Arterial stiffness and endothelial dysfunction in chronic kidney disease

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Publications

The following publications are relevant to the work described in this thesis. Papers are included in Appendix 5.

Dhaun, N., **Lilitkarntakul, P.**, MacIntyre, I. M., Muilwijk, E., Johnston, N. R., Kluth, D. C., Webb, D. J. & Goddard, J. (2009). Urinary endothelin-1 in chronic kidney disease and as a marker of disease activity in lupus nephritis. *Am J Physiol Renal Physiol*, 296: F1477-83.

Dhaun, N., MacIntyre, I. M., Melville, V., **Lilitkarntakul, P.**, Johnston, N. R., Goddard, J. & Webb, D. J. (2009). Blood pressure-independent reduction in proteinuria and arterial stiffness after acute endothelin-a receptor antagonism in chronic kidney disease. *Hypertension*, 54: 113-9.

Presentations

The following presentations are relevant to the work described in this thesis.

Lilitkarntakul, P., Dhaun, N., Goddard, J. & Webb, D.J. Arterial stiffness and endothelial dysfunction in chronic kidney disease. Oral presentation, the American Society of Nephrology 2007 meeting, San Francisco, CA, USA.

Lilitkarntakul, P., Dhaun, N., Melville, V., Goddard, J. & Webb, D.J. Determinant of arterial stiffness and endothelial dysfunction in chronic kidney disease. Poster presentation, the 10th International Conference on Endothelin - ET-10, 2007, Bergamo, Italy.

Lilitkarntakul, P., Dhaun, N., Goddard, J. & Webb, D.J. Risk factors for the metabolic syndrome independently predict arterial stiffness and endothelial dysfunction in chronic kidney disease. Poster presentation, the American Society of Nephrology 2008 meeting, Philadelphia, PA, USA.

Lilitkarntakul, P., Dhaun, N., Melville, V., Goddard, J. & Webb, D.J. Determinant of arterial stiffness and endothelial dysfunction in chronic kidney disease. Poster presentation, the British Renal Society/ Renal Association 2008 conference, Glasgow, UK.

Lilitkarntakul, P., Dhaun, N., Goddard, J. & Webb, D.J. Risk factors for the metabolic syndrome independently predict arterial stiffness and endothelial dysfunction in chronic kidney disease. Oral presentation, the Scottish Renal Association 2008 meeting, Dunfermline, UK.

Declaration

This thesis and the data presented within it are entirely the results of my own efforts, except where stated below. The work contains no material that has been accepted for the award of any other degree or diploma in any university. In addition, to the best of my knowledge, this work contains no material previously published or written by another person, except where stated in the text.

1. Studies

All clinical studies were performed by me with the exception of studies in Chapter 9 where systemic & renal haemodynamics and proteinuria parts were performed in conjunction with Dr Neeraj Dhaun.

2. Assays

All immediate processing of samples was undertaken by me. The subsequent analyses are divided into:-

1. Those performed by me were fetuin-A and oxidised low density lipoprotein.

2. Those largely analysed by the laboratory staffs but which I also performed to gain experience of the assays:-

> 2.1 Endothelin-1 and big endothelin-1 were analysed at the Clinical Pharmacology Unit, Centre for Cardiovascular Science, University of Edinburgh by Mr Neil Johnston, Miss Lorraine Bruce, and Miss Eilidh Cole.
>
> 2.2 Arginine, asymmetric dimethyl arginine, and symmetric dimethylarginine were analysed at the Department of Clinical Biochemistry & Metabolic Medicine, Royal Infirmary of Glasgow by Dr Scott Blackwell and Dr Dinesh K Talwar.

3. Those analysed entirely by the laboratory staff:-

3.1 High-sensitivity C-reactive protein was analysed at the Biochemistry & Haematology Laboratory at the Western General Hospital.

3.2 Interleukin-6 and isoprostane were analysed at the Vascular Biology Centre, Medical College of Georgia, USA.

3.3 All other routine laboratory measurements were analysed at the Biochemistry & Haematology Laboratory at the Western General Hospital and the Royal Infirmary of Edinburgh.

3.4 The computed tomography imaging for studies in Chapter 8 were performed and analysed by Dr Dilip Patel at the Radiology Department, Royal Infirmary of Edinburgh.

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Abbreviations

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
AIx	Augmentation index
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
ATP III	Adult Treatment Panel III
BMI	Body mass index
BP	Blood pressure
CAC	Coronary artery calcification
CAIx	Central augmentation index
CAIx@HR75	Central augmentation index adjusted to a standard heart rate of 75 bpm
CF-PWV	Carotid-femoral pulse wave velocity
C&G	Cockcroft & Gault
CI	Cardiac index
CKD	Chronic kidney disease
CrCl	Creatinine clearance
CRP	C-reactive protein
CR-PWV	Carotid-radial pulse wave velocity
CV	Coefficient of variation
DBP	Diastolic blood pressure
DDAH	Dimethylarginine dimethylaminohydrolase
ECE	Endothelin-converting enzyme
EDRF	Endothelium-derived relaxing factors
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbant assay

ERBF	Effective renal blood flow
ERPF	Effective renal plasma flow
ERVR	Effective renal vascular resistance
ESR	Erythrocyte sedimentation rate
ESRD	End-stage renal disease
ET	Endothelin
ET-1	Endothelin-1
FeET-1	Fractional excretion of endothelin-1
FF	Filtration fraction
FMD	Flow-mediated dilatation
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
GTN	Glyceryl trinitrate
HbA_1C	Haemoglobin A_1C
HDL	High density lipoprotein
hsCRP	High-sensitivity C-reactive protein
IgAN	IgA nephropathy
IL-6	Interleukin-6
IMT	Intima media thickness
Isop	Isoprostane
K/DOQI	Kidney Disease Outcomes Quality Initiative
LDL	Low density lipoprotein
L-NMMA	N^G-monomethyl L-arginine
MAP	Mean arterial pressure
MDRD	Modification of Diet in Renal Disease
NCEP	National Cholesterol Education Program
NO	Nitric oxide
NOS	Nitric oxide synthase

OxLDL	Oxidised low density lipoprotein
PAH	Para-aminohippurate
PP	Pulse pressure
PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acid
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RAIx	Radial augmentation index
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SDMA	Symmetric dimethylarginine
SVRI	Systemic vascular resistance index
TMB	Tetramethyl benzidine
WHO	World Health Organisation

Abstract

Patients with chronic kidney disease (CKD) have an increased risk of cardiovascular disease to which conventional cardiovascular risk factors and co-morbidity contribute. Increased arterial stiffness and impaired endothelial function are common features of CKD and recognised markers of cardiovascular risk. In recent years, emerging cardiovascular risk factors - including inflammation, oxidative stress, and a shift in the balance of the vasodilator nitric oxide and vasoconstrictor endothelin systems - have become increasingly important as major contributors to increased cardiovascular complications and may also contribute to arterial stiffness and endothelial dysfunction in CKD.

The overall aims of the work presented within this thesis were, therefore, to characterise the contribution of uraemia itself, and conventional and emerging cardiovascular risk factors, to arterial stiffness and endothelial dysfunction, as surrogates for cardiovascular risk, in a group of CKD patients, across a wide range of glomerular filtration rate (GFR) from normal to pre-dialysis, with relatively low co-morbidity. Arterial stiffness and endothelial dysfunction were measured by carotid-femoral pulse wave velocity (CF-PWV) and flow-mediated dilatation (FMD), respectively.

The first study aimed to assess the reproducibility for one observer with repeated measurements (intra-observer) and for two separate observers (inter-observer) of CF-PWV and FMD. I have shown that both inter-observer and intra-observer measurements of CF-PWV and FMD are highly reproducible. Hence, these techniques are therefore suitable to be incorporated into clinical studies.

The characteristics of the relationship of plasma and urinary endothelin-1 (ET-1) concentrations to renal function were studied. In this group of CKD patients, plasma ET-1 increased in a linear fashion, whereas fractional excretion of ET-1 increased exponentially as renal function declined. These findings support the role of renally derived ET-1 in renal pathophysiology.

In the next study, I showed that arterial stiffness increases incrementally as GFR declines whereas endothelial dysfunction is a feature only of late stage CKD (GFR ≤ 20 ml/min/1.73m^2). Age and blood pressure (BP) were the major determinants of both. However, GFR was not an independent predictor of either CF-PWV or FMD in this group of patients, suggesting that uraemia, on its own, is not the main driving force in the development of vascular complications in CKD.

Therefore, I further explored the role of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction. Whilst, BP remains the strongest determinant of arterial stiffness and endothelial dysfunction, inflammation and asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, are independent predictors of CF-PWV, and oxidative stress and plasma ET-1 independently predict FMD. Thus, the cardiovascular complications that occur in CKD may be substantially driven by these emerging risk factors.

Then, I examined the contribution of the metabolic syndrome to arterial stiffness and endothelial dysfunction in the same group of CKD patients. Irrespective of renal function, CKD patients with the metabolic syndrome have increased arterial stiffness and a trend to impaired endothelial function. Either the presence of the metabolic syndrome or the number of risk factors for it independently predicts CF-PWV and FMD. When risk factors for the metabolic syndrome are considered individually, BP remains an independent determinant of both CF-PWV and FMD. Additionally, waist circumference is also an independent predictor of CF-PWV. These findings suggest that the metabolic syndrome or its individual risk factors maybe targets for intervention to improve cardiovascular outcomes in all stages of CKD.

The contributions of arterial calcification to arterial stiffness and endothelial dysfunction were also assessed. I showed that, irrespective of renal function, CKD patients with arterial calcification have increased arterial stiffness and a trend to impaired endothelial function.

The findings from the observational studies presented in this thesis support the role of emerging cardiovascular risk factors on arterial stiffness and endothelial dysfunction in CKD. An interventional study using a peptide selective endothelin-A

(ET_A) receptor antagonist has confirmed this hypothesis. In a group of CKD patients, irrespective of renal function, selective ET_A receptor antagonism lowered BP, reduced proteinuria, improved arterial stiffness on top of a standard BP lowering treatment with renin-angiotensin system blockade, and appeared to reduced arterial stiffness independent of its effect on BP.

In summary, these studies show that in the absence of diabetes or established cardiovascular disease, CKD patients have increased arterial stiffness and endothelial dysfunction. However, arterial stiffness and endothelial dysfunction are not predicted by renal function. Although the conventional risk factor, BP, is the strongest determinant of CF-PWV and FMD in CKD, the contribution of several emerging cardiovascular risk factors on arterial stiffness and endothelial dysfunction is observed. On the basis of these findings, chronic interventional studies, for instance with endothelin receptor antagonists are now needed.

Chapter 1

Introduction

1.1 Chronic kidney disease

Chronic kidney disease (CKD) is a growing public health problem (Xue *et al.*, 2001). The increasing incidence of CKD is, to a large extent, being accelerated by the global epidemic of diabetes mellitus and hypertension, as well as by the ageing of the population (Fox *et al.*, 2008; McClellan *et al.*, 2003). The most widely used definition to diagnose and stratify the stages of CKD is the National Kidney Foundation, Kidney Disease Outcomes Quality Initiative (K/DOQI) guideline (Anonymous, 2002a). This guideline defines CKD according to the presence or absence of kidney damage and level of kidney function, irrespective of the type of kidney disease. CKD is defined as an individual with kidney damage for ≥3 months, as evidenced by pathological abnormalities or markers of damage including abnormalities in blood or urine tests or imaging test, with or without decreased glomerular filtration rate (GFR), or an individual with GFR <60 ml/min/1.73m^2 for ≥3 months, with or without kidney damage (Table 1.1).

Table 1.1 Stages of CKD as defined by K/DOQI classification.

Stages	Description	GFR (ml/min/1.73m^2)
Stage 1	Kidney damage* with normal or increased GFR	>90
Stage 2	Kidney damage* with mild reduction of GFR	60-89
Stage 3	Moderate reduction of GFR	30-59
Stage 4	Severe reduction of GFR	15-29
Stage 5	Kidney failure	<15 or dialysis

Key: *Kidney damage is defined as pathologic abnormalities or markers of damage including abnormalities in blood or urine tests or imaging studies.

In the general population with normal kidney function at baseline, the incidence of new CKD was 9.4% in 18.5 years in the Framingham Offspring study (Fox *et al.*, 2004) and 7% in 9 years in the Atherosclerosis Risk in Communities study (Kurella *et al.*, 2005). The prevalence of CKD in large community surveys by the National Health and Nutrition Examination Surveys (NHANES) III and IV for CKD stage 3 and 4, where kidney function was estimated by the Modification of Diet in Renal

Disease (MDRD) formula were 4.2% and 3.7%, respectively (Coresh *et al.*, 2005). Another study also reported that the prevalence of CKD stage 2 and 3 in over 10,000 adults, where kidney function was assessed by the Cockcroft & Gault (C&G) formula was as high as 11% (Chadban *et al.*, 2003).

CKD leads to kidney failure or so-called end-stage renal disease (ESRD), one of the highest cost conditions for treatment in clinical medicine. In the United Kingdom, only ~11% of all CKD patients are on renal replacement therapy but the management of this group consumes most of the renal health care budget (Nwankwo *et al.*, 2005). Despite advances in dialysis and transplantation, the prognosis of ESRD remains poor. One of the possible ways to prevent CKD patients progressing to the advanced stages of CKD is to focus on the people with earlier stages of CKD, and, through early diagnosis and treatment, slow the progression of their diseases to ESRD or other adverse outcomes.

1.2 Cardiovascular disease in chronic kidney disease

Cardiovascular disease, encompassing coronary heart disease, cerebrovascular disease, peripheral vascular disease, and congestive heart failure, is the leading cause of morbidity and mortality worldwide. The cardiovascular disease epidemic is occurring despite advances in the diagnosis and treatment of this condition. Cardiovascular disease is very common in CKD (Parfrey *et al.*, 1999; Sarnak *et al.*, 2000; Stack *et al.*, 2001). As a leading cause of mortality in the general population, it is not surprising that cardiovascular disease is also a leading cause of death in CKD patients. In fact, epidemiological and clinical studies have shown that cardiovascular disease accounts for nearly 50% of all-cause mortality in dialysis patients and cardiovascular mortality is at least 10-20 times greater in dialysis patients than in the general population (Foley *et al.*, 1998).

A pooled analysis of community-based studies combining data from the Framingham Heart study and the Atherosclerosis Risk in Communities study reported that subjects with moderate reduction of GFR (CKD stages 3 & 4) was

associated with greater risk for cardiovascular outcomes, including myocardial infarction, coronary heart disease, and stroke, compared to those of subjects with normal kidney function (Weiner *et al.*, 2004). The HOORN study showed that renal function was inversely associated with all-cause and cardiovascular mortality in CKD stages 2 to 5. In this study, risk of cardiovascular death was increased by ~20% for a decrease of 5 ml/min/1.73m^2 of GFR, calculated by the C&G formula, after adjustment for conventional cardiovascular risk factors and previous cardiovascular co-morbidity (Henry *et al.*, 2002). A recent study of ~34,000 ambulatory patients has shown a gradual increase in risk of cardiovascular mortality and decreasing GFR (adjusted hazard ratio 1.77, 3.75, and 3.83 for CKD stages 3, 4, and 5, respectively) (Ryan *et al.*, 2009). In addition, it is well recognised that individuals with impaired renal function are more likely to die from cardiovascular complications than to reach ESRD (Culleton *et al.*, 1999; Go *et al.*, 2004; Shulman *et al.*, 1989). Importantly, because of the high prevalence of, and mortality rate from, cardiovascular disease in CKD, it is now recommended that CKD patients are in the highest risk group for cardiovascular disease and that CKD itself is an independent risk factor for cardiovascular disease (Sarnak *et al.*, 2003).

There are three major pathologic forms of cardiovascular complications in CKD. The first is an alteration in the structure of the myocardium (left ventricular remodelling), resulting from pressure overload secondary to hypertension and arteriosclerosis and volume overload secondary to fluid retention, anaemia, or arteriovenous fistulae, leading to concentric and eccentric left ventricular hypertrophy, respectively. The second pattern is atherosclerosis and the third is arteriosclerosis which affects the large vessels such as the aorta, leading to the development of stiff arteries.

1.3 Cardiovascular disease risk factors in chronic kidney disease

Risk factors for cardiovascular disease in CKD can be divided into two broad categories: conventional and emerging risk factors (Table 1.2). The conventional risk factors for cardiovascular disease are defined as those that have primarily been described in the Framingham cohort including age, male gender, family history of

premature cardiovascular disease, smoking, left ventricular hypertrophy, hypertension and metabolic disorders such as hypercholesterolaemia and diabetes mellitus (Sarnak *et al.*, 2000). Emerging risk factors are defined as risk factors not included in the Framingham coronary risk equation and increased prevalence as renal function declines. These include inflammation, oxidative stress, a reduction of arterial compliance, and endothelial dysfunction (Sarnak *et al.*, 2000).

Table 1.2 Conventional and emerging cardiovascular disease risk factors in CKD.

Conventional (Framingham) risk factors	Emerging (uraemia-related) risk factors
Age	Anaemia
Diabetes mellitus	Arterial stiffness
Family history of premature cardiovascular disease	Calcium & phosphorus metabolism
Higher LDL cholesterol	Decreased GFR
Hypertension	Endothelial dysfunction (ET-1, ADMA)
Lower HDL cholesterol	Extra-cellular fluid volume overload
Male gender	Inflammation (CRP)
Menopause	Malnutrition
Physical inactivity	Metabolic syndrome
Psychological stress	Oxidative stress (Isop, OxLDL)
Smoking	Proteinuria
White race	Uraemic toxins

Key: Table modified from (Sarnak *et al.*, 2000). ADMA: asymmetric dimethylarginine; CRP: C-reactive protein; ET-1: endothelin-1; GFR: glomerular filtration rate; HDL: high density lipoprotein; Isop: isoprostane; LDL: low density lipoprotein; OxLDL: oxidised low density lipoprotein.

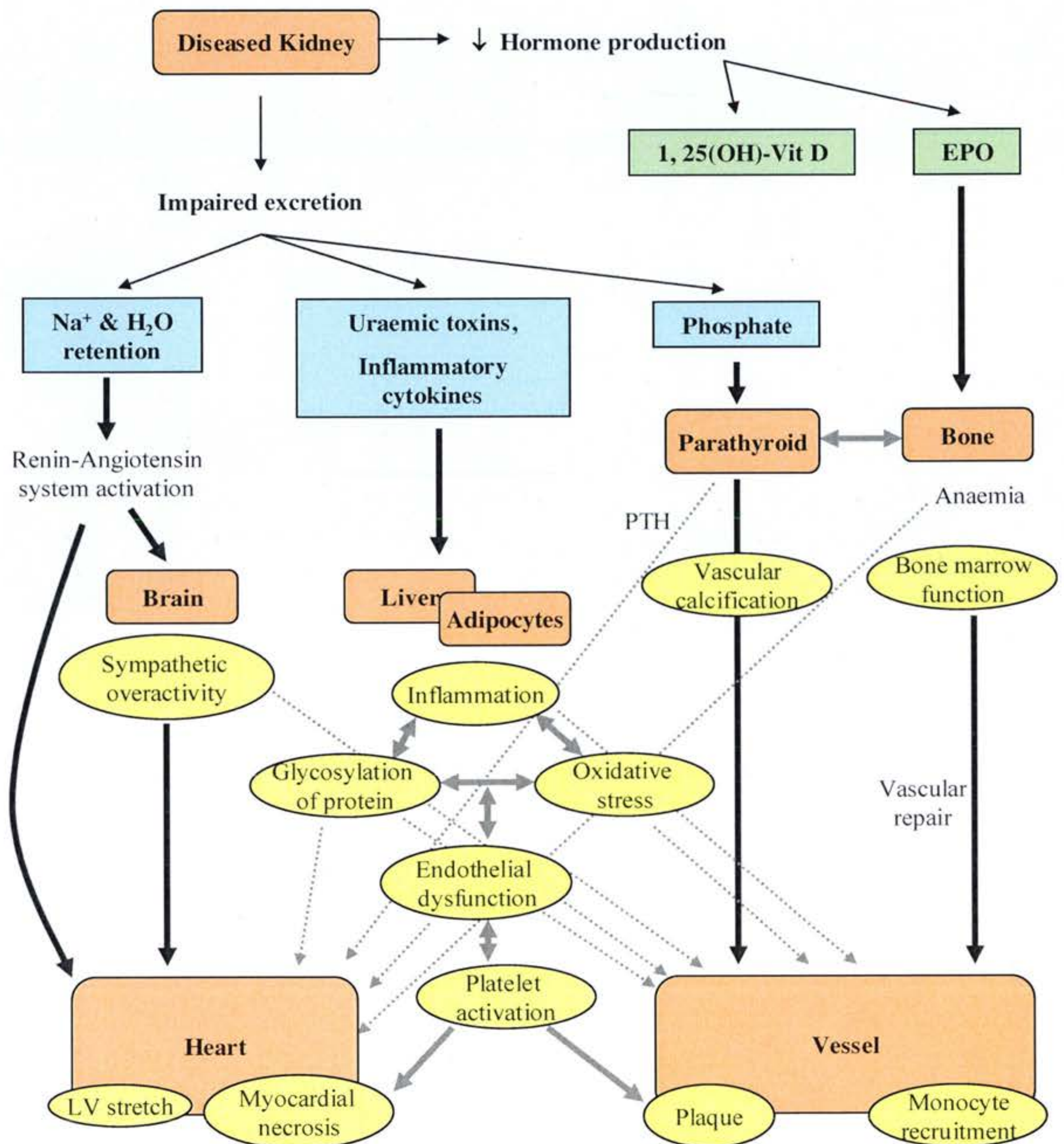
CKD patients have an increased prevalence of conventional risk factors compared to the general population (Vlagopoulos *et al.*, 2005). For example, it has been estimated that hypertension is present in 50-90% of dialysis patients and 70-80% of stage 1 to

4 CKD patients. Diabetes, a leading cause of ESRD, accounts for ~40% of pre-dialysis CKD. Dyslipidaemia (increased low density lipoprotein (LDL) cholesterol and decreased high density lipoprotein (HDL) cholesterol) was found in 50-70% of dialysis patients. The prevalence of left ventricular hypertrophy has been reported in up to 75% of both dialysis and non-dialysis patients. Additionally, ~25% of all CKD patients are smokers (Vlagopoulos *et al.*, 2005).

Conventional cardiovascular risk factors are believed to dominate the scene in CKD since most individuals who develop renal impairment usually present with a long history of hypertension, diabetes, dyslipidaemia, smoking, or a combination of these. However, these risk factors are not as strongly associated with subsequent cardiovascular morbidity and mortality in advanced stage CKD and are, therefore, insufficient to explain the high cardiovascular complications in these patients (Ikizler, 2002). This was evidenced by applying the Framingham risk equation to CKD patients which showed only a weak negative association between the calculated risk and baseline renal function, suggesting that the conventional risk factors account for some, but not all of the remarkable cardiovascular risk in CKD (Sarnak *et al.*, 2002).

Interestingly, evidence suggests that emerging cardiovascular risk factors become increasingly important as major contributors to increased cardiovascular complications in CKD as GFR declines (London *et al.*, 2004a; Schiffrin *et al.*, 2007; Zoccali *et al.*, 2003). These novel risk factors are being given emphasis not only because they could explain the high incidence of cardiovascular events in the renal population, but also because they may represent new targets for therapeutic interventions. Figure 1.1 demonstrates various pathological processes that give rise to the uraemia-related risk factors and some of the organ systems involved. Multiple interactions between processes are illustrated. Selected emerging cardiovascular risk factors will be described further in this chapter.

Figure 1.1 Diagram showing various pathological processes of uraemia-related risk factors and some of the organ systems involved.



Key: Diagram modified from (Roberts *et al.*, 2006), EPO: Erythropoietin; LV: left ventricular; PTH: parathyroid hormone.

1.4 Inflammation: C-Reactive Protein

Inflammation is an important process in the pathogenesis of atherosclerosis and its cardiovascular complications (Ross, 1999). A number of molecules involved in the atherosclerotic and inflammatory process can be measured as surrogates for inflammation. These include C-reactive protein (CRP) and soluble form of adhesion molecules, e.g. vascular cell adhesion molecule and endothelial-leucocyte adhesion molecule. Amongst these, CRP is the most widely used marker for inflammation. CRP is extremely sensitive, giving a very rapid response with a relatively short half-life. In inflammatory conditions, acute phase synthesis of CRP closely reflects disease activity. Despite the high sensitivity of CRP, it reflects only the inflammatory state but is not disease specific.

CRP is an endogenous nonglycosylated protein of a cyclic disc of 5 identical noncovalently-linked subunits with a molecular weight of 105,000 Da. CRP is produced mainly by the hepatocytes in response to infection, inflammation, or tissue damage. The formation of CRP is regulated mainly by interleukin-6 (IL-6) and, to a lesser degree, interleukin-1 and tumour necrosis factor-α. CRP was originally named for its ability to precipitate somatic C-polysaccharides of pneumococci. CRP elicits several effects on endothelial and vascular biology favouring pro-inflammatory and pro-atherogenic phenotypes (Schwedler *et al.*, 2006).

In the normal population, circulating CRP concentrations are very low (<1 mg/dl). In general, CRP levels of 1-10 mg/dl are considered moderately elevated and levels of >10 mg/dl are considered markedly elevated (van der Sande *et al.*, 2006). Recently, high-sensitivity assays for evaluating CRP levels (hsCRP) have been developed, yielding the detection of circulating CRP concentrations at a very low level (0.007 mg/L) (Rifai *et al.*, 1999). CRP is associated with increased risk for cardiovascular disease. This was evidenced in the Women's Health Study in which cardiovascular risk substantially increased in a linear fashion, across a range of hsCRP values from less than 0.5 mg/L to more than 20 mg/L, even after adjustment for the Framingham risk score (Ridker *et al.*, 2004).

In renal patients, inflammation is a common feature of ESRD (Stenvinkel *et al.*, 2005a). Elevated CRP levels have been reported in dialysis patients (Owen *et al.*, 1998; Spittle *et al.*, 2001; Stenvinkel *et al.*, 2002; Yeun *et al.*, 2000). CRP also independently predicts all-cause mortality in this group of patients (Spittle *et al.*, 2001; Yeun *et al.*, 2000; Zimmermann *et al.*, 1999). This is confirmed by a study from Wanner, *et al* in 280 haemodialysis patients. In this study, patients in the highest hsCRP quartile had a 2.4-fold higher risk for all-cause mortality and 1.7-fold higher risk for cardiovascular mortality compared with those of patients in the lowest hsCRP quartile (Wanner *et al.*, 2002). In addition, CRP yields a substantial increase in predictive value for all-cause and cardiovascular mortality on top of other biomarkers such as B-type natriuretic peptide, a cardiac hormone reflecting myocardial function, and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS) (Mallamaci *et al.*, 2005).

There is also evidence for the associations between elevated hsCRP and decreasing renal function in mild renal insufficiency (Gulcan *et al.*, 2007; Knight *et al.*, 2004; Romao *et al.*, 2006; Stuveling *et al.*, 2003; Yoshida *et al.*, 2007). Interestingly, hsCRP has recently been reported to incrementally increase from CKD stages 1 to 5, suggesting that low-grade inflammation is independently associated with a declining GFR (Romao *et al.*, 2006). It is important to note that, although CRP may be directly involved in the pathology of cardiovascular disease in CKD, it is usually regarded as a risk marker rather than a causative risk factor. Factors responsible for the high CRP concentrations in CKD patients are not fully understood. Nonetheless, co-morbidity, life style, and genetic variations must be taken into account.

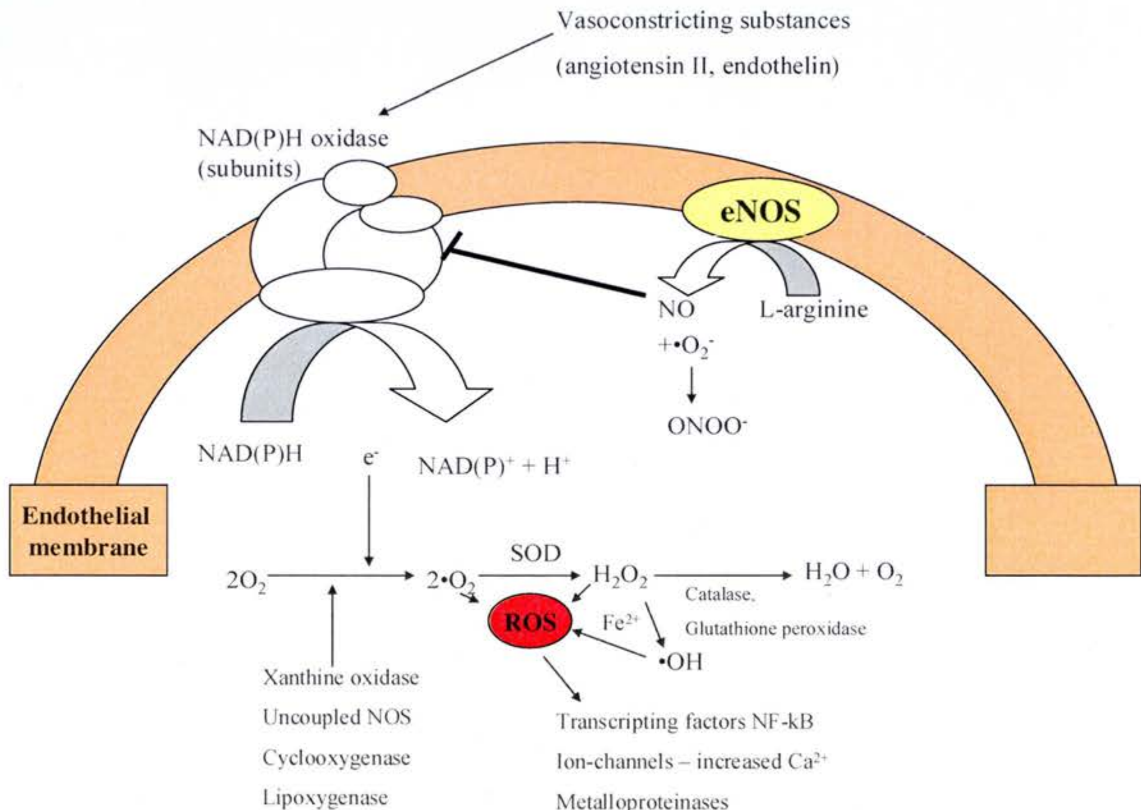
1.5 Oxidative stress

Oxidative stress is commonly described as a disturbance in the balance between oxidant production and anti-oxidant defences (Sies, 1997). A pro-oxidant state leads to the oxidation of macro-molecules such as lipids, carbohydrates, proteins, and DNA, resulting in tissue injury. Atherosclerotic conditions including diabetes mellitus, hypercholesterolaemia, and CKD are associated with increased oxidative stress (Himmelfarb, 2005).

Increased production of reactive oxygen species (ROS) in the vascular wall is a characteristic feature of atherosclerosis (Figure 1.2). ROS promote LDL oxidation, stimulate vascular smooth muscle cell proliferation and migration, and increase production of pro-inflammatory cytokines (Landmesser *et al.*, 2001; Steinberg *et al.*, 1989).

In general, oxidants are highly reactive compounds with a very short half-life (e.g. seconds). Lipids, proteins, carbohydrates, and nucleic acid can be modified by oxy-radicals and appear to have a longer half-life after being modified (e.g. hours to weeks). This long half-life makes the latter group an ultimate marker for oxidative stress. To prevent the harmful effects of ROS, both enzymatic and non-enzymatic anti-oxidant systems counteracting the free radicals are present (Table 1.3). Superoxide dismutase, catalase, and glutathione peroxidase are examples of enzymatic systems in the defence against ROS.

Figure 1.2 Reactive oxygen species generation in the cell.



Key: Figure modified from (Pechanova *et al.*, 2007). eNOS: endothelial nitric oxide synthase; NAD(P)H: nicotinamide adenine dinucleotide phosphate; NF-kB: nuclear factor kappa-B; NO: nitric oxide; NOS: nitric oxide synthase; SOD: superoxide dismutase; ROS: reactive oxygen species.

Table 1.3 Examples of markers of oxidative stress and anti-oxidants.

Markers of oxidative stress	Anti-oxidants
Carbohydrate oxidation	**Enzymatic**
Advanced glycation end products	Glutathione peroxidase
Lipid peroxidation	Superoxide dismutase
Advanced lipid peroxidation products	**Non-Enzymatic**
Isoprostanes	Glutathione
Malonyldialdehyde	Vitamin C
Oxidised low density lipoprotein	Vitamin E
Protein oxidation	
Advanced protein oxidation products	

Oxidative stress has been identified as a significant contributor to the accelerated vascular pathology associated with ESRD (Tetta *et al.*, 1999). CKD patients, in particular those who are on dialysis, are at risk of deficiencies of water-soluble vitamins and trace elements, as a results of restricted intake, impaired absorption, uraemia-induced alterations in metabolism and activity, as well as losses during haemodialysis or peritoneal dialysis. Therefore, they are subjected to enhanced oxidative stress as a result of reduced anti-oxidant defence due to a reduction of vitamin C, vitamin E, selenium levels, and reduced activity of the glutathione scavenging system. At the same time, their pro-oxidant activity is increased because of ageing, diabetes co-morbidity, chronic inflammation, and bio-incompatibility of dialysis membranes and solutions (Locatelli *et al.*, 2003).

1.5.1 Isoprostanes

Amongst markers for oxidative stress, isoprostanes (Isops) are an emerging family of compounds that, currently, are one of the most reliable markers for lipid peroxidation as recently evaluated by the National Institute of Environmental Health Science (Kadiiska *et al.*, 2005). First discovered by Morrow and colleagues (Morrow *et al.*, 1990), Isops are a group of bioactive eicosanoids derived from a non-specific free-radical attack of arachidonic acid in the cell membrane, producing four different series of regioisomers. These regioisomers are denoted as 5-, 8-, 12- or 15-series compound depending on the carbon atom to which the side chain hydroxyl is attached. Eight racemic diasteromers may be formed among each of those 4 regioisomers, giving 64 different Isops (Morrow, 2006; Taber *et al.*, 1997). Once Isops are generated, they are released by phospholipases, circulate as free Isops in blood and are excreted in urine as non-metabolised or metabolised compounds (Lynch *et al.*, 1994; Morrow, 2005).

From all of the myriad compounds produced through free radical peroxidation of arachidonic acid, the majority of studies have focused on the 15-series of Isop, especially, 15-F_{2t}-Isop or so called 8-iso-prostaglandin-F_2 (8-Isop) (Lawson *et al.*, 1999; Morrow *et al.*, 1999; Morrow *et al.*, 1997). 8-Isop is a vasoconstrictor. It stimulates mitogenesis, enhances monocyte adhesion to endothelial cells and induces endothelial cells necrosis. All of these effects are believed to be mediated by thromboxane A_2-prostanglandin H_2 receptor. 8-Isop also affects renal function by constricting the afferent renal arteriole leading to a decreased GFR (Fukunaga *et al.*, 1993; Takahashi *et al.*, 1992).

Plasma Isop concentrations are increased and correlate with the severity of congestive heart failure (Cracowski *et al.*, 2000) and coronary artery disease (Vassalle *et al.*, 2004). Urinary Isop concentrations are also elevated in patients with pulmonary arterial hypertension (Cracowski *et al.*, 2001), systemic sclerosis (Cracowski *et al.*, 2006), and coronary artery disease (Schwedhelm *et al.*, 2004), and are a strong independent risk marker for cardiovascular disease in patients with coronary artery disease (Schwedhelm *et al.*, 2004).

Several studies have indicated that plasma Isop concentrations are increased up to 6 folds in haemodialysis patients (Handelman *et al.*, 2001; Ikizler *et al.*, 2002; Kim *et al.*, 2004; Simmons *et al.*, 2005; Spittle *et al.*, 2001) and are accompanied by increased inflammation and reduced endogenous anti-oxidants such as vitamin C and vitamin E. In these studies, the effects of haemodialysis sessions on plasma Isop levels are not consistent. Recently, the increase in circulating Isop has also been shown in mild renal impairment (Cottone *et al.*, 2009). Importantly, studies have shown progressively increased plasma Isop concentrations in CKD patients from early stage to pre-dialysis (Cottone *et al.*, 2009; Dounousi *et al.*, 2006) and have reported that Isop independently predict GFR. Data regarding the relationship of these compounds to long-term outcomes, such as morbidity and mortality, remain to be collected.

1.5.2 Oxidised low density lipoprotein

The current concept for the mechanisms of atherosclerotic disease processes involve increased lipid peroxidation and formation of oxidised low density lipoprotein (OxLDL) particles leading to an inflammatory response which is a driving force in the development of atherosclerotic lesions (Harrison *et al.*, 2003).

In health, the central core of an LDL molecule contains cholesterol ester and triglyceride. The molecule is surrounded by a phospholipid monolayer, consisting mainly of lecithin. There is one apoB-100 molecule embedded in the outer layer. Approximately 50% of the fatty acids found in the LDL molecule are polyunsaturated fatty acids (PUFAs), including linoleic acid, arachidonic acid, and docosahexaenoic acid. A major role of PUFAs is to protect LDL from free radical attack and oxidation. LDL can be oxidised by three mechanisms (Figure 1.3): *in vitro* oxidation by metal ions, for example Cu^{2+}; *in vivo* oxidation by metal ion-dependent lipoxygenase enzyme; and *in vivo* oxidation by macrophage-secreted myleoperoxidase enzyme. NO inhibits $Cu^{2+}$and macrophage-mediated oxidation of LDL.

