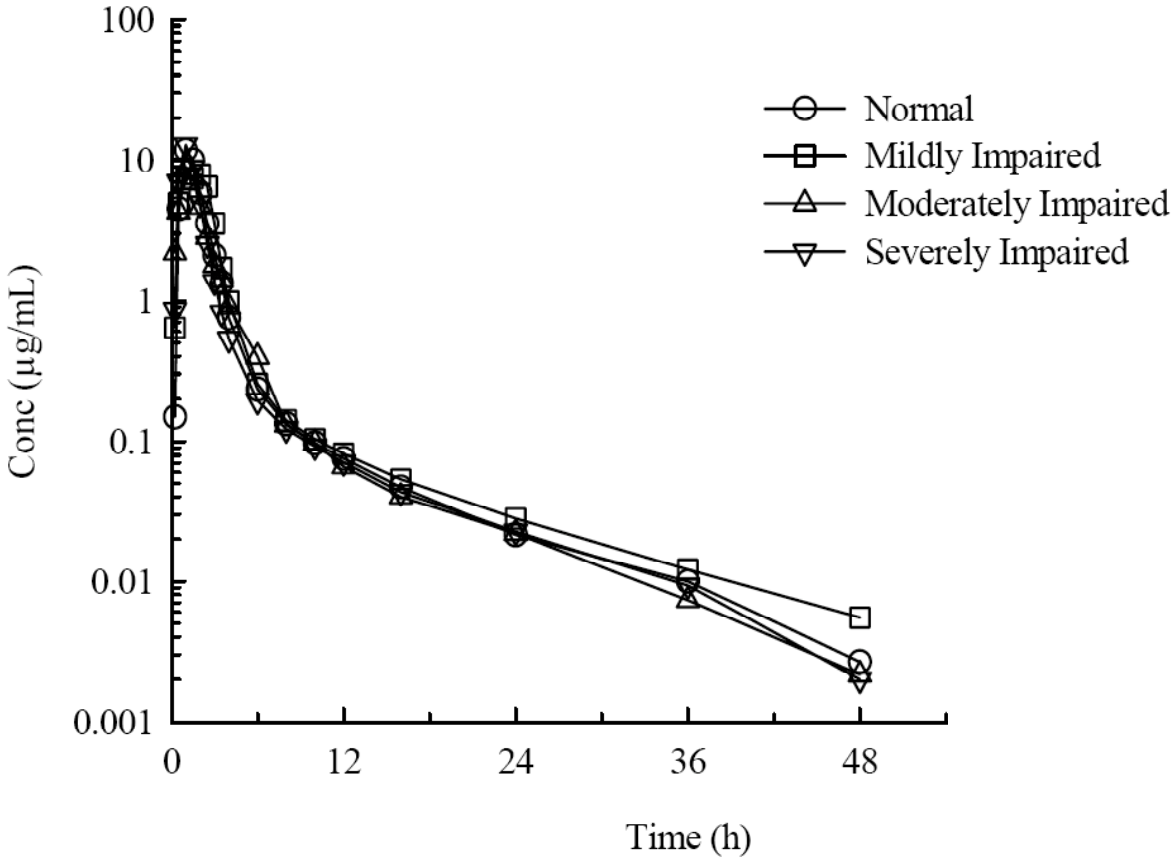


Figure 5.2: Relationship between CL/F and CrCl after oral administration of single 100mg doses to subjects with normal and impaired renal function (r^2 = 0.0255, p = 0.4560).

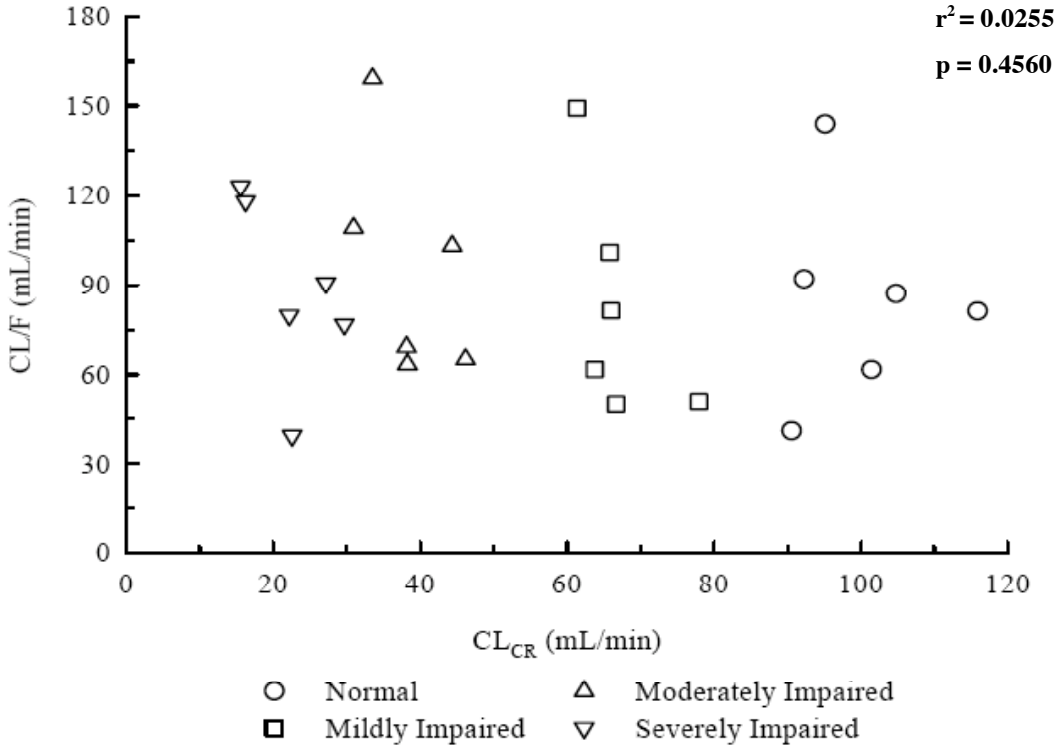
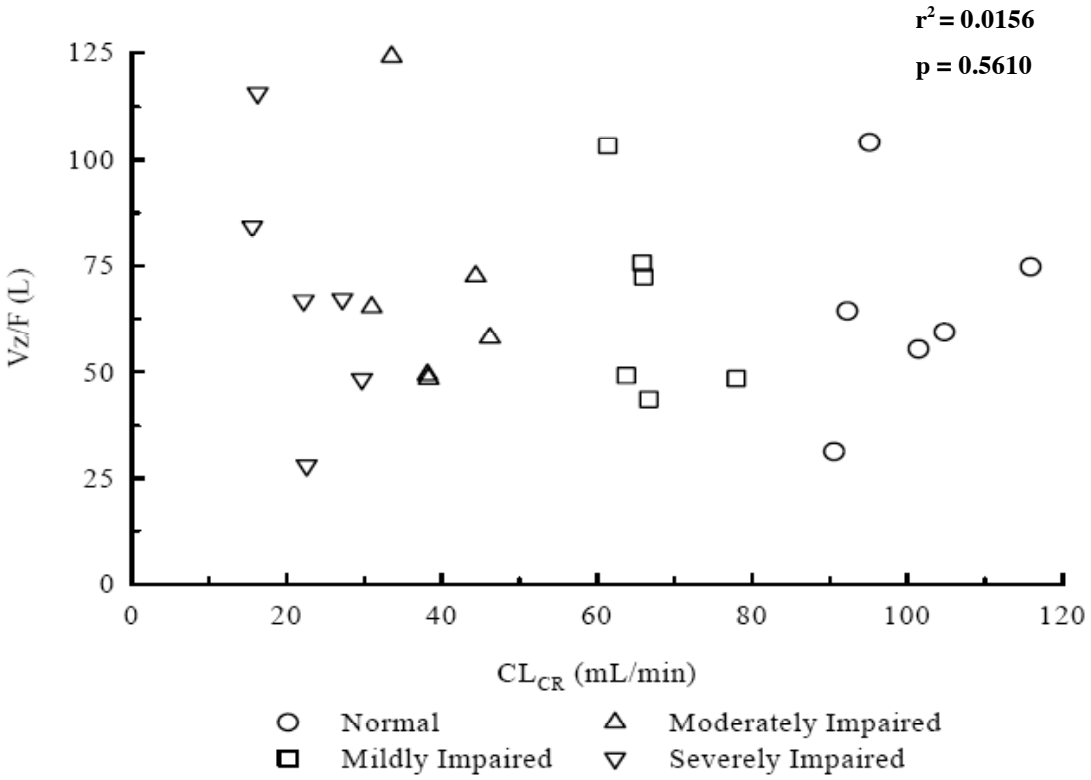


Figure 5.3: Relationship between Vz/F and CrCl after oral administration of single 100mg doses to subjects with normal and impaired renal function (r^2 = 0.0156, p = 0.5610).



Discussion

In this open label, two-centre study to determine the effects of renal impairment on the pharmacokinetics of sitaxsentan following a single 100 mg oral dose we have shown that plasma sitaxsentan concentrations were very similar between groups separated on the basis of CrCl. Furthermore, there was no apparent relationship between any of the sitaxsentan pharmacokinetic parameters and increasing renal impairment (Table 5.2).

Although initially licensed for the orphan area of primary pulmonary hypertension the indications for ET receptor antagonists are now expanding to include conditions such as scleroderma that may cause both pulmonary and renal disease[224]. Additionally, there is increasing interest in the ET system and its antagonism as a potential therapeutic target in CKD[181]. It is therefore, important to understand the pharmacokinetics of such drugs in the CKD population. Clinically, altered drug pharmacokinetics may require changes in drug dosing and/or frequency of dosing. The results of this study will inform dosing in any future studies in CKD, as it is clear that no dosing adjustment for sitaxsentan is required for declining glomerular filtration rate based on these pharmacokinetic findings for sitaxsentan.

The range of renal impairment in this study, as measured by Cockcroft and Gault CrCl, encompassed the full spectrum of CKD, allowing us to confidently state that sitaxsentan pharmacokinetics are unchanged. However, we are unable to comment on the degree of accumulation of sitaxsentan metabolites in renal impairment, and the potential therapeutic and toxicological consequences of this. Furthermore, study subjects had serum albumin concentrations that were within the normal range, and the nephrotic syndrome was a specific exclusion criterion. This is important as sitaxsentan is highly protein-bound (>99.5%), so the nephrotic syndrome may significantly increase free plasma drug concentrations. Finally, no patients established on dialysis were included in the study. One would not expect this population of patients, however, to have altered sitaxsentan pharmacokinetics, because the high degree of protein binding of drug would substantially limit clearance by dialysis.

Finally, although plasma concentrations of sitaxsentan did not vary significantly we cannot comment on whether the pharmacodynamics of ET system antagonism were affected similarly through the range of CKD. Increased activity of the ET system is recognised in CKD, and antagonism of ET in these patients may lead to beneficial cardiovascular and renal effects such as lowering of blood pressure, natriuresis and reduction in proteinuria[181]. If clinical trials using sitaxsentan, and ET antagonists more generally, are to proceed in CKD, these specific clinical parameters would need to be further studied.

Chapter 6

Selective endothelin-A receptor antagonism reduces proteinuria, blood pressure & arterial stiffness in chronic proteinuric kidney disease

Abstract

Background: We have shown that acute selective endothelin-A (ET_A) receptor antagonism reduces proteinuria, BP and arterial stiffness - key independent, surrogate markers of CKD progression and CVD risk, in patients with proteinuric CKD. We therefore examined if these effects are maintained longer term using the selective ET_A receptor antagonist sitaxsentan.

Methods: In a randomised double-blind, 3-way crossover study, 27 subjects receiving recommended renoprotective treatment, received 6 weeks of placebo, sitaxsentan 100mg od and nifedipine LA 30mg od. 24h proteinuria, protein:creatinine ratio (PCR), 24h ambulatory BP, and pulse wave velocity (PWV; as a measure of arterial stiffness), were measured at baseline and week 6 of each treatment period. In 13 subjects, renal blood flow and glomerular filtration rate were assessed at baseline and week 6 of each period.

Results: Compared to placebo, sitaxsentan significantly reduced proteinuria (24h proteinuria by -0.62±0.11g/d, $p<0.01$; PCR by -43±8mg/mmol, $p=0.01$), BP (24h mean arterial BP by -4±6mmHg, $p<0.01$), and PWV by -5±9% ($p<0.01$). Nifedipine matched the BP and PWV reductions seen with sitaxsentan, whereas sitaxsentan reduced proteinuria and nifedipine did not. Sitaxsentan alone reduced both glomerular filtration rate and filtration fraction. It caused no clinically significant side effects.

Conclusions: Sitaxsentan may provide additional cardiovascular and renal protection by reducing proteinuria, BP and arterial stiffness in CKD subjects already receiving optimal recommended treatment. The antiproteinuric effects of sitaxsentan likely relate to changes in BP and renal haemodynamics.

Introduction

We have shown that acute selective ET_A antagonism reduces proteinuria and arterial stiffness in those with non-diabetic proteinuric CKD. Importantly, these effects were not accounted for by BP reduction alone and were evident on top of standard treatment with ACE inhibitors and/or ARBs. The aim of the current study was to evaluate whether the oral ET_A receptor antagonist, sitaxsentan, currently available for the treatment of pulmonary artery hypertension, is able to reduce proteinuria, BP and arterial stiffness longer term in subjects with chronic non-diabetic proteinuric kidney disease.

Methods

Subjects

We enrolled subjects 18-70 years of age with stable CKD stages 1 to 4[201] and proteinuria (>300 mg/day). Subjects were on treatment with ACE inhibitors and/or ARBs for their proteinuria (Table 6.1). Explicitly, doses of one or both of drugs were titrated to the maximum tolerated, dependent on BP, renal function, serum potassium levels and side effects. All medications were unchanged over the 3 months preceding the studies.

Patients with significant co-morbidity, including diabetes mellitus, heart or lung disease, and peripheral vascular disease were excluded. To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis, other systemic inflammatory disease, polycystic kidney disease, and nephrotic syndrome were excluded. Furthermore, we excluded patients with abnormal liver enzymes, haemoglobin <8 g/dl, and women of childbearing potential.

Thirty-three patients with stable proteinuric CKD were screened, and 27 were recruited into the studies.

Study protocol

This was a single centre, 3-phase randomised, double-blind, placebo-controlled crossover study. Its purpose was to investigate the safety, tolerability and efficacy of

sitaxsentan 100mg once daily (od) versus placebo on reduction of proteinuria (primary endpoint), BP and arterial stiffness (co-secondary endpoints) in subjects with CKD. As previous studies with ET receptor antagonists have shown a reduction in BP[65, 214], and as BP reduction may contribute to changes in protein excretion and arterial stiffness, nifedipine LA 30mg od was used as an open-label active control. Our choice of active control agent was based, most importantly, on the need for the drug to match the antihypertensive profile of sitaxsentan and to be a clinically tolerable agent that is also a standard treatment in CKD patients[225]. A sub-study evaluated the effects of sitaxsentan 100mg od, placebo, and nifedipine LA 30mg od on renal haemodynamics.

Subjects were randomly assigned to receive sitaxsentan 100 mg, matched placebo, or nifedipine LA 30mg, once daily for 6 weeks in addition to their usual medications. Each phase was separated by a minimum 2 weeks washout period. Proteinuria, BP and arterial stiffness were assessed at baseline, week 3 and week 6 of each treatment period (Figure 6.1). Proteinuria was assessed using both the mean 24h protein excretion and the mean protein:creatinine ratio of 3 consecutive 24h urine collections. Ambulatory BP was recorded at the brachial artery using a validated Spacelabs 90217 ambulatory BP monitor[171]. Measurements were taken every 30m for 24h and mean systolic (SBP), mean arterial pressures (MAP) and diastolic (DBP) calculated. As measures of arterial stiffness, pulse wave velocity (PWV) and central augmentation index (cAIx) were recorded[90] and assessed pre-dose. Safety data were obtained at baseline and weeks 1, 2, 3, 4 and 6 for each treatment period. These included 'office' BP, weight, haemoglobin, haematocrit, liver enzymes, serum potassium and side effects.

Renal function sub-study

For those subjects taking part in the sub-study, PAH and inutest clearances were used to assess renal blood flow and GFR, respectively, at baseline and week 6 of each of the 3 study periods.

Plasma ET-1

Plasma ET-1 was measured at baseline, week 3 and week 6 of each treatment period.