OxLDL has a different biochemical composition from native LDL. It is characterised by a higher electrophoretic mobility on agarose gels and lower arachidonic and

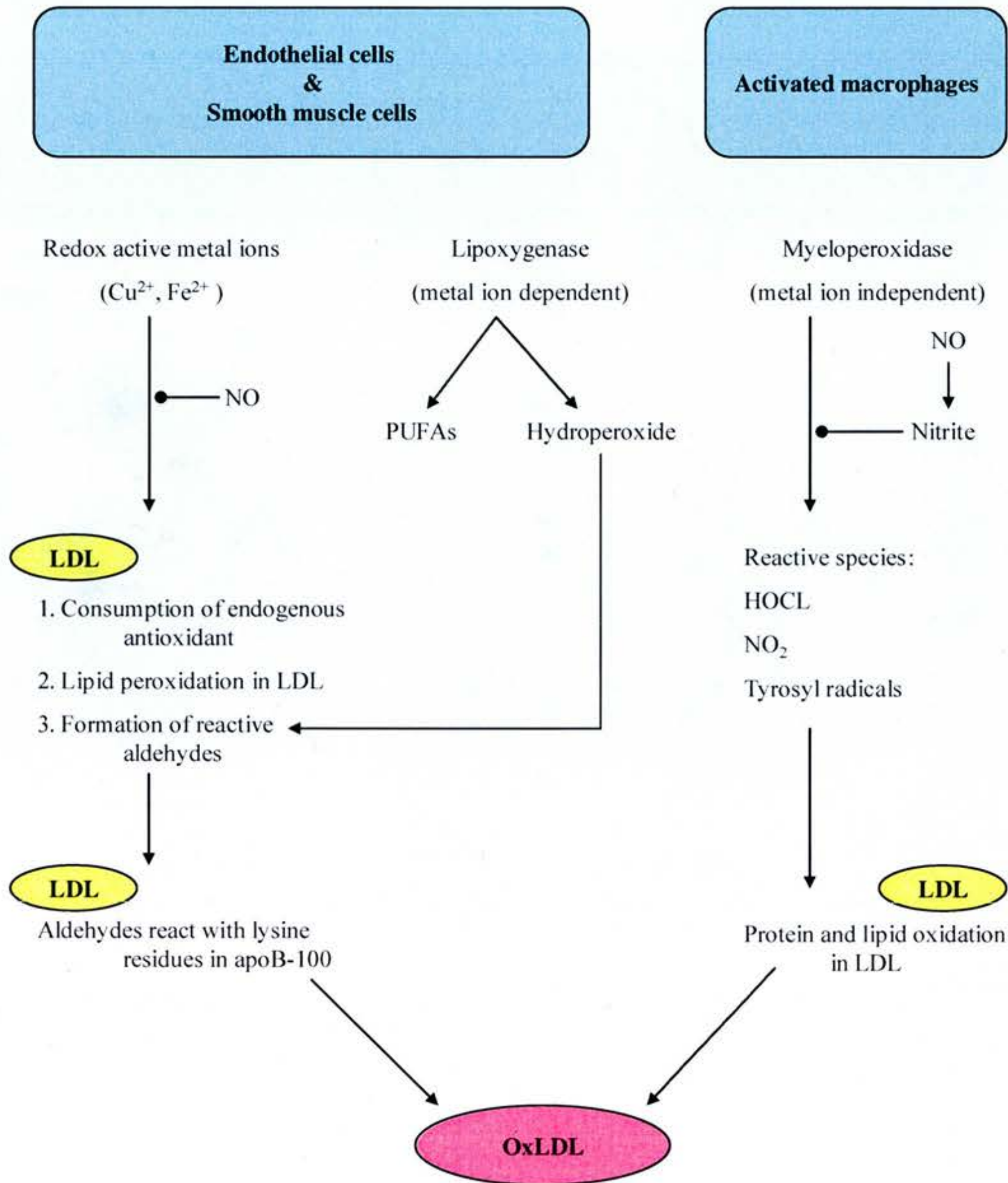
linoleic acid levels compared with native LDL. *In vitro* studies have shown that approximately 30-40% of lysine residues in the apoB-100 moiety of OxLDL are substituted by aldehyde. These characteristics suggested that most of the *in vivo* OxLDL is generated by oxidative enzymatic activity in the arterial wall, not by the metal ion-induced oxidation.

The main biological effect of OxLDL is to enhance the atherosclerotic process. OxLDL induces the endothelium to express adhesion molecules for monocytes, promoting fatty streak formation, and inducing smooth muscle cells migration leading to an increase in fibrous plaque formation. In addition, OxLDL promotes vasoconstriction through an inhibition of NO production and a release of endothelin-1 (ET-1) from the endothelium. Furthermore, OxLDL stimulates platelet adhesion and aggregation by decreasing endothelial production of NO and increasing prostaglandin production, enhancing endothelial dysfunction.

Higher circulating OxLDL concentrations are associated with subclinical atherosclerosis in healthy subjects (Metso *et al.*, 2004; Wallenfeldt *et al.*, 2004). Plasma levels of OxLDL are significantly elevated in patients with cardiovascular disease (Holvoet *et al.*, 1998a; Holvoet *et al.*, 1998b; Suzuki *et al.*, 2002). OxLDL concentrations correlate with the extent of coronary artery disease in heart transplant recipients, suggesting that OxLDL may be a marker for coronary artery disease (Holvoet *et al.*, 1998a). In addition, OxLDL is a good prognostic index for future cardiovascular events in a healthy population and in patients with coronary artery disease independent of conventional cardiovascular risk factors (Holvoet *et al.*, 2001; Meisinger *et al.*, 2005).

Plasma OxLDL concentrations are increased in dialysis patients (Maggi *et al.*, 1994; Van Tits *et al.*, 2003) and the concentrations gradually increase as GFR declines (Holvoet *et al.*, 1996). The mechanisms responsible for increasing OxLDL levels in CKD have not been well-characterised. However, vitamin E may be an important agent in the protection against free-radical induced oxidative damage of LDL and biological membranes (Cristol *et al.*, 1997; Galli *et al.*, 1998).

Figure 1.3 Mechanisms of LDL oxidation.



Key: NO: nitric oxide; LDL: low density lipoprotein; OxLDL: oxidised low density lipoprotein; PUFA: polyunsaturated fatty acid.

1.6 The metabolic syndrome

First described in 1988 by Reaven (Reaven, 1988) as *Syndrome X*, it is now recognised that the metabolic syndrome is a clustering of metabolic abnormalities and risk factors for cardiovascular disease including abdominal obesity, hyperglycaemia, hypertension, hypertriglyceridaemia, and reduced HDL cholesterol (Eckel *et al.*, 2005; Grundy *et al.*, 2004; Reaven, 1988). Several clinical criteria for diagnosis of the metabolic syndrome have been proposed by different groups including the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults – the Adult Treatment Panel III (ATP III), the World Health Organisation (WHO), and the American Association of Clinical Endocrinologists (AACE) (Grundy *et al.*, 2004). These criteria share the same pathological basis of metabolic syndrome: obesity, dyslipidaemia, and insulin resistance (Table 1.4).

In a population-based study, the metabolic syndrome, diagnosed using both NCEP ATP III and WHO criteria, is strongly associated with markers of atherosclerosis measured by carotid intima media thickness (IMT) and the presence of atherosclerotic plaques in carotid and femoral arteries (Ahluwalia *et al.*, 2006). In the West of Scotland Coronary Prevention (WOSCOPS) study in ~6,000 moderately hypercholesterolaemic men with no history of cardiovascular disease or diabetes, metabolic syndrome increases the risk of cardiovascular disease (hazard ratio 1.8, 95% confidence interval 1.4 – 1.3) and diabetes (hazard ratio 3.5, 95% confidence interval 2.5 – 4.9) (Sattar *et al.*, 2003). The increase in numbers of risk factors for the metabolic syndrome also independently predicts cardiovascular events (Sattar *et al.*, 2003). Since the metabolic syndrome is highly associated with increased risks of diabetes and cardiovascular disease, its treatment and prevention have become one of the major public health challenges worldwide (Eckel *et al.*, 2005).

Table 1.4 Summary of criteria for clinical diagnosis of the metabolic syndrome.

Criteria	Risk factors	Defining level
NCEP - ATP III	Blood pressure	≥130 / ≥ 85 mmHg
(Diagnosis made	Fasting glucose	≥ 6.0 mmol/L
when 3 out of 5	HDL cholesterol	
of the risk	Male	<1.0 mmol/L
factors are	Female	<1.3 mmol/L
present)	Triglycerides	≥1.7 mmol/L
	Waist circumference	
	Male	>102 cm
	Female	>88 cm
WHO	Insulin resistance, identified by one of the following:	
	Type 2 diabetes	
	Impaired fasting glucose	
	Impaired glucose tolerance	
	Plus any two of the following:	
	Antihypertensive medication and/or high blood pressure	≥140 / ≥ 90 mmHg
	Triglycerides	≥1.7 mmol/l
	HDL cholesterol	
	Men	<0.9 mmol/l
	Women	<1.0 mmol/l
	BMI and/or	>30 kg/m^2
	Waist/hip ratio	
	Men	>0.9
	Women	>0.85
	Urinary albumin excretion rate or albumin creatinine ratio	≥20 μg/min (>30mg/day) or ≥30 mg/g

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Criteria	Risk factors	Defining level
AACE	Overweight / obesity as BMI	≥25 kg/m^2
(Diagnosis	Triglycerides	≥1.69 mmol/L
depends on	HDL cholesterol	
clinical	Men	<1.04 mmol/L
judgement	Women	<1.29 mmol/L
based on risk	Blood pressure	≥130 / ≥ 85 mmHg
factors)	2-hour post-glucose challenge	>140 mg/dl
	Fasting glucose	110 – 126 mg/dl
	Other risk factors	
	Family history of type 2 diabetes	
	Polycystic ovary syndrome	
	Sedentary lifestyle	
	Ageing	
	Ethnic groups having high risk for type 2 diabetes or cardiovascular disease	

Key: Adapted from (Grundy *et al.*, 2004). AACE: the American Association of Clinical Endocrinologists; BMI: body mass index; HDL: high density lipoprotein; NCEP-ATP III: the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults – Adult Treatment Panel III; WHO: World Health Organisation.

Evidence has shown that the metabolic syndrome is prevalent in dialysis patients with the prevalence ranges from 30% to 70% (Young *et al.*, 2007; Zhe *et al.*, 2008). Recently, the metabolic syndrome, by itself, has been reported as a risk factor for CKD (Chen *et al.*, 2004; Hoehner *et al.*, 2002). The risk of developing CKD and microalbuminuria both increase progressively as the number of risk factors for the metabolic syndrome increases. Subjects with 2, 3, 4, and 5 risk factors for the metabolic syndrome had increased odds of 2.2, 3.4, 4.2, and 5.9, respectively, for CKD, compared to that of those with 0 or 1 risk factor. Subjects diagnosed with the metabolic syndrome had 2.6-fold increased odds of CKD compared to those without the metabolic syndrome. Likewise, subjects with 2, 3, 4, and 5 risk factors for the metabolic syndrome had increased odds of 1.2, 1.6, 2.5, and 3.2, respectively, for microalbuminuria, compared to that of those with 0 or 1 risk factor. Subjects diagnosed with the metabolic syndrome had 1.9-fold increased odds of microalbuminuria compared to those without the metabolic syndrome (Chen *et al.*, 2004).

1.7 The endothelium

The endothelium is a single layer of cells that lines the entire vascular system. Once thought to be inert, it is now recognised as an active organ with several important functions. The major functions include being a barrier between blood and underlying tissues, acting as a selective transport for essential molecules, regulating vascular tone, and regulating haemostasis and coagulation.

Maintaining vascular tone is a crucial role of the vascular endothelium. The vascular endothelium regulates vascular tone by producing and releasing vasoactive substances, for example vasodilators, including nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarising factor; and vasoconstrictors, including ET-1 and metabolites of arachidonic acid (Table 1.5). These vasoactive substances act either on endothelial cells in an autocrine function or in a paracrine manner on other vascular cells in order to control vascular tone (Cooke, 2000). Normally, functions of the endothelium are generally balanced on the side of promoting vasodilatation and inhibiting cellular proliferation. However, given that changes in environment are induced by diseases for example hypertension or hypercholesterolaemia, the balance can shift to vasoconstriction, thrombosis, and cell proliferation. These alterations of endothelial function, also known as endothelial dysfunction, are recognised to have an important role in the pathogenesis of vascular disease (Glasser *et al.*, 1996).

Table 1.5 A partial list of vasoactive substances produced by the endothelium.

Substances	Vascular effects
Endothelium-derived hyperpolarizing factor	Vasodilator
Nitric oxide	Vasodilator; inhibits vascular smooth muscle growth; anti-platelet; inhibits leucocyte adhesion
Prostacyclin	Vasodilator; inhibits vascular smooth muscle growth; anti-platelet; inhibits leucocyte adhesion
Angiotensin II	Vasoconstrictor; induces vascular smooth muscle growth; enhances leucocyte adhesion
Endothelin-1	Vasoconstrictor; induces vascular smooth muscle growth
Thromboxane A_2	Vasoconstrictor; activate platelet aggregation
Tissue plasminogen activator	Thrombolytic
Plasminogen activator inhibitor -1	Inhibits thrombolysis
Von Willebrand factor	Coagulation factor
Thrombomodulin	Inhibits coagulation

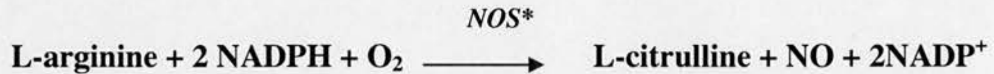
1.7.1 The endothelial nitric oxide pathway

NO is a potent vasodilator discovered as endothelium-derived relaxing factors (EDRF) by Furchgott and Zawadzki in 1980. Initially, it was demonstrated that the relaxation of rabbit thoracic aorta and other blood vessels by acetylcholine (ACh) required the presence of endothelial cells and this suggested that ACh acted on endothelial cell muscarinic receptors to stimulate the production of EDRF leading to the relaxation of vascular smooth muscle cells (Furchgott *et al.*, 1980). EDRF was later shown to be NO (Palmer *et al.*, 1987). To date, endothelium-derived NO is the most potent endogenous vasodilator in the body (Cooke, 2000). NO is

biosynthesised from the substrates amino acid L-arginine (Palmer *et al.*, 1988) which is in blood, extracellular fluid, and also within cells (Figure 1.4).

Figure 1.4 Biosynthesis of NO from L-arginine.

Overall reaction

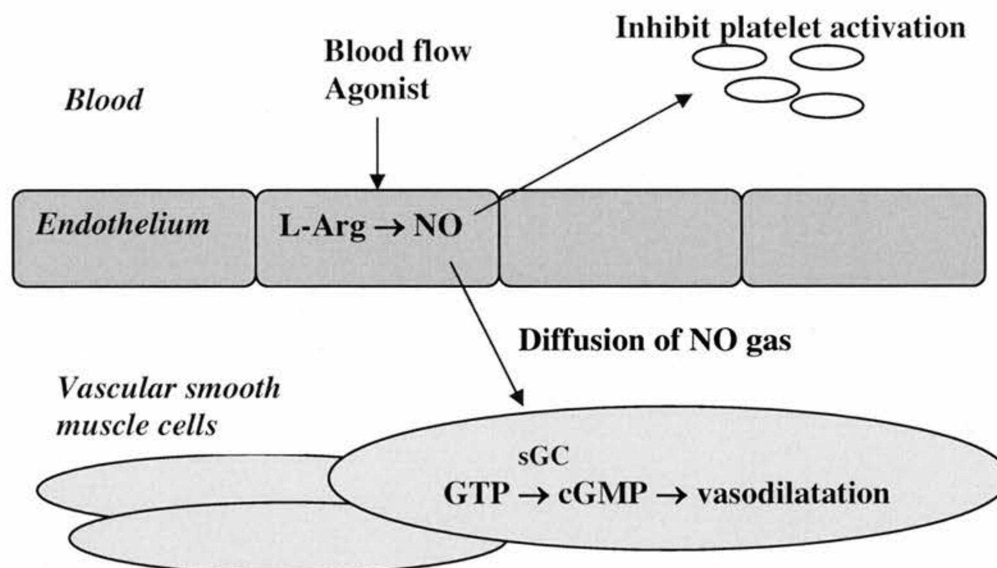


Key: *Cofactors for NOS are Calmodulin/Ca^{2+}, tetrahydrobiopterin, heam, flavin mononucleotide, and flavin adenine dinucleotide. NADPH: nicotine adenine dinucleotide phosphate; NO: nitric oxide; NOS: nitric oxide synthase.

Nitric oxide synthase (NOS) is the enzyme catalysing the synthesis of NO. There are 3 isoforms of NOS: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (Papapetropoulos *et al.*, 1999). NOS I and NOS III are constitutively expressed in the endothelium, platelets, and some neurones, and are calcium/calmodulin dependent whereas NOS II produces a large amount of NO (20 times or more higher than the constitutive production) after induction by various external stimuli for example bacterial lipopolysaccharide, independent of calcium (Nathan *et al.*, 1994). NOS III is located in the plasma membrane at the region called caveolae, structures associated with the accumulation of receptors for agents that regulate endothelial cell activity (Shaul, 2002). The most important physiological stimulus of NOS III is pulsatile blood flow. Normally, small amount of NO are produced constantly by the endothelium in large- and medium-size blood vessels to maintain blood flow by dilating the arteries. Some agonists such as ACh and bradykinin can also activate NOS III.

Endothelial NO diffuses across the cell membrane to the cytosol of vascular smooth muscle cells and into the lumen of the blood vessels reaching platelets. NO then reacts with its target protein, the enzyme soluble guanylyl cyclase, leading to the formation of cyclic guanosine monophosphate from the nucleotide guanosine triophosphate, resulting in vasodilatation (Figure 1.5).

Figure 1.5 NO in the regulation of blood flow and platelet activation.



Key: cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; L-Arg: L-arginine; NO: nitric oxide; sGC: soluble guanylyl cyclase.

There are many factors leading to an inhibition of biological activity of NO including decreased L-arginine uptake; reduction of co-factors such as Ca^{2+}, calmodulin, BH_4; inhibition of electron flow by reduced NADPH and flavins; inhibition of NOS expression; inhibition of substrate binding to NOS such as the endogenous inhibitor of NOS, and increased NO scavengers.

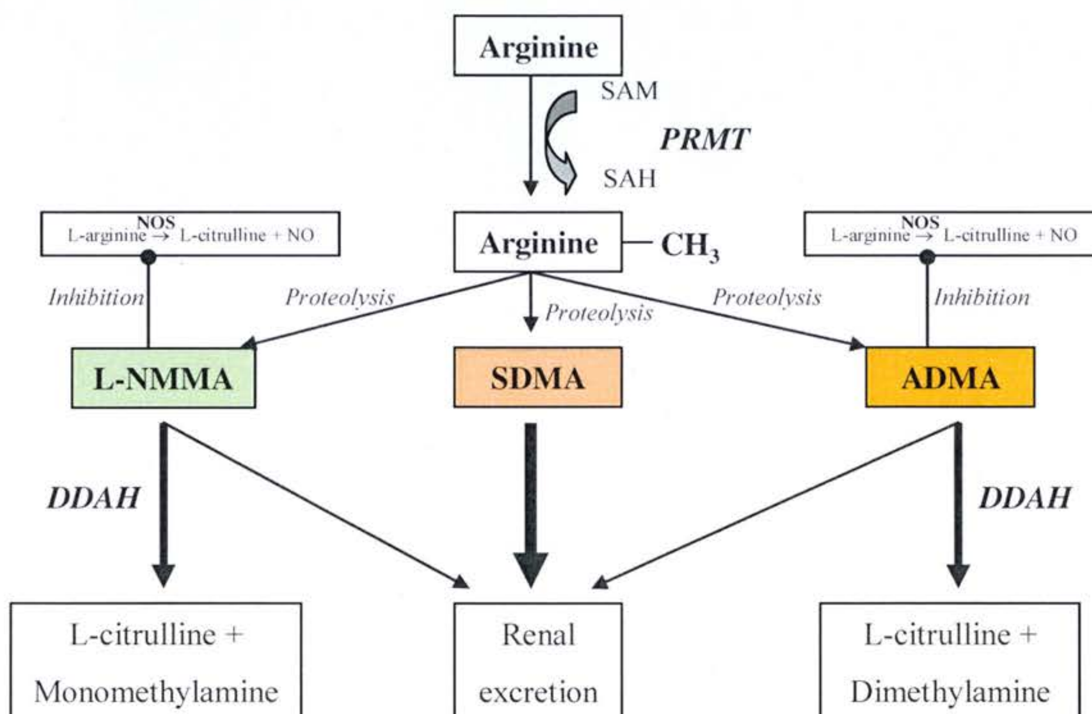
1.7.2 Asymmetric dimethylarginine

ADMA is an endogenous competitive inhibitor of NOS (Vallance *et al.*, 2004). In the cardiovascular system, ADMA is produced in the heart, endothelium, and smooth muscle cells. It is synthesised via methylation of arginine residues within the cell by the action of protein-arginine methyltransferases (PRMT). S-adenosylmethionine is the methyl donor in this reaction, and S-adenosylhomocysteine is produced. Two types of PRMT were identified. PRMT type I and II both form N^G-monomethyl L-arginine (L-NMMA). ADMA is only produced by PRMT type I and symmetric dimethylarginine (SDMA) is produced by PRMT type II. L-NMMA and ADMA are equipotent, competitive inhibitors of all 3 isoforms of NOS, whereas SDMA has no inhibitory activity on NOS. It has been

estimated that, per day, ~300 μmol of ADMA is generated in the human body (Achan *et al.*, 2003).

L-NMMA and ADMA are metabolised by dimethylarginine dimethylaminohydrolase (DDAH) enzyme to citrulline and monomethylamine or dimethylamine, respectively. DDAH is classified into 2 types, DDAH I and II, which differ in tissue expression. DDAH I is highly expressed in the brain and kidney whereas DDAH II is found in the heart, placenta, and kidney (Leiper *et al.*, 1999). SDMA is excreted via the kidneys while ADMA is eliminated by a combination of a renal excretion and a metabolism by DDAH (Boger *et al.*, 2003; Vallance *et al.*, 2004). Brief metabolic pathways for ADMA and SDMA is summarised in Figure 1.6.

Figure 1.6 Generation and metabolism of methylarginines.



Key: ADMA: asymmetric dimethylarginine; DDAH: dimethylarginine dimethylaminohydrolase; L-NMMA: N^G-monomethyl L-arginine; NO: nitric oxide; NOS: nitric oxide synthase; PRMT: protein arginine methyltransferases; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SDMA: symmetric dimethylarginine.

ADMA leads to impaired endothelial function by increasing endothelial cell adhesion and enhancing atherogenesis. Several clinical studies in non-renal patients including patients with coronary artery disease (Lu *et al.*, 2003; Valkonen *et al.*, 2001) and those with peripheral vascular disease (Mittermayer *et al.*, 2006) have demonstrated a strong correlation between increased plasma ADMA concentrations and cardiovascular morbidity and mortality.

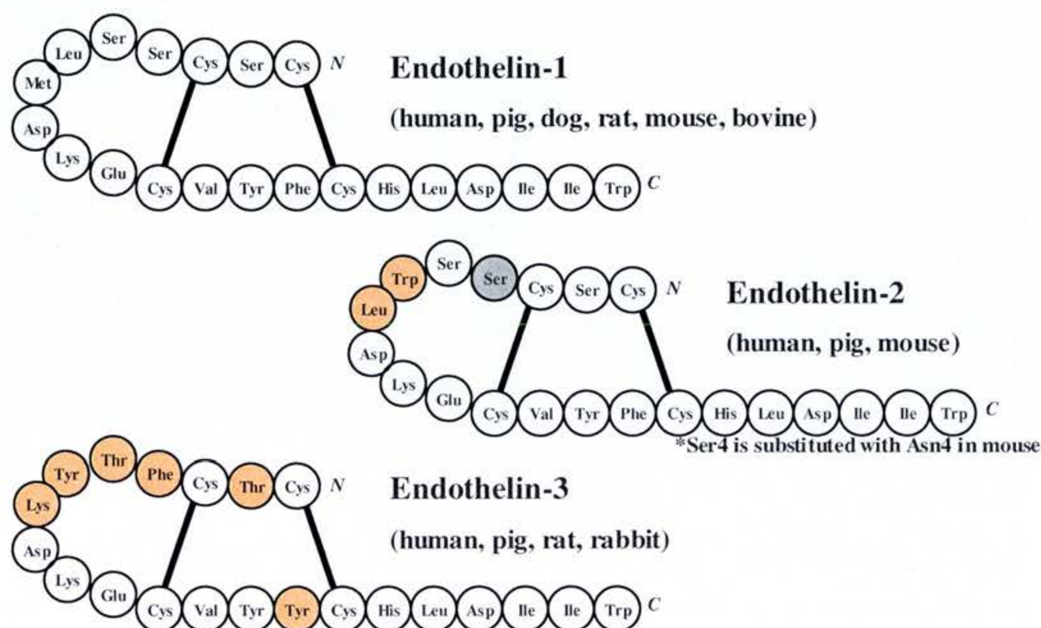
Plasma ADMA and SDMA concentrations are increased in dialysis patients compared to healthy controls, ranging from 1.4- to 10.3-fold and 5.0- to 11.7-fold, respectively [Vallance *et al.*, 1992; MacAllister *et al.*, 1996; Anderstam *et al.*, 1997; Kielstein *et al.*, 1999; Schmidt *et al.*, 1999; Cross *et al.*, 2001; Fleck *et al.*, 2001; Wahbi *et al.*, 2001; Osanai *et al.*, 2002; Raj *et al.*, 2002; Bergamini *et al.*, 2004; Martens-Lobenhoffer *et al.*, 2004; Mochizuki *et al.*, 2005; Morimoto *et al.*, 2005; Siroka *et al.*, 2005; Yano *et al.*, 2005; Aslam *et al.*, 2006; Yilmaz *et al.*, 2006]. The increase in plasma SDMA concentrations is substantially higher than plasma ADMA reflecting renal clearance as the main elimination process for SDMA. Most studies have shown that plasma ADMA levels appear to be higher in haemodialysis patients compared to patients on peritoneal dialysis. Plasma ADMA also independently predicts all-cause mortality in dialysis patients with an increase in concentration of plasma ADMA by 1 μmol/L leading to a 26% increase in risk of death (Zoccali *et al.*, 2001). In CKD patients who are not in ESRD, plasma ADMA and SDMA levels are also significantly elevated, ranging from 1.1- to 3.4-fold and 1.4- to 8.2-fold, respectively, compared to controls (Busch *et al.*, 2006; Caglar *et al.*, 2006; Fleck *et al.*, 2001; Kielstein *et al.*, 2002; MacAllister *et al.*, 1996; Marescau *et al.*, 1997; Nanayakkara *et al.*, 2005; Ravani *et al.*, 2005; Saran *et al.*, 2003; Schmidt *et al.*, 2000; Tarnow *et al.*, 2004; Wahbi *et al.*, 2001; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2007). Importantly, plasma ADMA concentrations has been shown to correlate with GFR ($r = -0.59$, $p < 0.01$), and independently predict the progression of renal failure (odds ratio 1.5, 95% confidence interval 1.1 – 1.9, $p < 0.006$) (Fliser *et al.*, 2005).

1.7.3 The endothelial endothelin-1 pathway

The endothelins (ETs) are a family of potent vasoconstrictive peptides consisting of 21 amino acid residues. Initially, ET was isolated and identified from the medium of

cultured porcine endothelial cells in 1988 (Yanagisawa *et al.*, 1988). After the discover of ET, three endogenous isoforms of ET were identified as ET-1, ET-2, and ET-3. Each isoform contains two intra-chain disulphide bridges linking paired cysteine amino acid residues, producing a semi-conical structure. ET-2 differs from ET-1 by two amino acids residues while ET-3 differs by 6 amino acids (Figure 1.7). ETs possess molecular structural homology with sarafotoxin peptides extracted from the venom of *Atractaspis engaddensis* (Kloog *et al.*, 1988).

Figure 1.7 Structure of the three isoforms of ET.



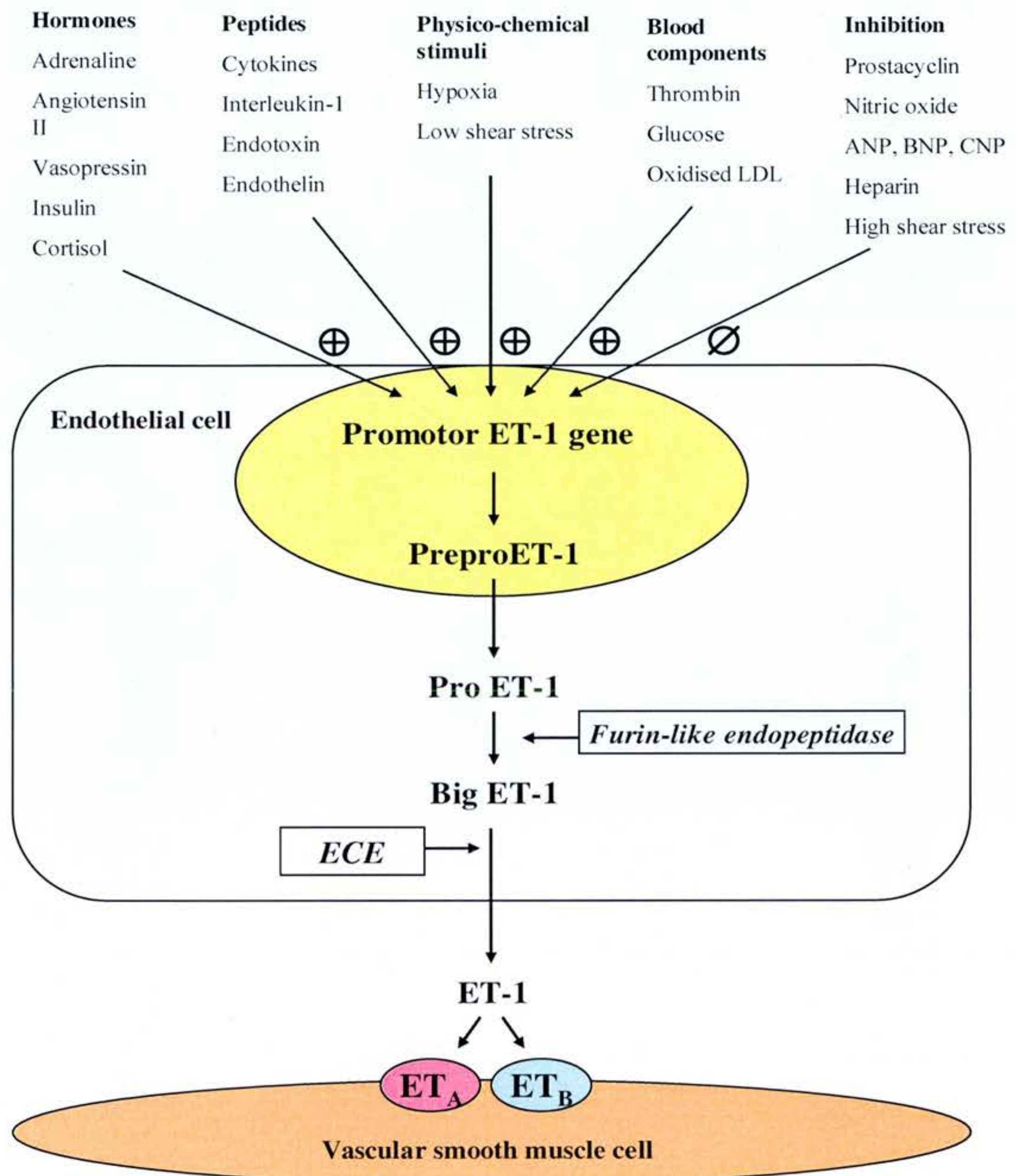
ET-1 is the principal cardiovascular isoform of ET produced by the endothelial cells (Inoue *et al.*, 1989). Synthesis of ET-1 is enhanced in response to low shear stress, turbulent blood flow, hypoxia, cytokines, angiotensin II, adrenaline, and LDL. In contrast, high shear stress, NO, and vasodilating agents, including prostaglandins and natriuretic peptides, suppress ET-1 production (Gray *et al.*, 1996; Motte *et al.*, 2006).

ET-1 is generated within the endothelial cells via a two-step proteolytic pathway (Gray *et al.*, 1996) (Figure 1.8). After the transcription of a gene on chromosome 6, a large precursor peptide of 212 amino acid residues called preproET-1 is formed. PreproET-1 is then translated and secreted into the cytoplasm as proET-1. ProET-1

is further processed by an enzyme, a furin-like endopeptidase, to the 38-amino-acid precursor molecule named big ET-1. Big ET-1 is a biologically inactive intermediate peptide and is further converted into an active ET-1 (21-amino-acid ET-1) by an endothelin-converting enzyme (ECE) in the cytoplasm of the endothelial cells. ECE is a family of metalloprotease enzymes with two main isozymes, ECE-1 and ECE-2, of which ECE-1 is the physiologically active ECE in humans.

The endothelium releases ET-1 through both constitutive and regulated (rapid release) pathways. The constitutive pathway is regulated mainly at the level of gene transcription. The rapid release pathway is that ET-1 released from vesicles originating in the Weibel-Palade bodies and the Golgi network (Barnes *et al.*, 1998). ET-1 abluminally released from endothelial cells acts primarily as a local autocrine and paracrine substance rather than as an endocrine hormone. In health, low levels of ET-1 (1-10 pmol/L) are detected in plasma, suggesting that circulating levels of ET-1 represent an overflow of tissue-bound ET-1 and do not truly reflect the overall ET-1 activity (Haynes *et al.*, 1998).

Figure 1.8 Generation of ET-1 in the vascular endothelial cell.



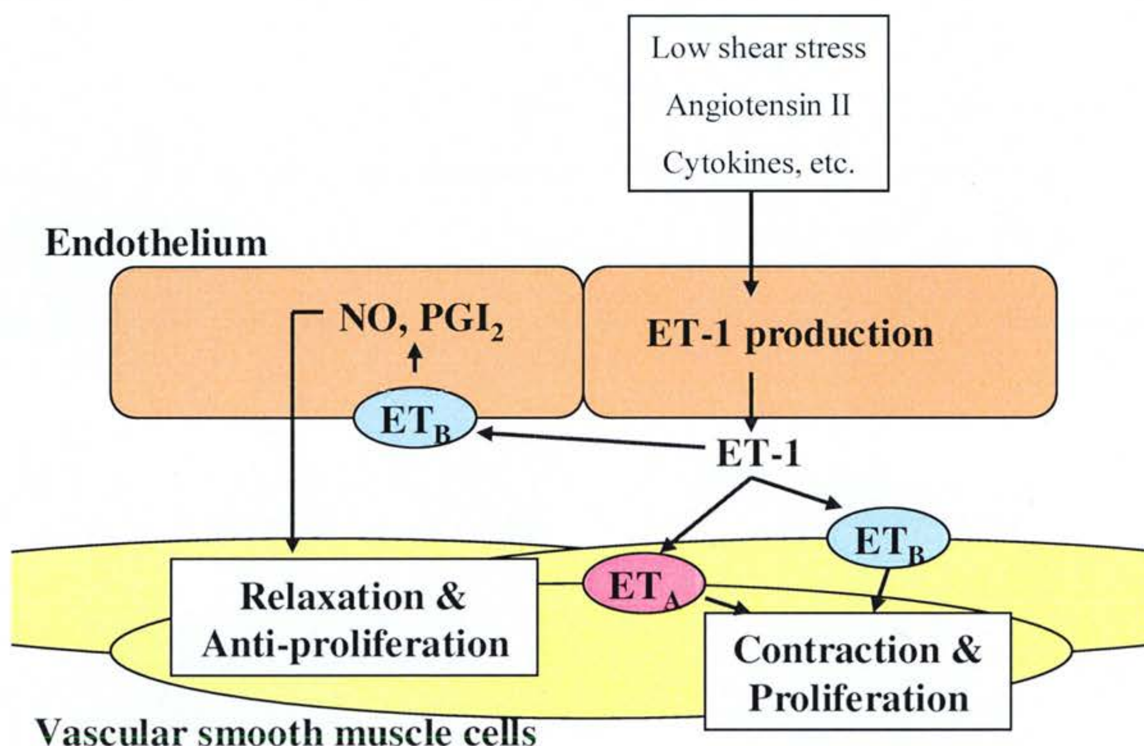
Key: ECE: endothelin-converting enzyme.

In humans, ET-1 exerts its action via two types of ET receptors, ET_A and ET_B receptors. ET receptors are members of the heptahelical G-protein-coupled receptor superfamily. ET-1 binds ET_A and ET_B receptors with equal affinity. By contrast, ET-

3 has at least 100-fold less affinity for ET_A receptors than ET_B receptors (Davenport, 2002). In the vasculature, vascular smooth muscle cells express both ET_A and ET_B receptors while the endothelial cells express mainly only ET_B receptors (Molenaar *et al.*, 1993). Direct actions of ET-1 on ET_A and ET_B receptors on the vascular smooth muscle cell are contraction, proliferation, and migration of vascular cells. On the other hand, the effects of ET-1 via the ET_B receptors on the endothelium are a release of vasodilating compounds such as NO, leading to vascular relaxation and anti-proliferation (Figure 1.9) (Kirkby *et al.*, 2008). Binding of ET-1 to ET_A receptors stimulates phospholipase C to further hydrolyse phosphotidyl inositol 4, 5 biphosphate into inosital triphosphate and diacylglycerol, leading to increased intracellular Ca^{2+} and vasoconstriction. The first phase of response is rapid (2-5 seconds), followed by the second phase of sustained increase intracellular Ca^{2+} which may last up to 20 minutes. The effects of ET-1 binding to ET_B receptors are similar to those effects of stimulating ET_A receptors via activation of phospholiphase C. However, ET_B receptor binding also activates phospholipase A_2 to release vasodilating substances such as prostaglandin (Kirkby *et al.*, 2008; Simonson *et al.*, 1992). Clinical studies have demonstrated the role of ET-1 in the control of vascular tone in healthy subjects (Haynes *et al.*, 1994), hypertension (Cardillo *et al.*, 1999; Taddei *et al.*, 1999), and CKD (Hand *et al.*, 1999).

To date, ET receptors have been identified in many major organs including the lungs, heart, and kidneys. In the kidneys, both ET_A and ET_B receptors are widely distributed with ET_B to ET_A ratio = 2:1. ET_A receptors are located in the glomeruli, vasa recta and arcuate arteries, while ET_B receptors are more widespread and highly found in the collecting systems. In animal, exogenous ET-1 causes vasoconstriction of afferent and efferent arterioles, with a greater effect on the former. In human, ET-1 regulates renal blood flow and glomerular haemodynamics leading to a fall in total renal blood flow and a consequence reduction of GFR. ET-1 also regulates sodium and water homeostasis and acid-base balance (Dhaun *et al.*, 2006; Karet *et al.*, 1996).

Figure 1.9 Actions of ET-1 on vascular smooth muscle cells.



Key: ET-1: endothelin-1; ET_A: endothelin receptor type A; ET_B: endothelin receptor type B; NO: nitric oxide; PGI_2: prostacyclin.

Clearance of ET-1 from plasma is very rapid. It occurs largely in the lungs via ET_B receptors by endocytosis and degradation of the ET_B receptor-ligand complex (Dupuis *et al.*, 1996). The pulmonary circulation extracts ~50% of the circulating ET-1 per a single pass of blood through the lungs (Dupuis *et al.*, 1996). The speed of this clearance and the polarised abluminal nature of ET-1 secretion by the endothelial cells highlight the poor predictive value of plasma ET-1 concentration as a measure of ET-1 systhesis and the difficulty of assessing altered ET-1 activity in disease. The kidney also plays a role in ET-1 clearance. It was shown that 10% of circulating ET-1 is removed from the human circulation via the kidneys (Gasic *et al.*, 1992) either by glomerular filtration or enzymatic degradation. Neutral endopeptidase, the enzyme located in the brush border vesicle of the proximal tubule catabolised ET-1 and inhibition of this enzyme leads to an increase in plasma and urinary ET-1 concentrations (Abassi *et al.*, 1992). Renal ET-1 clearance was also indicated by a study in rats where rats with bilateral nephrectomy had impaired exogenous ET-1 removal (Shi *et al.*, 1994).

Plasma ET-1 concentrations are elevated in patients with pulmonary arterial hypertension (Stewart *et al.*, 1991), diabetes (Ferri *et al.*, 1997; Schneider *et al.*, 2002; Takahashi *et al.*, 1990), hypertension (Ferri *et al.*, 1997; Goddard *et al.*, 2000; Shichiri *et al.*, 1990), acute myocardial infarction (Miyauchi *et al.*, 1991), chronic heart failure (Lerman *et al.*, 1992; McMurray *et al.*, 1992), and metabolic syndrome (Ferri *et al.*, 1997). Plasma ET-1 concentrations are also increased in dialysis patients (Blazy *et al.*, 1994; Dammers *et al.*, 2005; Demuth *et al.*, 1998; Deray *et al.*, 1992; Koyama *et al.*, 1989; Mallamaci *et al.*, 1993; Miyauchi *et al.*, 1991; Saito *et al.*, 1991; Totsune *et al.*, 1989; Vlassopoulos *et al.*, 1995). Circulating ET-1 is predictive of cardiovascular remodelling presented by increased left ventricular mass and increased common carotid artery IMT in ESRD (Demuth *et al.*, 1998). In non-dialysis patients, plasma ET-1 concentrations are also increased (Blazy *et al.*, 1994; Deray *et al.*, 1992; Koyama *et al.*, 1989; Mallamaci *et al.*, 1993; Saito *et al.*, 1991; Vlachojannis *et al.*, 1997). Additionally, plasma ET-1 correlates with renal function (Blazy *et al.*, 1994; Dammers *et al.*, 2005; Goddard *et al.*, 2007; Mallamaci *et al.*, 1993). A study has shown a progressive increase in plasma ET-1 in hypertensive patients with GFR ranging from CKD stage 1 to pre-dialysis and also showed that ET-1 independently predicts GFR, and is superior to inflammation (CRP) or oxidative stress (Isop) in this respect (Cottone *et al.*, 2009).

One mechanism proposed to be responsible for increased circulating ET-1 concentrations in CKD includes reduced ET-1 renal clearance as a consequence of reduced GFR. However, this may not be true as evidence suggests that renal and vascular ET-1 are two independent systems (Serneri *et al.*, 1995). A study in rats has shown that after systemic infusion of radiolabeled ET-1, less than 1% of radiolabelled ET-1 is recovered in the urine (Benigni *et al.*, 1991). This suggests that neither glomerular filtration nor tubular secretion of plasma ET-1 account for urinary ET-1, which is therefore assumed to be primarily of renal origin and, thus, urinary ET-1 excretion reflects renal ET-1 production. Physiological studies in dogs and humans have shown no difference between renal arterial and venous ET suggesting that there is no ET clearance across the kidneys (Deray *et al.*, 1992). This is also supported by a study in healthy subjects which demonstrated that plasma ET-1 and urinary ET-1 did not relate to each other, but both correlated inversely with GFR (Goddard *et al.*, 2007).

1.7.4 Measurements of endothelial function

A major characteristic of endothelial dysfunction is a reduction of NO produced or released by the endothelium (Cooke, 2000). Several methodologies have been established to indirectly quantify the amount of NO production by assessing the degree of vasodilatation that occurs when endothelial NO production is stimulated.

1.7.4.1 Invasive measurements of endothelial function

The gold standard clinical measure of endothelium-dependent vasomotor function is a local intra-arterial administration, either into the forearm or coronary circulations, using a substance that stimulates endothelial NO production such as ACh (Ludmer *et al.*, 1986; Newby *et al.*, 2001). The vasodilatation can be measured as the change of blood flow to the forearm, using venous occlusive plethysmography (Wilkinson *et al.*, 2001), or assessed by quantitative angiography or Doppler ultrasound in the coronary circulations (Ludmer *et al.*, 1986; Newby *et al.*, 2001). An impaired endothelium-dependent vasomotor function may be a result of either impaired production and release of NO, increased degradation of NO, or impaired sensitivity of the vascular smooth muscle cell to NO. Hence the endothelial-independent vasomotor function by a NO-donor substance such as glyceryl trinitrate (GTN) or sodium nitroprusside, is usually measured as a control (Wilkinson *et al.*, 2001). These methodologies have major limitations. They are invasive, expensive, time consuming, carry with them the risks of vascular injury and have limited applicability for large-scale studies.

1.7.4.2 Non-invasive measurements of endothelial function

Flow-mediated dilatation (FMD) is a widely used non-invasive measure of endothelial-dependent vasomotor function of a conduit artery such as the brachial artery (Corretti *et al.*, 2002), using the shear stress from the flow of blood as a physiological stimulus for NO generated vasodilatation. Impaired FMD has been demonstrated in subjects with atherosclerosis and those with cardiovascular risk factors (Brunner *et al.*, 2005; Celermajer *et al.*, 1992). In most studies, the endothelium-independent GTN responses are reported to be preserved. This

technique is safer and faster than invasive methods and can be applied to large groups of patients. It can also be used to make repeated measurements over time. Studies suggest that endothelial function assessed non-invasively in the brachial artery correlates well with endothelial function in the coronary arteries (Anderson *et al.*, 1995). However, FMD is operator-dependent and results are markedly dependent on the resolution of the ultrasound image.

Another non-invasive technique used to measure endothelial function is the assessment of arterial pressure waveforms or pulse wave analysis (PWA) after β_2 adrenoreceptor agonist administration. PWA is one of several non-invasive methods commonly used to measure arterial stiffness (Oliver *et al.*, 2003b). PWA has been adapted to measure endothelial function, by giving patients a β_2-adrenoreceptor agonist, such as salbutamol, which is a NO-dependent vasodilating agent in the peripheral circulation. When given systemically salbutamol reduces peripheral artery wave reflection and this response is NO-dependent. This methodology was first described in 1999 by Chowienczyk *et al.*, where salbutamol was shown to reduce the height of the inflection point of the digital volume pulse (Chowienczyk *et al.*, 1999). Subsequently, salbutamol was also shown to reduce the most relevant parameter obtained from PWA known as augmentation index (AIx) (both central (aortic) AIx (Wilkinson *et al.*, 2002a) and peripheral (radial) AIx (Hayward *et al.*, 2002)). As with FMD, the change of the arterial waveforms that occurs with salbutamol measured by PWA is compared to that following sublingual GTN, as a measure of endothelium-independent vasodilatation (Wilkinson *et al.*, 2002a). A number of studies confirm the potential of evaluating endothelial function with this technique. The reduction of AIx after salbutamol is impaired in patients with diabetes mellitus (Chowienczyk *et al.*, 1999), hypercholesterolaemia (Wilkinson *et al.*, 2002a), and coronary artery disease (Hayward *et al.*, 2002). This methodology is a simple, non-invasive technique that could be applied to large population studies and potentially be used in clinical practice.

1.8 Arterial stiffness

Arterial stiffening is a dynamic process involving functional and structural, including cellular elements of the vessel walls. It is caused by fracture and fragmentation of the elastic lamellae within the media of the vessel walls, principally in central elastic arteries such as the aorta (O'Rourke, 1999; Zieman *et al.*, 2005). It is an ageing phenomenon which is accelerated by hypertension and arterial disease.

Arterial stiffening is of interest since it has been shown that it is pathologically relevant to cardiovascular disease. Arterial stiffness is associated with a number of conventional cardiovascular risk factors including age, gender, smoking, hypertension, hypercholesterolaemia, and diabetes mellitus (Cockcroft *et al.*, 1997; Nichols, 2005; Wilkinson *et al.*, 2000; Wilkinson *et al.*, 2002b; Yasmin *et al.*, 2004). It also correlates with the severity of cardiovascular disease and is an independent predictor for cardiovascular disease in hypertension and ESRD (Blacher *et al.*, 1999a; Boutouyrie, 1999; London, 2001; Safar, 2002; Sutton-Tyrrell *et al.*, 2005). A study has shown that survival rate is higher in patients whose blood pressure (BP) and arterial stiffness were well controlled, compared to those with BP control only (Guerin, 2001).

1.8.1 Arteries and arterial function

The arterial wall has three layers. The tunica externa is the outermost, composed of connective tissue. The tunica media is a middle layer containing smooth muscle. The tunica interna is the innermost which includes three parts: the endothelium that lines the lumen of blood vessels; the layer of glycoprotein called the basement membrane; and the layer of elastic fibres known as elastin. Arteries can be divided into 2 major types: elastic and resistance arteries.

The elastic arteries, such as the aorta and the large central arteries are highly distensible because they contain numerous layers of elastin fibres between smooth muscle cells in the tunica media. The large elastic arteries have two main functions: to act as conduits and as buffers. As conduit arteries, their role is to deliver blood to

tissue and organs with minimal loss of perfusion pressure. The buffer function aims to smooth flow pulsations from ventricular ejection to the target organs. In order to achieve the continuous needs of peripheral tissue oxygen demand these two functions must be balanced and performed effectively during both systole and diastole. Large arterial functions are altered by the degenerative process that occurs with ageing and diseases. However, it should be emphasised that the stiffening of major arteries, particularly in hypertension and ageing, mostly affects the buffer function (O'Rourke, 1995; Zieman *et al.*, 2005).

Yet, the resistance arteries, for example radial, brachial, and femoral arteries, which are sometimes referred to as peripheral arteries, are less elastic. They have a thick layer of smooth muscle with narrow lumina. Contraction or relaxation of this smooth muscle has a considerable effect on the lumen of the resistance arteries, altering blood flow to the relevant distal organs.

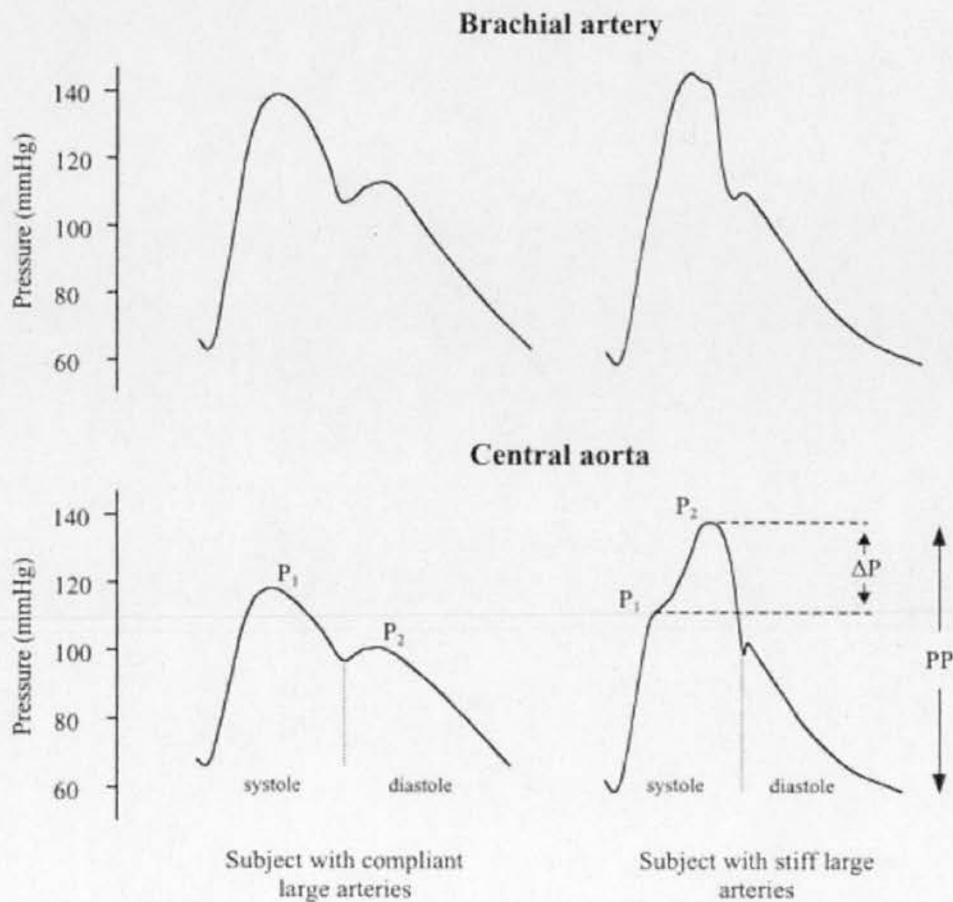
1.8.2 Arterial wave reflection

After being pumped from the heart, blood travels along the arteries to the peripheral tissues and pressure waves and flow waves are produced. The behaviour and contours of arterial pressure waves and flow waves vary in different arteries. These changes in the pressure waveforms are because of the "reflected" pressure waves (Nichols, 2005). Basically, by absorbing a proportion of the energy in systole and releasing it in diastole, peripheral blood flow is smoothed and diastolic coronary artery flow maintained. However, some pressure waves are reflected back from the periphery because of discontinuity in calibre or elastic properties along the arterial tree. Possible reflecting sites are branching points, areas of alteration in arterial distensibility and high-resistance arterioles. These reflected waves summate with the forward-going waves and produce the typical resultant waveforms (Nichols, 2005).

When the large arteries are compliant, the arterial waveform is amplified as it travels towards the periphery (Figure 1.10, waveforms on the left). Ageing, diabetes, or other cardiovascular diseases stiffen the arteries resulting in a reduction of the arterial waveform amplification (Figure 1.10, waveforms on the right) (Kelly *et al.*, 1989; McEniery *et al.*, 2005). These changes in the pressure waveforms caused by

arterial stiffening negatively affect the circulation. Normally, in young adults, the reflected wave arrives in the central artery after the closure of the aortic valve. Therefore, it does not have any effect on central systolic pressure but it improves coronary diastolic blood flow by augmenting diastolic pressure. In elderly individuals with poor arterial elasticity, the reflected wave arrives earlier, occurs in systole, augmenting systolic pressure and results in a decrease in coronary blood flow (Figure 1.10) (Nichols, 2005).

Figure 1.10 Typical pressure waveforms of the brachial artery and central aorta.



Key: Figure from (Oliver *et al.*, 2003b). Waveforms on the left: subjects with compliant large arteries. Waveforms on the right: subjects with stiff large arteries. When the large arteries are compliant, the initial systolic pressure wave (P_1) travelling from the heart to the periphery is responsible for the peak systolic blood pressure. Reflected pressure wave (P_2) arrives at the central aorta in the diastole, augmenting diastolic blood pressure and coronary filling. In stiff large arteries, wave reflection occurs earlier so that systolic blood pressure is augmented and diastolic blood pressure falls. PP: pulse pressure; $\Delta P = P_2 - P_1$; Augmentation index (%) = $[\Delta P/PP] \times 100$. More detail regarding augmentation index can be found in Chapter 2, section 2.5.1.

1.8.3 Measurements of arterial stiffness

The aorta is the major vessel of interest for determining arterial stiffness because it makes the largest contribution to the arterial buffering function (Nichols, 2005). Importantly, stiffness of the aorta, measured by aortic pulse wave velocity (PWV), is an independent predictor of outcomes in various populations (Blacher *et al.*, 1999a; Boutouyrie *et al.*, 2002; Cruickshank *et al.*, 2002; Laurent *et al.*, 2001). To date, a number of invasive and non-invasive techniques have been developed for the assessment of arterial stiffness *in vivo*. Invasive clinical measurements, such as 2-site intra-arterial catheterisation for measuring PWV, are suitable for experimental animal laboratories. These methods are not appropriate for clinical use. Non-invasive measurements of arterial stiffness are well-established and entail measurement of surrogate parameters which are associated with arterial stiffness. Many are reproducible, non-invasive and relatively easily accessible to anyone without special knowledge and without a detailed exploration of arterial mechanics. These methodologies are in three main groups. The first group is a measure of PWV. The second group is an assessment of arterial pressure waveforms or PWA with indices such as AIx. The third group is a relation of changes in the diameter or an area of an artery to distending pressure (Laurent *et al.*, 2006; Oliver *et al.*, 2003b).

1.8.3.1 Pulse wave velocity

PWV is the most simple, non-invasive, robust, and reproducible method for measuring arterial stiffness. PWV is defined as the speed in which the pulse wave travels along a measured arterial segment (Laurent *et al.*, 2006). PWV is calculated by dividing the distance between the two sites at which the pressure wave is being recorded, by the time taken from the first to the second site. The proximal arterial site is usually a common carotid artery and the distal sites are femoral artery or radial artery (carotid-femoral PWV (CF-PWV) or carotid-radial PWV (CR-PWV)). CF-PWV is a gold standard measure of arterial stiffness according to the current guideline (Laurent *et al.*, 2006). CF-PWV represents the measurement of arterial stiffness of the aorto-iliac pathway, which is the most clinically relevant for the assessment of arterial stiffness of the aorta since the aorta and its first branches

(femoral arteries) are what the left ventricle 'sees' and thus are responsible for the pathophysiological effects of arterial stiffness of the central artery.

Higher PWV reflects stiffer arteries and several conventional cardiovascular risk factors, for instance hypertension, hypercholesterolaemia, and diabetes mellitus, are associated with increased PWV (Nichols, 2005). However, this technique carries some limitations. The pressure waveforms at the femoral site may be difficult to record especially in obese patients. Additionally, the inaccuracy of the measurement of the distance between two recording sites may affect the calculation of PWV.

1.8.3.2 Pulse wave analysis

The parameter obtained from PWA is AIx which is defined as the ratio of the difference of the first and second systolic pressure to the pulse pressure (see Chapter 2, section 2.5.1 for detail) (Laurent *et al.*, 2006; Oliver *et al.*, 2003b). It reflects the degree to which central arterial pressure is augmented by wave reflections.

According to the current guideline, arterial pressure waveforms should be analysed at the central level. The central AIx (CAIx) is usually estimated from either the radial artery waveform (RAIx) using a transfer function or from the common carotid waveform. AIx increases with age. Diseases such as hypertension and diabetes mellitus are also associated with increased AIx (Nichols, 2005). AIx is also influenced by PWV and other factors such as body height and heart rate (O'Rourke *et al.*, 1996; Yasmin *et al.*, 1999). Importantly, comparison between AIx and aortic PWV has shown a significant association, but a relatively low positive correlation. This suggests that although AIx is a measurement of arterial stiffness, it is not simply a surrogate measure of PWV (Yasmin *et al.*, 1999). This technique is easy to perform and highly reproducible (Wilkinson *et al.*, 1998) but it has some limitations. The generalised transfer function used is derived from patients with coronary heart disease and therefore it may not be accurate for some diseases. Although, the transfer function is not necessary when recording the waveforms at the common carotid artery, this requires a higher degree of technical expertise.

1.8.3.3 Arterial compliance or arterial distensibility

Local stiffness of the arteries can be measured using an ultrasound or magnetic resonance imaging device. With these techniques, the change in pressure driving the change in volume of the artery is measured, providing a direct measurement of stiffness (Laurent *et al.*, 2006; Oliver *et al.*, 2003b). Of the superficial arteries, carotid stiffness is of particular interest since it is one of the most common sites for atherosclerosis. Using the magnetic resonance imaging technique yields the ability to measure local arterial stiffness at the aorta. However, this technique takes longer than measuring PWV and requires a high degree of clinical expertise, therefore it is not suitable for epidemiological studies (Laurent *et al.*, 2006). Of note, although carotid stiffness and CF-PWV provide similar information on the impact of ageing on large artery stiffness in normal subjects, this is not the case for patients with hypertension or diabetes. With diseases, the aorta stiffens more than the carotid artery and thus aortic stiffness and carotid stiffness cannot be used interchangeably in these patients (Laurent *et al.*, 2006).

1.8.4 Arterial calcification and arterial stiffness in CKD

Arterial calcification has been recognised for many years as a common complication in CKD (Salusky *et al.*, 2002). In fact, arterial disease in CKD patients is characterised by extensive calcification (London *et al.*, 2005). The precise mechanisms responsible for this are not well understood, but arterial calcification appears to be a similar process to bone formation. Abnormalities in mineral metabolism and disturbances in phosphate metabolism are believed to be particularly important determinants (Demer *et al.*, 2008; Goodman *et al.*, 2004). Arterial calcification occurs in two sites: the tunica intima and tunica media. Intimal calcification is a marker of atherosclerotic disease. Yet, medial calcification promotes arterial stiffening, leading to left ventricular hypertrophy and reduced coronary perfusion (Table 1.6).

Table 1.6 Types and characteristics of vascular calcification.

Types	Location and features	Associated conditions
Calcific atherosclerosis	Tunica intima	Atherosclerosis, hyperlipidaemia, osteoporosis, hypertension, inflammation
Calcific medial vasculopathy (Monckenberg's medial calcific sclerosis)	Tunica media	Type 2 diabetes mellitus, end-stage renal disease

Key: Table modified from (Demer *et al.*, 2008).

In ESRD, the presence of arterial calcifications is associated with increased arterial stiffness (Sigrist *et al.*, 2007). The extent of arterial calcifications increases with age and the duration of dialysis (Guerin *et al.*, 2000; Sigrist *et al.*, 2007). In addition, the progression of arterial calcification in a period of 12 months is an independent predictor of survival (hazard ratio 1.03, 95% confidence interval 1.01-1.05) (Sigrist *et al.*, 2007). Intimal calcification, which is associated with generalised atherosclerosis, is not specifically attributable to haemodialysis whereas medial calcification is much more closely associated (Guerin *et al.*, 2000). Nevertheless, both intimal and medial calcification are associated with PWV (Guerin *et al.*, 2000; Haydar *et al.*, 2004).

Abnormalities in calcium and phosphate metabolism have emerged as important determinants of arterial calcification. Recently, attention has been focused on the potential importance of serum fetuin-A, a potent calcification inhibitor. The absence of fetuin-A in fetuin-A knock-out mice leads to massive extra-osseous calcification (Schafer *et al.*, 2003). Low circulating fetuin-A levels are associated with increased all-cause and cardiovascular outcomes in ESRD (Ketteler *et al.*, 2003; Stenvinkel *et al.*, 2005b). It is unclear whether fetuin-A levels are inversely related to arterial stiffness markers (Hermans *et al.*, 2006).

1.9 Relationship between arterial stiffness and endothelial dysfunction

Arterial stiffness and endothelial dysfunction commonly co-exist in patients with cardiovascular disease. Conditions associated with endothelial dysfunction, such as diabetes mellitus (Calver *et al.*, 1992; Wilkinson *et al.*, 2000) and hypercholesterolaemia (Chowienczyk *et al.*, 1992; Wilkinson *et al.*, 2002b) are associated with increased arterial stiffness. Evidence supports a role for the endothelium in regulating arterial stiffness by the release of vasoactive mediators such as NO (Boutouyrie *et al.*, 1997; Levy *et al.*, 1990; Wilkinson *et al.*, 2002c). Several interventions that reduce arterial stiffness also improve endothelial function and *vice versa* (Oliver *et al.*, 2003a; Van Bortel *et al.*, 2001).

1.10 Arterial stiffness and endothelial dysfunction in chronic kidney disease

Increased arterial stiffness is a common feature of CKD (Guerin *et al.*, 2008). In ESRD, patients have reduced arterial compliance compared to the general population of the same age and BP level (Konings *et al.*, 2004). Indices of arterial stiffness are substantially higher than those recorded in non-renal subjects (Benetos *et al.*, 2002; Blacher *et al.*, 1999b; Konings *et al.*, 2002; Stancanelli *et al.*, 2007). Increases of PWV and AIx are strong independent predictors of all-cause and cardiovascular mortality in ESRD. Studies have shown that, for each increase of 1 m/s in PWV, the all-cause mortality-adjusted odd ratio was 1.4 (95% confidence interval 1.2-1.6). Likewise, for every 10% increase in AIx, the risk ratio was 1.5 (95% confidence interval 1.2-1.9) (Blacher *et al.*, 1999a; London, 2001). In addition, aortic PWV is an independent predictor of survival in ESRD (Guerin, 2001).

Shinohara, *et al.* compared aortic PWV of CKD patients before starting haemodialysis with that of patients on maintenance haemodialysis. The aortic PWV was significantly higher in pre-dialysis and haemodialysis groups (9.7 and 8.9 m/s, respectively, $p < 0.01$ for both), compared with 7.4 m/s in healthy controls. This

suggests that aortic stiffening occurs in CKD before starting dialysis (Shinohara *et al.*, 2004). Interestingly, a study also indicated that even a mild to moderate deterioration in renal function was associated with increased arterial stiffness. In normotensive and hypertensive patients, those with normal serum creatinine levels but who were in the lowest tertile of creatinine clearance (CrCl) had increased arterial stiffness, independent of BP and other cardiovascular risk factors. The negative association between PWV and CrCl was stronger in subjects who were younger than 55 years old (Mourad *et al.*, 2001). Furthermore, evidence suggests that arterial stiffness increases correspond with the stages of CKD. In a cross-sectional study of 102 CKD patients, Wang *et al* demonstrated a trend for a stepwise increase in PWV from CKD stages 1 to 5. However, they did not find a significant difference in the PWV between CKD stages 1 to 2 and the aged-matched control group (Wang *et al.*, 2005). A recent study has also confirmed the parallel decline in GFR and arterial stiffness measured by distensibility and PWV in mild-to-moderate CKD patients (Briet *et al.*, 2006).

Endothelial dysfunction is evidenced in ESRD (van Guldener *et al.*, 1998; van Guldener *et al.*, 1997). A prospective study in dialysis patients has demonstrated that endothelial dysfunction measured by an invasive forearm blood flow technique is an independent predictor of all-cause mortality (London *et al.*, 2004b). Endothelial dysfunction has also been found in CKD patients with mild renal insufficiency suggesting a role for uraemia directly promoting the early development of cardiovascular complications in CKD patients (Annuk *et al.*, 2001; Foster *et al.*, 2008; MacKinnon *et al.*, 2008; Mourad *et al.*, 2001; Perticone *et al.*, 2004; Stam *et al.*, 2006; Thambyrajah *et al.*, 2000; Yilmaz *et al.*, 2006).

1.11 Relationship of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction

1.11.1 C-reactive protein

A number of studies have shown associations between the circulating levels of CRP and arterial stiffness in the general population (Kullo *et al.*, 2005; Mattace-Raso *et al.*, 2004; Nagano *et al.*, 2005; Vlachopoulos *et al.*, 2005; Yasmin *et al.*, 2004), hypertension (Amar *et al.*, 2005; Mahmud *et al.*, 2005), familial hypercholesterolaemia (Cheng *et al.*, 2007), and the metabolic syndrome (Tomiyama *et al.*, 2005). A substudy of the regression of arterial stiffness - the controlled doubled-blinded REASON study suggested the associations between arterial stiffness and CRP by showing that in a group of hypertensive patients whose baseline CRP was high (>3 mg/L), those with a greater reduction in arterial stiffness after 12 months of antihypertensive treatment, were more likely to have a reduction of CRP to the normal range at the end of the 12-month treatment (Amar *et al.*, 2005).

In renal patients, the association between arterial stiffness and inflammation has been shown in dialysis (London *et al.*, 2003) and renal transplant patients (Verbeke *et al.*, 2007). A prospective study in haemodialysis patients has shown that subjects who respond to the antihypertensive treatment had less arterial stiffness, less left ventricular hypertrophy, and lower CRP levels compared to these of the non-responders. The improvement of arterial stiffness and left ventricular hypertrophy in the responder group was independently associated with CRP levels (London *et al.*, 2003).

With regards to endothelial dysfunction, CRP correlated with the endothelium-dependent vasodilatory response in a forearm blood flow study in untreated hypertensive patients (Zoccali *et al.*, 2006). In dialysis patients, evidence has shown associations between circulating markers of endothelial damage and increased CRP concentrations (Bolton *et al.*, 2001; Vaccaro *et al.*, 2007). These effects are also

present in patients with mild renal insufficiency (Annuk *et al.*, 2005; Bolton *et al.*, 2001; Landray *et al.*, 2004; Stam *et al.*, 2003; Vaccaro *et al.*, 2007). However, one study in CKD stages 3 to 5 has shown no associations between endothelial function and inflammation, assessed using FMD, and CRP (Annuk *et al.*, 2005).

1.11.2 Isoprostanes

Data regarding the relationship between Isop and arterial stiffness is limited. However, a study has reported their associations in patients with chronic fatigue syndrome (Spence *et al.*, 2008). Yet, several lines of evidence have shown the relationship between both plasma and urinary Isop and endothelial dysfunction in healthy subjects (Sarabi *et al.*, 1999) and those with systemic sclerosis (Cracowski *et al.*, 2006), hypercholesterolaemia (Martino *et al.*, 2008), and coronary artery disease (Lavi *et al.*, 2008). The relationship between Isop and arterial stiffness and endothelial dysfunction in renal patients remains to be investigated.

1.11.3 Oxidised low density lipoprotein

Reports concerning serum lipoproteins as related to arterial stiffness are controversial. Some studies have found positive associations whereas some report negative associations (Hopkins *et al.*, 1993; Kupari *et al.*, 1994; Spence *et al.*, 2008; Tomochika *et al.*, 1996; Wilkinson *et al.*, 2002b). Nonetheless, it has been shown that high plasma OxLDL concentrations are associated with increased arterial stiffness in healthy men and familial hypercholesterolaemic patients (Toikka *et al.*, 1999). Adjusted for age, BP, and other cardiovascular risk factors, OxLDL is the only independent determinant of compliance of the carotid artery in healthy men (Toikka *et al.*, 1999). In addition, in borderline hypertensive individuals, reduced arterial distensibility is related to increased OxLDL (Toikka *et al.*, 2002).

Data regarding the association of OxLDL and endothelial dysfunction is limited and unclear. Holvoet, *et al.* reported that OxLDL is associated with endothelial dysfunction in CKD (Holvoet *et al.*, 1996). Moreover, OxLDL of uraemic patients appears to be more atherogenic since it is associated with greater monocyte-endothelial cell adhesion (O'Byrne *et al.*, 2001). However, Bolton, *et al.* found no

relationship between OxLDL and endothelial dysfunction, measured by FMD, in CKD patients (Bolton *et al.*, 2001).

1.11.4 The metabolic syndrome

The metabolic syndrome and its risk factors are substantially associated with arterial stiffness in healthy subjects (Ferreira *et al.*, 2007; Ferreira *et al.*, 2005; Kovaite *et al.*, 2007; Li *et al.*, 2005; Nakanishi *et al.*, 2003; van Popele *et al.*, 2000), hypertensive (Mule *et al.*, 2006; Schillaci *et al.*, 2005; Seo *et al.*, 2005), and diabetes patients (Martens *et al.*, 2008; Yokoyama *et al.*, 2007). The metabolic syndrome is an independent predictor for arterial stiffness in many studies (Ferreira *et al.*, 2005; Kovaite *et al.*, 2007; Mule *et al.*, 2006; Schillaci *et al.*, 2005; Yokoyama *et al.*, 2007). Recently, a study has shown relationships between the increase in numbers of metabolic syndrome risk factors and arterial stiffness in peritoneal dialysis patients (Zhe *et al.*, 2008).

With regards to endothelial dysfunction, data is not consistent. Some studies in healthy subjects reported that the metabolic syndrome is associated with endothelial dysfunction (Kovaite *et al.*, 2007; Lind, 2008) whereas others report no associations (Mattsson *et al.*, 2008; Wendelhag *et al.*, 2002). In disease, the metabolic syndrome has been shown to correlate with endothelial dysfunction in subjects with a family history of diabetes and obesity (Ghiadoni *et al.*, 2008) and patients with peripheral vascular disease (Golledge *et al.*, 2008). To date, the contribution of the metabolic syndrome or its risk factor to arterial stiffness and endothelial dysfunction especially in non-dialysis CKD patients is not well-investigated.

1.11.5 Asymmetric dimethylarginine

In healthy subjects, infusion of ADMA significantly decreased total cerebral infusion and increased AIx (Kielstein *et al.*, 2006). ADMA was associated with arterial stiffness and was proposed to play a role in the regulation of vascular tone in young and healthy subjects (Kals *et al.*, 2007; Paiva *et al.*, 2008). However, ADMA was not an independent predictor of PWV in a large population-based study (Chirinos *et al.*, 2008). In CKD, an association between plasma ADMA and arterial stiffness has

been shown in haemodialysis patients (Soveri *et al.*, 2007). In this study, haemodialysis sessions reduced ADMA levels and this reduction was associated with an improvement in CAIx. More recently, plasma ADMA was reported to be associated with arterial stiffness in patients with mild to moderate renal insufficiency (Fujii *et al.*, 2008).

Since ADMA reduces NO generation through the inhibition of NOS, elevated plasma ADMA concentrations in patients with hypertension, diabetes mellitus, congestive heart failure, and patients who are on dialysis (Abbasi *et al.*, 2001; Boger, 2003; Vallance *et al.*, 1992; Zoccali *et al.*, 2001) may contribute to endothelial dysfunction seen in these conditions (Boger, 2003; Endemann *et al.*, 2004). Moreover, correlations between ADMA and endothelial dysfunction have been shown in non-dialysis proteinuric CKD patients (Yilmaz *et al.*, 2008b), stage 1 to 5 CKD patients including those on dialysis (Yilmaz *et al.*, 2006), and renal transplant patients (Yilmaz *et al.*, 2005) but not in a study in peritoneal dialysis patients (Mittermayer *et al.*, 2005). Plasma ADMA was an independent predictor of FMD in some of these studies (Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008b).

1.11.6 Endothelin-1

An animal study has shown that infusion of exogenous ET-1 via the ovine iliac artery increases PWV and administration of BQ123, a selective ET_A receptor antagonism, attenuates this effect, suggesting that ET-1, acting via the ET_A receptor, regulates large artery stiffness (McEniery *et al.*, 2003). Similar findings were shown in healthy subjects where ET-1 caused an increase in arterial stiffness which could be blocked by concomitant administration of ET_A receptor blockade (Vuurmans *et al.*, 2003). The effect of ET-1 on arterial stiffness was also investigated in patients with coronary artery disease where plasma ET-1 concentrations correlated substantially with stiffness index of the thoracic aorta, abdominal aorta, and common iliac artery (Heintz *et al.*, 1993). In haemodialysis patients, plasma ET-1 concentrations were associated with PWV, independent of age, BP and indices of cardiovascular remodelling including left ventricular hypertrophy and IMT (Demuth *et al.*, 1998).

ET-1 is also implicated in endothelial dysfunction seen in atherosclerotic conditions, diabetes mellitus, and pulmonary arterial hypertension (Iglarz *et al.*, 2007). The proposed mechanisms of ET-1 induced endothelial dysfunction are that ET-1 decreases NO bioavailability either by diminishing NO production via an inhibition of eNOS activity or by enhancing NO degradation via a formation of oxygen radicals (Iglarz *et al.*, 2007). In haemodialysis patients, plasma ET-1 concentrations inversely correlated with endothelial function, measured using FMD, independent of age and BP (Demuth *et al.*, 1998). In addition, plasma ET-1 was independently related to von Willebrand factor, a circulating marker of endothelial damage, in stage 5 CKD patients who are not on dialysis (Dammers *et al.*, 2005).

1.12 Hypotheses and aims

This thesis explores the degree of arterial stiffness and endothelial dysfunction in relatively low risk CKD patients without diabetes or cardiovascular co-morbidity, across the wide range of renal function from normal GFR to pre-dialysis, and investigates a contribution of conventional as well as different types of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction in this group of patients. Furthermore, the effects of selective ET_A receptor antagonism on arterial stiffness and endothelial dysfunction in the same group of patients are examined.

Chapter 3 (Study 1): This study aimed to assess the reproducibility of non-invasive measurements of arterial stiffness and endothelial function.

> ***Hypotheses:*** CF-PWV and AIx, as measures of arterial stiffness, and FMD of the brachial artery, as a measure of endothelial function, are reproducible for one observer with repeated measurements, and for two separate observers.

Chapter 4 (Study 2): This study investigated the relationship of plasma and urinary ET-1 concentrations to renal function in CKD patients.

Hypotheses: As a result of reduced renal clearance and increased renal production, plasma and urinary ET-1 concentration will increase as renal function declines.

Chapter 5 (Study 3): This study sought to investigate the role of uraemia and conventional cardiovascular risk factors including age, gender, smoking, high BP, glucose, and dyslipidaemia on arterial stiffness and endothelial dysfunction in CKD patients.

Hypotheses: As renal function declines, arterial stiffness and endothelial dysfunction gradually increase. The increase in arterial stiffness and endothelial dysfunction will significantly and independently relate to renal function.

Chapter 6 (Study 4): This study assessed the contribution of inflammation, oxidative stress, and a shift in the balance between the vasodilator (NO) and vasoconstrictor (ET) systems to arterial stiffness and endothelial dysfunction in CKD patients.