Data and statistical analysis

Data were stored and analysed using SAS® Version 8.2 or higher. The planned sample size was based on logistical and clinical considerations. The approximate target was a total of 30 subjects to be enrolled in the main study, with around 15 of these to be included in the sub-study, with the aim of 24 and 12 subjects, respectively, completing.

For efficacy endpoints, the change from baseline to week 6, and from baseline to week 3, was calculated using analysis of covariance (ANCOVA). The ANCOVA model was implemented with terms for treatment group (fixed effect, categorical variable), baseline value (fixed effect, continuous variable, as appropriate for the endpoint), period effect (fixed effect, categorical variable), and subject effect (random effect, categorical variable). Restricted Maximum Likelihood (REML) estimates for the treatment differences (sitaxsentan 100 mg minus placebo, sitaxsentan 100 mg minus nifedipine LA 30 mg, and nifedipine LA 30 mg minus placebo), the associated standard error and p value were calculated. The assumptions of the ANCOVA model were checked by investigation of a normal probability plot of standardised residuals and a plot of standardised residuals versus fitted values. Differences between the least squares means, standard errors and p values are presented. Statistical analysis of percentage change from baseline was performed similarly.

Table 6.1: Individual subject characteristics. Doses are total per day. IgAN: immunoglobulin A nephropathy; FSGS: focal & segmental glomerulosclerosis; Membranous: membranous glomerulopathy; EPO: erythropoietin.

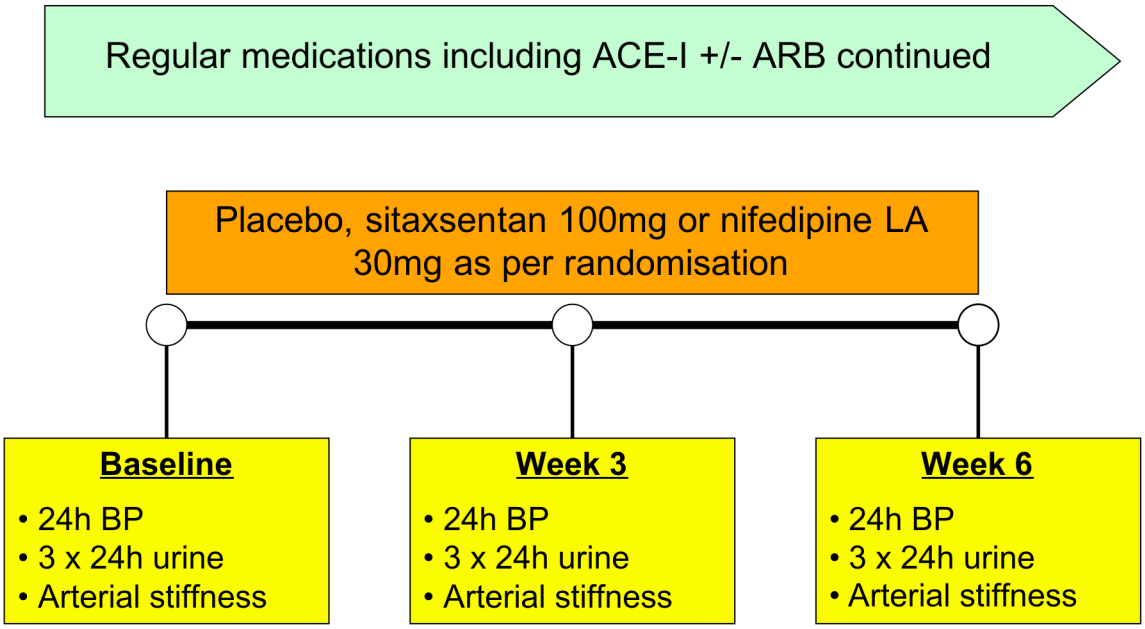
	Diagnosis	Creatinine (mg/dl)[a]	Proteinuria (g/d)	BP (mmHg)		ACE inhibitor	ARB	Other drugs
				Systolic	Diastolic			
1	IgA nephropathy	1.86	0.59	119	79	Ramipril 10mg	-	
2	IgA nephropathy	1.85	1.50	124	84	Lisinopril 10mg	-	Simvastatin 20mg
3	Reflux nephropathy	2.15	4.05	134	87	Ramipril 10mg	-	Ciprofloxacin 100mg Zopliclone 7.5mg Ibuprofen 400mg (once) Simvastatin 10mg Trimethoprim 400mg Fluconazole 150mg Cefalexin 250mg
4	Membranous nephropathy	1.10	1.38	130	74	Enalapril 30mg	-	Ibuprofen 400mg (once)
5	IgA nephropathy	3.70	1.08	132	87	-	Valsartan 160mg	Amlodipine 5mg
6	IgA nephropathy	2.23	0.43	112	67	Lisinopril 10mg	-	Atenolol 50mg (stopped) Amlodipine 10mg Aspirin 75mg Simvastatin 40mg

7	IgA nephropathy	2.08	3.90	145	83	Ramipril 10mg	-	Atorvastatin 40mg Aspirin 75mg Atenolol 50mg Ferrous Sulphate 600mg
8	IgA nephropathy	2.03	1.30	119	85	Lisinopril 40mg	-	Omeprazole 20mg Metoprolol 50mg Flucloxacillin 250mg
9	Unknown	1.04	0.50	125	78	-	-	Pseudoephedrine 60mg (once)
10	FSGS	1.48	5.46	109	68	Lisinopril 20mg	-	Ezetimibe 10mg Atorvastatin 60mg
11	Hypertensive nephropathy	1.88	1.79	125	79	Lisinopril 20mg	Valsartan 40mg	Doxazosin 8mg Atenolol 100mg Atorvastatin 20mg Allopurinol 100mg
12	IgA nephropathy	4.27	2.59	129	79	Ramipril 2.5mg	-	Erythropoetin 4000IU Alfacalcidol 0.25mcg Atorvastatin 20mg Ferrous Sulphate 600mg Sodium Bicarbonate3g
13	IgA nephropathy	1.87	4.49	155	91	-	Irbesartan 225mg	Metoprolol 100mg Doxazosin 4mg Simvastatin 20mg Aspirin 75mg Sodium Bicarbonate 2g
14	IgA nephropathy	1.24	0.64	139	77	-	Candesartan 4mg	Rosuvastatin 5mg Folic Acid 5mg Citirizine 10mg

15	IgA nephropathy	1.73	0.50	122	74	Lisinopril 20mg	-	Simvastatin 20mg Indapamide 2.5mg
16	FSGS	2.70	1.87	129	81	-	Candesartan 32mg	Atenolol 50mg Aspirin 75mg Salbutamol 200mcg PRN Beclamethasone 200mcg
17	IgA nephropathy	1.56	3.28	127	79	Ramipril 10mg	Candesartan 4mg	Atorvastatin 20mg Cod-liver oil
18	FSGS	1.33	5.37	129	76	Ramipril 5mg	-	Atorvastatin 20mg Allopurinol 300mg
19	Membranous nephropathy	0.74	2.58	107	69	Perindopril 8mg	Candesartan 16mg	Atorvastatin 80mg Ezetimibe 10mg
20	FSGS	1.34	0.47	139	89	Ramipril 10mg	-	Diltiazem 360mg Simvastatin 20mg Citirizine 2.5mg Aspirin 75mg
21	Membranous nephropathy	0.75	0.82	112	76	-	-	Stemetil 5mg Sumatriptan 50mg
22	IgA nephropathy	0.98	1.13	131	83	-	-	
23	Hypertensive nephropathy	1.25	0.15	106	72	Ramipril 10mg	Irbesartan 300 mg,	Doxazosin 4mg Hydrochlorothiazide 12.5 mg Prednisolone 5mg (2 courses) Beclamethasone 200mg Salbutamol 200mg
24	FSGS	0.92	1.67	108	66	Lisinopril 10mg	Irbesartan 150mg	Simvastatin 40mg Doxazosin 2mg Metoprolol 50mg Ezetimibe 10mg

								Folic Acid 5mg Omeprazole 20mg Ferrous sulphate 600mg
25	IgA nephropathy	1.38	0.27	116	74	Lisinopril 40mg	-	Simvastatin 10mg Fish Oil, Colchicine 1500mcg Aspirin 600mg (once)
26	FSGS	2.50	5.12	136	83	-	Candesartan 32mg	Allopurinol 100mg Alfacalcidol 0.25mcg Doxazosin 4mg Atenolol 50mg Atorvastatin 40mg
27	IgA nephropathy	0.61	1.88	128	72	-	Candesartan 16mg	Simvastatin 20mg Bendroflumethiazide 2.5mg Doxazosin 4mg

Figure 6.1: Study outline.