Hypotheses: The increase in arterial stiffness and endothelial dysfunction seen in study 3 is also substantially explained by inflammation, oxidative stress, and a shift in the balance of NO/ET systems (evidenced by increased ADMA and ET-1 levels, respectively) independent of conventional cardiovascular risk factors and renal function.

Chapter 7 (Study 5): This study assessed the prevalence of the metabolic syndrome and the contribution of the metabolic syndrome and the risk factors for it on arterial stiffness and endothelial dysfunction in CKD patients.

Hypothesis: The metabolic syndrome, by itself, or through its risk factors is substantially associated to the increased arterial stiffness and endothelial dysfunction seen in study 3, independent of conventional cardiovascular risk factors and renal function.

Chapter 8 (Study 6): This study assessed the prevalence of cardiovascular calcification in CKD patients and investigated the contribution of cardiovascular calcification to arterial stiffness and endothelial dysfunction in CKD patients.

> ***Hypotheses:*** Cardiovascular calcification occurs in the early stages of CKD and the calcification score is substantially related to arterial stiffness and endothelial dysfunction, independent of conventional cardiovascular risk factors and renal function.

Chapter 9 (Study 7): This study examined the effects of BQ123, a selective ET_A receptor antagonism, on arterial stiffness and endothelial function in CKD patients.

> ***Hypotheses:*** Indices of arterial stiffness and endothelial dysfunction will improve with blockade of the ET system at the ET_A receptor. These effects will be greater with ET blockade than those seen with equivalent BP lowering using nifedipine, indicating specific beneficial effects of ET receptor antagonism on arterial stiffness and endothelial function independent of BP. All of these effects will occur on top of standard treatment with renin-angiotensin system blockade.

Chapter 2

Materials and methods

2.1 General requirements

2.1.1 Subjects

Subjects abstained from alcohol for at least 24 hours and any caffeine-containing products for at least 12 hours before the study. All of them were fasted for at least 8 hours before the study except for study 7 (Chapter 9) in which subjects had a light breakfast on the morning of the study. Smoking was not permitted on the morning of the study.

Healthy subjects in a study of the reproducibility of CF-PWV and AIx (Chapter 3) were recruited from staff at the Clinical Research Centre and medical students at the University of Edinburgh. They were male or female, aged 18-50 years old, with no history of major cardiac, respiratory, neurological, diabetic, and renal disease. None of them were on any medication.

For a study of the reproducibility of FMD (Chapter 3) and studies 2 to 6 (Chapter 4 to 8) CKD patients were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh. The inclusion criteria for CKD patients were: male or female CKD patients, 18-65 years old, BP ≤160/100 mmHg, whether or not on anti-hypertensive medication. Patients with a renal transplant or on dialysis, patients with systemic vasculitis or connective tissue disease, those with a history of established cardiovascular disease, peripheral vascular disease, diabetes mellitus, respiratory disease, or neurological disease, those with current alcohol abuse or those who were pregnant, and patients treated with an organic nitrate or β-agonist were excluded. CKD patients enrolled for study 7 (Chapter 9) were 18-70 years old with the remainder of the inclusion criteria similar to that previously described for studies 2 to 6. For studies 2 to 6, patients continued their usual medication but omitted the morning dose on the day of the study. Hypercholesterolaemic patients were not excluded but they had to be established on statin medication with good cholesterol control for at least 3 months before taking part in the study. Healthy subjects were enrolled from the community and those who had taken any medication in the previous 2 weeks were excluded from the studies.

2.1.2 Study environment

All studies were performed in the Clinical Research Centre, University of Edinburgh, in a quiet, temperature-controlled room, at 22-24°C.

2.1.3 Research governance and ethics

Studies were approved either by a Local Research Ethics Committee or the Multi-centre Research Ethics Committee for Scotland. Studies were performed in accordance with the Declaration of Helsinki of the World Medical Association. All subjects provided their written informed consent.

2.2 Haemodynamic measurements

2.2.1 Blood pressure and heart rate

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate were recorded in duplicated using a validated oscillometric sphygmomanometer, the Omron HEM-705CP (Omron Healthcare (UK) Ltd, Milton Keynes) (O'Brien *et al.*, 1996), except for the study investigating the effect of a selective ET_A receptor antagonist (BQ123) on arterial stiffness and endothelial dysfunction (Chapter 9) in which a Dinamap Pro 100 BP monitor (GE Healthcare, Chalfont St Giles) was used (Chang *et al.*, 2003). Recordings were required to be within 10 mmHg of each other (both SBP and DBP). If not, BP was repeated until two consecutive readings fulfilled the criterion. BP was recorded on the non-dominant arm but if the patients had an arterio-venous fistula (some of the stage 5 patients), BP was measured on the non-fistula arm. Brachial mean arterial pressure (MAP) was calculated as MAP (mmHg) = DBP + [(SBP-DBP) / 3]. Pulse pressure (PP) was calculated as PP (mmHg) = SBP – DBP.

2.2.2 Assessment of left ventricular hypertrophy

Left ventricular hypertrophy was assessed using gender-specific electrocardiogram (ECG) criteria (Alfakih *et al.*, 2004). Cornell criteria was used in males: left ventricular hypertrophy present if the sum of the R-wave in lead aVL and the S-wave in lead V3 is greater than 25 mm, and Sokolow-Lyon criteria was applied in females: left ventricular hypertrophy presents if the QRS duration multiplied by the sum of the S-wave in lead V1 and the R-wave in lead V5 or V6 is greater than 2970.

2.3 Anthropometric measurements

Height (m) and weight (kg) were measured using a digital scale (GEC/Avery digital scales, model number 824/890, Avery Weigh-Tronix, West Midlands, UK). Body mass index (BMI) was calculated as: weight / $[\text{height}]^2$ and reported in kg/m^2. Waist and hip circumference (cm) were measured on the subject in a standing position with feet 15 cm apart. Waist circumference was measured at the midpoint between iliac crest and lowest rib. Hip circumference was measured at the widest part between the waist and groin. During the measurements, the tape measure was parallel to the ground (Wahrenberg *et al.*, 2005).

2.4 Estimation of glomerular filtration rate

CrCl, as an estimate of glomerular filtration rate (eGFR), was calculated according to the C&G equation (Mafham *et al.*, 2007). GFR ($ml/min/1.73m^2$) = [(140- age (years) x weight (kg) x 1.23 for male or 1.05 for female] / serum creatinine (μmol/L). The C&G equation was selected to assess renal function in this study because it is more accurate than the MDRD equation when used to assess mild renal insufficiency (Froissart *et al.*, 2005; Mafham *et al.*, 2007). It was further corrected by body surface area as described by Du Bois, *et al* (Du Bois D *et al.*, 1916).

2.5 Arterial stiffness measurements

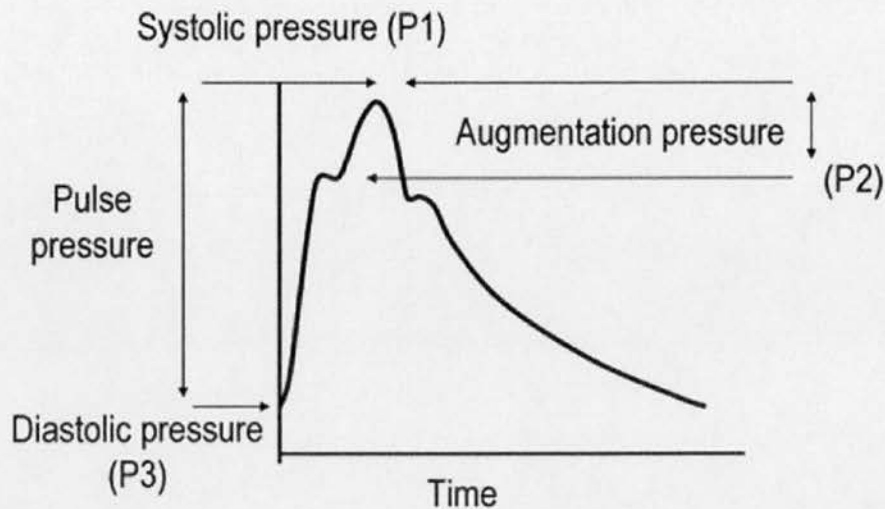
The well-validated techniques of PWA and CF-PWV were used in this thesis as surrogate measures of arterial stiffness.

2.5.1 Pulse wave analysis

Peripheral pressure waveforms were recorded from the radial artery at the wrist by applanation tonometry using a high fidelity micromanometer (SPC-301, Millar Instruments, Texas, USA) and the SphygmoCor apparatus (AtCor Medical Pty Ltd, West Ryde, Australia) with SphygmoCor software version 6.3 (Figure 2.1). Averaged peripheral and corresponding central (ascending aortic) pressure waveforms were generated from the last 10 seconds of the radial artery recordings. AIx was defined as: AIx (%) = [(P1 – P2) / PP] x 100 (Figure 2.2).

Figure 2.1 The peripheral pressure waveform is recorded using a tonometer applied lightly over the radial artery at the wrist.

Figure 2.2 The central pressure waveform and the calculation of Aix.



Key: Figure from (Laurent *et al.*, 2006). Augmentation pressure = P1 – P2. Augmentation index = [P1-P2/ pulse pressure] x 100%. P1: late systolic peak; P2: systolic peak. In young healthy subjects AIx is negative. AIx becomes increasingly positive in stiff arteries.

RAIx was determined from the average peripheral waveform. CAIx and CAIx adjusted to a standard heart rate of 75 bpm (CAIx@HR75) were calculated from the averaged central waveform which was derived from peripheral arterial pressure waveform by a transfer function. To derive CAIx@HR75, the SphygmoCor software adjusts CAIx at an inverse rate of 4.8% for each 10 bpm increment in HR. All PWA measurements were made in duplicate with CAIx values within 5% of each other. If not, further recordings were made until two consecutive readings fulfilled the criterion. The quality control for PWA including average pulse height >100, pulse height variation <5%, and diastolic variation <5% were followed.

2.5.2 Carotid-femoral pulse wave velocity

The SphygmoCor apparatus was also used to measure CF-PWV. During continuous ECG monitoring, pulse wave recordings were made first at the common carotid artery (proximal site) and then at the femoral artery (distal site) using a high fidelity micromanometer. The software identified the foot of the pulse wave as the beginning of the sharp systolic upstroke. The wave transit time between arterial sites was determined in relation to the R-wave of the ECG. Surface distance between the two recording sites was measured in mm from the suprasternal notch to the carotid

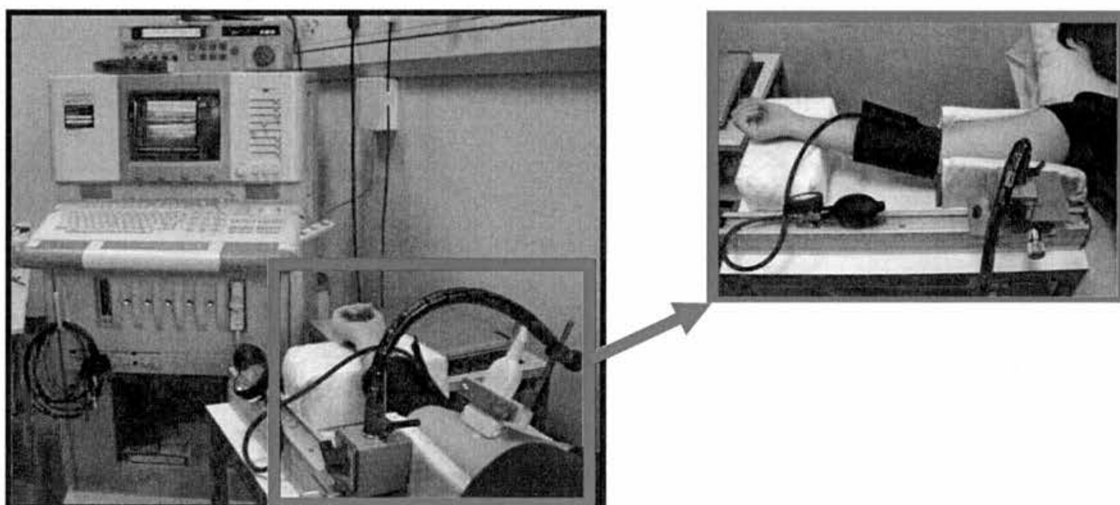
measurement site (proximal distance) and to the femoral measurement site (distal distance) and the distance for PWV calculation was calculated as distance travelled (mm) = distal distance – proximal distance. CF-PWV was calculated as: CF-PWV (m/s) = distance travelled / wave transit time. All CF-PWVs were recorded in duplicate with values within 0.5 m/s of each other. If not, further recordings were made until two consecutive readings fulfilled the criterion. The same quality control for PWA was applied to the waveforms of CF-PWV.

2.6 Endothelial function measurements

2.6.1 Flow-mediated dilatation

FMD uses the shear stress from the flow of blood as a physiological stimulus for NO generated vasodilatation. In this thesis, FMD of the brachial artery was measured. The experimental set-up for FMD is shown in Figure 2.3.

Figure 2.3 An experimental set-up for FMD.



With individuals in a supine position and their arms out-stretched perpendicular to the body, the brachial artery was imaged longitudinally with B-mode ultrasound (Acuson XP 128, Siemen plc, Bracknell, UK) 5 cm above the antecubital fossa using a linear array transducer with an imaging frequency of 11 MHz. The ultrasound probe was held in place with a stereotactic clamp throughout the study. A segment with clear anterior and posterior intimal interfaces between the lumen and vessel

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

Baseline diameter was recorded for 1 minute. To create a flow stimulus in the brachial artery, a BP cuff, which was placed around the upper forearm, was inflated to 50 mmHg above SBP in order to occlude blood flow into the forearm for 5 minutes. Following deflation of the cuff the artery was scanned for a further 5 minutes. The changes with endothelium-independent vasomotor function after a sublingual administration of GTN was performed after finishing FMD. The brachial artery was scanned at the baseline for 1 minute. 25μg of GTN (Oliver *et al.*, 2005) was administered sublingually and the artery was scanned for a further 5 minutes. All the ultrasound recordings were stored on videotape. Brachial artery diameter was calculated off-line from the stored images using semi-automated wall tracking software (Brachial Analyzer, Medical Imaging Application, Iowa, USA). The endothelium-dependent vasodilatation (FMD) and endothelium-independent vasodilatation (GTN) were reported as a percentage change of the brachial artery diameter from baseline (FMD% and GTN%).

2.6.2 A reduction of arterial wave reflection induced by inhaled β_2-adrenoreceptor agonist

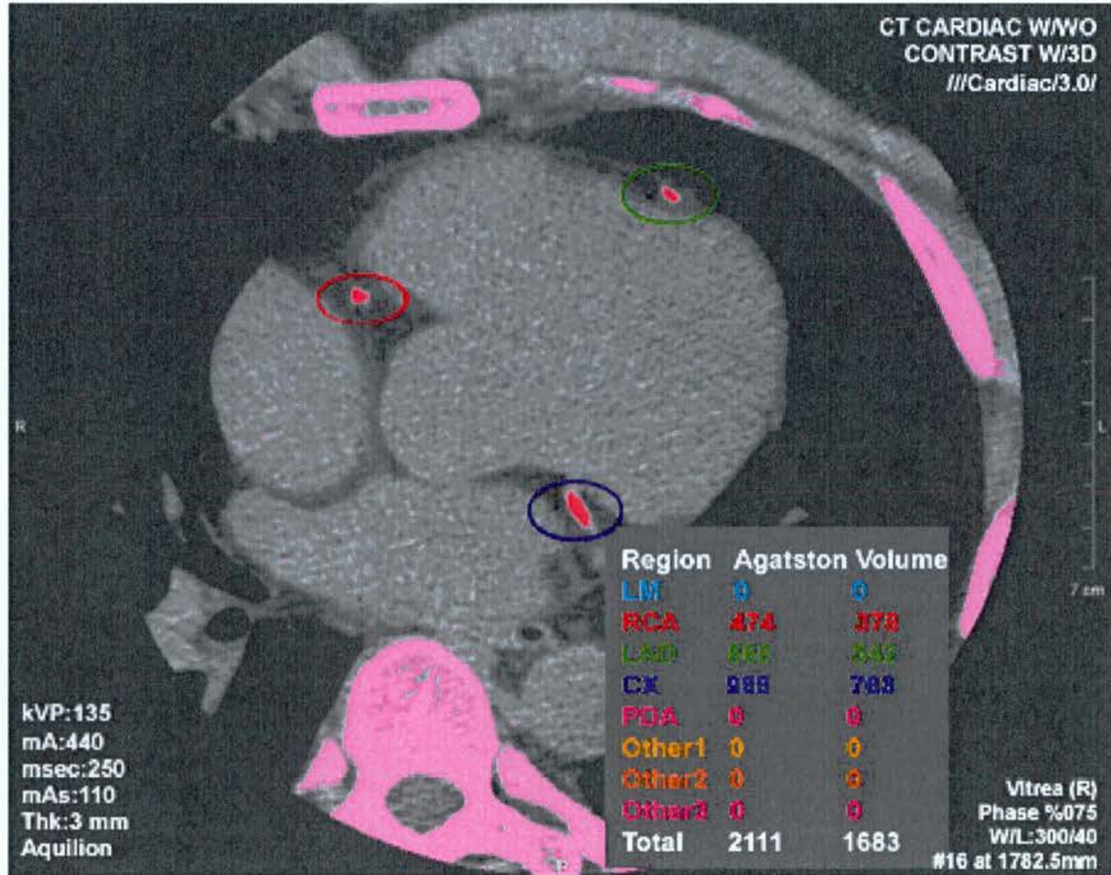
Inhaled β_2-adreneroreceptor agonist, salbutamol (Allen & Hanbury, Stockley Park, UK) was administered to patients through a spacer device (Volumetric® Allen & Hanbury, Stockley Park, UK). Two puffs (200 μg of salbutamol) were initially placed into the spacer and were inhaled via a full inspiration. Subjects then held their breath for 10 seconds, exhaled, and once again fully inhaled through the spacer and held their breath for another 10 seconds. This procedure was then repeated with a further 2 puffs. The SphygmoCor apparatus was used to measure PWA at the peripheral site (radial artery) at 5, 10, 15, and 20 minutes of salbutamol administration. CAIx was derived and the maximum change of CAIx from the

baseline was compared to that following 25 μg sublingual GTN, as a measure of endothelium-independent vasodilatation (Wilkinson *et al.*, 2002a). The PWA recordings for GTN were made at 3, 5, 10, 15, and 20 minutes post dose.

2.7 Assessment of arterial calcification

The presence and quantity of coronary artery calcification (CAC) was determined by a 16-slice multi-detector computed tomography (MDCT) (Toshiba, Aquilion 16). Imaging was applied when the patient lies supine. No contrast was used. Tomographic imaging was preceded from the level of the lower margin of the bifurcation of the main pulmonary artery to the diaphragm. The CAC score was calculated using a semi-automated computer software programme (3D Vitrea Workstation, Vital system) based on the total amount and density of the calcific deposits found in the four branches of the coronary arteries (left main, left anterior descending, circumflex, and right coronary artery). The final CAC score of each patient was reported as a combination of the CAC scores of the four branches of the coronary arteries as previously described by Agatston (Agatston *et al.*, 1990) (Figure 2.4, Table 2.1). The radiologist who performed the test was blinded to the stages of CKD and all of the procedures were conducted by one radiologist. This technique is the most accurate technique available for calcium quantification and is reproducible. It provides a quantitative evaluation of the extent of plaque burden as the calcification of the coronary arteries closely correlates with the presence of calcification in the aorta (Dellegrottaglie *et al.*, 2005).

Figure 2.4 Coronary artery calcium score calculation.



Key: Circles indicate coronary arteries. CX; circumflex artery; LAD: left anterior descending artery; LM: left main artery; RCA: right coronary artery.

Table 2.1 An example of a coronary artery calcium score report in one subject of the study presented in this thesis (Agatston score is the final score reported to physician).

Name	Voxel	Volume(mm^3)	Plaque	Agatston score
LM	0	0	0	0
LAD	223	163.92	2	218.6
LCX	14	10.29	3	5.9
RCA	161	118.35	7	117.9
Total	398	292.56	12	342.4

Key: LAD: left anterior descending artery; LCX; left circumflex artery; LM: left main artery; RCA: right coronary artery; Voxel: volumetric pixel (a volume element, representing a value on a regular grid in three dimensional space).

2.8 Diagnosis of the metabolic syndrome

The metabolic syndrome was diagnosed using the NCEP-ATP III criteria (Table 2.2) (Anonymous, 2002b). The diagnosis of the metabolic syndrome was made when subjects had ≥3 risk factors for the metabolic syndrome. Subjects with 0-1 risk factors for the metabolic syndrome were regarded as those without the metabolic syndrome. Subjects with 2 risk factors for the metabolic syndrome were regarded as those at risk of developing metabolic syndrome.

Table 2.2 The NCEP ATP III criteria for clinical diagnosis of the metabolic syndrome.

Risk factors	**Defining level**
Blood pressure	≥130 / ≥ 85 mmHg
Fasting glucose	≥ 6.0 mmol/L
High density lipoprotein cholesterol	
Male	<1.0 mmol/L
Female	<1.3 mmol/L
Triglycerides	≥1.7 mmol/L
Waist circumference	
Male	>102 cm
Female	>88 cm

Key: No metabolic syndrome: subjects with 0-1 risk factor; risk for developing the metabolic syndrome: subjects with 2 risk factors; metabolic syndrome: subjects with ≥3 risk factors.

2.9 Assays

2.9.1 General blood and urine samples

Blood samples were taken from subjects into the appropriate blood tubes (Sarstedt) and analysed for full blood count, erythrocyte sedimentation rate (ESR), folate, urea,

creatinine, sodium, potassium, bicarbonate, albumin, calcium, phosphate, glucose, haemoglobin A_1C (HbA_1C), insulin, urate, total cholesterol, triglycerides, and HDL cholesterol. These samples were analysed by the Haematology and Biochemistry laboratories at the Western General Hospital. Plasma concentrations of parathyroid hormone (PTH) were analysed by the Haematology and Biochemistry laboratories at the Royal Infirmary of Edinburgh. Plasma concentrations of vitamins (vitamin A, B1, B2, B6, C, and E) and trace elements (Zn, Cu, Se, and Mn) were analysed by the Department of Clinical Biochemistry, Glasgow Royal Infirmary.

LDL cholesterol was calculated using Friedewald's formula (Friedewald *et al.*, 1972). LDL cholesterol (mmol/L) = total cholesterol – HDL cholesterol– [triglycerides / 5]. Insulin resistance was calculated using a homeostatic model assessment of insulin resistance (HOMA-IR) equation (Wallace *et al.*, 2004). HOMA-IR = [fasting plasma insulin (mU/L) x fasting plasma glucose (mmol/L)] / 22.5

Urine samples were taken in the plain tubes from subjects and a urinary dipstick was used to analyse for blood, glucose and protein (Multistix 10 SG reagent strips for urinalysis, Siemens Healthcare Diagnostics Inc). Urine samples were also analysed for urinary albumin and creatinine, by the Haematology and Biochemistry laboratories at the Western General Hospital.

2.9.2 Specific blood and urine samples

For the measurement of IL-6, ADMA, SDMA, ET-1, and big ET-1, blood samples were collected into EDTA tubes (Sarstedt). For the measurements of hsCRP and OxLDL blood samples were collected into plain tubes (Sarstedt). For the measurement of 8-Isop, blood sample were collected into ice-cold EDTA tubes (Sarstedt) containing 1 mg/ml of reduced glutathione (Mori *et al.*, 1999). All blood samples were centrifuged immediately at 2500*g* at 4°C for 20 minutes. Plasma for 8-Isop analysis was stored in plain tubes containing 200 μg/ml of butylated hydroxy toluene to prevent further oxidation (Mori *et al.*, 1999) and stored at -80°C until analysis. The remainder of the plasma and serum samples were also stored in plain

tubes at -80°C until analysis. 20 ml aliquots of urine were collected into plain tubes containing 2.5 ml of acetic acid for the measurement of urinary ET-1 and store at -80°C until analysis.

For all assays, reagents and samples were brought to room temperature prior to assay. The water used to prepare all reagents was deionised and free of trace organic contaminants. For enzyme-linked immunosorbant assay (ELISA), all standards, quality controls and samples were assayed in duplicate.

2.9.2.1 Plasma interleukin-6

Plasma IL-6 was quantified in the Vascular Biology Centre, Medical College of Georgia, USA, using a commercially available sandwich ELISA (Cayman Chemical, Ann Arbor, MI). Prior to addition to the assay, the plasma samples were diluted with a dilution buffer (2x). Also, plasma samples and standards were purified by the addition of a non-specific mouse immunoglobin (25 μl of the mouse serum for each 500 μl of sample or standard) in order to compensate for the effects of human anti-mouse IgG which may be present in the samples.

100 μl of standards, quality controls, dilution buffer (= blank), and diluted samples were added to appropriate wells of a microplate pre-coated with monoclonal antibody specific for IL-6 which will bind any IL-6 introduced into the well. 100 μl of an acetylcholinesterase conjugate, which binds selectively to a different epitope on the IL-6 molecule, was then added to the well. The microplate was covered with plastic film and incubated overnight at 25°C. After being washed 5 times with an automatic washer, the concentration of the analyte was then determined by measuring the enzyme activity of the acetylcholinesterase by adding 200 μl of Ellman's reagent to each well. The product of the acetylcholinesterase-catalysed reaction has a distinct yellow colour. The optimum assay development was detected after 180 minutes incubation. The intensity of this colour was determined by spectrophotometry (wavelength 405 nm) and was directly proportional to the amount of bound conjugate which in turn was proportional to the concentration of IL-6. The concentration of IL-6 was obtained by automatic calculations. Concentrations of

plasma IL-6 were automatically calculated from the standard curve and multiplied by a dilution factor of 2. The final IL-6 concentration was reported in pg/ml. The detection limit of the assays was 1 pg/ml. The intra-assay coefficient variation was 5% and inter-assay coefficient variation was 15%.

2.9.2.2 Plasma isoprostane

Plasma 8-Isop was quantified in the Vascular Biology Centre, Medical College of Georgia, USA. Free 8-Isop was determined by a commercially available competitive ELISA (Cayman Chemical, Ann Arbor, MI). This method was based on the competition between 8-Isop and 8-Isop-acetylcholinesterase conjugate (8-Isop tracer) for a limited number of 8-Isop-specific rabbit antiserum binding sites. As the concentration of the 8-Isop tracer was held constant while the concentration of the 8-Isop varied, the amount of 8-Isop tracer that was able to bind to the rabbit antiserum was inversely proportional to the concentration of 8-Isop in the well.

Samples were purified using a solid phase extraction purification method with a recovery of more than 90% before starting ELISA. 50 μl of standards, quality controls, dilution buffer (= blank), and diluted samples were added to appropriate wells of a microplate pre-coated with rabbit IgG mouse monoclonal antibody. 50 μl of 8-Isop acetylcholinesterase tracer and then 50 μl of 8-Isop antiserum were added to each well. The microplate was incubated at 4°C for 18 hours. After being washed 5 times with an automatic washer to remove any unbound reagents, 200 μl of Ellman's reagent which contained the substrate to acetylcholinesterase was added to the wells. Optimum assay development was detected after 90 minutes of incubation. The intensity of the colour, determined spectrophotometrically at 405 nm, was proportional to the amount of 8-Isop tracer bound to the well, which was inversely proportional to the amount of free 8-Isop present in the well during the incubation - absorbance $\propto$ [bound 8-Isop tracer] $\propto$ 1 / [8-Isop]. The detection limit of the assays was approximately 2 pg/ml. The intra-assay coefficient variation was 9% and inter-assay coefficient variation was 13%.

2.9.2.3 Plasma dimethylarginines

Plasma ADMA, and SDMA were determined in the Department of Clinical Biochemistry at Glasgow Royal Infirmary using an optimised and fully validated high performance liquid chromatography (HPLC) method (Blackwell *et al.*, 2007). Briefly, the methylarginines were extracted from plasma using solid phase extraction on a cation exchange resin. They then underwent isocratic reverse-phase HPLC (Waters, Watford, UK) utilising pre-column derivatisation with ortho-phthaldialdehyde and fluorescence detection. Quantification was performed by the method of internal standardisation using a single level calibration. The detection limits were 0.04 μmol/L for arginine and 0.004 μmol/L for ADMA and SDMA. The intra-assay coefficients of variation were 1.9% for ADMA and 1.6% for SDMA. The inter-assay coefficients of variation were 2.3% for ADMA and 3.1% for SDMA.

2.9.2.4 Plasma and urinary endothelin-1

Plasma ET-1, big ET-1, and urinary ET-1 concentrations were analysed using a standard radioimmunoassay (Peninsular Laboratories Europe, St. Helens, UK) at the Clinical Pharmacology Unit, Centre for Cardiovascular Science, Queen Medical Research Institute, University of Edinburgh.

The very low concentrations of ET-1 and big ET-1 in plasma preclude the direct measurement of these peptides by radioimmunoassay. Therefore, ET-1 and big ET-1 were extracted from plasma using a sample preparation column, concentrated, and then the subsequent extract was analysed by radioimmunoassay. The extraction technique used was an acetic acid extraction described by Rolinski, *et al* which gave approximately 89% and 91% of a recovery of ET-1 and big ET-1 from plasma, respectively (Rolinski *et al.*, 1994). Following the extraction, 100 μl of standards, samples and controls were incubated overnight with the appropriate antibody. In the following day, a known concentration of radio-labelled ET-1 or big ET-1 was added and the tubes were incubated for further 16 hours. On the third day, the immune complex was precipitated with Amerlex-M donkey anti-rabbit antibody (Amersham Biosciences UK Ltd, Buckinghamshire, UK). The precipitated were counted in a gamma counter. A standard curve was constructed and the unknown values were

calculated from the standard curve. The detection limit was 0.25 pg/ml for ET-1 and 1 pg/ml for big ET-1. This technique had the intra- and inter-assay coefficient variations of 6.3% and 7.2%, respectively. Urine ET-1 was determined with the same extraction and radioimmunoassay methods as plasma ET-1.

2.9.2.5 Serum high-sensitivity C-reactive protein

Serum hsCRP was analysed by the Haematology and Biochemistry laboratories at the Western General Hospital, using a validated latex particle enhanced immunoturbidimetry technique (Vitros® 5, 1 FS Chemistry Systems, Ortho-Clinical Diagnostics, Inc., New York, USA). Briefly, the reaction sequence was performed in two steps. First, samples, standards, and quality controls were mixed with Reagent 1 containing a buffer. Then, to allow the formation of a CRP antigen-antibody complex, anti-CRP mouse monoclonal antibodies coupled to latex microparticles (Reagent 2) were added. The turbidity was measured spectrophotometrically at 600 nm. This technique detects CRP at the range of 0.10-15.00 mg/L. The intra- and inter-assay variations were 2.3% and 5% respectively.

2.9.2.6 Serum oxidised low density lipoprotein

Serum OxLDL was determined in the Clinical Pharmacology Unit, Centre for Cardiovascular Science, University of Edinburgh, using a commercially available sandwich ELISA (Mercodia AB, Uppsala, Sweden). Serum samples were diluted (6,561x) with a dilution buffer. 25 μl of standards, quality controls, dilution buffer (= blank), and diluted samples were added to appropriate wells of a microplate pre-coated with mouse monoclonal anti-OxLDL. 100 μl of assay buffer was then added to each well. The microplate was incubated at 25°C, on an orbital microplate shaker (600 rpm) for 2 hour. After being washed 6 times (0.35 ml of wash solution per well) with an automatic washer, 100 μl of enzyme conjugate solution (peroxidise conjugated mouse monoclonal anti-apoB) was added to the wells and incubated at 25°C, on an orbital microplate shaker (600 rpm) for another 1 hour. Following another washing step, 100 μl of tetramethyl benzidine (TMB) substrate solution was added to the well and the microplate was incubated at 25°C for 15 minutes without

shaking. The reaction was then stopped by the addition of 50 μl stop solution (0.5 M H_2SO_4). The optical density was then read at 450 nm within 30 minutes. The absorbance was proportional to the concentration of OxLDL. The concentration of OxLDL was obtained by automatically calculating of the absorbance for the standards *versus* the concentration, using cubic spline regression. The concentrations obtained from a standard curve (mU/L) were multiplied by the dilution factor of 6,561. The final OxLDL concentrations were reported in U/L. The limit of detection of the assays was 1 mU/L. The intra-assay coefficient variation was 7.3% and inter-assay coefficient variation was 8.3%.

2.9.2.7 Serum fetuin-A

Serum fetuin-A was analysed in the Clinical Pharmacology Unit, Centre for Cardiovascular Science, University of Edinburgh, using a commercially available sandwich ELISA (BioVendor GmbH, Heidelberg, Germany). 100 μl of diluted standards, quality controls, dilution buffer (= blank), and diluted samples were added to appropriate wells of a microplate pre-coated with polyclonal anti-human fetuin-A antibody. The microplate was then incubated at 25°C, on an orbital microplate shaker (300 rpm) for 1 hour. After being washed 3 times (0.35 ml of wash solution per well) with an automatic washer, 100 μl of polyclonal anti-human fetuin-A antibody, conjugated with horseradish peroxidise was added to the wells and incubated at 25°C, on an orbital microplate shaker (300 rpm) for another 1 hour with captured fetuin-A. Following another washing step, the remaining horseradish peroxidise conjugate was allowed to react with 100 μl of substrate solution TMB (incubation at 25°C for 10 minutes, without shaking). The reaction was then stopped by addition of 100 μl acidic stop solution. The absorbance of the resulting yellow product was measured spectrophotometrically at 450 nm within 5 minutes. The absorbance was proportional to the concentration of fetuin-A. The microplate reader performed automatic calculations of analyte concentration. The standard curve was constructed by plotting the mean absorbance at 450 nm of standards against a log of the known concentration of standards. Results calculated from a standard curve were concentrations of fetuin-A in ng/ml and these were multiplied by their respective dilution factor of 10,000 (e.g. 21.5 ng/ml (from standard curve) x 10,000 (dilution

factor) = 215.0 ug/ml or 0.215 g/L). The final fetuin-A concentrations were reported in g/L. The limit of detection of the assays was 0.35 ng/ml. The intra-assay coefficient variation was 4.8% and the inter-assay coefficient variation was 5.4%.

2.10 Drugs

2.10.1 BQ123

BQ123 (Clinalfa AG) was used as a selective ET_A receptor antagonist. 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 (IC50: ET_A = 7.3 nM, ET_B = 18 μM) (Ihara *et al.*, 1992). Studies with radiolabelled BQ123 demonstrate that it binds competitively to the ET_A receptor, achieving steady state within 7 minutes of injection and dissociates with a half-life of 1.4 minutes (Ihara *et al.*, 1995). It is extracted from the circulation by the hepatic anion transport system (Fukami *et al.*, 1996). The dose of BQ123 infused (1000 nmol/min for 15 minutes) was selected from previous studies as being ET_A selective based on plasma BQ123 concentrations and relating these to the binding constant for the ET_A receptor, as well as by a lack of rise in plasma ET-1 concentration after its infusion (Goddard *et al.*, 2004b). BQ123 was dissolved in 15 ml of 0.9% physiological saline (Baxter Healthcare Ltd) and infused intravenously at a constant rate of 1 ml/min.

2.10.2 Glyceryl trinitrate

GTN was administered sublingually for the assessment of the endothelium-independent vasomotor function following FMD and a reduction of arterial wave reflection after inhaled salbutamol. The dose of 25 μg was used according to the study which showed that this dose gave a response that was comparable to that of the higher dose (Oliver *et al.*, 2005).

2.10.3 Salbutamol

The salbutamol 400 μg inhaler (Allen & Hanbury, Stockley Park, UK) was used in the studies. Salbutamol was administered through a spacer device (Volumetric® Allen & Hanbury, Stockley Park, UK). The dose of salbutamol was based on a previous study by Wilkinson, *et al* (Wilkinson *et al.*, 2002a).

2.10.4 Nifedipine

The calcium channel blocker, nifedipine (10 mg Adalat, Bayer; immediate release), was obtained from the Pharmacy Department at the Western General Hospital, and was used as an active control for the study in Chapter 9. It was administered orally.

2.10.5 Para-aminohippurate

Para-aminohippurate (PAH) sodium (Clinalfa) was used for the measurement of renal plasma flow for studies in Chapter 9 by standard clearance techniques (Levinsky *et al.*, 1973). PAH 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 by clearance), but about 80-90%, thus measurements are quoted as 'effective' renal plasma flow (ERPF). This extraction is not affected by BQ123 in man (Bohm *et al.*, 2003).

Chemical name: Sodium salt of para-amino hippuric acid

Formulation: Injection, 1.1g in 5.5 ml (protect from light)

Renal Blood Flow:

Effective renal plasma flow (ERPF) was estimated, using the Fick principle, from measurement of PAH clearance. Effective renal blood flow (ERBF) was then calculated as:

$$ERBF = ERPF / 1 - haematocrit$$

A constant infusion technique was used for the measurement of PAH clearance. A bolus dose in 100 ml of 5% dextrose was given over 15 minutes. This was followed by a continuous infusion of PAH in 5% dextrose at 2 ml/min (= 120 ml/hr). The aim was to achieve a constant plasma concentration of between 15 and 20 mg/l, at which concentration, the above extraction ratio applied (ie. almost complete extraction). Higher concentrations would saturate tubular transport capacity and led to progressive reductions in clearance and extraction. Assuming a volume of distribution of 28% of body weight for PAH (Smith, 1951), the loading dose was calculated as:

Plasma concentration = Dose / Volume of distribution = Dose / Weight x 0.28

Thus for an 80 kg volunteer, the loading dose was 15 to 20 x (80 x 0.28) = 336 to 448 mg.

Assuming that all PAH is cleared renally from the plasma compartment, the maintenance dose was calculated as:

Clearance = ERPF = (Infusion concentration x Infusion rate)/ plasma concentration

Using a normal ERPF value of 675 ml/min (for men), an infusion rate of 2 ml/min and a plasma concentration of 15 to 20 mg/l, the infusion concentration was therefore,

(675 ml/min x 15 to 20 mg/l) / 2 ml/min

= 5063 to 6750 mg/l

= 5.06 to 6.75 g/l (=10.1 to 13.5 mg/min)

An equilibration period of 120 minutes was allowed to achieve a steady state and thus enable venous sampling rather than arterial sampling to be employed. Previous

work has shown that 100 minutes was sufficient to achieve a stable plasma concentration of PAH.

In summary:

- Loading dose of PAH = 0.4 g (2ml) in 100ml of 5% dextrose over 15 min
- Maintenance infusion of PAH = 6.6 g/l (33ml) at an infusion rate of 2 ml/min

For subjects with a GFR >30 to <50 ml/min, the maintenance dose was reduced by a third.

- Loading dose of PAH: 0.4 g (2ml) in 100ml of 5% dextrose over 15 min
- Maintenance infusion of PAH: 4.4 g/l (22ml) at an infusion rate of 2 ml/min

For subjects with a GFR <30 ml/min the maintenance dose was reduced by two thirds.

- Loading dose of PAH: 0.4 g (2ml) in 100ml of 5% dextrose over 15 min
- Maintenance infusion of PAH: 2.2 g/l (11ml) at an infusion rate of 2 ml/min

2.10.6 Inulin

Inutest (Fresenius Pharma, Austria, GmbH) was used for the measurement of GFR for studies in Chapter 9 by standard clearance techniques (Levinsky *et al.*, 1973). 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 nor reabsorbed within the tubules, nor metabolised within the kidney and thus fulfils the criteria for the measurement of GFR by clearance measurements. Its problems with solubility have been over come by the introduction of Sinistrin (Inutest) arelated polysaccharide with identical clearance.

Chemical name: Sinistrin

Formulation: Injection, 5g in 20 ml (25%)

Glomerular Filtration Rate (GFR)

GFR was calculated as follows:

GFR = (Urine concentration of Inutest x Urine flow rate) / Plasma concentration of Inutest

A constant infusion technique was used for the measurement of Inutest clearance. A bolus dose in 100 ml of 5% dextrose was given over 15 minutes. This was followed by a continuous infusion of Inutest in 5% dextrose at 2 ml/min (= 120 ml/hr). The aim was to achieve a constant plasma concentration of between 200-250 mg/l. Assuming a volume of distribution of 12-18% of body weight for Inutest, (Inutest 25% Summary of product characteristics, Fresenius-GmbH, Linz) the loading dose was calculated as:

Plasma concentration = Dose / Volume of distribution = Dose / Weight x 0.12 to 0.18

Thus for an 80 kg volunteer, the loading dose was 250 x (80 x 0.12 to 0.18) = 2400 to 3600 mg = 2.4 - 3.6 g/l

Assuming that all Inutest is cleared renally from the plasma compartment, the maintenance dose was calculated as:

Clearance (= GFR) = (Infusion concentration x Infusion rate) / Plasma concentration

Using a normal GFR value of 100 ml/min (for men), an infusion rate of 2 ml/min and a plasma concentration of 200-250 mg/l, the infusion concentration was thus

(100 ml/min x 200 to 250 mg/l) / 2 ml/min

= 10000 to 12500 mg/l

= 10 - 12.5 g/l (= 20 - 25 mg/min)

An equilibration period of 120 minutes was allowed to achieve a steady state and thus enable venous sampling rather than arterial sampling to be employed. Previuos work has shown that 100 minutes was sufficient to achieve a stable plasma concentration of Inutest.

In summary

- Loading dose of inutest = 3.5 g (14ml) in 100ml 5% dextrose over 15 min

- Maintenance dose of inutest = 10 g/l (40ml) at an infusion rate of 2 ml/min

For subjects with a GFR >30 to <50 ml/min, the maintenance dose was reduced by a third.

- Loading dose of inutest = 3.5 g (14ml) in 100ml 5% dextrose over 15 min

- Maintenance dose of inutest: 6.7 g/l (26.7ml) at an infusion rate of 2 ml/min

For subjects with a GFR <30 ml/min the maintenance dose was reduced by two thirds.

- Loading dose of inutest: 3.5 g (14ml) in 100ml 5% dextrose over 15 min

- Maintenance dose of inutest: 3.3 g/l (13.3ml) at an infusion rate of 2 ml/min

2.11 Data analysis

Descriptive statistics were presented as mean ± SD (range) unless otherwise indicated. Microsoft Excel 2003 for Windows and SPSS version 15.0 for Windows (SPSS Inc., Chicago, USA) were used for statistical analyses. For categorical data, means were compared by a one-way analysis of variance (ANOVA), Kruskal-Wallis test, the unpaired Student's *t*-test, and the Mann-Whitney test where appropriate.

For Chapters 5 to 7, all data were entered into univariate analysis. This was to explore associations of each variable in the data set separatedly. Correlation coefficeints were calculated using the Pearson method. Significant correlations seen with univariate analysis were presented graphically.

They were then further analysed by multivariate analysis using a stepwise linear regression to assess how dependent variable (CF-PWV or FMD) change when anyone of the independent variable (each of the traditional or emerging cardiovascular risk factors or GFR entered into a regression model) is varied. Approximately 10 independent variables were allowed to enter into one multivariate analysis model. Therefore, data were grouped and presented in different chapter (Chapter 5 to 7). Each chapter focuses on different aspects of emerging cardiovascular risk factors (as independent variables) on CF-PWV and FMD (as dependent variables) (Table 2.3). More details were described in the methodology section of each chapter.

Table 2.3 Independent variables on focus of Chapters 5 to 7.

Chapter	Independent variables on focus
Chapter 5	Traditional cardiovascular risk factors and GFR
Chapter 6	Traditional cardiovascular risk factors, GFR and emerging cardiovascular risk factors (NO/ET-1 balance, inflammation and oxidative stress)
Chapter 7	Traditional cardiovascular risk factors, GFR and emerging cardiovascular risk factors (metabolic syndrome and its components)

In order to assess the contribution of emerging cardiovascular risk factors on CF-PWV and FMD, the first multivariate analysis model was set to comprise only the traditional cardiovascular risk factors and GFR. Then the emerging cardiovascular risk factors were added to the multivariate anaylsis and presented in a separate model. The multiple coefficient of determination (r^2) of each model was reported. Multivariate analysis data were reported for both CF-PWV and FMD using standard

regression coefficient (*β* value) of independent variables. For all analyses, a p value of < 0.5 was considered significant.

For the reproducibility test (Chapter 3), 95% limits of agreement were used to assess the repeatability of the two measurements. This means that only 5% of the pairs of measurements on the same subject would fall outside of the 95% limit of agreement. Means and SDs of the differences between the two measurements were calculated and the 95% limit of agreement was defined as mean difference ± 2SDs. Reproducibility data was also presented graphically as plots of the difference between the two measurements against the mean of the measurements (Bland-Altman plots) (Bland *et al.*, 1986).

Chapter 3

Reproducibility

Two reproducibility studies were performed and they are considered separately in this chapter. The first study assessed the intra- and inter-observers reproducibility of PWV and AIx determined using the SphygmoCor system. The second study investigated the intra- and inter-observers reproducibility of the brachial artery FMD.

3.1 Reproducibility of carotid-femoral pulse wave velocity and central augmentation index

3.1.1 Background

Arterial stiffness is highly associated with cardiovascular disease and its risk factors (Cockcroft *et al.*, 1997; Nichols, 2005; Wilkinson *et al.*, 2000; Wilkinson *et al.*, 2002b; Yasmin *et al.*, 2004). Therefore, measuring or monitoring arterial stiffness may allow early identification of a subject at risk, to whom intervention might be of most benefit (Cockcroft *et al.*, 1997; Guerin, 2001). Two of the most widely used non-invasive techniques for measuring arterial stiffness are PWV and AIx, assessed using the SphymoCor system (Oliver *et al.*, 2003b). These techniques were previously shown to be highly reproducible (Wilkinson *et al.*, 1998). However, prior to applying the techniques to the series of clinical studies in this thesis, the reproducibility of the techniques should be tested to assure the validity of the measurements.

3.1.2 Aims and hypotheses

The aims of this study were to assess the reproducibility of CF-PWV and CAIx determined using the PWA technique. The hypotheses were that CF-PWV and CAIx were reproducible for one observer with repeated measurements, and for two separate observers.

3.1.3 Methods

3.1.3.1 Subjects

Healthy volunteers were recruited from staff at the Clinical Research Centre and medical students at the University of Edinburgh. The inclusion criteria were healthy males or females, aged 18 - 50 years. Subjects with a history of major cardiac, respiratory, neurological, diabetic, or renal disease were excluded. None of them were on any medication.

3.1.3.2 Study protocol

CF-PWV and CAIx were measured using the PWA technique, after 20 minutes of supine rest. Two observers made two measurements of each, in a random order. First, CAIx, was measured, followed by CF-PWV. For CF-PWV, each observer recorded a surface distance independently.

3.1.3.3 Data analyses

Values are presented as mean ± SD (range). A paired Student's *t*-test was used to compare differences in the measurements between observers 1 & 2. Intra- and inter-observer variabilities were reported as mean difference ± SD of the difference and the reproducibility was also presented graphically as Bland-Altman plots (plots of the difference between the two measurements against the mean of the measurements). 95% limit of agreement (mean difference ± 2SDs of the difference) was used to assess the reproducibility of the measurements within the graph (Bland *et al.*, 1986). If two measurements are reproducible, the mean difference should be close to zero and all data should be within 2SDs of the difference.

3.1.4 Results

17 healthy subjects (male/female = 6/11), aged 28±7 years (range 21 – 40 years), were recruited. Baseline SBP and DBP were 105±10 and 60±1 mmHg, respectively.

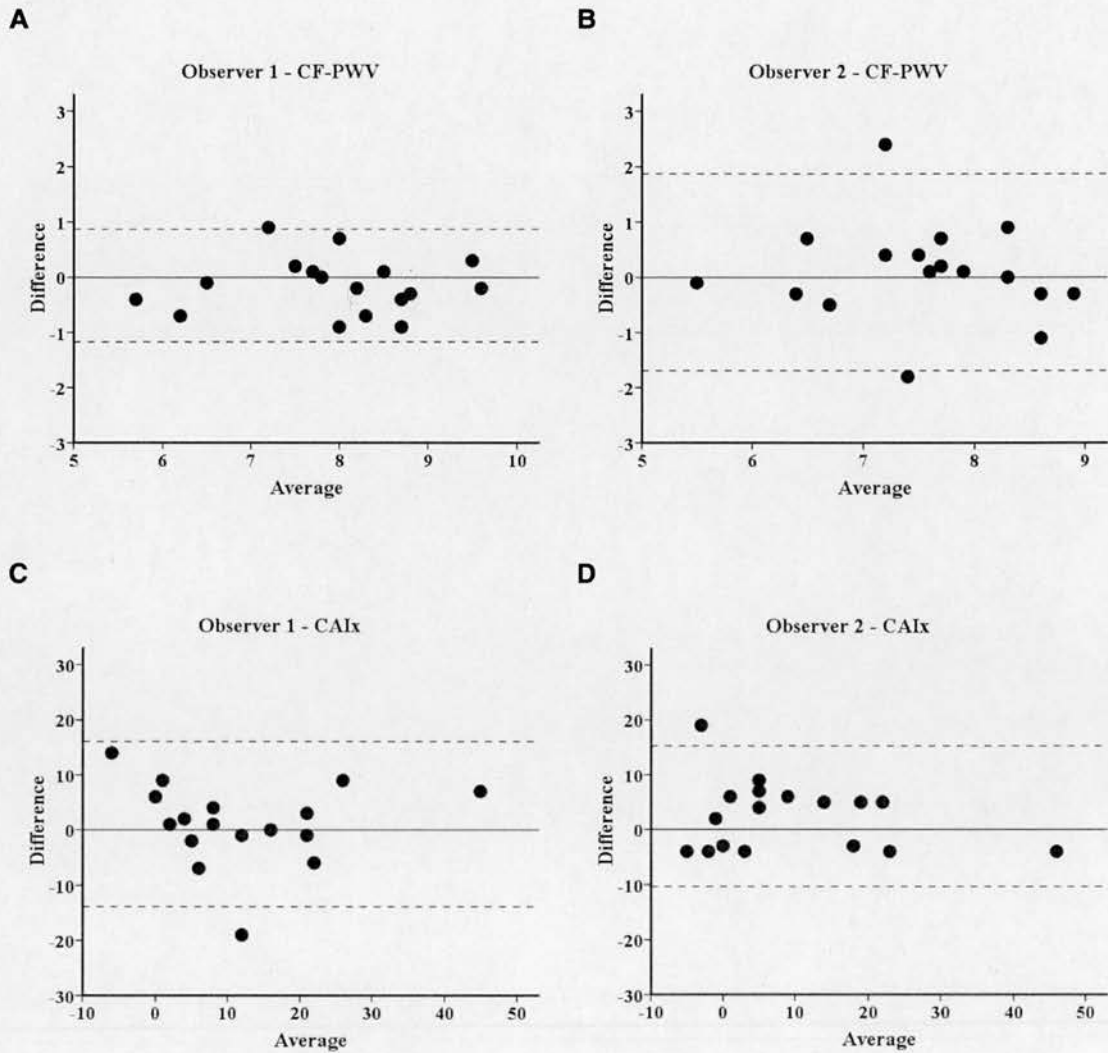
3.1.4.1 Intra-observer reproducibility

There were no differences between the means of the 1st & 2nd CF-PWV and CAIx measurements for both observers (Table 3.1). The intra-observer variability for CF-PWV was -0.2±0.5 m/s (95% limit of agreement: -1.2 to 0.9) and 0.1±0.9 m/s (95% limit of agreement: -1.7 to 1.9) for observer 1 and observer 2, respectively. The intra-observer variability for CAIx was 1±8 % for observer 1 (95% limit of agreement: -14 to16) and 2±6 % for observer 2 (95% limit of agreement: -10 to 15). Bland-Altman plots showed that most of CF-PWV and CAIx measured by both observers were within 95% limit of agreement (Figures 3.1 A-D).

Table 3.1 CF-PWV and CAIx measured by 2 observers.

	CF-PWV (m/s)		CAIx (%)	
	Observer 1	**Observer 2**	**Observer 1**	**Observer 2**
1st measurement	7.9±1.1	7.6±1.0	12±13	11±13
	(5.5 - 9.6)	(5.4 - 8.8)	(2 - 49)	(-7 - 45)
2nd measurement	8.0±1.1	7.5±1.0	11±13	8±14
	(5.9 - 9.7)	(5.6 - 9.2)	(-13 - 42)	(-13 - 48)

Figure 3.1 Bland-Altman plots presenting intra-observer reproducibility of CF-PWV and CAIx.



Key: Dotted lines represent the 95% limit of agreement (mean difference ± 2SDs of the difference).

3.1.4.2 Inter-observer reproducibility

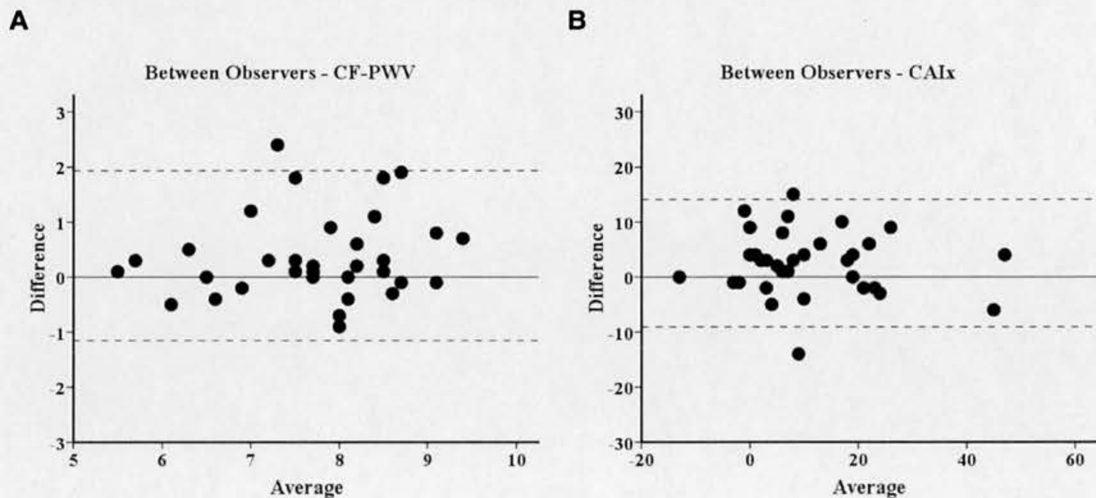
The pooled data of each observer was analysed for inter-observer reproducibility. Overall, CF-PWV and CAIx measurements of observer 1 were higher than those of observer 2 (Table 3.2). The inter-observer variability for CF-PWV was -0.4±0.8 m/s (95% limit of agreement: -1.2 to 1.9). The inter-observer variability for CAIx was 3±6 % (95% limit of agreement: -9 to 14). The Bland-Altman plots showed that most data was within 95% limit of agreement (Figures 3.2 A&B).

Table 3.2 Pooled CF-PWV and CAIx measured by 2 observers.

	CF-PWV (m/s)*	CAIx (%)*
Observer 1	7.9±1.1 (5.5 - 9.7)	12±13 (-13 - 49)
Observer 2	7.5±1.0 (5.4 - 9.2)	9±13 (-13 - 48)

Key: *$p < 0.01$ observer 1 *versus* observer 2.

Figure 3.2 Bland-Altman plots presenting inter-observers reproducibility of CF-PWV and CAIx.



Key: Dotted lines represent the 95% limit of agreement (mean difference ± 2SDs of the difference).

3.1.5 Discussion

Main findings: The findings indicate that CF-PWV and CAIx determined by the PWA technique are reproducible.

The reproducibility shown in this study is in accordance with previous reports on healthy subjects (Crilly *et al.*, 2007; Liang *et al.*, 1998; Papaioannou *et al.*, 2007; Paul *et al.*, 2009; Siebenhofer *et al.*, 1999; Wilkinson *et al.*, 1998), renal patients (Frimodt-Moller *et al.*, 2008; Savage *et al.*, 2002) and patients with cardiovascular disease (Adji *et al.*, 2006; Papaioannou *et al.*, 2004; Paul *et al.*, 2009; Wilkinson *et al.*, 1998).

In several previous studies, reproducibility of the measurements was reported as a coefficient of variation (CV). However, it has been demonstrated that CV can be misleading and is, therefore, a less satisfactory method for assessing reproducibility (Bland *et al.*, 1986). The reproducibility reported as SD of the difference between the paired measurements and the Bland-Altman plots which were used in this study are more reliable (Bland *et al.*, 1986). Table 3.3 summarises reproducibility data from previous studies which use SD of the difference to evaluate reproducibility. From this data, it is clear that the reproducibility of PWV in the current study is better than others.

Of note, in this study, PWV assessed using the PWA technique proved to be more reproducible than AIx for either the inter- or intra-observer measurements. This is in contrast to data from a previous study in which PWV was reported to be less reproducible than AIx (Wilkinson *et al.*, 1998), but in keeping with recent findings from a study in CKD patients (Frimodt-Moller *et al.*, 2008). The higher variability of AIx seen in this study could probably be explained by the variability in the quality of the pressure waveforms obtained between the two observers.

Summary: PWV and AIx obtained by the PWA technique are reproducible and, with well-trained individuals, are suitable to be incorporated into large-scale clinical studies as simple, non-invasive measurements of arterial stiffness.

Table 3.3 Intra- and inter-observer reproducibility of PWV and AIx, reported as SD of the difference.

Studies	Intra-observer reproducibility	Inter-observer reproducibility
PWV (m/s)		
This study: healthy subjects	***Observer 1: 0.5***	***0.8***
	Observer 2: 0.9	
(Wilkinson *et al.*, 1998): healthy subjects and patients with hypertension and hypercholesterolaemia	1.2	1.3
(Frimodt-Moller *et al.*, 2008): CKD stages 3 - 5	Observer 1: 3.9	3.2
	Observer 2: 3.6	
AIx (%)		
This study: healthy subjects	***Observer 1: 8.0***	***6.0***
	Observer 2: 6.0	
(Wilkinson *et al.*, 1998): healthy subjects and patients with hypertension and hypercholesterolaemia	5.4	3.8
(Siebenhofer *et al.*, 1999): healthy subjects	-	6.4
(Savage *et al.*, 2002): CKD stages 1 – 5, dialysis, and renal transplant patients	4.0	3.0
(Papaioannou *et al.*, 2004): patients with cardiogenic shock and acute myocardial infarction	5.8	-
(Crilly *et al.*, 2007): healthy subjects	Observer 1: 2.7	2.0
	Observer 2: 3.7	
(Frimodt-Moller *et al.*, 2008): CKD stages 3 - 5	Observer 1: 18.8	15.8
	Observer 2: 10.6	

Key: AIx: augmentation index; PWV: pulse wave velocity.

3.2 Reproducibility of brachial artery flow-mediated dilatation

3.2.1 Background

Endothelial dysfunction is involved in the pathogenesis of a vascular disease (Glasser *et al.*, 1996). Brachial artery FMD is a widely used non-invasive measure of the endothelial-dependent vasomotor function of a conduit artery (Corretti *et al.*, 2002). This technique uses a shear stress from the flow of blood as a physiological stimulus for NO generated vasodilatation. Impaired FMD is associated with atherosclerosis and cardiovascular disease risk factors (Brunner *et al.*, 2005; Celermajer *et al.*, 1992). Although FMD was shown to be reproducible in some studies (Bots *et al.*, 2005; Donald *et al.*, 2006; Simova *et al.*, 2008), the limitations of FMD are the variability among study centres in both the procedural technique and the analysis of an ultrasound image. Therefore, the reproducibility of FMD was assessed to assure the validity of the measurements in the series of clinical studies presented in this thesis.

3.2.2 Aims and hypotheses

The aim of this study was to assess the reproducibility of the brachial artery FMD in CKD patients. The hypothesis was that FMD was reproducible for one observer with repeated measurements, and for two separate observers.

3.2.3 Methods

3.2.3.1 Subjects

Patients were sub-recruited from a study to investigate arterial stiffness and endothelial dysfunction in CKD. The inclusion and exclusion criteria for the patients was fully described in Chapter 2, section 2.1.1.

3.2.3.2 Study protocol

The brachial artery FMD was recorded after 20 minutes of supine rest. Two observers performed FMD measurements on the same subjects but on different days. One observer repeated FMD measurements on the same subjects 2 hours after the first measurements were made, in order to assess intra-observer reproducibility.

3.2.3.3 Data analyses

Values are presented as mean ± SD (range). A paired Student's *t*-test was used to compare the differences in the measurements between observers 1 & 2. Intra- and inter-observer variability was reported as the mean difference ± SD of the difference and the reproducibility was also presented graphically as the Bland-Altman plots (plots of the difference between the two measurements against the mean of the measurements). 95% limit of agreement (mean difference ± 2SDs of the difference) was used to assess reproducibility of the measurements within the graph (Bland *et al.*, 1986). If two measurements are reproducible, the mean difference should be close to zero and all data should be within 2SDs of the difference.

3.2.4 Results

Sixteen CKD patients (male/female = 13/3) with eGFR of 50 ± 24 ml/min/1.73m^2 (range: 22 – 108 ml/min/1.73m^2) were enrolled onto the study. Age was 44 ± 10 years. SBP and DBP were 118 ± 11 and 76 ± 9 mmHg, respectively.

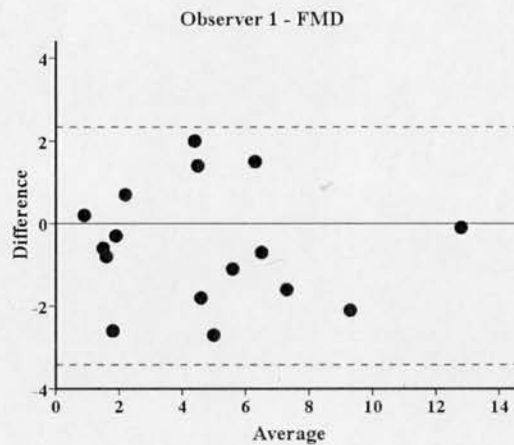
3.2.4.1 Intra-observer reproducibility

There were no differences between the means of the first and second FMD measured by the same observer (Table 3.4). The intra-observer variability for FMD was -0.5 ± 1.4 m/s (95% limit of agreement: -3.4 to 2.3). The Bland-Altman plots showed that data was within 95% limit of agreement (Figure 3.3).

Table 3.4 FMD measured by observer 1 at 0 and 2 hours.

	FMD (%)
1st measurement (at 0 hour)	4.5 ± 3.2 (0.6 – 12.7)
2nd measurement (at 2 hour)	5.0 ± 3.3 (0.8 – 12.8)

Figure 3.3 Bland-Altman plot presenting intra-observer reproducibility of FMD.



Key: Dotted lines represent the 95% limit of agreement (mean difference ± 2SDs of the difference).

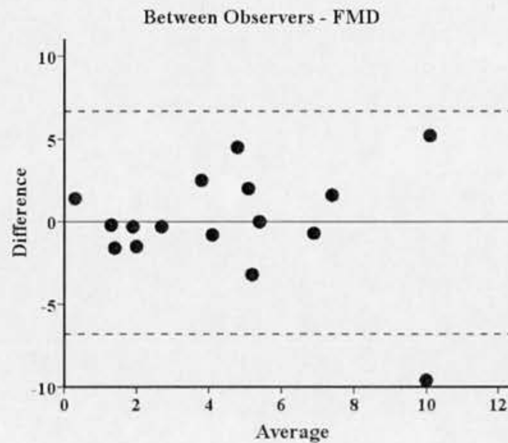
3.2.4.2 Inter-observer reproducibility

There were no differences between the FMD measured by observer 1 & 2 (Table 3.5). The inter-observer variability for FMD was -0.1±3.4 m/s (95% limit of agreement: -6.8 to 6.7). The Bland-Altman plots showed that most data was within 95% limit of agreement (Figure 3.4).

Table 3.5 FMD measured by observer 1 and 2.

	FMD (%)
Observer 1	4.5±3.2 (0.6-12.7)
Observer 2	4.6±3.6 (-0.4–14.8)

Figure 3.4 Bland-Altman plot presenting inter-observer reproducibility of FMD.



Key: Dotted lines represent the 95% limit of agreement (mean difference ± 2SDs of the difference).

3.2.5 Discussion

Main findings: The findings indicate that the brachial artery FMD is a reproducible technique to assess endothelial function in CKD patients.

Evidence has shown that the brachial artery FMD technique is a reproducible non-invasive technique for assessing endothelial vasomotor function. These include studies on healthy subjects (De Roos *et al.*, 2003; Donald *et al.*, 2006; Donald *et al.*, 2008; Hardie *et al.*, 1997; Harris *et al.*, 2006; Liang *et al.*, 1998; Malik *et al.*, 2004; Sorensen *et al.*, 1995), overweight subjects (Harris *et al.*, 2007), and patients with cardiovascular disease (Simova *et al.*, 2008). Unfortunately, most previous studies reported their reproducibility data in CV, with a wide range of CV from very low (1.8%) (Sorensen *et al.*, 1995) to very high (50.3%) (De Roos *et al.*, 2003), and that

data, therefore, cannot be compared directly to the findings from this study. Moreover, as discussed in section 3.1.5 of this Chapter the reproducibility presented in SD of the difference between paired measurements and the Bland-Altman plots as used in this study are more reliable than CV (Bland *et al.*, 1986). Table 3.6 presents reproducibility data from previous studies that use SD of the difference to evaluate reproducibility. Here, the reproducibility data of the current study is comparable to others.

Table 3.6 Intra- and inter-observer reproducibility of FMD, reported as SD of the difference.

Studies	Intra-observer reproducibility	Inter-observer reproducibility
FMD (%)		
This study: CKD patients	***1.4***	***3.4***
(Hardie *et al.*, 1997): healthy subjects	2.1	2.2
(Donald *et al.*, 2008): healthy subjects	1.1	-
(Simova *et al.*, 2008): patients with cardiovascular disease	1.2	1.6

Key: FMD: flow-mediated dilatation.

Summary: This study has demonstrated that FMD is a reproducible technique for assessing endothelial vasomotor function in CKD patients and it is suitable to be applied to future clinical studies.

Chapter 4

Plasma endothelin-1 and fractional excretion of endothelin-1 in chronic kidney disease

4.1 Introduction

The ETs are a family of peptides consisting of 21 amino acid residues first identified in 1988 (Yanagisawa *et al.*, 1988). ET-1 is produced both within the vascular endothelial cells (Dhaun *et al.*, 2006) and is the principal endogenous vasoconstrictor among the ET family (Yanagisawa *et al.*, 1988). Todate, it is now recognised that vascular smooth muscle cells, macrophages, leucocytes, cardiomyocytes and fibroblasts are all capable of ET-1 production (Kirkby *et al.*, 2008). Direct actions of ET-1 on vascular smooth muscle cell are contraction, proliferation, and migration of vascular cells (Kirkby *et al.*, 2008). Several clinical studies have demonstrated the role of ET-1 in the control of vascular tone, at least in healthy subjects (Haynes *et al.*, 1994) and hypertension (Cardillo *et al.*, 1999; Taddei *et al.*, 1999). ET-1, abluminally released from endothelial cells, acts primarily as a local autocrine and paracrine substance. Thus, circulating levels of ET-1 represent an overflow of tissue-bound ET-1. This means that plasma ET-1 concentrations are not a reliable measure of vascular ET-1 production in the vasculature (Haynes *et al.*, 1998).

ET-1 is also produced within the kidney. Tubular epithelial cells, mesangial cells and podocytes are capable of ET-1 production (Kohan, 1997). Infact, the kidney contains the highest concentration of ET-1 compared to any other organs. Importantly, evidence suggest that vascular and renal ET-1 are two independent systems and urinary ET-1 may represent ET-1 renal production (Benigni *et al.*, 1991).

Clearance of ET-1 occurs largely in the lungs by endocytosis and degradation of the ET_B receptor-ligand complex (Dupuis *et al.*, 1996). However, 10% of circulating ET-1 is removed from the human circulation via the kidneys (Gasic *et al.*, 1992) either by glomerular filtration or enzymatic degradation such as neutral endopeptidase, the enzyme located in the brush border vesicle of the proximal tubule (Abassi *et al.*, 1992). The renal ET-1 clearance was also indicated by a study in rats where rats with bilateral nephrectomy had impaired exogenous ET-1 removal (Shi *et al.*, 1994).

In renal patients, studies have shown that plasma ET-1 concentrations are increased in dialysis (Blazy *et al.*, 1994; Demuth *et al.*, 1998; Deray *et al.*, 1992; Koyama *et*

al., 1989; Mallamaci *et al.*, 1993; Miyauchi *et al.*, 1991; Saito *et al.*, 1991; Totsune *et al.*, 1989; Vlassopoulos *et al.*, 1995; Warrens *et al.*, 1990) and pre-dialysis patients (Blazy *et al.*, 1994; Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; Koyama *et al.*, 1989; Mallamaci *et al.*, 1993; Saito *et al.*, 1991; Vlachojannis *et al.*, 1997; Vlassopoulos *et al.*, 1995; Warrens *et al.*, 1990). Studies also showed increased urinary ET-1 excretion in CKD (Cantaro *et al.*, 2001; Goddard *et al.*, 2007; Ohta *et al.*, 1991). In addition, some studies, but not all (Vlachojannis *et al.*, 1997; Warrens *et al.*, 1990), suggest that plasma ET-1 concentrations correlate with renal function (Blazy *et al.*, 1994; Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; Goddard *et al.*, 2007; Mallamaci *et al.*, 1993). A recent study within the Clinical Research Centre, University of Edinburgh, has demonstrated increases in plasma and urinary ET-1 concentrations across a range of GFR. Interestingly, this study also showed a rise in FeET-1 as GFR fell (Goddard *et al.*, 2007). As previously mentioned that vascular and renal ET-1 are two independent systems. Assessment of a fractional excretion of ET-1 (FeET-1) would help understand this hypothesis. FeET-1 is a measure of the amount of ET-1 that leaves the body through urine compared to the amount of ET-1 filtered and reabsorbed by the kidney. However, this and other studies were performed on relatively small numbers of subjects and some were confounded by co-morbidities (Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; Koyama *et al.*, 1989; Ohta *et al.*, 1991; Vlachojannis *et al.*, 1997; Warrens *et al.*, 1990).

Based on the previous work, it is worth exploring the relationships between plasma and urinary ET-1 concentrations, FeET-1 and GFR in a larger group of CKD patients.

4.1.1 Aims and hypotheses

The aims of this study were to investigate the relationship of plasma and urinary ET-1 concentrations to renal function in a group of CKD patients with minimal co-morbidity from CKD stage 1 to pre-dialysis. The hypotheses were that, as a result of reduced renal clearance and increased renal production, plasma and urinary ET-1 concentrations would substantially increase as renal function declined.

4.2 Methods

4.2.1 Subjects

Subjects were recruited from the renal outpatients clinic at the Royal Infirmary of Edinburgh. Subjects were categorised into 5 groups on the basis of the K/DOQI classification (Anonymous, 2002a). The aim was to recruit around 30 subjects in each of the CKD stages 1 to 3, and 30 subjects in stages 4 and 5 combined. Around 30 age-matched healthy volunteers were also enrolled as a control group. The inclusion and exclusion criteria were as previously described in Chapter 2, section 2.1.1.

4.2.2 Study design and protocol

This was a prospective, cross-sectional study. Subjects refrained from alcohol for at least 24 hours and caffeinated drinks, food and smoking for at least 12 hours before the study. Subjects were asked to withhold their current medications on the morning of the study. All studies were conducted in a quiet, temperature-controlled room. Following a brief medical inquiry, the body weight and height of the subjects were recorded. After 30 minutes of supine rest, participants underwent BP measurements. Blood samples were then taken and urine was collected and tested for plasma and urinary ET-1, respectively. All sample collection procedures and assays for plasma and urinary ET-1 were performed as described in Chapter 2.

4.2.3 Data analyses

Descriptive data are given as mean ± SD. FeET-1 was calculated by the equation: FeET-1 (%) = [(urine ET-1 / plasma ET-1) / (urine creatinine / plasma creatinine)] x 100. Means were compared by the unpaired Student's *t*-test and one-way ANOVA where appropriate. Correlation coefficients were calculated using the Pearson method. A significant level was p value < 0.05.

4.3 Results

One hundred and forty two subjects took part in the study (115 CKD and 27 matched non-renal controls). Causes of CKD were autosomal dominant polycystic kidney disease (n = 26), IgA nephropathy (n = 24), reflux nephropathy (n = 11), chronic glomerulonephritis (n = 10), noninflammatory glomerular disease (n = 8), obstructive nephropathy (n = 5), thin basement membrane disease (n = 2), cystinuria (n = 2), Alport disease (n =1), medullary cystic kidney (n = 1), and CKD with unknown cause (n = 25).

The characteristics of the subjects are given in Table 4.1. Age, BMI, and BP were comparable between CKD and non-CKD subjects. CKD patients had higher concentrations of plasma ET-1, big ET-1, and FeET-1 than of non-CKD subjects. There was no difference in urinary ET-1 concentrations between CKD and non-CKD subjects.

Plasma ET-1 and plasma big ET-1 linearly correlated with eGFR (Table 4.2, Figures 4.1 A & B). FeET-1 increased exponentially as eGFR declined (Table 4.2, Figure 4.1C). These correlations hold true even after adjustment for age, SBP, DBP, gender, total cholesterol, plasma glucose, and BMI. While plasma ET-1 and big ET-1 did not correlate with age, SBP, DBP, and gender, FeET-1 positively correlated with SBP ($r^2 = 0.04$, $p < 0.05$). Men had higher FeET-1 than women (3.0 ± 3.3 *versus* 2.0 ± 2.2 %, $r^2 = 0.03$, $p < 0.05$).

Table 4.1 Subject characteristics.

	Non-CKD	CKD
n	27	115
Age (year)	48±9 (32-64)	47±10 (23-65)
Gender: Male/Female (n)	13/14	77/38
Body mass index (kg/m^2)	26±5 (18-46)	28±5 (19-41)
Systolic blood pressure (mmHg)	110±17 (83-152)	119±15 (85-159)
Diastolic blood pressure (mmHg)	70±10 (54-90)	74±9 (52-96)
Creatinine (mmol/L)*	78±13 (55-98)	193±164 (55-825)
Estimated glomerular filtration rate ($ml/min/1.73m^2$)*	94±18 (68-131)	63±35 (8-154)
Glucose (mmol/L)	5.0±0.6 (3.5-5.9)	5.0±0.5 (2.5-6.5)
Total cholesterol (mmol/L)**	4.9±0.8 (3.4-6.5)	4.6±0.7 (3.0-8.2)
Plasma ET-1 (pg/ml)*	4.6±1.0 (2.8-7.5)	5.5±1.1 (3.4-9.8)
Plasma big ET-1 (pg/ml)**	42±16 (25-80)	62±41 (17-320)
Urinary ET-1 (pg/ml)	5.7±2.9 (1.4-11.3)	6.6±3.8 (0.9-36.1)
FeET-1 (%)*	1.1±0.7 (0-3.3)	3.0±3.1 (0.2-14.7)

Key: Values are mean±SD (range). *$p < 0.01$, **$p < 0.05$ for the unpaired Student's *t*-test. ET-1: endothelin-1; FeET-1: fractional excretion of ET-1.

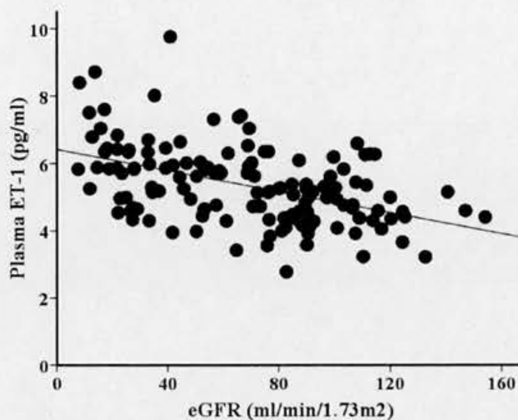
Table 4.2 Plasma and urinary ET-1 at different eGFRs.