Results

All 27 subjects completed all 3 phases of the study. Patient diagnoses were IgA nephropathy (n = 14, 52%), focal segmental glomerulosclerosis (n = 6, 22%), membranous nephropathy (n = 3, 11%), hypertensive nephrosclerosis (n = 2, 7%), reflux nephropathy, microhaematuria of presumed glomerular origin (n = 1, 4%, for both) and one subject with an unknown cause for their CKD. Subject baseline parameters are shown in Table 6.2. For all subjects, baseline parameters did not differ between the 3 study phases. Individual subject characterisation is provided in Table 6.1.

Table 6.2: Baseline patient characteristics for main study and sub-study. Values are given as mean of 3 baseline pre-treatment periods ± SD. GFR: glomerular filtration rate; PCR: protein:creatinine ratio; PWV: pulse wave velocity; cAIx: central augmentation index; ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker.

	Main study (n = 27)	**Sub-study (n = 13)**
Demographic		
Age, y	48 ± 12	46 ± 13
Male sex (%)	23 (85)	12 (92)
Caucasian	27 (100)	13 (100)
Clinical		
Body mass index, kg/m^2	29.3 ± 4.6	28.2 ± 4.7
24h BP, mmHg		
Systolic	125 ± 12	127 ± 10
Diastolic	78 ± 7	80 ± 8
Mean	94 ± 78	95 ± 7
Creatinine, μmol/l	153 ± 75	152 ± 67
Estimated GFR, $ml/min/1.73m^2$	54 ± 26	55 ± 26
Haemoglobin, g/l	136 ± 18	132 ± 16
Serum potassium, mmol/l	4.6 ± 0.4	4.6 ± 0.4
Cholesterol, mmol/l	4.6 ± 0.8	4.4 ± 1.0

Urinary protein excretion		
g/24h	2.03 ± 1.7	2.01 ± 1.6
PCR, mg/mmol	156 ± 143	150 ± 144
Arterial stiffness		
PWV, m/s	8.3 ± 2.4	7.4 ± 1.4
AIx, %	28 ± 12	24 ± 14
Medications, n (%)		
ACE inhibitor	18 (67)	10 (77)
ARB	11 (41)	3 (23)
ACE inhibitor + ARB	5 (19)	2 (15)
No ACE inhibitor or ARB	3 (11)	1 (8)
α blocker	6 (22)	1 (8)
β blocker	8 (30)	4 (31)
Calcium channel blocker	3 (11)	3 (23)
Diuretic	2 (7)	0 (0)
Statin	18 (67)	8 (62)

Main study (n = 27, Table 6.3)

Sitaxsentan vs. placebo

Proteinuria (Figures 6.2a, b & c)

Placebo was associated with no changes in 24h urinary protein excretion or PCR from baseline to week 6. Sitaxsentan, however, significantly reduced both 24h proteinuria and PCR by ~30% by study end. These effects of sitaxsentan on proteinuria were apparent at week 3 of the study period (24h proteinuria: 2.07 ± 0.34 *vs.* 1.35 ± 0.22 g/d; PCR: 157 ± 28 *vs.* 110 ± 21 mg/mmol, $p < 0.01$ for both).

Sitaxsentan reduced proteinuria by 25% or more in 19 out of 27 (70%) subjects, and by 40% or more in 9 out of 27 (33%) subjects. Only 2 subjects failed to show a reduction in 24h urine protein excretion, and only 1 in PCR. Furthermore, the degree of proteinuria reduction closely related to the baseline urinary protein excretion with subjects with higher baseline proteinuria achieving a greater reduction ($r^2 = 0.67$, $p < 0.01$). This effect was seen across all levels of GFR (data not shown).

Blood pressure (Figures 6.3a, b & c) & arterial stiffness (Figures 6.4a & b)

Whereas placebo did not affect MAP, SBP, or DBP between baseline and week 6 of the study period, sitaxsentan reduced all three parameters by ~ 4 mmHg following 6 weeks dosing.

Placebo had no effects on PWV or cAIx over the 6 weeks study period, whereas, sitaxsentan reduced both by study end. PWV fell by ~5% compared to baseline, a difference of ~ 10% compared to placebo.

Sitaxsentan vs. nifedipine LA 30mg

Over 6 weeks dosing nifedipine matched the reductions in SBP (-4.9 ± 1.6 *vs.* -3.6 ± 1.5 mmHg), MAP (nifedipine *vs.* sitaxsentan: -3.8 ± 1.1 *vs.* -3.7 ± 1.0 mmHg), and DBP (-3.2 ± 1.0 *vs.* -3.6 ± 1.0 mmHg) seen with sitaxsentan. Despite this, sitaxsentan reduced proteinuria to a greater extent than nifedipine (24h proteinuria: -0.6 ± 0.1 *vs.* 0.0 ± 0.1

g/d, $p < 0.01$; PCR: -43 ± 8 *vs.* -3 ± 11 mg/mmol, $p = 0.01$). For sitaxsentan, the reduction in proteinuria correlated with the fall in MAP at week 6 ($r^2 = 0.16$, $p = 0.04$). However, there were no relationships for the changes in proteinuria and BP for the placebo and nifedipine phases. Whereas PWV fell to a similar degree with nifedipine as with sitaxsentan (-0.4 ± 0.2 *vs.* -0.4 ± 0.2 m/s), nifedipine did not reduce cAIx following 6 weeks dosing.

All changes in proteinuria, BP and arterial stiffness had returned to baseline before starting the next phase of the study (minimum 2 weeks).

Plasma ET-1

There were no changes in plasma ET-1 concentrations with placebo, sitaxsentan or nifedipine.

Table 6.3: Main study data at baseline and week 6 of each study period. Values are given as pre-dosing baseline ± SEM. PCR: protein:creatinine ratio; MAP: mean arterial pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; PWV: pulse wave velocity; cAIx: central augmentation index; ET-1: endothelin-1. * $p < 0.01$, † $p = 0.01$, + $p < 0.05$ for week 6 *vs*. baseline.

	Placebo		**Sitaxsentan**		**Nifedipine**	
	Baseline	Week 6	Baseline	Week 6	Baseline	Week 6
24h proteinuria (g/d)	2.06 ± 0.38	2.00 ± 0.33	**2.07 ± 0.34**	**1.46 ± 0.26***	1.95 ± 0.30	1.99 ± 0.33
PCR (mg/mmol)	155 ± 31	153 ± 27	**157 ± 28**	**114 ± 23†**	155 ± 27	152 ± 29
MAP (mmHg)	94.6 ± 2.2	94.3 ± 1.7	**94.4 ± 1.8**	**90.7 ± 1.8+**	**95.5 ± 2.0**	**91.7 ± 1.7+**
SBP (mmHg)	125.4 ± 2.7	124.2 ± 1.9	**124.3 ± 2.2**	**120.7 ± 1.9+**	**125.7 ± 2.4**	**120.7 ± 1.6+**
DBP (mmHg)	77.9 ± 1.5	77.5 ± 1.2	**77.9 ± 1.3**	**74.3 ± 1.3***	**78. 9 ± 1.5**	**75.7 ± 1.2***
PWV (m/s)	7.7 ± 0.3	8.0 ± 0.4	**8.0 ± 0.3**	**7.6 ± 0.3+**	**7.9 ± 0.3**	**7.6 ± 0.3+**
cAIx (%)	20 ± 2	20 ± 2	**20 ± 2**	**15 ± 2***	19 ± 2	17 ± 2
Plasma ET-1 (pg/ml)	3.6 ± 0.5	3.7 ± 0.6	3.6 ± 0.5	3.7 ± 0.5	3.5 ± 0.5	3.5 ± 0.5

Figure 6.2: Effects of placebo, sitaxsentan and nifedipine LA 30mg on the co-primary endpoints of (A) 24h proteinuria, and (B) protein:creatinine ratio (PCR). Values are given as mean % change from baseline ± SEM at week 3 and week 6. Gray block, placebo; hashed block, sitaxsentan; black block, nifedipine. * $p < 0.001$ for sitaxsentan *vs.* both placebo and nifedipine. (C) Effect of baseline protein excretion on maximal proteinuria reduction (g/d) with sitaxsentan, $r^2 = 0.67$, $p < 0.01$.