	Estimated glomerular filtration rate (ml/min/1.73m^2)		
	< 30	30-60	> 60
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)
Plasma big ET-1 (pg/ml)*	73±58 (24-320)	73±39 (28-188)	48±26 (17-178)
Urinary ET-1 (pg/ml)	6.8±2.4 (3.0-13.2)	7.2±5.8 (2.6-36.1)	6.1±2.8 (0.9-13.4)
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)

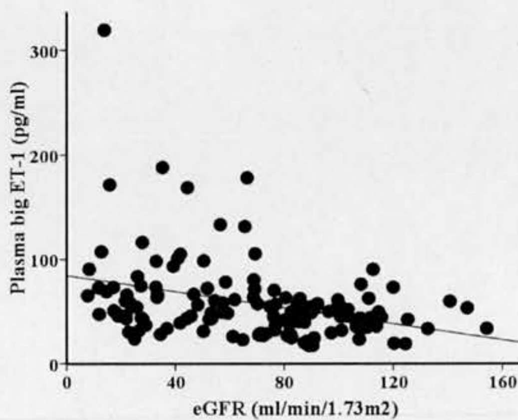
Key: *$p < 0.01$ for one-way ANOVA by estimated glomerular filtration rate groups. ET-1: endothelin-1; FeET-1: fractional excretion of ET-1.

Figure 4.1 Relationships of eGFR to plasma ET-1, big ET-1, and FeET-1.

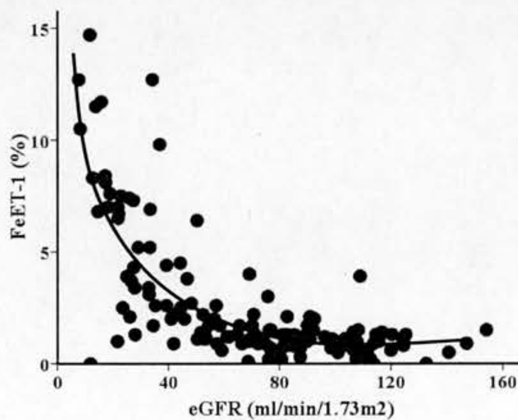
A **$r^2 = 0.22, p < 0.01$**



B **$r^2 = 0.12, p < 0.01$**



C **$r^2 = 0.47, p < 0.01$**



4.4 Discussion

4.4.1 Main findings

In a large cohort of CKD patients without dialysis or established cardiovascular co-morbidity, plasma ET-1 increases linearly as renal function declines, whereas FeET-1 increases exponentially.

The finding that plasma ET-1 concentrations are increased in CKD patients is in keeping with results from previous studies on dialysis (Blazy *et al.*, 1994; Demuth *et al.*, 1998; Deray *et al.*, 1992; Koyama *et al.*, 1989; Mallamaci *et al.*, 1993; Miyauchi *et al.*, 1991; Saito *et al.*, 1991; Totsune *et al.*, 1989; Vlassopoulos *et al.*, 1995; Warrens *et al.*, 1990) and pre-dialysis patients (Blazy *et al.*, 1994; Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; Koyama *et al.*, 1989; Mallamaci *et al.*, 1993; Saito *et al.*, 1991; Vlachojannis *et al.*, 1997; Vlassopoulos *et al.*, 1995; Warrens *et al.*, 1990). Selected previous studies in which the CKD stages were well defined were presented in Table 4.3 and plasma ET-1 concentrations were compared to those of this study. The levels of plasma ET-1 in the current study are comparable to most of the studies shown in Table 4.3.

In addition, this study demonstrates a clear picture of an inverse linear correlation between plasma ET-1 and renal function, across a wide range of GFR, in a homogenous group of non-diabetic, low co-morbid CKD population that is consistent with others (Blazy *et al.*, 1994; Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; Goddard *et al.*, 2007; Mallamaci *et al.*, 1993). The correlation between plasma ET-1 and plasma big ET-1 as well as the association between plasma big ET-1 and GFR is consistent with a previous report (Miyauchi *et al.*, 1991) and could be explained by the fact that plasma big ET-1 is an intermediate molecule in the synthesis of active ET-1 (Gray *et al.*, 1996). These data support increased plasma ET-1 concentration either by increased renal or extra renal ET-1 production (from increased levels of big ET-1 seen here) or reduced renal filtration of ET-1 and thus renal clearance.

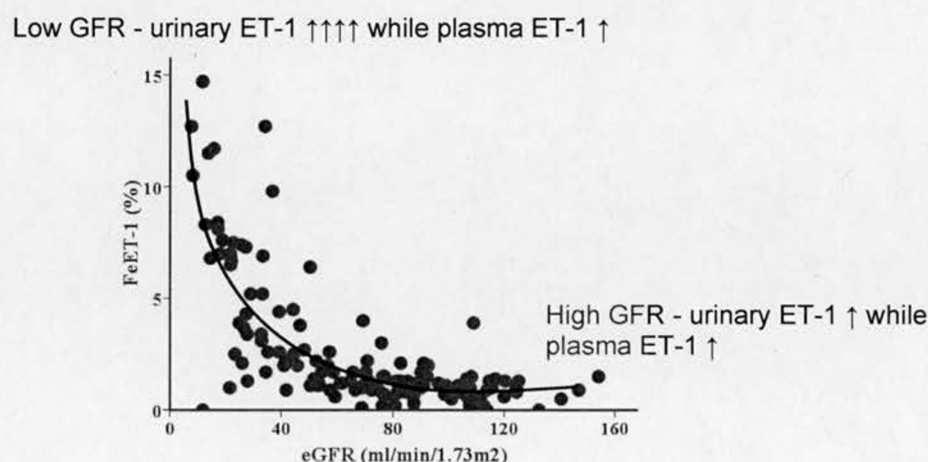
Table 4.3 Comparing plasma ET-1 and FeET-1 with data from previous studies in pre-dialysis CKD.

<table>
<tr><th>Studies</th><th>Control</th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Stage 4</th><th>Stage 5</th></tr>
<tr><td colspan="7">Plasma ET-1 (pg/ml)</td></tr>
<tr><td>This study</td><td>4.6</td><td colspan="2">← 5.1 →</td><td>5.8</td><td colspan="2">← 6.3 →</td></tr>
<tr><td>(Cottone et al., 2009)</td><td>100</td><td colspan="2">← 3.3 →</td><td>3.9</td><td>4.5</td><td>5.7</td></tr>
<tr><td>(Dammers et al., 2005)</td><td>1.9</td><td>-</td><td>-</td><td>-</td><td>-</td><td>14.9</td></tr>
<tr><td>(Goddard et al., 2007)</td><td>4.2</td><td>-</td><td colspan="4">← 5.6 →</td></tr>
<tr><td>(Vlachojannis et al., 1997)</td><td>29</td><td>54</td><td>-</td><td>-</td><td>60</td><td>-</td></tr>
<tr><td colspan="7">FeET-1 (%)</td></tr>
<tr><td>This study</td><td>1.1</td><td colspan="2">1.1</td><td>3.2</td><td colspan="2">6.6</td></tr>
<tr><td>(Goddard et al., 2007)</td><td>3.5</td><td>-</td><td colspan="4">← 15.2 →</td></tr>
<tr><td>(Vlachojannis et al., 1997)</td><td>10.2</td><td>12.0</td><td>-</td><td>-</td><td>64.0</td><td>-</td></tr>
</table>

Furthermore, this study shows an exponential rise in FeET-1 as GFR falls which is consistent with previous reports (Table 4.3) (Goddard *et al.*, 2007; Vlachojannis *et al.*, 1997). This important observation supports the view that there are two independently acting ET systems in the systemic circulation and kidney as shown in animal models (Benigni *et al.*, 1991; Brooks *et al.*, 1991; Wesson, 2001). Benigni, *et al* performed isotope studies on rats and found that plasma ET-1 does not account for urinary ET-1 (Benigni *et al.*, 1991). Brook, *et al* has demonstrated that plasma ET-1 concentrations were similar between rats with chronic renal failure and control rats, but there was an increased urinary ET-1 excretion in rats with chronic renal failure, likely reflecting increased renal production (Brooks *et al.*, 1991). Later, Wesson, *et al.* found that in rats, renal cortical interstitial ET-1 levels correlate with urinary ET-

1 excretion (Wesson, 2001). This data suggests that urinary ET-1 concentrations represent renal ET-1 production. FeET-1 is theoretically a measure of the amount of ET-1 that leaves the body through urine compared to the amount of ET-1 filtered and reabsorbed by the kidney. Although evidence has shown that ET-1 is also cleared renally, this is not the major route of ET-1 elimination and thus a substantial increase in FeET-1 in a declining GFR may be more influenced by a marked increase in renal ET-1 production (Figure 4.2).

Figure 4.2 FeET-1 *versus* GFR represents that increased urinary ET-1 could be a result of a reduction in ET-1 renal clearance or an increase in ET-1 renal production.



Key: ↑↑↑↑: marked increased, ↑: increased.

However, the results also show a positive correlation between BP and FeET-1 and increasing BP as renal function declines, which may be one of the factors for increased renal ET-1 production. Moreover, in this study, men had higher FeET-1 than women suggesting that gender may influence renal ET-1 production.

4.4.2 Limitations

As this was a cross-sectional study, the causal relationships behind the associations described cannot be fully defined. Urinary ET-1 was measured from spot urine, and therefore urinary ET-1 clearance cannot be calculated.

4.4.3 Summary

In CKD patients with GFRs ranging between 8 and 154 ml/min/1.73m^2, plasma ET-1 increases in a linear fashion, whereas FeET-1 increases exponentially as GFR declines. These findings support the view that the upregulation of renal ET-1 generation plays an important role in renal pathophysiology, and therapeutic strategies such as ET-1 antagonists may offer benefits in CKD.

Chapter 5

Relationships of renal function to arterial stiffness and endothelial dysfunction in chronic kidney disease

5.1 Introduction

Increased arterial stiffness, as measured by PWV, is a common feature of CKD (Guerin *et al.*, 2008), a recognised marker of cardiovascular risk (Blacher *et al.*, 1999a; Guerin *et al.*, 2008), and an independent predictor of mortality and survival in dialysis patients (Blacher *et al.*, 1999a; Guerin, 2001). The endothelium is an important regulator of arterial stiffness (Wilkinson *et al.*, 2004), and endothelial dysfunction is also a common feature of CKD (Endemann *et al.*, 2004; van Guldener *et al.*, 1998; van Guldener *et al.*, 1997) and a predictor of cardiovascular disease (Perticone *et al.*, 2001).

Most studies of arterial stiffness and endothelial dysfunction in CKD involve dialysis patients, or those with complex co-morbidity, including diabetes mellitus and established cardiovascular disease, which are themselves associated with arterial stiffening and endothelial dysfunction (Blacher *et al.*, 1999a; Guerin, 2001; van Guldener *et al.*, 1998; van Guldener *et al.*, 1997). Evidence suggests an increase in arterial stiffness and endothelial dysfunction in the early stages of CKD with progression as GFR falls (Thambyrajah *et al.*, 2000; Wang *et al.*, 2005). However, these and other such studies again include patients with conventional cardiovascular risk factors as well as major cardiovascular co-morbidity (Annuk *et al.*, 2001; Matsuda *et al.*, 2009; Thambyrajah *et al.*, 2000; Wang *et al.*, 2005). Hence, the contribution of uraemia to arterial stiffness and endothelial dysfunction in CKD remains unclear.

5.1.1 Aims and hypotheses

The aims of this study were to investigate the relationship of arterial stiffness and endothelial dysfunction to renal function in a group of CKD patients with minimal co-morbidity from CKD stage 1 to pre-dialysis.

Hypotheses:

1. Arterial stiffness and endothelial dysfunction would worsen as renal function declines.

2. Renal function would predict arterial stiffness and endothelial dysfunction, independent of conventional risk factors such as age and BP.

5.2 Methods

5.2.1 Subjects

Subjects were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh. Subjects were categorised into 5 groups on the basis of the K/DOQI classification (Anonymous, 2002a). The aim was to recruit around 30 subjects in each of the CKD stages 1 to 3, and 30 subjects in stages 4 and 5 combined. Around 30 age-matched healthy volunteers were also enrolled as a control group. The inclusion and exclusion criteria were as previously described in Chapter 2, section 2.1.1.

5.2.2 Study design and protocol

The study was a prospective, cross-sectional study. Subjects refrained from alcohol for at least 24 hours and caffeinated drinks, food and smoking for at least 12 hours before the study. Subjects were asked to withhold their current medications on the morning of the study. All studies were conducted in a quiet, temperature-controlled room. After 30 minutes of supine rest, participants underwent BP measurements and blood samples were taken. Then CAIx, CF-PWV, FMD, and PWA techniques for measuring endothelial function were made, in that order. All techniques were performed as described in Chapter 2, sections 2.5.1, 2.5.2, 2.6.1, and 2.6.2, respectively.

5.2.3 End points

Primary end points were arterial stiffness measured by CF-PWV and endothelial dysfunction measured by FMD (percentage change from baseline: FMD (%)), and renal function as an independent determinant of CF-PWV and FMD. Secondary end

points were arterial wave reflection measured by CAIx and endothelial dysfunction measured by maximal changes in CAIx after salbutamol administration.

5.2.4 Data analyses

Descriptive data is given as mean ± SD. Means of the subject characteristics of all CKD stages were compared using one-way ANOVA. Means of CF-PWV and FMD were compared using one-way ANOVA with Bonferroni's correction for multiple comparisons. Correlation coefficients for univariate analysis were calculated using the Pearson method. Multivariate analysis was performed using a stepwise linear regression. Dependent variables were CF-PWV, CAIx, and FMD. Independent variables were age, gender, MAP, smoking status, plasma glucose, total cholesterol and GFR. The multiple coefficient of determination (r^2) of each model was reported. Multivariate analysis data were reported for CF-PWV, CAIx and FMD using standard regression coefficient (β value) of independent variables. A significant level was p value < 0.05.

5.3 Results

5.3.1 Subject characteristics

One hundred and thirty eight subjects were recruited. Causes of CKD and medication use in the study population and characteristics of the subjects are given in Tables 5.1 and 5.2 respectively (for full subject characteristics, see Appendix 1).

Table 5.1 Causes of CKD and medication use in the study population.

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Causes of chronic kidney disease (n)					
ADPKD	3	4	8	10	1
Congenital cystinuria	1	1	0	0	0
GN-unspecified	1	6	7	2	2
Hereditary nephritis	0	0	0	0	1
IgA nephropathy	4	9	7	3	1
Medullary cystic kidney	0	1	0	0	0
Obstructive nephropathy	2	1	1	1	0
Reflux nephropathy	2	1	3	3	2
TBM disease	2	0	0	0	0
Unknown	13	7	4	1	0
Medication (n)					
α-blockers	2	6	2	5	3
ACE inhibitors	6	15	22	8	4
ARBs	2	3	7	6	0
Aspirin	1	3	5	0	0
β-blockers	2	9	8	8	3
Calcium channel blockers	2	5	13	14	5
Diuretics	1	2	9	9	2
Statins	3	7	14	6	3

Key: ACE: angiotensin converting enzyme; ADPKD: autosomal dominant polycystic kidney disease; ARB: angiotensin receptor blocker; GN: glomerulonephritis; TBM: thin basement membrane.

Table 5.2 Subject characteristics.

	Controls	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
n	23	28	30	30	20	7
Gender: Male/Female (n)	13/10	17/11	18/12	22/8	16/4	4/3
Smoker: Yes/No (n)	2/21	9/19	5/25	6/24	4/16	0/7
Serum creatinine (mmol/L)	78±13	77±13	102± 21	177±5	364±105	632±181
eGFR (ml/min/1.73m^2)	97±19	108±17	77±9	45±9	23±4	12±3
Age (year)	47±8	43±11	49±9	51±10	45±9	51±12
SBP (mmHg)*	113±16	114±15	116±13	120±12	121±12	132±16
DBP (mmHg)	71±10	71±10	74±9	76±9	76±7	76±7
MAP (mmHg)	85±12	86±11	88±10	90±10	91±8	95±8
PP (mmHg)*	42±9	43±8	41±7	45±10	45±9	56±17
Heart rate (bpm)	58±7	63±8	62±10	60±8	58±9	63±13
BMI (kg/m^2)	26±6	29±5	28±4	28±6	28±5	25±7
Plasma glucose (mmol/L)	5.0±0.5	5.0±0.5	5.0±0.6	5.1±0.4	5.1±0.5	4.8±0.7
Total cholesterol (mmol/L)	4.7±0.9	4.9±0.8	4.7±1.0	4.8±0.8	4.5±0.8	4.4±0.8

Key: *$p < 0.05$ for one-way ANOVA by CKD stage. BMI: body mass index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; MAP: mean arterial pressure; PP: pulse pressure; SBP: systolic blood pressure.

5.3.2 Relationships to renal function

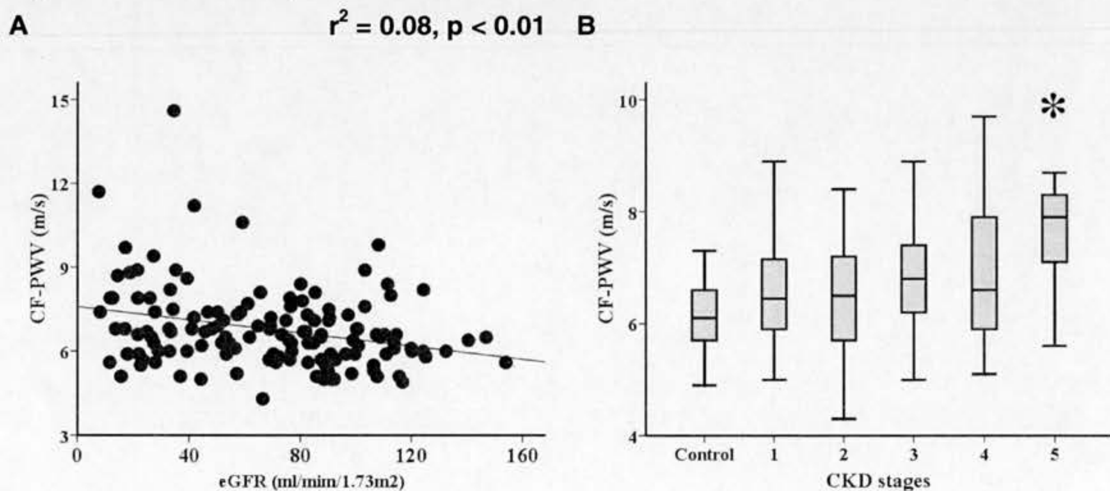
SBP and PP increased as renal function declined (Table 5.2). There was an inverse correlation between CF-PWV and eGFR (Figure 5.1A) with an incremental increase of CF-PWV with increasing CKD stages (Table 5.3, Figure 5.1B). Yet, RAIx, CAIx, and CAIx@HR75 had no relationship to eGFR (Table 5.3).

Table 5.3 Arterial stiffness and wave reflections.

	Controls	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CF-PWV (m/s)*	6.15±0.82	6.61±1.14	6.51±1.04	7.29±1.96	6.98±1.41	8.00±1.90
RAIx (%)	74±20	72±14	79±16	78±20	77±16	81±20
CAIx (%)	21±15	20±10	25±11	24±14	24±12	24±15
CAIx@HR75 (%)	13±14	13±11	18±19	16±13	15±11	19±19

Key: *p < 0.05 for one-way ANOVA. CAIx: central augmentation index; CAIx@HR75: central augmentation index at heart rate of 75 bpm; CF-PWV: carotid-femoral pulse wave velocity; RAIx: radial augmentation index.

Figure 5.1 Relationships of eGFR to CF-PWV.



Key: Figure B: *p < 0.01 for stage 5 *versus* control, stage 1 and 2.

In terms of FMD, baseline diameters of the brachial artery are not different across CKD stages (Table 5.4). FMD showed a positive correlation with eGFR (Figure 5.2A). When considered by stage, FMD only fell significantly in stage 5 (Table 5.4, Figure 5.2B). Whilst there was generally wide variability between patients in a given CKD stage, subjects with an eGFR <20 ml/min/1.73m^2 uniformly had an FMD of <1.5% (Figure 5.2A). A receiver operating characteristic (ROC) curve was plotted to identify the sensitivity and specificity of the GFR cut point. Although the specificity was very high (98%), the sensitivity of this GFR cut point was poor (57%) (see Appendix 2).

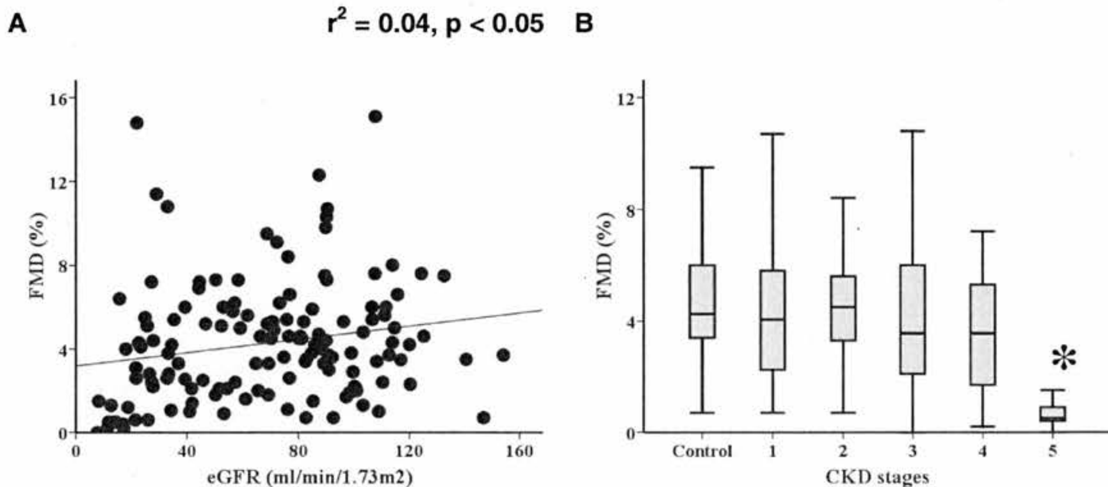
As expected, the GTN response was higher than the FMD response in all stages. Endothelium-independent vasodilatation by GTN did not show an association with eGFR, although the GTN response tended to be lower in stage 5 patients compared to the other groups (Table 5.4). The change in CAIx after inhaled salbutamol and sublingual GTN administrations had no relationship with eGFR (Table 5.4)

Table 5.4 Endothelial function.

	Controls	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
FMD						
Baseline arterial diameter (mm)	4.19±0.81	4.35±0.72	4.15±0.75	4.34±0.75	4.09±0.73	4.40±1.17
Peak arterial diameter (mm)	4.38±0.77	4.54±0.73	4.34±0.75	4.50±0.75	4.25±0.71	4.42±1.15
Percentage diameter change (FMD%)*	4.93±3.01	4.56±2.63	4.68±2.67	4.01±2.55	4.17±3.69	0.66±0.54
GTN						
Baseline arterial diameter (mm)	4.16±0.75	4.31±0.70	4.14±0.81	4.36±0.89	4.12±0.70	4.12±1.27
Peak arterial diameter (mm)	4.68±0.75	4.77±0.70	4.61±0.78	4.84±0.87	4.55±0.71	4.42±1.29
Percentage diameter change (GTN%)	13.26±5.53	10.97±2.93	12.16±4.72	11.74±6.27	10.74±4.31	7.58±3.55
PWA technique (maximal changes in CAIx after salbutamol or GTN administrations)						
With salbutamol	-7±9	-6±6	-7±4	-7±6	-6±9	-4±6
With GTN	-13±6	-14±8	-12±4	-13±6	-13±6	-13±8

Key: *$p < 0.05$ for one-way ANOVA. CAIx: central augmentation index; FMD: endothelium-dependent flow-mediated dilatation; GTN: endothelial-independent flow-mediated dilatation with glyceryl trinitrate; RAIx: radial augmentation index.

Figure 5.2 Relationships of eGFR to FMD.



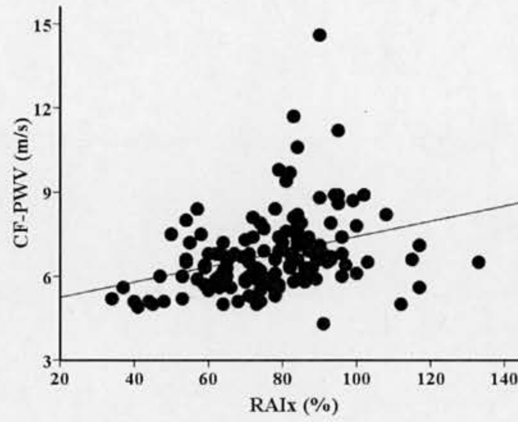
Key: Figure B: **p < 0.01 for stage 5 *versus* control, stages 1, 2, 3, and 4.

5.3.3 Relationships and predictors of carotid-femoral pulse wave velocity and central augmentation index

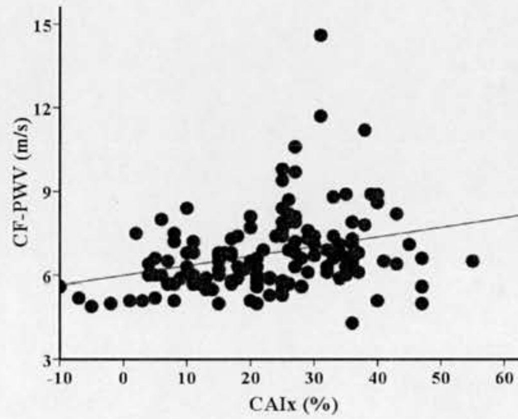
CF-PWV correlated with RAIx, CAIx, and CAIx@HR75 (Figures 5.3 A-C). CF-PWV also correlated with age, MAP, and plasma glucose (Figures 5.4 A-C), and renal function (Figures 5.1 A & B), but not with gender, smoking status, total cholesterol, or BMI. However, CAIx correlated with age, MAP, total cholesterol (Figures 5.4 D-F), gender ($r^2 = 0.07$, $p < 0.01$), and smoking status ($r^2 = 0.09$, $p < 0.01$) but not with plasma glucose, BMI, or renal function.

Figure 5.3 Correlations between CF-PWV and AIx.

A $r^2 = 0.11, p < 0.01$



B $r^2 = 0.09, p < 0.01$



C $r^2 = 0.14, p < 0.01$

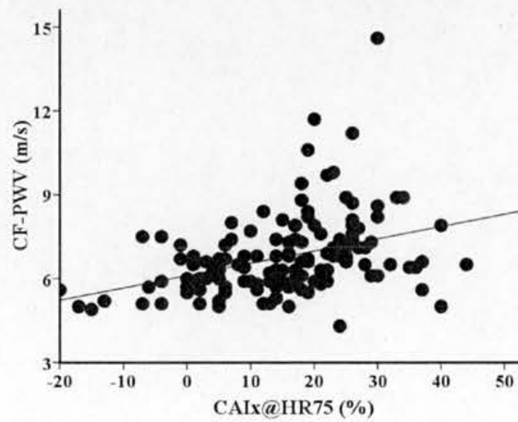
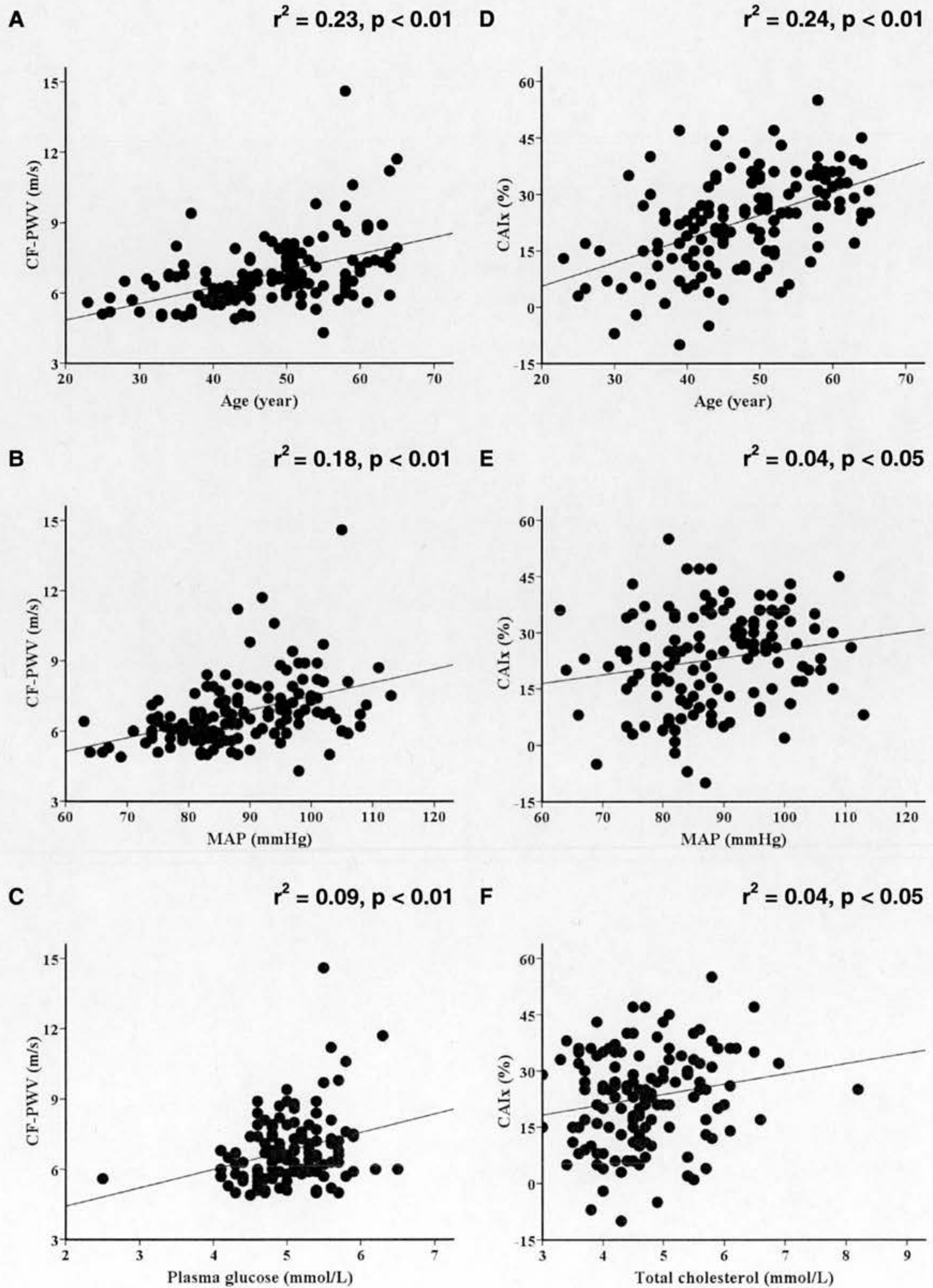


Figure 5.4 Significant positive correlations between CF-PWV & CAIx and conventional risk factors.



In multivariate analysis, considering renal function alongside conventional risk factors, age and BP were the independent determinants of both CF-PWV and CAIx. Additionally, gender and smoking status also independently predicted CAIx. Importantly, renal function was not a predictor of either CF-PWV or CAIx (Table 5.5).

Table 5.5 Multivariate analysis of renal function and conventional cardiovascular risk factors, as independent predictors of CF-PWV and CAIx.

	CF-PWV	CAIx
Age	**0.06***	**0.51***
Gender (male/female)	-0.12	**8.27***
Mean arterial pressure	**0.05***	**0.22****
Smoking status (yes/no)	0.07	**10.04***
Glucose	0.14	-0.08
Total cholesterol	-0.12	0.03
Estimated glomerular filtration rate	-0.13	-0.07
r^2	**0.35***	**0.41***

Key: The table gives standard regression coefficients (β values); r^2: multiple coefficient of determination; *$p < 0.01$; **$p < 0.05$.

5.3.4 Relationships and predictors of flow-mediated dilatation

In univariate analysis, FMD correlated with MAP (Figure 5.5) and renal function (Figures 5.2 A & B) but not with age, gender, smoking status, plasma glucose, total cholesterol, and BMI. In multivariate analysis, with conventional risk factors and eGFR, MAP was the only predictor of FMD (Table 5.6). The change in CAIx after administration of β_2-agonist had no relationship with FMD, renal function, or conventional cardiovascular risk factors.

Figure 5.5 A relationship between FMD and MAP.

A **$r^2 = 0.07, p < 0.01$**

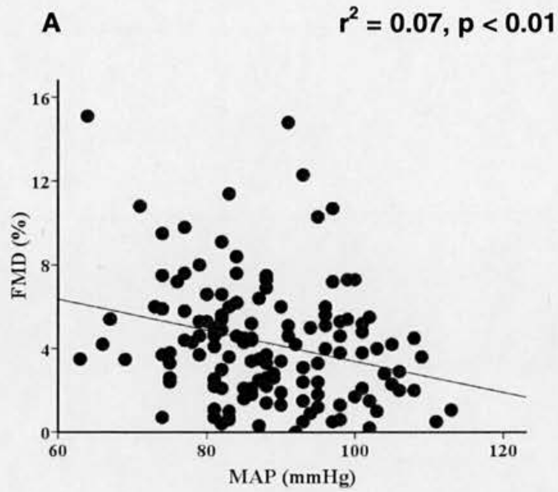


Table 5.6 Multivariate analysis of renal function and conventional cardiovascular risk factors, as independent predictors of FMD.

	FMD
Age	-0.06
Gender (male/female)	0.08
Mean arterial pressure	**-0.07***
Smoking status (yes/no)	-0.02
Glucose	-0.08
Total cholesterol	0.09
Estimated glomerular filtration rate	0.11
r^2	**0.07***

Key: The table gives standard regression coefficients (β values); r^2: multiple coefficient of determination; *p < 0.01.

5.4 Discussion

5.4.1 Main findings

In a minimally co-morbid cohort of CKD patients, arterial stiffness, measured by CF-PWV, increases incrementally as renal function declines whereas endothelial dysfunction, measured by FMD of the brachial artery, is a feature only of late stage CKD. However, renal function is not an independent predictor of either CF-PWV or FMD in this group of patients.

5.4.2 Relationships of carotid-femoral pulse wave velocity & central augmentation index to renal function

The finding of an inverse and graded association between CF-PWV and eGFR is in keeping with previous data (Kawamoto *et al.*, 2008; Kimoto *et al.*, 2006; Matsuda *et al.*, 2009; Mourad *et al.*, 2001; Ohya *et al.*, 2006; Schillaci *et al.*, 2006; Shinohara *et al.*, 2004; Stancanelli *et al.*, 2007; Wang *et al.*, 2005). However, those studies included patients with dialysis, diabetes, or cardiovascular disease. Moreover, in the current study PWV measurements are lower than in previous studies that used a similar technique for measuring arterial stiffness (Figure 5.6, Table 5.7). The finding that CAIx has no association with renal function is consistent with previous reports (Lacy *et al.*, 2006; Stancanelli *et al.*, 2007; Takenaka *et al.*, 2005). Again, CAIx of this study are lower than in those studies (Table 5.7). Taken together, this likely reflects the lack of co-morbidity in this CKD cohort, specifically chosen to minimise confounding by conventional cardiovascular risk factors.

Of note, although PWV and CAIx were used as surrogates for arterial stiffness (Oliver *et al.*, 2003b), these two technique are not identical because their determinants are different. AIx is dependent on the speed of the travelling wave (which is regarded as PWV itself), the amplitude of the reflected wave, the reflection point, heart rate, and ventricular contractility, whereas PWV represents intrinsic

arterial stiffness (Oliver *et al.*, 2003b; Yasmin *et al.*, 1999). These differences probably explain the discrepancy between CF-PWV and CAIx findings and the lack of relationship between CAIx and renal function seen in the current study.

Figure 5.6 Comparing PWV across CKD stages with data from a previous study.

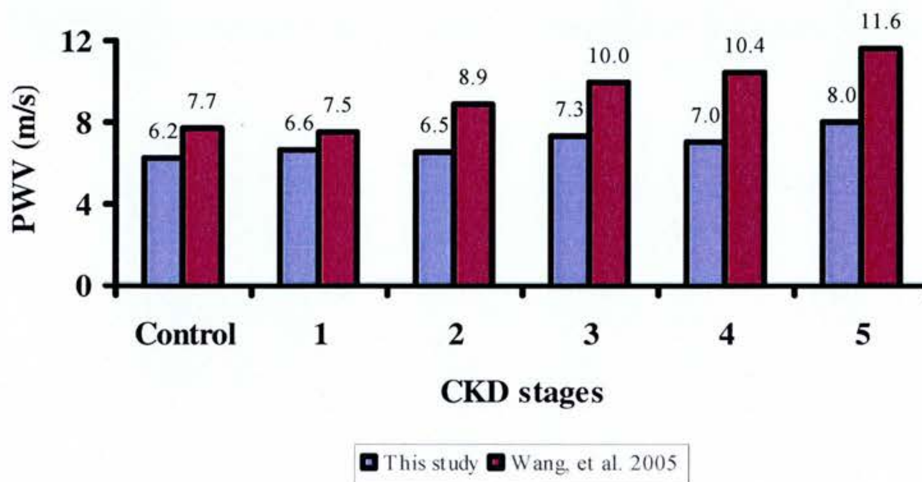


Table 5.7 Comparing PWV and CAIx measurements with data from previous studies (measurements obtained using the SphymoCor system).

<table>
<tr><th>Studies</th><th>Control</th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Stage 4</th><th>Stage 5</th></tr>
<tr><td colspan="7">PWV (m/s)</td></tr>
<tr><td>This study</td><td>6.2</td><td>6.6</td><td>6.5</td><td>7.3</td><td>7.0</td><td>8.0</td></tr>
<tr><td>(Wang et al., 2005)</td><td>7.7</td><td>7.5</td><td>8.9</td><td>10</td><td>10.4</td><td>11.6</td></tr>
<tr><td>(Lacy et al., 2006)</td><td>-</td><td>-</td><td>-</td><td colspan="2">7.7</td><td>9.1</td></tr>
<tr><td>(Briet et al., 2006)</td><td>9.5</td><td>-</td><td>-</td><td>11.3</td><td>-</td><td>-</td></tr>
<tr><td>(Stancanelli et al., 2007)</td><td>6.2</td><td>-</td><td colspan="3">8.0</td><td>-</td></tr>
<tr><td colspan="7">CAIx (%)</td></tr>
<tr><td>This study</td><td>21</td><td>20</td><td>25</td><td>24</td><td>24</td><td>24</td></tr>
<tr><td>(Lacy et al., 2006)</td><td>-</td><td>-</td><td colspan="3">28</td><td>-</td></tr>
<tr><td>(Briet et al., 2006)</td><td>35</td><td>-</td><td>-</td><td>32</td><td>-</td><td>-</td></tr>
<tr><td>(Stancanelli et al., 2007)</td><td>29</td><td>-</td><td>-</td><td>-</td><td>29</td><td>-</td></tr>
</table>

Key: The values presented are the mean values.