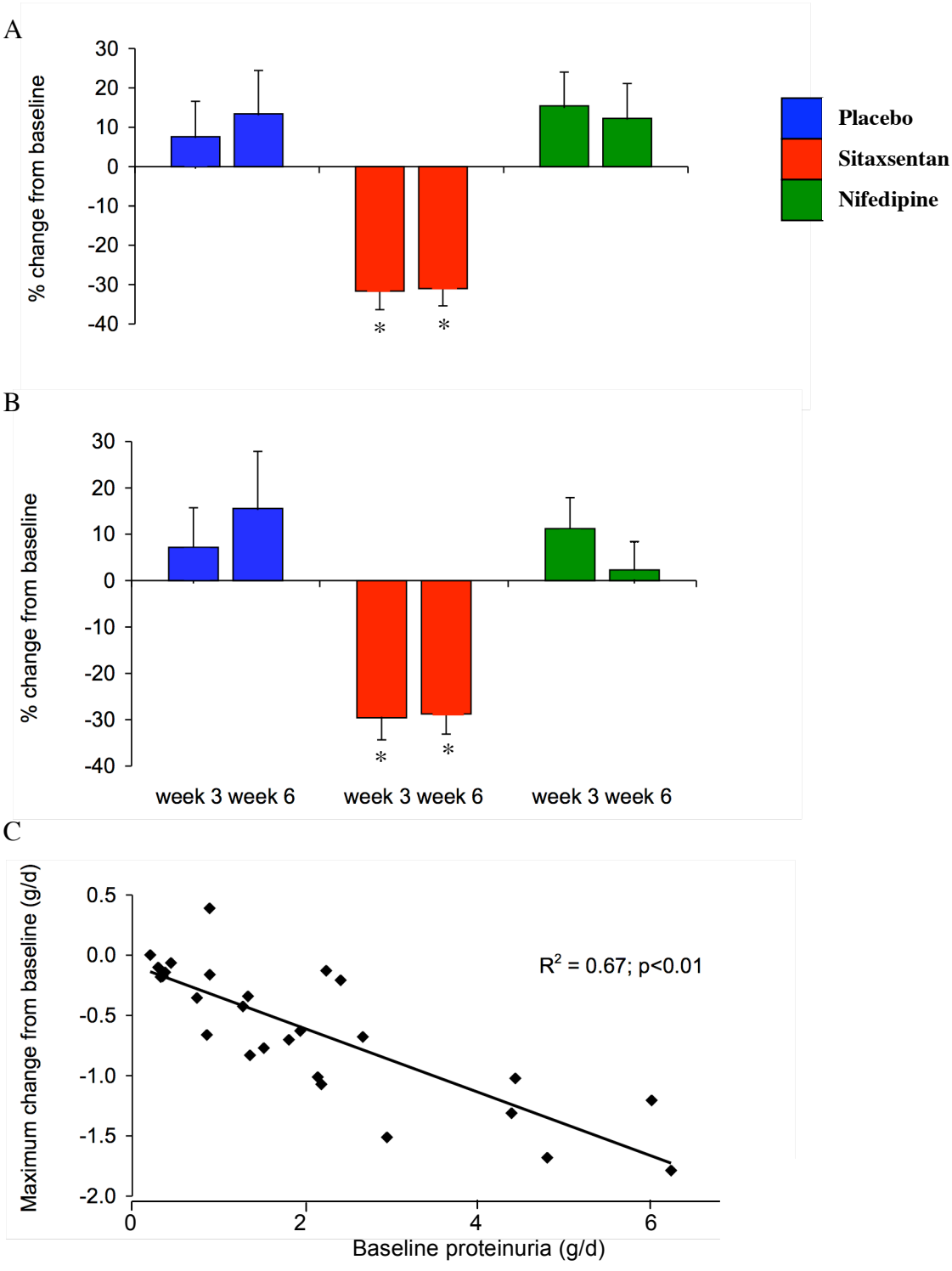


Figure 6.3: Effects of placebo, sitaxsentan and nifedipine LA 30mg on 24h (A) mean arterial pressure, (B) systolic blood pressure, and (C) diastolic blood pressure. Legend as for Figure 1. * $p < 0.01$ and + $p < 0.05$ for sitaxsentan and nifedipine *vs.* placebo.

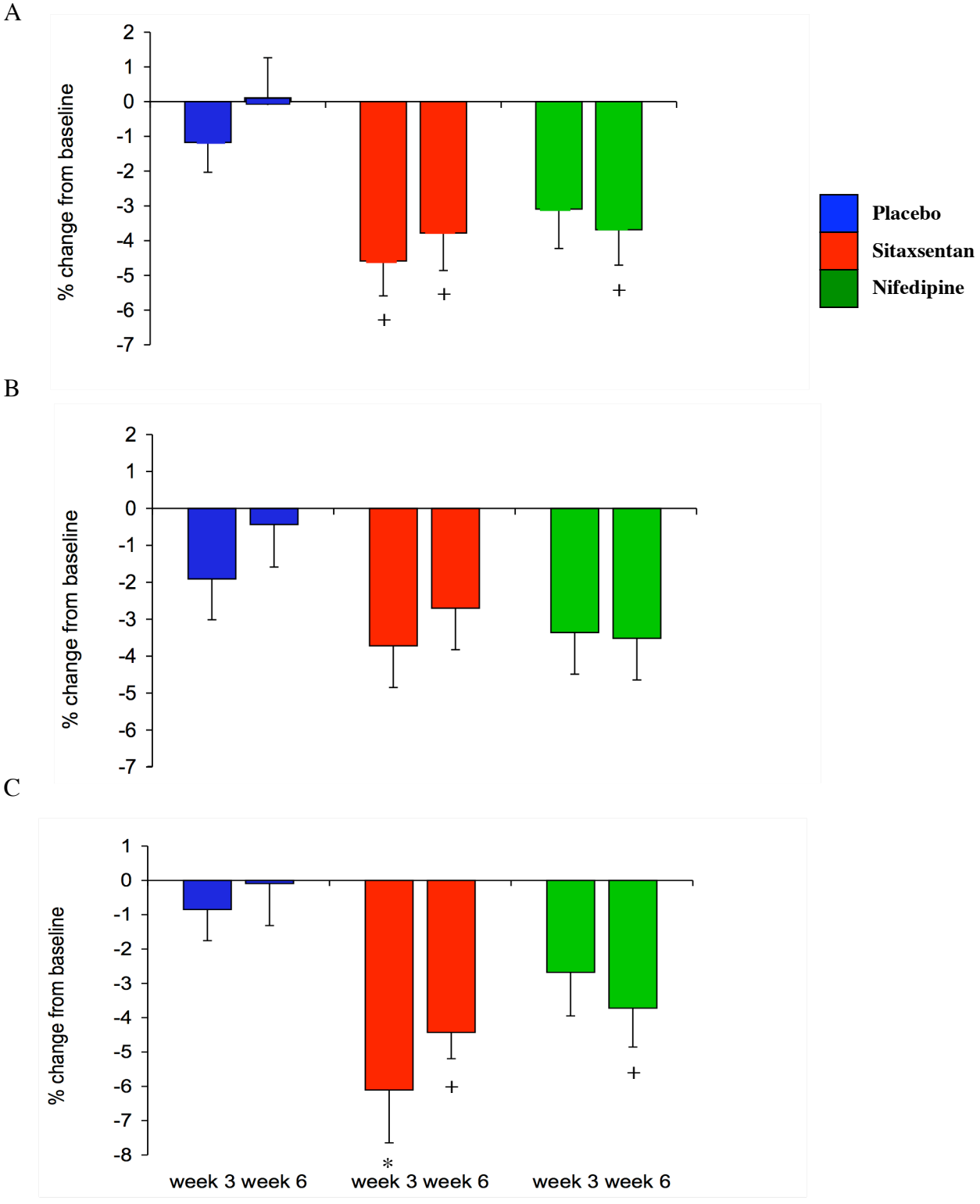
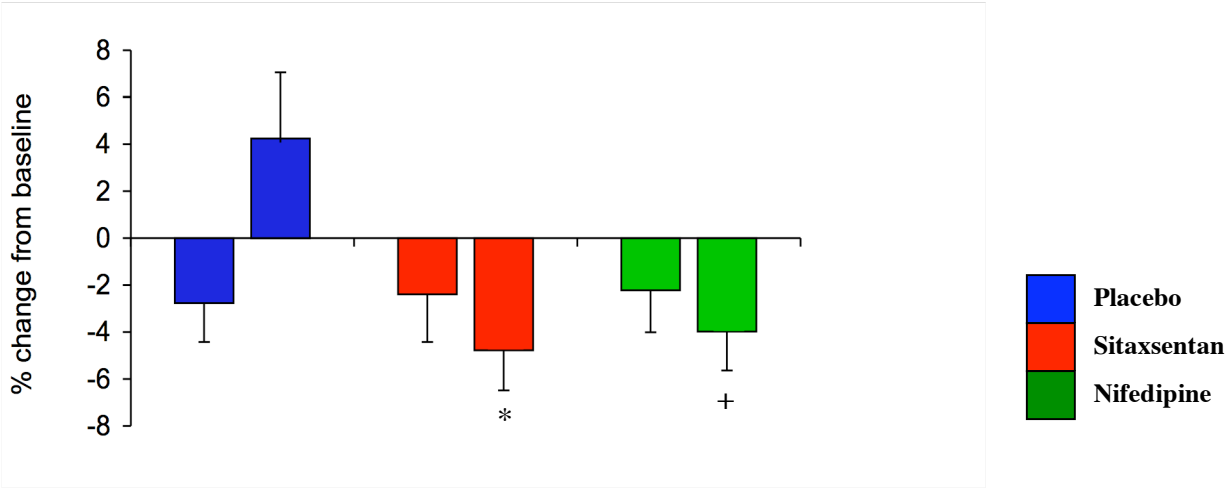
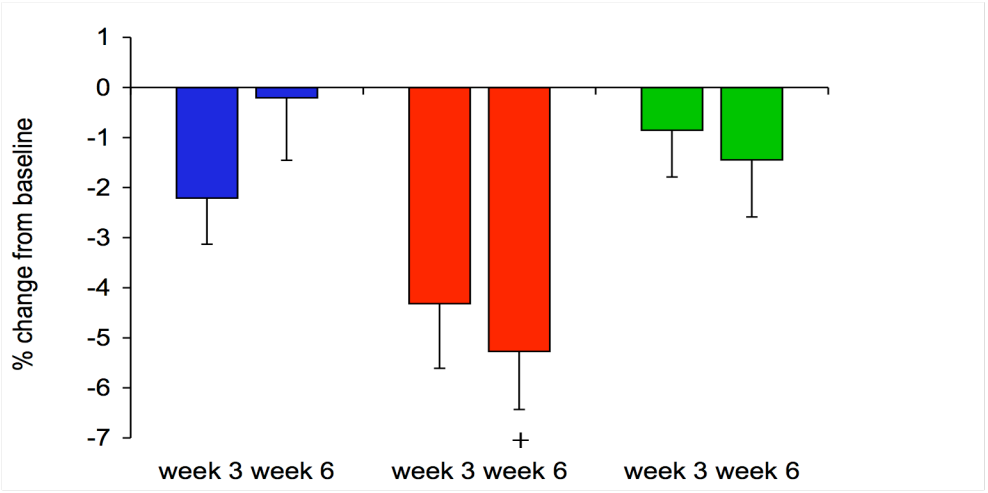


Figure 6.4: Effects of placebo, sitaxsentan and nifedipine LA 30mg on (A) pulse wave velocity, and (B) central augmentation index. Legend as for Figure 1. For (A), * $p < 0.01$ and + $p < 0.05$ for sitaxsentan and nifedipine *vs.* placebo. For (B), + $p < 0.05$ for sitaxsentan *vs.* both placebo and nifedipine.

A



B



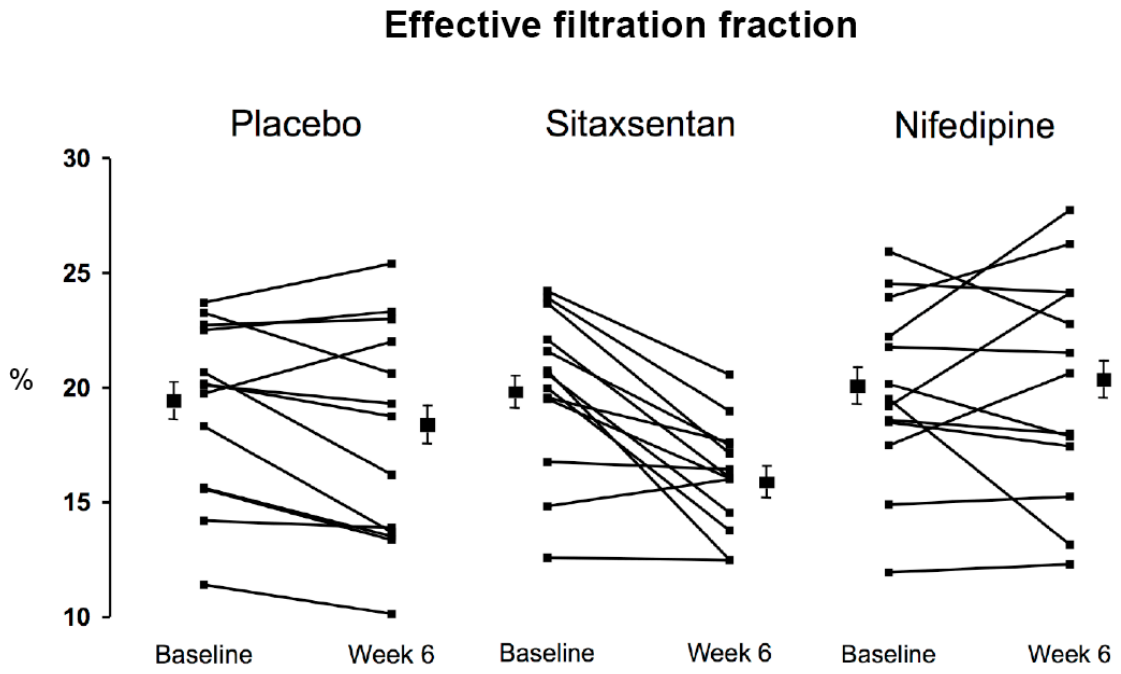
Renal sub-study (n = 13, Table 6.4 & Figure 6.5)

ERBF did not change from day 0 to week 6 with placebo, sitaxsentan or nifedipine. Whilst GFR was similar at day 0 and week 6 with placebo and nifedipine, sitaxsentan produced a substantial fall in GFR by week 6. Effective filtration fraction (EFF) remained 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).

Table 6.4: Renal sub-study data from clearance studies performed at baseline and week 6 of each study period. Values are given as pre-dosing baseline ± 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		**Sitaxsentan**		**Nifedipine**	
	Baseline	Week 6	Baseline	Week 6	Baseline	Week 6
GFR (ml/min)	56 ± 7	54 ± 8	**57 ± 8**	**48 ± 8**$^{+}$	59 ± 8	58 ± 9
ERBF (ml/min)	533 ± 66	552 ± 65	511 ± 63	543 ± 73	562 ± 82	530 ± 72
ERVR (mmHg/min/l)	230 ± 52	206 ± 39	236 ± 44	232 ± 48	248 ± 58	254 ± 56
EFF (%)	19.1 ± 1.1	17.9 ± 1.3	**20.8 ± 1.0**	**16.6 ± 0.7***	20.3 ± 1.1	20.5 ± 1.4

Figure 6.5: Effect of placebo, sitaxsentan and nifedipine LA 30mg on effective filtration fraction (EFF). Individual subject data is presented as well as the mean ± SEM at baseline and 6 weeks.