5.4.3 Predictors of carotid-femoral pulse wave velocity and central augmentation index

When conventional risk factors (age, gender, BP, smoking, plasma glucose, and hypercholesterolaemia) were considered alongside renal function, age and BP, as expected, were the strongest determinants of arterial stiffness (Guerin *et al.*, 2008). The finding that renal function is not a predictor for either PWV or CAIx is contrary to several previous reports on studies including dialysis patients and CKD patients with cardiovascular co-morbidity (Kawamoto *et al.*, 2008; Kimoto *et al.*, 2006; Matsuda *et al.*, 2009; Mourad *et al.*, 2001; Ohya *et al.*, 2006; Schillaci *et al.*, 2006; Shinohara *et al.*, 2004; Wang *et al.*, 2005) but is in accordance with some studies

(Briet *et al.*, 2006; Stancanelli *et al.*, 2007; Takenaka *et al.*, 2005). However, in those studies, patients with lower GFR had a higher percentage of co-morbidity which would affect PWV and might, therefore, drive the presumed association with renal function. Importantly, as CKD patients in this study were highly selected to have very low comorbidity, the finding that GFR was not a predictor of PWV suggests that uraemia *per se* has minimal role on enhancing cardiovascular disease in CKD. This is contrast to the hypothesis of this study. However, this finding underscores the importance of the well-established cardiovascular risk factor, BP, on cardiovascular disease in CKD and emphasises the fact that all CKD patients should have tight controlled of their BP.

Although it proved impossible to recruit a sufficient number of non-smokers to exclude this risk factor, the results from this study suggest that smoking does not independently contribute to CF-PWV. Despite a low number of smokers, smoking is still an independent predictor of CAIx, in line with other studies (Polonia *et al.*, 2009; Rehill *et al.*, 2006). Gender independently predicts CAIx. This could be explained by differences of body height on wave reflections (the shorter the body height, the higher the AIx and women are generally shorter than men) (O'Rourke *et al.*, 1996; Yasmin *et al.*, 1999).

5.4.4 Relationships of flow-mediated dilatation to renal function

The finding of endothelial dysfunction, measured by FMD, being a feature only of late stage CKD appears to be at odds with previous studies on the early stages of CKD (Annuk *et al.*, 2001; Bolton *et al.*, 2001; Caglar *et al.*, 2008a; Caglar *et al.*, 2008b; Chan *et al.*, 2006; Dogra *et al.*, 2006; Ghiadoni *et al.*, 2004; Perticone *et al.*, 2004; Thambyrajah *et al.*, 2000; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008a; Yilmaz *et al.*, 2007). Amongst these, three studies have measured brachial artery FMD across 5 stages of CKD and found that FMD gradually reduces as GFR falls (Figure 5.7, Table 5.8).

However, the results of the current study are in line with a recent report from the Framingham Offspring cohort study in which the endothelium-dependent vasomotor function was not different between subjects with CKD (GFR 50 ml/min/1.73m^2) and those without CKD (FMD: 2.3 *versus* 2.9 %, respectively) (Table 5.8) (Foster *et al.*, 2008). The finding that endothelial dysfunction occurs only at late stage CKD was also confirmed by the ROC curve with the specificity of 98%. Thus, the endothelial dysfunction seen in early stages of CKD in other studies is once again likely to be a reflection of the co-morbidities of the subjects studied, including atherosclerotic vascular disease (Thambyrajah *et al.*, 2000), hypertension (Perticone *et al.*, 2004), diabetes mellitus (Annuk *et al.*, 2001; Chan *et al.*, 2006; Dogra *et al.*, 2006), and dialysis (Annuk *et al.*, 2001; Caglar *et al.*, 2008b; Dogra *et al.*, 2006; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008a).

Figure 5.7 Comparing FMD (%) across CKD stages with data from previous studies.

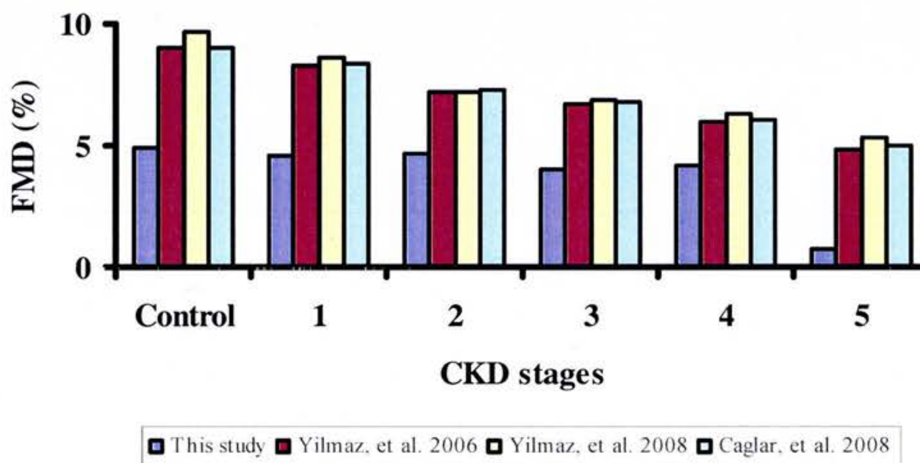


Table 5.8 Comparing FMD (%) measurement with data from previous studies.

<table>
<tr><th>Studies</th><th>Control</th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Stage 4</th><th>Stage 5</th></tr>
<tr><td>This study</td><td>4.9</td><td>4.6</td><td>4.7</td><td>4.0</td><td>4.2</td><td>0.7</td></tr>
<tr><td>(Yilmaz et al., 2006)</td><td>9.0</td><td>8.3</td><td>7.2</td><td>6.7</td><td>6.0</td><td>4.8</td></tr>
<tr><td>(Yilmaz et al., 2008a)</td><td>9.7</td><td>8.6</td><td>7.2</td><td>6.9</td><td>6.3</td><td>5.3</td></tr>
<tr><td>(Caglar et al., 2008b)</td><td>9.0</td><td>8.4</td><td>7.3</td><td>6.8</td><td>6.1</td><td>5.0</td></tr>
<tr><td>(Thambyrajah et al., 2000)</td><td>6.5</td><td>-</td><td>-</td><td colspan="2">2.6
◄──►</td><td>-</td></tr>
<tr><td>(Bolton et al., 2001)</td><td>6.4</td><td>-</td><td>-</td><td colspan="2">2.3
◄──►</td><td>-</td></tr>
<tr><td>(Ghiadoni et al., 2004)</td><td>6.9</td><td>-</td><td>-</td><td colspan="3">5.3
◄────►</td></tr>
<tr><td>(Dogra et al., 2006)</td><td>5.7</td><td>-</td><td>-</td><td colspan="3">3.8
◄────►</td></tr>
<tr><td>(Chan et al., 2006)</td><td>4.3</td><td>-</td><td>-</td><td>-</td><td>1.9</td><td>-</td></tr>
<tr><td>(Yilmaz et al., 2007)</td><td>8.9</td><td>-</td><td>-</td><td>6.7</td><td>-</td><td>-</td></tr>
<tr><td>(Foster et al., 2008)</td><td>2.9</td><td>-</td><td>-</td><td>2.3</td><td>-</td><td>-</td></tr>
<tr><td>(Caglar et al., 2008a)</td><td>8.8</td><td>-</td><td>-</td><td>-</td><td>5.9</td><td>-</td></tr>
</table>

Key: The values presented are the mean values.

The change in CAIx after inhaled β_2-agonist is a novel technique for measuring endothelial function (Hayward *et al.*, 2002). Although the technique is relatively easy compared to FMD, it has not been applied much in clinical research compared to FMD. In addition, the technique has only been tested on a small number of healthy subjects (Hayward *et al.*, 2002; Wilkinson *et al.*, 2002a) and in patients with coronary artery disease (Weber *et al.*, 2007) but not in renal patients. Our finding that the change in CAIx after salbutamol does not correlate with FMD may imply

that this technique might not be suitable for assessing endothelial function in uraemic patients. However, one study has shown a higher degree of endothelial dysfunction, assessed by this technique, in dialysis patients compared to hypertensive patients (Covic *et al.*, 2004).

5.4.5 Predictors of flow-mediated dilatation

Of all the risk factors studied here, BP is the only predictor of FMD. Results from this study both confirm and contrast with previous data. In contrast to the current findings, previous studies have shown uraemia to be an independent predictor of endothelial dysfunction (Annuk *et al.*, 2001; Stam *et al.*, 2003; Yilmaz *et al.*, 2008a). In support of the present study is a study of stage 3 to 5 CKD patients where neither GFR nor oxidative stress were shown to independently predict endothelial function (Annuk *et al.*, 2005).

Again, the finding that FMD was not determined by renal function in this low-comorbidity cohort is contrast to the hypothesis that uraemia, on its own, would contribute to endothelial dysfunction in CKD. In fact, uraemia has minimal role on endothelial dysfunction unless GFR substantailly drops to the level that requires a renal replacement therapy. As with PWV, the FMD finding indicates the importance of a modifiable risk factor, BP, in CKD.

5.4.6 Limitations

Some of the medications taken by the patients, such as ACE inhibitors, ARBs, β-blockers, and statins, may have had effects on both arterial stiffness and endothelial function. However, all patients were stabilised on their therapy and did not receive their medication on the day of the study until after measurements were made. In addition, as this was a cross-sectional study, the causal relationships behind the associations described cannot be fully defined, though they do have a sound mechanistic basis.

5.4.7 Summary

The present study shows that, in the absence of established cardiovascular disease and diabetes, deterioration in renal function is associated with increased arterial stiffness and endothelial dysfunction. Age and BP remain the major determinants of both. However, when renal function and conventional cardiovascular risk factors are considered together, renal function is not a determinant of either arterial stiffness or endothelial dysfunction. Thus, in this relatively healthy CKD population, uraemia, on its own, is not the main driving force of the development of vascular complications. These complications would mainly be driven by BP or other emerging cardiovascular risk factors. Hence, a study assessing the role of emerging cardiovascular risk factors, such as inflammation, oxidative stress, and imbalance of NO/ET, on arterial stiffness and endothelial dysfunction would be of great interest.

Chapter 6

Relationships of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction in chronic kidney disease

6.1 Introduction

CKD patients have an increased risk of cardiovascular disease, with cardiovascular mortality at least 10-20 times higher in dialysis patients than in the general population (Foley *et al.*, 1998). Indeed, CKD patients have a substantially higher chance of dying from cardiovascular disease than of progressing to end-stage renal disease (Sarnak *et al.*, 2003). Conventional Framingham cardiovascular risk factors including smoking, hypertension, hypercholesterolaemia, and diabetes mellitus, are common in CKD patients. However, these only partly explain the high cardiovascular risk. Thus, the study of emerging cardiovascular risk factors has been an area of intense investigation (Zoccali, 2006).

Arterial stiffness and endothelial dysfunction are recognised markers of cardiovascular risk (Blacher *et al.*, 1999a; Guerin *et al.*, 2008; Perticone *et al.*, 2001). Importantly, arterial stiffness is an independent predictor of mortality and survival in renal patients (Blacher *et al.*, 1999a; Guerin AP, 2001). Emerging cardiovascular risk factors such as inflammation, oxidative stress, and a shift in the balance of the vasodilator NO and vasoconstrictor ET systems may contribute to arterial stiffness and endothelial dysfunction (Gusbeth-Tatomir *et al.*, 2007). These are all common in a typical CKD population (Schiffrin *et al.*, 2007; Zoccali, 2006), but are also increased by conventional cardiovascular risk factors and by established cardiovascular disease (Dhaun *et al.*, 2006; Schiffrin *et al.*, 2007). Hence, the contribution of these emerging risk factors to arterial stiffness and endothelial dysfunction, in CKD remains unclear.

6.1.1 Aims and hypotheses

The major findings of Chapter 5 are that arterial stiffness and endothelial dysfunction increase as renal function declines, and these are driven by conventional cardiovascular risk factors, such as age and BP. Based on these findings, it is worth exploring the role of emerging cardiovascular risk factors in helping to explain the augmented arterial stiffness and endothelial dysfunction, and high cardiovascular risk in CKD patients.

Thus, the aims of this study were to investigate the relationship of both conventional and emerging cardiovascular risk factors including inflammation, oxidative stress and markers of NO/ET activity to arterial stiffness and endothelial dysfunction in a group of CKD patients with minimal co-morbidity from CKD stage 1 to pre-dialysis. The prevalence of emerging cardiovascular risk factors in this group was also assessed.

Hypotheses:

1. In this minimally co-morbid CKD group, the measured emerging risk factors would accumulate, as renal function declined.

2. These emerging risk factors would, at least in part, independently explain the increase in arterial stiffness and endothelial dysfunction seen in this group of patients.

6.2 Methods

6.2.1 Subjects

All subjects recruited from a study investigating the relationship of arterial stiffness and endothelial dysfunction to renal function (Chapter 5) were further enrolled on this study.

6.2.2 Study design and protocol

This was a prospective, cross-sectional study. Subjects refrained from alcohol for at least 24 hours and caffeinated drinks, food, and smoking for at least 12 hours before the study. Subjects were asked to withhold their current medications on the morning of the study. All studies were conducted in a quiet, temperature-controlled room. After 30 minutes of supine rest, participants underwent BP measurements and then blood samples were taken for creatinine, glucose, total cholesterol, ESR, hsCRP,

OxLDL, IL-6, 8-IsoP, ADMA, and ET-1. After that, CF-PWV and FMD were measured. All techniques were performed as described in Chapter 2.

6.2.3 Laboratory investigations

Serum creatinine, plasma glucose, total cholesterol, and ESR were quantified in the hospital biochemistry laboratory. All sample collections and analyses of serum hsCRP and OxLDL, and plasma IL-6, 8-IsoP, ADMA, and ET-1 were performed as previously described in Chapter 2, section 2.9.2. Although not regarded as an emerging cardiovascular risk factors, SDMA was also measured.

6.2.4 Data analyses

Descriptive data is given as mean ± SD. The means of the subject characteristics and the emerging cardiovascular risk factors of all CKD stages were compared using one-way ANOVA. Correlation coefficients for univariate analysis were calculated using the Pearson method. Multivariate analysis was performed using a stepwise linear regression. Dependent variables were CF-PWV and FMD. First, GFR and traditional cardiovascular risk factors including age, gender, MAP, smoking status, plasma glucose, and total cholesterol were entered into the first model. Thereafter, emerging cardiovascular risk factors including hsCRP, IL-6, ESR, 8-Isop, OxLDL, arginine, ADMA and ET-1 were entered into the second model. Multiple coefficient of determination (r^2) of each model was reported. Multivariate analysis data were reported for both CF-PWV and FMD using standard regression coefficient (β value) of independent variables. A significant level was p value < 0.05.

6.3 Results

6.3.1 Subject characteristics

A total of 138 subjects were studied. The characteristics of the studied population are shown in Table 6.1 (for full subject characteristics, see Appendix 1). Cause of CKD and the medication used by the patients were similar to those summarised in Table 5.1, Chapter 5.

Age was comparable between stages while MAP increased as renal function declined but this did not reach significance. CF-PWV incrementally increased with increasing CKD stage ($r^2 = 0.08$, $p < 0.01$). FMD positively correlated with eGFR ($r^2 = 0.04$, $p < 0.05$) with those whose GFR <20 ml/min/1.73m^2 having a substantial reduction of FMD.

Table 6.1 Subject characteristics.

	Controls	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
n	23	28	30	30	20	7
Male/Female (n)	13/10	17/11	18/12	22/8	16/4	4/3
Smoker: Yes/No (n)	2/21	9/19	5/25	6/24	4/16	0/7
Creatinine (mmol/L)	78±13	77±13	102± 21	177±5	364±105	632±181
eGFR (ml/min/1.73m^2)	97±19	108±17	77±9	45±9	23±4	12±3
Age (year)	47±8	43±11	49±9	51±10	45±9	51±12
MAP (mmHg)	85±12	86±11	88±10	90±10	91±8	95±8
BMI (kg/m^2)	26±6	29±5	28±4	28±6	28±5	25±7
Glucose (mmol/L)	5.0±0.5	5.0±0.5	5.0±0.6	5.1±0.4	5.1±0.5	4.8±0.7
Total cholesterol (mmol/L)	4.7±0.9	4.9±0.8	4.7±1.0	4.8±0.8	4.5±0.8	4.4±0.8
CF-PWV (m/s)*	6.15±0.82	6.61±1.14	6.51±1.04	7.29±1.96	6.98±1.41	8.00±1.90
FMD (%)**	4.93±3.01	4.56±2.63	4.68±2.67	4.01±2.55	4.17±3.69	0.66±0.54
GTN (%)	13.26±5.53	10.97±2.93	12.16±4.72	11.74±6.27	10.74±4.31	7.58±3.55

Key: *p < 0.01, **p < 0.05 for one-way ANOVA by CKD stage. BMI: body mass index; CF-PWV: carotid-femoral pulse wave velocity; eGFR: estimated glomerular filtration rate; FMD: flow-mediated dilatation; GTN: endothelium-independent vasodilatation response to glyceryl trinitrate; MAP: mean arterial pressure.

6.3.2 Relationships of emerging risk factors to renal function

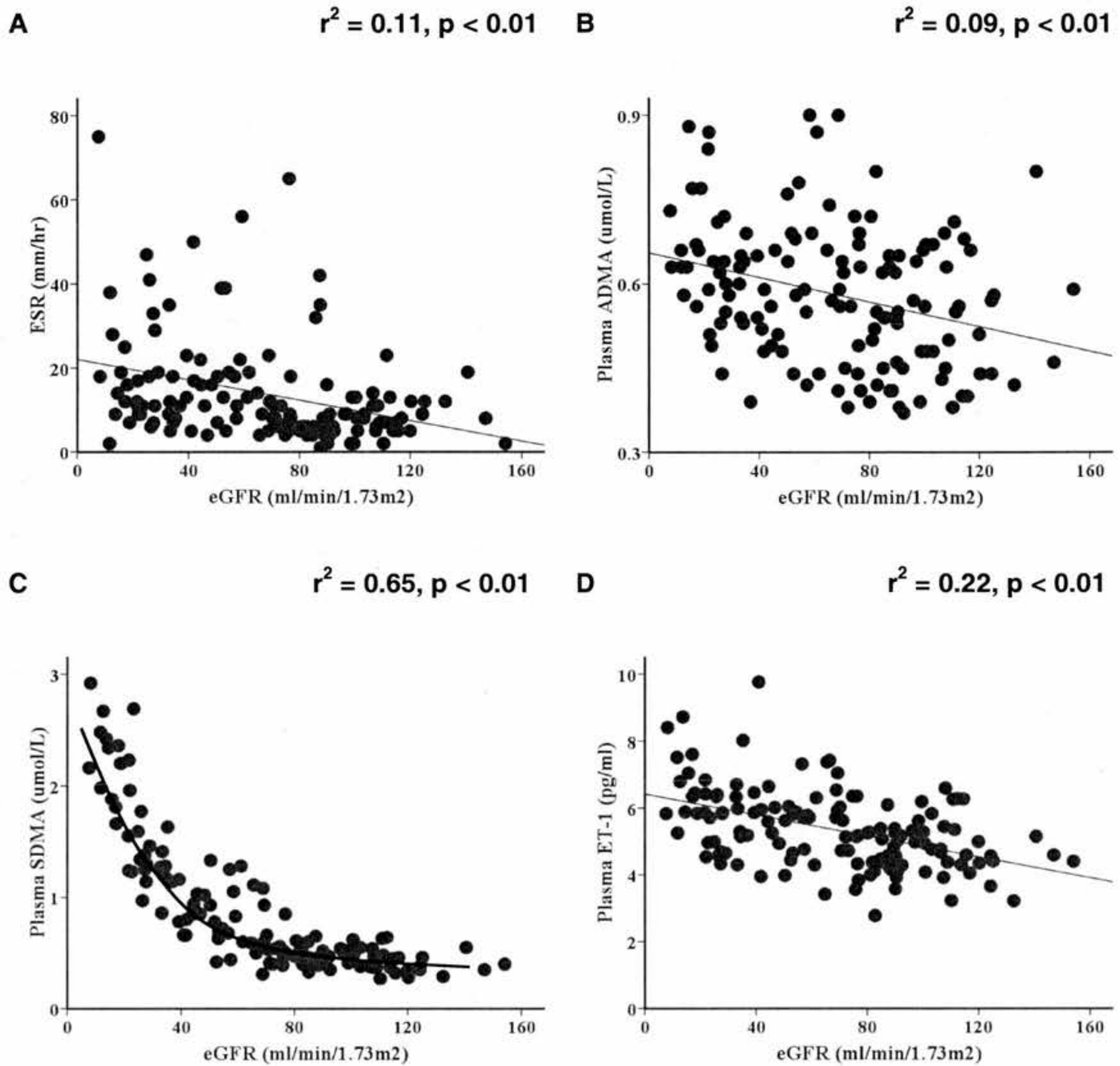
ESR, ADMA, SDMA, and ET-1 all increased as eGFR declined (r^2 = 0.11, 0.09, 0.65, 0.22, respectively, $p < 0.01$ for all, Table 6.2, Figures 6.1 A-D) whereas hsCRP, IL-6, 8-Isop and OxLDL showed no relationship to renal function (Table 6.2).

Table 6.2 Circulating concentrations of variables identified as emerging cardiovascular risk factors.

	Controls	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
hsCRP (mg/L)	1.8±3.0	2.8±2.5	3.3±3.5	4.9±4.5	2.8±3.0	3.4±3.4
IL-6 (pg/ml)	8.7±8.9	9.9±10.6	11.5±11.8	12.3±12.7	6.9±6.9	6.4±5.0
ESR (mm/hr)*	7±4	9±5	14±14	18±13	18±12	26±25
8-Isop (pg/ml)	35±38	33±28	32±35	37±34	38±24	32±25
OxLDL (U/L)	54±13	52±18	55±17	57±16	51±15	53±18
Arginine (μmol/L)	79±22	72±11	75±16	72±15	77±20	76±18
ADMA (μmol/L)*	0.51±0.12	0.56±0.10	0.58±0.13	0.60±0.11	0.64±0.12	0.68±0.10
SDMA (μmol/L)*	0.41±0.08	0.48±0.09	0.60±0.23	0.95±0.29	1.65±0.46	2.42±0.31
ET-1 (pg/ml)*	4.6±0.9	5.0±0.8	5.2±1.1	5.8±1.2	5.7±0.9	6.9±1.3

Key:*p < 0.01 for one-way ANOVA by CKD stage. ADMA: asymmetric dimethylarginine; ET-1: endothelin-1; ESR; erythrocyte sedimentation rate; hsCRP: high-sensitivity C-reactive protein; IL-6: interleukin-6; 8-Isop: 8-isoprostaglandin F_2; OxLDL: oxidised low density lipoprotein; SDMA: symmetric dimethylarginine.

Figure 6.1 Relationships between eGFR and ESR, ADMA, SDMA, and ET-1.



Additionally, in univariate analysis, age correlated positively with ESR ($r^2 = 0.04$, $p < 0.05$) and ADMA ($r^2 = 0.07$, $p < 0.01$); smoking status correlated with arginine and ET-1 ($r^2 = 0.03$ and 0.05, respectively, $p < 0.05$); plasma glucose correlated with 8-Isop ($r^2 = 0.03$, $p < 0.05$); and LDL highly correlated with OxLDL ($r^2 = 0.32$, $p < 0.01$) and ET-1 ($r^2 = 0.08$, $p < 0.01$). MAP and gender showed no associations with any emerging risk factors.

Amongst markers of inflammation, hsCRP correlated with ESR ($r^2 = 0.13$, $p < 0.01$) but not IL-6. For measures of oxidative stress, 8-Isop correlated with OxLDL ($r^2 = 0.06$, $p < 0.01$). With regards to NO/ET systems, ADMA correlated with arginine and ET-1 ($r^2 = 0.19$ and 0.12, respectively, $p < 0.01$).

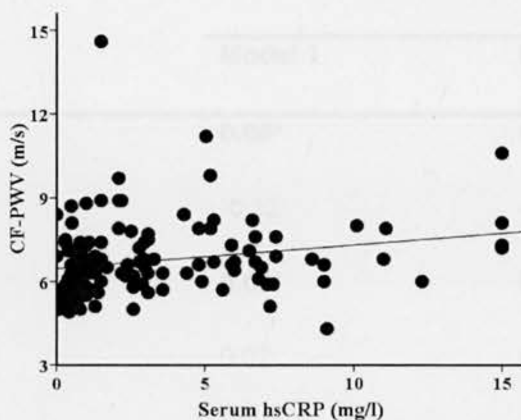
6.3.3 Relationships and predictors of carotid-femoral pulse wave velocity

In univariate analysis, CF-PWV correlated with age, MAP, plasma glucose and eGFR ($r^2 = 0.23, 0.18, 0.09$, and 0.08, respectively, $p < 0.01$ for all), but not with gender, smoking status, total cholesterol, or BMI. With regards to emerging risk factors, hsCRP, ESR, and ADMA correlated with CF-PWV (Figures 6.2 A-C) whereas IL-6, 8-Isop, OxLDL, and ET-1 did not.

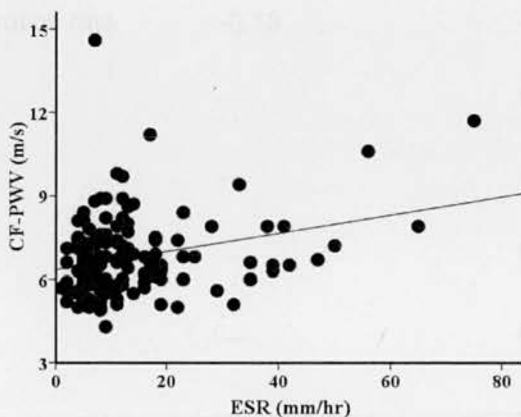
When considering renal function alongside conventional risk factors, age and BP were the independent determinants of CF-PWV (Table 6.3, Model 1). When emerging risk factors were added to the multivariate model, age and BP remained the major predictors of CF-PWV but hsCRP and ADMA also predicted CF-PWV (Table 6.3, Model 2). For both multivariate models, renal function, smoking, and gender were not predictive of PWV.

Figure 6.2 Relationships between CF-PWV and hsCRP, ESR, and ADMA.

A $r^2 = 0.05$, $p < 0.05$



B $r^2 = 0.08$, $p < 0.01$



C $r^2 = 0.10$, $p < 0.01$

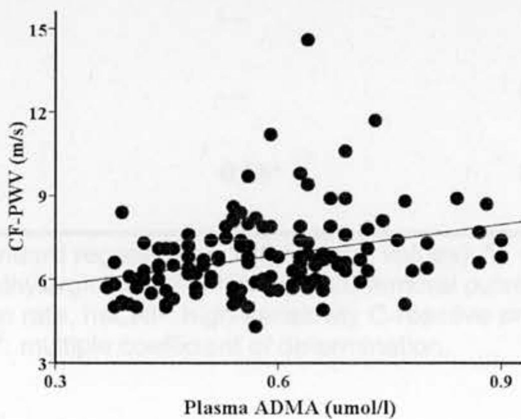


Table 6.3 Multivariate analysis of renal function, conventional and emerging cardiovascular risk factors, as independent predictors of CF-PWV.

	CF-PWV	
	Model 1	**Model 2**
Age	**0.06***	**0.05***
Gender (male/female)	-0.12	-0.12
Mean arterial pressure	**0.05***	**0.04***
Smoking status (yes/no)	0.07	0.09
Glucose	0.14	0.10
Total cholesterol	-0.12	-0.05
Estimated glomerular filtration rate	-0.13	-0.06
hsCRP	—	**0.07****
Interleukin-6	—	-0.05
ESR	—	0.02
8-Isop	—	0.09
OxLDL	—	-0.04
Arginine	—	-0.10
ADMA	—	**1.86****
Endothelin-1	—	0.06
r^2	**0.35***	**0.40***

Key: The table gives standard regression coefficients (β values); *p < 0.01; **p < 0.05. ADMA: asymmetric dimethylarginine; CF-PWV: carotid-femoral pulse wave velocity; ESR; erythrocyte sedimentation rate; hsCRP: high-sensitivity C-reactive protein; OxLDL: oxidised low density lipoprotein; r^2: multiple coefficient of determination.

6.3.4 Relationships and predictors of flow-mediated dilatation

In univariate analysis, FMD correlated with MAP ($r^2 = 0.07$. $p < 0.01$) and renal function ($r^2 = 0.04$, $p < 0.05$) but not with age, gender, smoking status, plasma glucose, total cholesterol, and BMI. With regards to emerging cardiovascular risk factors, FMD only correlated with plasma ET-1 (Figure 6.3) but not with markers of inflammation, oxidative stress, or plasma ADMA.

In multivariate analysis, with conventional risk factors and eGFR, MAP was the only predictor of FMD (Table 6.4, Model 1). When both conventional and emerging risk factors were considered together, MAP remained a determinant of FMD but 8-Isop and ET-1 were also independent predictors of FMD (Table 6.4, Model 2). For both multivariate models, renal function, smoking, and gender were not predictive of FMD.

Figure 6.3 A relationship between FMD and plasma ET-1.

$r^2 = 0.04$, $p < 0.05$

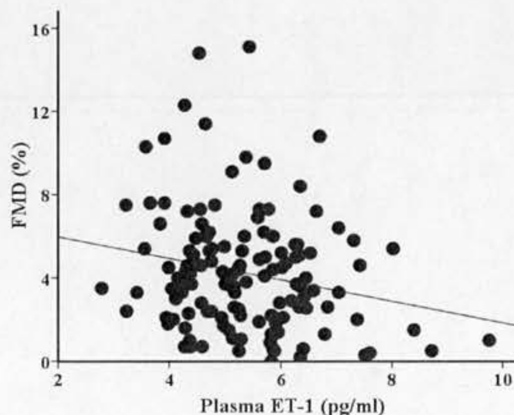


Table 6.4 Multivariate analysis of renal function, conventional and emerging cardiovascular risk factors, as independent predictors of FMD.

	FMD	
	Model 3	**Model 4**
Age	-0.06	-0.04
Gender (male/female)	0.08	0.10
Mean arterial pressure	**-0.07***	**-0.07***
Smoking status (yes/no)	-0.02	0.01
Glucose	-0.08	-0.08
Total cholesterol	0.09	0.09
Estimated glomerular filtration rate	0.11	0.04
hsCRP	—	-0.07
Interleukin-6	—	-0.07
ESR	—	0.02
8-Isop	—	**-0.02****
OxLDL	—	-0.04
Arginine	—	0.00
ADMA	—	-0.04
Endothelin-1	—	**-0.53****
r^2	**0.07***	**0.15***

Key: The table gives standard regression coefficients (β values), *p < 0.01, **p < 0.05
ADMA: asymmetric dimethylarginine; FMD: flow-mediated dilatation; hsCRP: high-sensitivity C-reactive protein; OxLDL: oxidised low density lipoprotein; r^2: multiple coefficient of determination.

6.4 Discussion

6.4.1 Main findings

CKD patients without diabetes or established co-morbidity have increased arterial stiffness and endothelial dysfunction. They also have a shift in the balance between the NO/ET systems towards an upregulation of the vasoconstrictor ET system but have low prevalence of inflammation and oxidative stress. Based on multivariate analysis, amongst the conventional cardiovascular risk factors, BP is the major determinant of arterial stiffness and endothelial dysfunction. Additionally, when conventional and emerging cardiovascular risk factors are considered together, hsCRP and ADMA independently predict arterial stiffness, and 8-Isop and ET-1 independently predict endothelial dysfunction.

6.4.2 Relationships of emerging risk factors to renal function

The finding of an incremental increase of CF-PWV across CKD stages and a reduction of FMD, especially in CKD stage 5, are in accordance with other studies (Foster *et al.*, 2008; Kawamoto *et al.*, 2008; Kimoto *et al.*, 2006; Matsuda *et al.*, 2009; Mourad *et al.*, 2001; Ohya *et al.*, 2006; Schillaci *et al.*, 2006; Shinohara *et al.*, 2004; Stancanelli *et al.*, 2007; Wang *et al.*, 2005) (see the relationship of CF-PWV and FMD to renal function in Chapter 5, sections 5.4.2 and 5.4.4, respectively).

In this study, neither hsCRP nor IL-6 was related to GFR. These are in contrast to previous data (Figure 6.4, Table 6.5) (Caglar *et al.*, 2008b; Cottone *et al.*, 2009; Dogra *et al.*, 2006; Landray *et al.*, 2004; Panichi *et al.*, 2002; Perticone *et al.*, 2004; Romao *et al.*, 2006; Shlipak *et al.*, 2003; Stam *et al.*, 2003) but in keeping with a recent study in Japanese non-diabetic CKD patients (Matsuda *et al.*, 2009). Although the results showed that ESR increased linearly as renal function declined, this is likely due to anaemia associated with renal impairment (Borawski *et al.*, 2001). In consistent with previous reports on pre-dialysis patients, none of the measures of oxidative stress were associated with renal function (Figures 6.5 & 6.6) (Annuk *et*

al., 2005; Bolton *et al.*, 2001; Cottone *et al.*, 2009; Dounousi *et al.*, 2006). Additionally, measures of inflammation and oxidative stress in the current study were relatively low compared to other studies (Figures 6.4-6.6, Table 6.5). Taken together, these are all in contrast to the hypothesis that inflammation and oxidative stress are substantially increased in CKD and contribute significantly to its accelerated vascular pathology (Himmelfarb *et al.*, 2002; Zimmermann *et al.*, 1999). However, the findings of the present study suggest that uraemia is not a pro-inflammatory or pro-oxidant state itself, but that these features are driven largely by co-morbidity.

Figure 6.4 Comparing circulating CRP concentrations with data from previous studies.

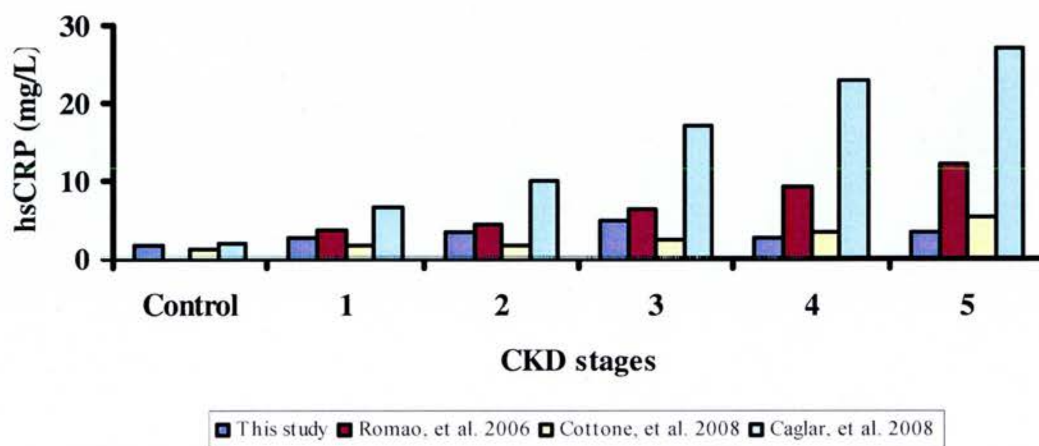


Figure 6.5 Comparing plasma Isop concentrations with data from previous studies.

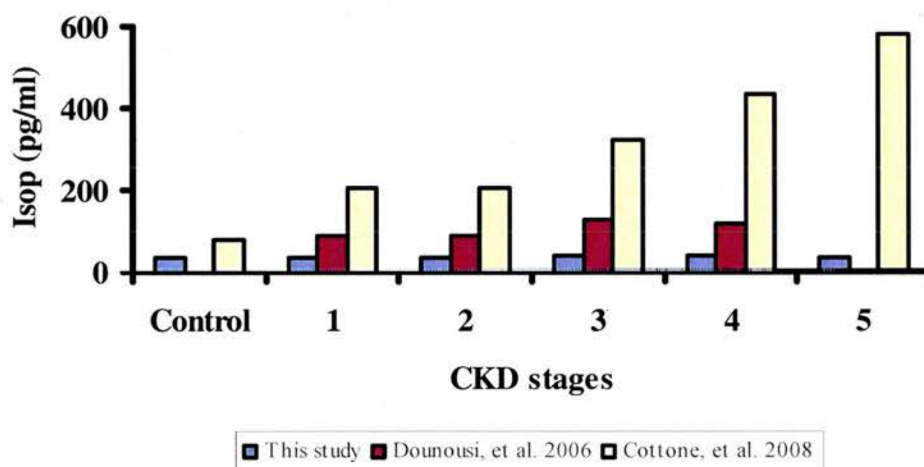


Figure 6.6 Comparing plasma OxLDL concentrations with data from previous studies.

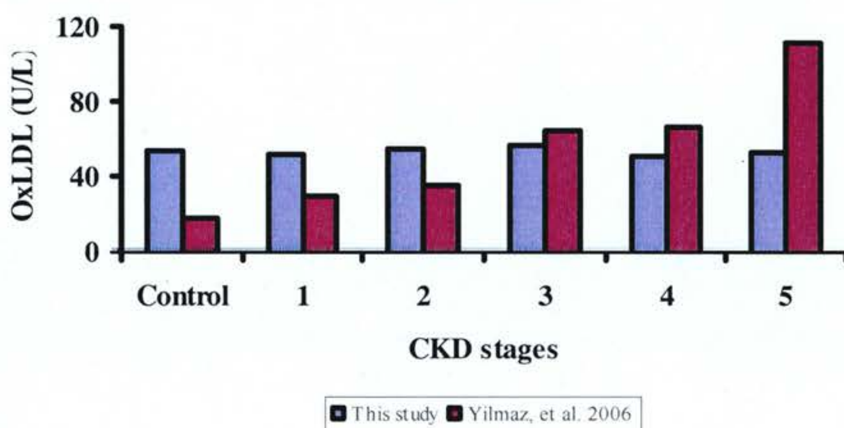


Table 6.5 Comparing circulating CRP and IL-6 with data from previous studies.