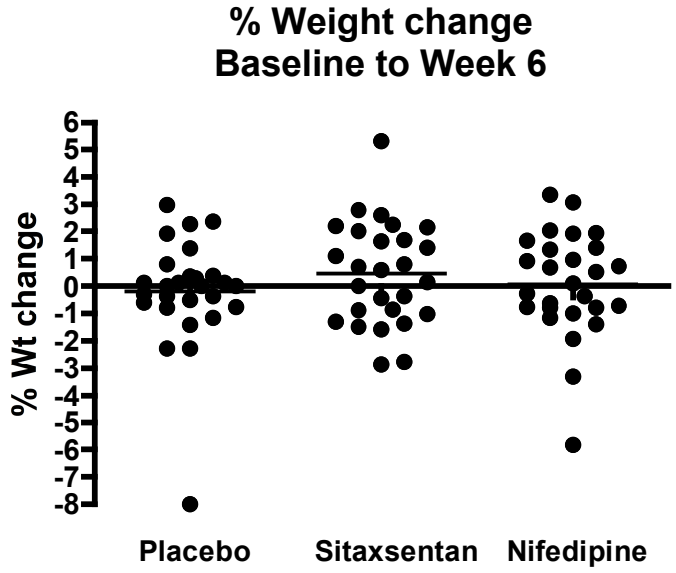
Adverse events (Table 6.5)

There was no difference in the overall incidence of adverse events between the sitaxsentan and placebo groups. Of note, there was no significant weight gain (Figure 6.6), fall in haemoglobin or haematocrit, or rise in serum potassium associated with sitaxsentan treatment compared to placebo.

Table 6.5: Adverse events reported in the study.

	Placebo (n = 27)	**Sitaxsentan (n = 27)**	**Nifedipine (n = 27)**
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)
Adverse events reported >5%, n (%)			
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)

Figure 6.6: Effects of placebo, sitaxsentan and nifedipine on weight.



Discussion

We have demonstrated that sitaxsentan, an oral selective ET_A receptor antagonist reduces proteinuria, BP and arterial stiffness in patients with varying degrees of proteinuric nephropathy. These effects on proteinuria were seen in patients already receiving optimal treatment with ACE inhibitors and ARBs, and were at least in part BP independent. These findings suggest a potential role for ET_A receptor antagonism in conferring longer-term cardiovascular and renal benefits in patients with CKD.

In the current study all subjects had renal diagnoses that are classically associated with proteinuria and all were established on maximally tolerated treatment with ACE inhibitors and/or ARBs with good BP control. Despite this, mean baseline proteinuria was still significant at ~2g/d (range 0.3 – 7.8g/d). Importantly, the data presented here support a potential role for ET receptor antagonists as a novel class of drug to help further reduce proteinuria in these patients on top of standard therapy. This should have the capacity to reduce CKD progression, and the associated CVD, morbidity and mortality.

We have recently shown that *acute* ET_A receptor antagonism can reduce proteinuria by a further ~30% on top of that achieved with optimal treatment with inhibitors of the renin-angiotensin system in subjects with proteinuric CKD. The current study, in a similar cohort of subjects, suggests that these effects are maintained longer term and are of a similar magnitude. Interestingly, the size and time course of this effect is similar to that seen with blockers of the renin-angiotensin system[226-228]. Furthermore, of those subjects showing ≥ 40% reduction in urinary protein leak (9 of 27), 4 were on dual ACE inhibitor / ARB therapy, supporting a role for ET receptor antagonists as adjunctive treatments for CKD patients already established on renin-angiotensin system inhibitors. As has been previously shown with ACE inhibitors[119], the reduction in proteinuria was related to baseline proteinuria, with subjects with a higher level of baseline urinary protein leak achieving greater reductions. This effect was seen across the range of renal function.

The effects of sitaxsentan on proteinuria described here are likely explained by changes in both systemic and renal haemodynamics. As expected, the change in BP following 6 weeks' sitaxsentan dosing correlated with the reduction in proteinuria (r^2 = 0.16, p = 0.04), supporting a role for BP reduction in reducing proteinuria. However, sitaxsentan also reduces proteinuria through effects that are independent of BP. In the current study our active control nifedipine matched the fall in BP seen with sitaxsentan but despite this sitaxsentan reduced proteinuria to a greater degree. Furthermore, for the reduction in BP seen with sitaxsentan (~4 mmHg) a less impressive fall in proteinuria than the observed ~30% would be expected. ACE inhibitors which reduce proteinuria by a similar degree to the effect seen here with sitaxsentan have more impressive effects on BP, reducing it by ~10 mmHg[65].

Our current sub study data support a renal haemodynamic mechanism for the reduction in proteinuria seen with sitaxsentan. ET_A receptor antagonism had no effect on renal blood flow or renal vascular resistance. However, as in previous studies[53, 221], there was a very consistent fall in filtration fraction (-4%), suggesting 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 renin-angiotensin system 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' sitaxsentan treatment. In patients already prescribed blockers of the renin-angiotensin system these effects, despite an initial fall in GFR, should correlate with longer term slowing of the rate of CKD progression.

The current study confirms the concept that blocking the ET_A receptor reduces BP in CKD. Sitaxsentan reduced BP modestly (a fall in MAP of ~4 mmHg). This effect may have been more impressive had the subjects not had such good baseline BP control (see Table 6.1), which meets current CKD management guidelines[204]. There are only three

studies of the longer term antihypertensive effects of ET receptor antagonism. These suggest that both selective ET_A[214, 229] and mixed $ET_{A/B}$ antagonists[65] are effective at reducing BP in untreated hypertensive patients or those with resistant hypertension. Our current data suggest that, at least in patients with CKD, where BP control is often difficult[202], ET receptor antagonism may provide a novel strategy to lower BP to a greater extent than that achieved with existing treatments.

Sitaxsentan also significantly improved arterial stiffness as measured by PWV and cAIx compared to placebo. This is likely to be due largely to the reduction in BP seen with sitaxsentan[90], a view supported by the similar change in BP and PWV seen with nifedipine. Interestingly, despite similar BP effects, sitaxsentan reduced cAIx to a greater extent than nifedipine. In the current study, unlike for BP and proteinuria, the reductions in PWV and cAIx were higher at 6 weeks' than after 3 weeks' sitaxsentan treatment. It is possible that longer treatment with an ET_A receptor antagonist might reduce PWV further, and perhaps to a greater degree than nifedipine. There are few clinical trials demonstrating that differential lowering of PWV with medical treatment results in different cardiovascular or renal outcomes[88, 217] but the importance of such studies is underscored by epidemiological data suggesting that PWV is an independent risk factor for CVD morbidity and mortality[87, 218].

6 weeks' sitaxsentan dosing in subjects with varying degrees of proteinuric CKD was not associated with any more adverse events than placebo. Importantly, we observed no weight gain, clinically significant oedema, fall in haemoglobin or haematocrit, or rise in serum potassium. Furthermore, the changes in renal haemodynamics were not associated with sodium retention (data not shown). Fluid retention has been observed in several trials with ET receptor antagonists though its mechanism remains unclear. From a renal perspective, the lack of rise in serum potassium with sitaxsentan is clinically significant as this is a troublesome side effect with both ACE inhibitors and ARBs limiting their use. Indeed, 3 of the 27 subjects studied here were intolerant of either agent due to

problematic hyperkalemia, and so selective ET_A receptor antagonists may provide an alternative proteinuria lowering strategy in such patients.

We recognise some limitations to the current work. The study was crossover by design. This may lead to subjects dropping out limiting its power, as well as having the issue of carry over effects between different treatment phases. However, we observed no such problems here. Furthermore, whilst the small study number is reasonable to show benefits of treatment much larger studies are required to highlight potentially important but infrequent adverse events. In summary, the current data support a role for selective ET_A receptor antagonism as a novel and worthwhile therapeutic target in CKD to lower proteinuria, BP and arterial stiffness on top of standard treatment, and on this basis, larger and longer term studies are now justified.

Chapter 7

Conclusions

The studies presented in this thesis have explored the role of ET-1 in CKD and the effects of selective ET_A receptor antagonism, both acutely and longer term, in chronic proteinuric nephropathy. They have helped establish a clearer picture of ET in renal pathophysiology and support a clinical potential for ET receptor antagonists in the treatment of renal dysfunction.

Urinary ET-1 in CKD

The studies in Chapter 3 have shown that plasma and urinary ET-1 concentrations increase as renal function declines. Importantly, whereas plasma ET-1 increases linearly, urinary ET-1 shows an exponential increase. This suggests that plasma and urinary ET-1 act as two separate systems. Indeed, this is supported by other data which show that when the administration of ET antagonists dynamically affect plasma ET-1, urinary ET-1 is unaffected[183]. The prominent rise in urinary ET-1, which is assumed to be of renal origin, with declining GFR supports its role as an important mediator of CKD development and progression.

We have also shown that urinary ET-1 is a marker of active renal inflammation. Its levels being high in those with active, biopsy-proven lupus nephritis, and falling with successful immunosuppressive treatment. It would be of great interest to extend these findings to other more inflammatory renal diseases such as ANCA-associated vasculitis to see if the same holds true. Also, further investigation as to whether the rise in urinary ET-1 precedes any obvious clinical or biochemical decline would make it a potentially powerful biomarker of disease relapse allowing earlier diagnosis and treatment of disease than is currently possible.

The role of ET-1 and the ET_A receptor in the maintenance of vascular tone in CKD

The studies in Chapters 4 and 6 support a role for ET-1 acting via the ET_A receptor in contributing to systemic and renal vascular tone in patients with CKD. However, whereas the fall in BP seen acutely (Chapter 4) is maintained longer term (Chapter 6) the increase in renal blood flow is not. This is likely due to adaptive changes in renal haemodynamic autoregulatory mechanisms.

The current data are in keeping with earlier work where the magnitudes of the BP reduction and increase in renal blood flow were similar[53]. These earlier studies compared systemic and renal haemodynamic responses in both health and CKD and suggested that the effects of selective ET_A receptor antagonism on lowering BP are greater in CKD than in health and the increase in renal blood flow is *only* seen in CKD. Thus, the presence of renal failure and (treated) hypertension may alter vascular sensitivity to selective ET_A receptor antagonists and reflect an enhanced activity of the ET system in renal disease, particularly in the renal circulation. The observed increased production of urinary ET-1 in CKD (Chapter 3) would support this argument, at least in respect of the kidney.

Renoprotective profile of selective ET_A receptor antagonism in CKD

The studies presented here suggest that selective ET_A receptor antagonism offers a potentially renoprotective profile by reducing both BP and proteinuria. Hypertension is an independent risk factor for CKD progression[200], and is a frequent finding in patients with CKD. Despite treatment with multiple antihypertensive agents the majority of CKD patients fail to reach target BP[202]. Proteinuria is a common feature of CKD and its presence is independently associated with an adverse renal outcome[203]. Current treatments for proteinuria focus on BP reduction[119]. Nevertheless, many CKD patients have significant residual proteinuria despite optimal treatment[206]. Importantly, both the reductions in BP and proteinuria seen acutely (Chapter 4) with selective ET_A receptor blockade are maintained longer term (Chapter 6).

These studies have demonstrated that ET_A receptor blockade produces a clinically relevant fall in BP in CKD patients. Importantly, this is without sodium retention, weight gain or troublesome adverse effects. Interestingly, the subjects studied already had excellent BP control, essentially achieving current recommended targets for CKD and it may be that if given to those with higher BP and more difficult to control hypertension the effects of ET_A receptor antagonism may be even more impressive.

Whereas the BP lowering effect of ET_A antagonism was lower longer term than acutely (4 *vs.* 10 mmHg), the reduction in proteinuria was similar (~30%). Current guidelines for treating proteinuria recommend the use of ACE inhibitors[230] and angiotensin receptor blockers[121], both of which are inhibitors of the renin-angiotensin system and thought to reduce proteinuria to a greater extent than accounted for by BP-lowering alone[205]. Importantly, the effects seen in the current studies, both acute and chronic, were in patients that were optimally treated for their proteinuria in terms of ACE inhibitors and/or angiotensin receptor blockers. Thus, selective ET_A receptor antagonism offers a potentially novel approach to reducing proteinuria in CKD.

Future work

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

1. Selective ET_A vs. mixed $ET_{A/B}$ receptor antagonism in CKD

The studies presented here have used a selective ET_A blocking approach. 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 (proteinuria, BP and arterial stiffness 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 blockade[64], 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[231, 232]. Although both approaches may be of benefit in CKD there are no head-to-head studies.

2. ET receptor antagonism and CKD progression

We have shown that ET receptor antagonists reduce BP and proteinuria over medium term dosing. These data suggest that these agents may attenuate CKD progression. If true, this would clearly be of major therapeutic interest and benefit. This would be best assessed by long term clinical trials using progression to dialysis or doubling of serum creatinine as current validated end points. Whilst there are currently no human data, ET receptor antagonists have been shown to attenuate the progression of renal insufficiency in a rat remnant-kidney model[136]. While both selective and mixed ET receptor antagonists have produced positive results[137], the effect appears to be greater with selective ET_A receptor antagonism[138]. Indeed, concomitant administration of an ET_B receptor antagonist can abolish the beneficial effects of ET_A receptor antagonism[139].

3. Natriuresis and salt sensitivity

We clearly demonstrated a natriuresis with ET_A receptor antagonism in Chapter 4. This may be of particular interest in salt sensitive hypertension, a condition where an inability to excrete sodium effectively contributes to hypertension. Animal models of salt sensitive hypertension have suggested an important pathogenic role for ET-1[233] and the therapeutic potential for ET_A receptor antagonism in this condition[234-236]. In man, plasma and urinary ET concentrations have been shown to be elevated in salt-sensitive individuals[237, 238], though this is not a uniform finding[70]. Examining the effects of ET receptor antagonism in two hypertensive groups, one clearly identified as salt sensitive, one not, on both high and low salt diets would clarify the role of ET, particularly of renal origin in salt sensitivity. Because salt sensitivity may be linked to a blunted increase in endothelial nitric oxide (NO) in response to a high salt diet[236], the comparison of ET_A with $ET_{A/B}$ blockade may be interesting in this respect.

4. Interaction of selective ET_A receptor antagonists with PDE5 inhibitors

CKD is associated with a downregulation of the NO system and this contributes to the development of hypertension and disease progression[239, 240]. NO causes vasodilatation by stimulating vascular smooth muscle soluble guanylate cyclase to convert guanosine 5'-

triphosphate to cGMP[241], which leads to a reduction in intracellular calcium concentration[242]. cGMP is degraded by cGMP-specific, cGMP-binding phosphodiesterase 5 (PDE5), and intracellular concentrations of cGMP are tightly controlled by this enzyme via a number of negative feedback mechanisms[243]. Inhibitors of PDE5 increase the intracellular concentration of cGMP and thus promote NO-mediated cellular responses.

PDE5 inhibitors have been investigated in animal models of CKD where they have been shown to reduce BP, proteinuria, inflammation and, importantly, retard CKD progression[244]. There are no human data to date. This would be of great interest given that these are currently available licensed drugs. Furthermore, as the ET system works in balance with the NO system and ET receptor antagonism restores NO bioavailability, it would be worthwhile to compare the effects of PDE5 inhibition both in the presence and absence of ET receptor antagonism as there may be additional effects of a combined approach.

5. Acute intervention in scleroderma hypertensive crisis

Scleroderma associated pulmonary arterial hypertension is treated with ET receptor antagonism[245, 246]. Though not directly studied in this thesis, given the vasoactive nature of these drugs, and the pro-fibrotic effects of ET-1 experimentally, this condition would represent a potential target for ET_A receptor antagonists, particularly given that the current treatment for this condition is ACE inhibition.

6. The role of the ET system in podocyte and macrophage biology

We have shown a reduction in proteinuria with selective ET_A receptor antagonism. The mechanism for this, in part, relates to a reduction in GFR (Chapter 6). However, proteinuria is associated with damage to podocytes and the longer term effects of ET receptor antagonism may also relate to alterations in podocyte function. Indeed, selective ET_A receptor blockade is protective in a rodent model of glomerulosclerosis that exhibits both podocyte injury and proteinuria[247].

Glomerular infiltration by macrophages (Mϕ) is common in many forms of proteinuric nephropathy. Mϕ have the potential to cause death of intrinsic renal cells including mesangial and tubular cells and this contributes to disease progression[248]. Mϕ are likely to interact with podocytes but data are limited. Strategies that reduce the pro-inflammatory activity of Mϕ reduce proteinuria in experimental models[249].

There are few data on the role of ET-1 in either podocyte or Mϕ biology. Murine podocytes exposed to protein de-differentiate and produce ET-1[126]. Furthermore, treatment of these podocytes with exogenous ET-1 induces a similar de-differentiated phenotype[126]. Mϕ have been shown to produce ET-1 in response to LPS stimulation[250] and in human monocytes ET-1 stimulation resulted in TNF-α and IL-1β secretion[251]. It would thus be of great interest as a corollary to our clinical studies to address the effects of ET-1 on podocyte and Mϕ function, to examine how these cells interact, and to assess the impact of ET receptor antagonism on glomerular inflammation and proteinuria in animal models.

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