Studies	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CRP (mg/L)						
This study	***1.8***	***2.8***	***3.3***	***4.9***	***2.8***	***3.4***
(Dogra *et al.*, 2006)	1.3	-	-	←	3.9	→
(Dounousi *et al.*, 2006)	-	3.0	3.5	3.8	3.4	-
(Landray *et al.*, 2004)	2.2	-	-	←	3.7	→
(Matsuda *et al.*, 2009)	2.0	← 0.9	→	←	1.8	→
(Panichi *et al.*, 2002)	-	-	3.2	← 4.0	→	7.9
(Shlipak *et al.*, 2003)	4.4	-	-	-	5.5	-
(Stam *et al.*, 2003)	-	1.1	2.3	4.5	← 5.0	→
IL-6 (pg/ml)						
This study	***8.7***	***9.9***	***11.5***	***12.3***	***6.9***	***6.4***
(Dogra *et al.*, 2006)	1.6	-	-	←	3.7	→
(Panichi *et al.*, 2002)	-	-	2.8	← 5.3	→	7.5
(Shlipak *et al.*, 2003)	2.7	-	-	-	3.1	-

Plasma ADMA, an endogenous inhibitor of NOS (Boger, 2003), and plasma ET-1 were measured as components of the NO and ET systems, respectively (Boger, 2003; Dhaun *et al.*, 2006). Both increased linearly as eGFR fell in accordance with the literature (Figures 6.7, Table 6.6) (Blazy *et al.*, 1994; Busch *et al.*, 2006; Cottone *et al.*, 2009; Dammers *et al.*, 2005; Deray *et al.*, 1992; El-Mesallamy *et al.*, 2008; Fleck *et al.*, 2001; Goddard *et al.*, 2007; Kielstein *et al.*, 2002; Koyama *et al.*, 1989; MacAllister *et al.*, 1996; Mallamaci *et al.*, 1993; Panichi *et al.*, 2008; Uzun *et al.*, 2008; Vlachojannis *et al.*, 1997; Vlassopoulos *et al.*, 1995; Wahbi *et al.*, 2001; Warrens *et al.*, 1990; Xiao *et al.*, 2001; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008b; Young *et al.*, 2009). Interestingly, the concentrations of plasma ADMA and ET-1 seen here are lower than previously published in subjects with similar levels of renal function but added co-morbidity (Fleck *et al.*, 2001; Koyama *et al.*, 1989; MacAllister *et al.*, 1996; Saito *et al.*, 1991; Xiao *et al.*, 2001; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008b; Young *et al.*, 2009), suggesting that cardiovascular co-morbidity may contribute significantly to endothelial dysfunction in these other studies (Table 6.6).

There is often reciprocal upregulation of the ET system in circumstances where there is downregulation of NO system activity (Dhaun *et al.*, 2006). In this study, plasma ADMA and ET-1 did, indeed, correlate highly with each other, confirming the reciprocal relationship between the NO and ET systems. The increases in plasma ADMA, SDMA, and ET-1 with deteriorating renal function may, in part, be due to their reduced renal clearance (Boger, 2003; Dhaun *et al.*, 2006), although not excluding an increase in their synthesis.

Figure 6.7 Comparing plasma ADMA concentrations with data from previous studies.

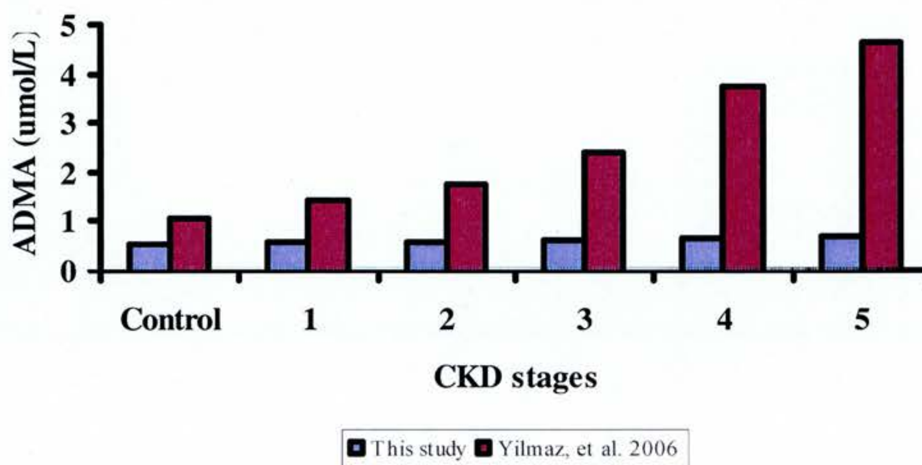


Table 6.6 Comparing plasma ADMA and ET-1 with data from previous studies.

Studies	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
ADMA (μmol/L)						
This study	***0.51***	***0.56***	***0.58***	***0.60***	***0.64***	***0.68***
(Kielstein *et al.*, 2002)	1.40	4.00	3.80	-	4.70	-
(MacAllister *et al.*, 1996)	0.36	-	-	-	-	0.90
(Panichi *et al.*, 2008)	0.69	-	← 0.84 (Stage 2–3) →		-	-
(Uzun *et al.*, 2008)	1.05	-	-	-	← 1.26 (Stage 4–5) →	
(Xiao *et al.*, 2001)	0.40	-	-	-	1.10	-
ET-1 (pg/ml)						
This study	***4.6***	***5.0***	***5.2***	***5.8***	***5.7***	***6.9***
(Cottone *et al.*, 2009)	1.6	← 3.3 (Stage 1–2) →		3.9	4.5	5.7
(Dammers *et al.*, 2005)	1.9	-	-	-	-	14.9
(Goddard *et al.*, 2007)	4.2	-	← 5.6 (Stage 2–5) →			
(Vlachojannis *et al.*, 1997)	29	54	-	-	60	-

6.4.3 Relationships and predictors of carotid-femoral pulse wave velocity

Here, hsCRP, a marker of inflammation is associated with CF-PWV. This relationship has been shown in several large population studies in subjects with normal renal function (Mattace-Raso *et al.*, 2004; Nagano *et al.*, 2005; Yasmin *et al.*, 2004) and in smaller studies in haemodialysis (Haydar *et al.*, 2004; London *et al.*, 2003), hypertensive (Mahmud *et al.*, 2005), and metabolic syndrome patients (Tomiyama *et al.*, 2005). Of all these studies, some have also shown that hsCRP is an independent predictor of PWV (London *et al.*, 2003; Mahmud *et al.*, 2005; Matsuda *et al.*, 2009; Mattace-Raso *et al.*, 2004; Nagano *et al.*, 2005; Tomiyama *et al.*, 2005; Yasmin *et al.*, 2004; Yoshida *et al.*, 2007). Our findings are in keeping with this, suggesting that even low-grade inflammation may influence vascular function in CKD (Stenvinkel *et al.*, 2005a).

The current study has shown that neither 8-Isop nor OxLDL is independently associated with CF-PWV. To date, there is no study that directly examines the contribution of Isop or OxLDL to arterial stiffness in renal patients. However, a study in patients with peripheral arterial disease has shown that oxidative stress, measured by urinary Isop, was independently associated with arterial stiffness, measured by AIx, while OxLDL had no association with AIx (Kals *et al.*, 2008). In contrast to this, a recent study in the elderly with diabetes and cardiovascular co-morbidity has shown that OxLDL independently predicted PWV (Brinkley *et al.*, 2009).

There is conflicting data regarding the relationship of arterial stiffness and ADMA. Several studies showed associations in univariate analysis (Fujii *et al.*, 2008; Kals *et al.*, 2007; Kielstein *et al.*, 2006; Paiva *et al.*, 2008; Soveri *et al.*, 2007; Weber *et al.*, 2007), while some did not (Chirinos *et al.*, 2008; Paiva *et al.*, 2006). Here, I also propose that plasma ADMA is an independent predictor of arterial stiffness in CKD patients with varying GFR. This independent association has been shown in CKD patients on dialysis (Soveri *et al.*, 2007) and, more recently, in hypertensive patients with renal insufficiency (Fujii *et al.*, 2008). In contrast to the finding of this study,

ET-1 was shown to be related to arterial stiffness (Demuth *et al.*, 1998; Heintz *et al.*, 1993; Karakitsos *et al.*, 2007; McEniery *et al.*, 2003; Vuurmans *et al.*, 2003). The absence of plasma ET-1 as an independent predictor of arterial stiffness is also contrary to other studies. However, these included patients on dialysis (Demuth *et al.*, 1998), those with ischaemic heart disease (Heintz *et al.*, 1993) and diabetes (Karakitsos *et al.*, 2007). Thus, co-morbidity may again partly explain these findings. Overall, our findings strongly support the contribution of ADMA and the shift in vasodilator-vasoconstrictor balance in the development of vascular dysfunction in CKD.

6.4.4 Relationships and predictors of flow-mediated dilatation

In this study, BP, 8-Isop, and ET-1 were shown to be independent predictors of endothelial function. These are in contrast to previous studies in which uraemia is an independent predictor of endothelial dysfunction (Annuk *et al.*, 2001; Stam *et al.*, 2003). A study in haemodialysis and stage 3 to 4 CKD patients (Bolton *et al.*, 2001) and in hypertensive patients with mild to moderate renal impairment (Zoccali *et al.*, 2006), inflammation independently predicts FMD. OxLDL is a predictor for FMD in stage 1 to 5 CKD patients including those on dialysis. Nonetheless, the findings of the current study are in accordance with the studies of stage 3 to 5 CKD patients in which neither GFR nor OxLDL were shown to independently predict endothelial function (Annuk *et al.*, 2005; Bolton *et al.*, 2001).

Our finding that ET-1 predicts FMD, again, strongly supports the contribution of the shift in vasodilator-vasoconstrictor balance of NO/ET in CKD. Two studies have suggested that ADMA is an independent predictor of endothelial dysfunction in non-dialysis proteinuric patients (Yilmaz *et al.*, 2008b) and in stage 1 to 5 CKD patients including those on dialysis (Yilmaz *et al.*, 2006). Of note, however, these studies included patients with co-morbidity.

To sum up, as it was shown in Chapter 5 that uraemia has no role as a predictor of arterial stiffness and endothelial dysfunction in this group of CKD paients with very

low comorbidity. The finding Chapter 6 strongly confirms the impact of BP on cardiovascular complications in CKD and, as hypothesised, further emphasises the contribution of emerging risk factors especially inflammation and ADMA on PWV and oxidative stress and ET-1 on FMD. Although the contribution of these emerging risk factors on cardiovascular complications on top of traditional risk factors in the multivariate analysis is not massive (5% increase for PWV and 0.07% increase for FMD), these changes are of significance. As these risk factors are modifiabled it could serve as target for interventions in cardiovascular risk and disease reduction in CKD patients.

6.4.5 Limitations

Some of the medications taken by the patients, such as ACE inhibitors, ARBs, β-blockers, and statins, may have had effects on both arterial stiffness and endothelial function. However, all patients were stabilised on their therapy and did not receive their medications on the day of the study until after measurements had been made. In addition, as this was a cross-sectional study, the causal relationships behind the associations described cannot be fully defined, although they do have a sound mechanistic basis.

6.4.6 Summary

In the absence of established cardiovascular disease and diabetes, arterial stiffness and endothelial dysfunction increase as GFR falls. Amongst conventional risk factors, BP determines arterial stiffness and endothelial dysfunction while renal function is not a predictor of either. Additionally, hsCRP and ADMA are independent predictors of arterial stiffness, and 8-Isop and ET-1 independently predict endothelial dysfunction. Thus, the vascular complications occur in a uraemic population may substantially be driven by inflammation, oxidative stress, and NO/ET imbalance, instead of the uraemia itself, and measures of these could help identify those at a higher risk of vascular disease. Interventional studies to examine the influence of these emerging risk factors on arterial stiffness and endothelial dysfunction, using potential therapeutic agents such as ET antagonists or

phosphodiesterase type 5 inhibitors, would be of great interest and may offer benefits to CKD patients.

Chapter 7

Risk factors for the metabolic syndrome independently predict arterial stiffness and endothelial dysfunction in chronic kidney disease

7.1 Introduction

The metabolic syndrome is a clustering of metabolic abnormalities and risk factors for cardiovascular disease featuring abdominal obesity, hyperglycaemia, hypertension, hypertriglyceridaemia, and reduced HDL cholesterol (Grundy *et al.*, 2004; Reaven, 1988). As the metabolic syndrome is associated with increased risks of diabetes and cardiovascular disease (Ahluwalia *et al.*, 2006; Sattar *et al.*, 2003), its treatment and prevention have become one of the major public health challenges worldwide. The risk factors for the metabolic syndrome, either together or individually, are also associated with arterial stiffness and endothelial dysfunction, in health (Achimastos *et al.*, 2007; Ferreira *et al.*, 2007; Ferreira *et al.*, 2005; Li *et al.*, 2005; Nakanishi *et al.*, 2003; van Popele *et al.*, 2000) and disease (Martens *et al.*, 2008; Mule *et al.*, 2006; Schillaci *et al.*, 2005; Seo *et al.*, 2005; Stehouwer *et al.*, 2008; Vyssoulis *et al.*, 2008; Yokoyama *et al.*, 2007).

The metabolic syndrome is widely prevalent in CKD (Johnson *et al.*, 2007) and the metabolic syndrome is itself a risk factor for CKD (Chen *et al.*, 2004). There is no study directly assessing the contribution of the metabolic syndrome to arterial stiffness and endothelial dysfunction in non-dialysis CKD patients. However, a recent study suggests the metabolic syndrome and its risk factors do contribute to arterial stiffness and endothelial dysfunction in dialysis patients (Zhe *et al.*, 2008).

7.1.1 Aims and hypotheses

The major findings of Chapter 5 are that arterial stiffness and endothelial dysfunction increase as renal function declines, and these are mainly driven by conventional cardiovascular risk factors, such as age and BP. In Chapter 6, I have extended these findings and showed that inflammation and the NO/ET systems also contribute to arterial stiffness and endothelial dysfunction, and high cardiovascular risk in CKD patients.

Based on data from Chapters 5 and 6, it is now interesting to explore the role of the metabolic syndrome on arterial stiffness and endothelial dysfunction. The aims of the

current study are to investigate the relationships of the metabolic syndrome and its individual components to arterial stiffness and endothelial dysfunction in relatively low co-morbid CKD patients across a wide range of renal function from early CKD to pre-dialysis.

Hypotheses:

In this group of patients with minimal co-morbidity, arterial stiffness and endothelial dysfunction would still relate to the presence of the metabolic syndrome or its components independent of renal function and other well-established risk factors for cardiovascular disease.

7.2 Methods

7.2.1 Subjects

All subjects recruited from studies in Chapters 5 and 6 were further enrolled into this study.

7.2.2 Study design and protocol

This was a prospective, cross-sectional study. Subjects refrained from alcohol for at least 24 hours and caffeinated drinks, food, and smoking for at least 12 hours before the study. Subjects were asked to withhold their current medications on the morning of the study. All studies were conducted in a quiet, temperature-controlled room. On arrival, the subject's height, weight, waist and hip circumference were measured as described in Chapter 2. After 30 minutes of supine rest, participants underwent BP measurements, and then blood samples were taken for creatinine, glucose, triglycerides, and HDL cholesterol. After that, CF-PWV and FMD were measured. All techniques were performed as described in Chapter 2.

7.2.3 Laboratory investigations

Serum creatinine, plasma glucose, triglycerides, and HDL cholesterol were quantified in the hospital biochemistry laboratory.

7.2.4 Diagnosis of the metabolic syndrome

The metabolic syndrome was diagnosed according to the criteria from the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel (ATP) III) (2002; Grundy *et al.*, 2004). The diagnosis of the metabolic syndrome was made when subjects had ≥3 risk factors for the metabolic syndrome. Subjects with 0 - 1 and those with 2 risk factors for the metabolic syndrome were classified as no metabolic syndrome and risk for developing metabolic syndrome, respectively (see Table 2.2, Chapter 2, section 2.8).

7.2.5 Outcomes

Primary outcome variables were the presence of the metabolic syndrome and the number of risk factors for the metabolic syndrome in relation to eGFR, CF-PWV, and FMD, and the relationships of each risk factor for the metabolic syndrome (waist circumference, SBP, DBP, glucose, triglycerides, and HDL cholesterol) to eGFR, CF-PWV, and FMD.

7.2.6 Data analyses

Descriptive data is given as mean ± SD unless otherwise stated. The means of the categorical data (subjects without metabolic syndrome, subjects with risk of developing metabolic syndrome, subjects with the metabolic syndrome) were compared by one-way ANOVA. Continuous data (risk factors for the metabolic syndrome as 0 to 5 risk factors) were analysed by correlation coefficients for univariate analysis using the Pearson method. Multivariate analysis was performed using a stepwise linear regression. Dependent variables were CF-PWV and FMD.

First, GFR and traditional cardiovascular risk factors that were not regarded as the metabolic syndrome components including age, gender and smoking status were entered into the first model. Thereafter, along with GFR and traditional cardiovascular risk factors in the first model, the presence of the metabolic syndrome (yes/no) and number of the metabolic syndrome risk factors (1 to 5 risk factors) were entered into the second and third models, respectively. Finally, in the fourth model, each components of the metabolic syndrome risk factors were consider individually. Multiple coefficient of determination (r^2) of each model was reported. Multivariate analysis data were reported for both CF-PWV and FMD using standard regression coefficient (β value) of independent variables. A significant level was p value < 0.05.

7.3 Results

7.3.1 Subject characteristics

One hundred and fifteen CKD patients and 23 age-matched non-CKD controls were enrolled into the study. The characteristics of the studied subjects are given in Table 7.1 (for full subject characteristics, see Appendix 1). Causes of CKD and the medication used by the patients were similar to those summarised in Table 5.1, Chapter 5.

Table 7.1 Subject characteristics.

	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
n	23	28	30	30	20	7
eGFR (ml/min/1.73m^2)*	97±19	108±17	77±9	45±9	23±4	12±3
Male/ Female (n)	13/10	17/11	18/12	22/8	16/4	4/3
Age (year)	47±8	43±11	49±9	51±10	45±9	51±12
Smoker/ Non-smoker (n)	2/21	9/19	5/25	6/24	4/16	0/7
Subjects without MS (n (%))	19 (83)	17 (61)	14 (47)	21 (70)	9 (72)	3 (42)
Subjects at risk of MS (n (%))	3 (13)	5 (18)	9 (30)	6 (20)	5 (25)	2 (29)
Subjects with MS (n (%))	1 (4)	6 (21)	7 (23)	3 (10)	6 (3)	2 (29)
BMI (kg/m^2)	26±6	29±5	28±4	28±6	28±5	25±7

Key: *$p < 0.01$ for one-way ANOVA by CKD stage. Subjects without the metabolic syndrome: subjects with 0 – 1 risk factor for the metabolic syndrome; subjects at risk of developing metabolic syndrome: subjects with 2 risk factors for the metabolic syndrome; subjects with the metabolic syndrome: subjects with ≥3 risk factors for the metabolic syndrome. BMI: body mass index; eGFR: estimated glomerular filtration rate; MS: metabolic syndrome.

Subjects were classified into 3 categories according to the number of risk factors for the metabolic syndrome (no metabolic syndrome: subjects with 0-1 risk factor; risk of developing metabolic syndrome: subjects with 2 risk factors; and metabolic syndrome: subjects with ≥3 risk factors). 19 subjects (18%) had the metabolic syndrome and 29 subjects (21%) were regarded as at risk of developing it. All three categories were comparable in respect of age and eGFR (Table 7.2). As expected, subjects with the metabolic syndrome had higher BMI, waist circumference, SBP, DBP, glucose, triglycerides, and had lower HDL cholesterol compared to those without the metabolic syndrome or only at risk for developing it (Table 7.2).

With regards to the relationship of the risk factors for the metabolic syndrome to renal function, only SBP increased as eGFR declined ($r^2 = 0.11$, $p < 0.01$). Waist circumference, DBP, glucose, triglycerides, and HDL cholesterol showed no relationship to renal function.

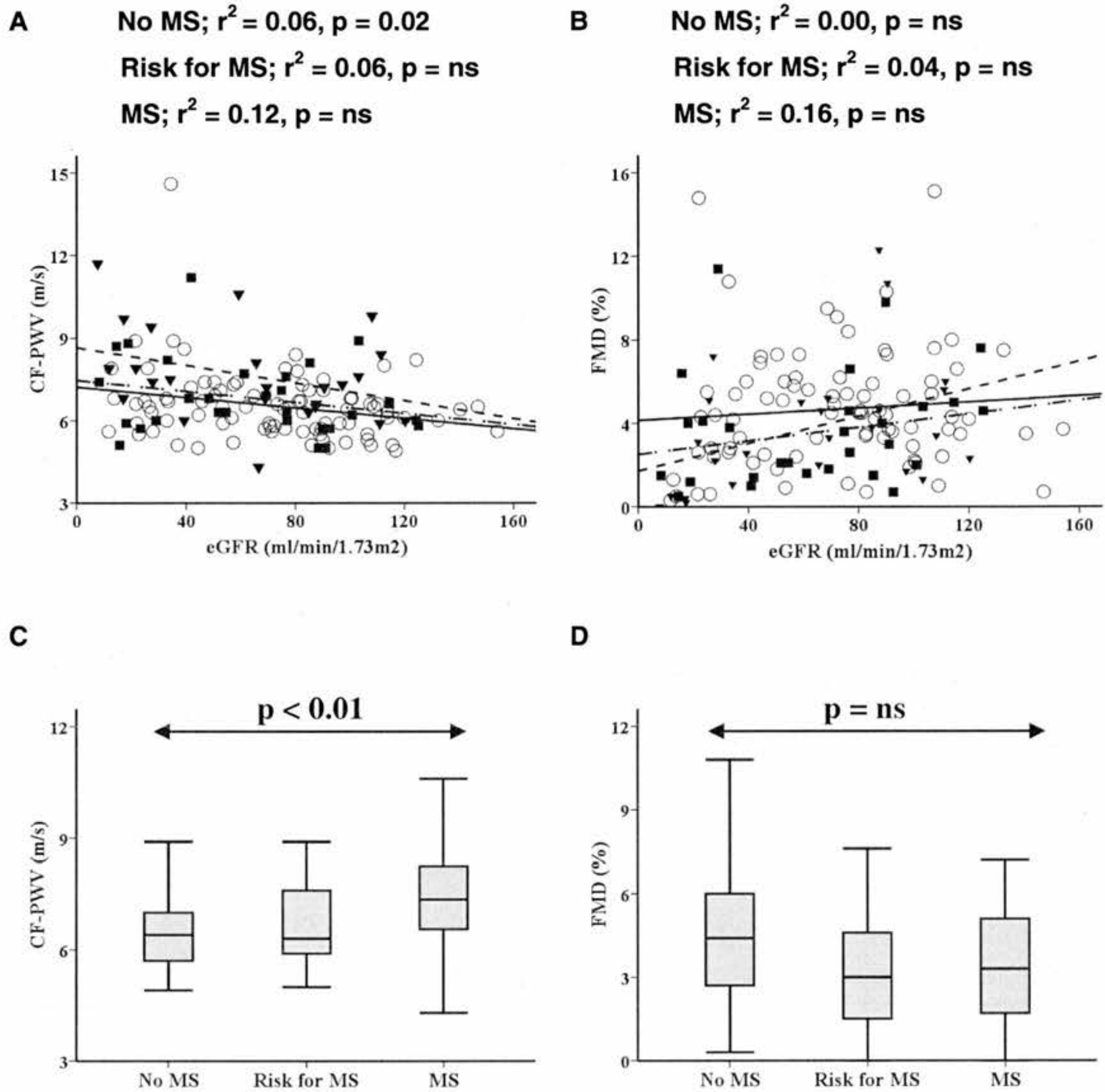
CF-PWV was higher in the metabolic syndrome group (Table 7.2, Figures 7.1 A & C) whereas RAIx, CAIx, showed no relationship to the metabolic syndrome (Table 7.2). FMD was lower in the metabolic syndrome group (Table 7.2, Figures 7.1 B & D) but this was not found to be significant. The endothelium-independent response to GTN had no relationship to the metabolic syndrome (Table 7.2).

Table 7.2 Risk factors for the metabolic syndrome, arterial stiffness and endothelial function.

	No MS	Risk for MS	MS
n	84	29	25
Male/ Female (n)	52/32	19/10	19/6
Smoker/ Non-smoker (n)	16/68	4/25	6/19
Age (year)	46±9	48±11	51±8
eGFR (ml/min/1.73m^2)	73±34	64±35	62±37
Body mass index (kg/m^2)*	26±4	29±5	33±4
Risk factors for the metabolic syndrome			
Waist circumference (cm)*	90±11	96±13	111±12
Systolic blood pressure (mmHg)*	113±14	122±15	126±13
Diastolic blood pressure (mmHg)*	72±9	75±8	77±7
Glucose (mmol/L)*	4.9±0.5	5.0±0.4	5.5±0.5
Triglycerides (mmol/L)*	1.1±0.4	1.6±0.9	2.3±1.2
HDL cholesterol (mmol/L)*	1.4±0.3	1.1±0.3	0.9±0.2
Arterial stiffness and endothelial function			
CF-PWV (m/s)*	6.6±1.3	6.8±1.4	7.6±1.7
RAIx (%)	75±18	76±17	81±16
CAIx (%)	22±13	23±13	26±11
FMD (%)	4.7±2.9	3.5±2.8	3.8±3.1
GTN (%)	12.3±5.2	10.5±3.9	10.5±4.8

Key: *$p < 0.05$ for one-way ANOVA by the metabolic syndrome groups. No MS: subjects with 0-1 risk factor for the metabolic syndrome; risk for MS: subjects with 2 risk factors for the metabolic syndrome; MS: subjects with ≥3 risk factors for the metabolic syndrome. CAIx: central augmentation index; CF-PWV: carotid-femoral pulse wave velocity; eGFR: estimated glomerular filtration rate; FMD: flow-mediated dilatation; HDL: high density lipoprotein; MS: metabolic syndrome; GTN: endothelium-independent vasodilatation response to glyceryl trinitrate; RAIx: radial augmentation index.

Figure 7.1 Relationships of the number of the metabolic syndrome risk factors to CF-PWV and FMD.



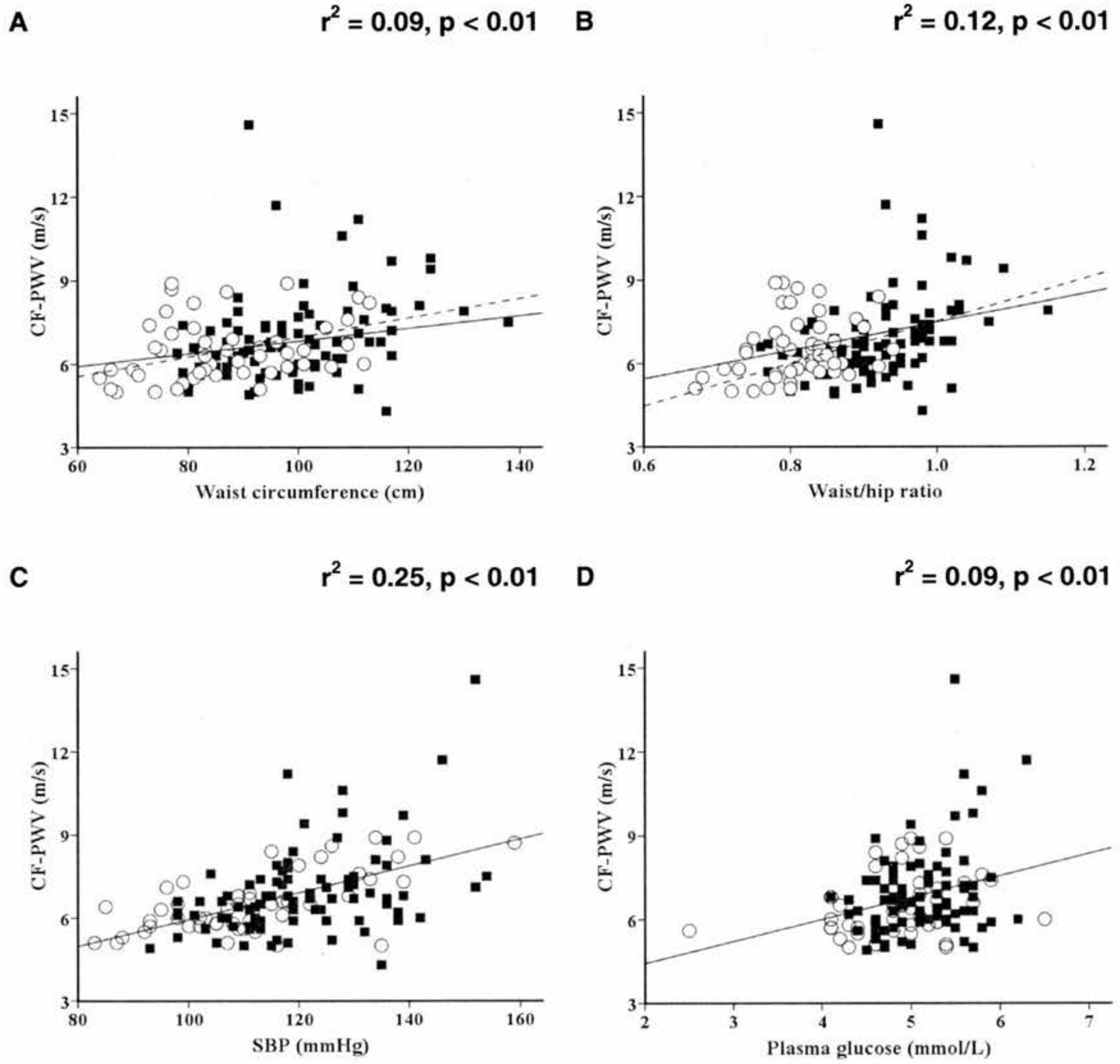
Key: No MS (white circle, — fitted line): subjects without metabolic syndrome (risk factors = 0-1); Risk for MS (black square, ‾ ˙ ‾ fitted line): subjects at risk of developing metabolic syndrome (risk factors = 2); MS (black triangle, --- fitted line): subjects with metabolic syndrome (risk factors ≥ 3). For C & D, p values are for one-way ANOVA; eGFR: estimated glomerular filtration rate; ns: non-significant.

7.3.2 Relationships and predictors of carotid-femoral pulse wave velocity

Univariate analysis was performed to assess the relationship of CF-PWV to the number of the metabolic syndrome risk factors, each of the risk factor for the metabolic syndrome, and related parameters which are not included in the NCEP ATIP III criteria (eGFR, age, BMI, and waist/hip ratio). CF-PWV correlated with the number of the metabolic syndrome risk factors (Figures 7.1 A & C). For each of the risk factors for the metabolic syndrome, CF-PWV increased with waist circumference, SBP, DBP ($r^2 = 0.10$, $p < 0.01$), and glucose (Figures 7.2 A, C, D). CF-PWV also increased with triglycerides and inversely correlated to HDL cholesterol but these relationships did not reach significance. Additionally, CF-PWV correlated with eGFR ($r^2 = 0.08$, $p < 0.01$), age ($r^2 = 0.23$, $p < 0.01$), and waist/hip ratio (Figure 7.2B) but not with BMI.

In multivariate analysis, when renal function and conventional risk factors including age, gender, and smoking status were entered into the model, renal function and age were independent predictors of arterial stiffness (Table 7.3, Model 1). When either the presence of the metabolic syndrome or the number of risk factors for the metabolic syndrome a subject had were considered, both of them, together with renal function and age, independently predicted CF-PWV (Table 7.3, Models 2 & 3). When the individual risk factors for the metabolic syndrome were considered, renal function was replaced by waist circumference and SBP as independent predictors of CF-PWV (Table 7.3, Model 4).

Figure 7.2 Correlations between CF-PWV and SBP, plasma glucose, waist circumference, and waist/hip ratio.



Key: Male: black square; female: white circle. Figures A and B: male: --- fitted line, female: — fitted line. Figures C and D: — fitted line for total.

Table 7.3 Multivariate analysis of renal function, conventional cardiovascular risk factors, and risk factors for the metabolic syndrome, as independent predictors of CF-PWV.

	CF-PWV			
Predictors	**Model 1**	**Model 2**	**Model 3**	**Model 4**
Estimated glomerular filtration rate	**-0.01****	**-0.01****	**-0.01****	-0.11
Age	**0.06***	**0.06***	**0.06***	**0.05***
Gender (male/ female)	-0.14	-0.12	-0.13	0.01
Smoking status (yes/no)	0.02	0.00	0.02	-0.02
The presence of MS (yes/no)	-	**0.73****	-	-
Number of MS risk factors (0 to 5)	-	-	**0.25***	-
Waist circumference	-	-	-	**0.02***
Systolic blood pressure	-	-	-	**0.03***
Diastolic blood pressure	-	-	-	-0.21
Glucose	-	-	-	0.04
Triglycerides	-	-	-	0.06
HDL cholesterol	-	-	-	-0.08
r^2	**0.26***	**0.30***	**0.31***	**0.45***

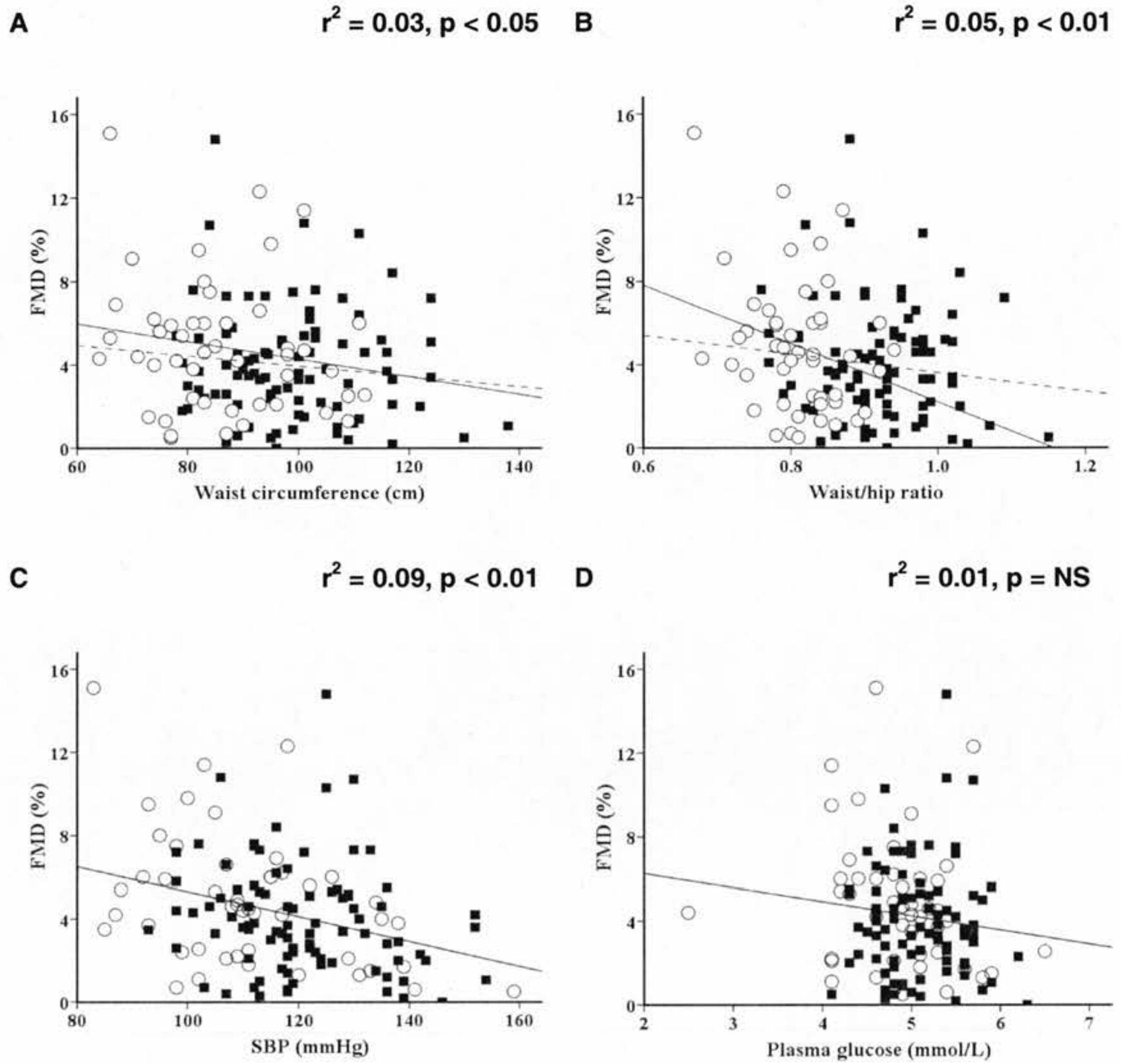
Key: The table gives standard regression coefficients (β values).*p < 0.01, **p < 0.05. CF-PWV: carotid-femoral pulse wave velocity; HDL: high density lipoprotein; MS: metabolic syndrome; r^2: multiple coefficient of determination.

7.3.3 Relationships and predictors of flow-mediated dilatation

In univariate analysis, FMD correlated with eGFR ($r^2 = 0.04$, $p < 0.05$) but not the number of the metabolic syndrome risk factors (Figures 7.1 B & D), age, or BMI. In terms of each of the risk factors for the metabolic syndrome, FMD correlated with waist circumference, SBP, and DBP but not plasma glucose, triglycerides, and HDL cholesterol (Figures 7.3 A, C, D). FMD also correlated with waist/hip ratio (Figure 7.3B).

In multivariate analysis assessing renal function and conventional risk factors, eGFR was an independent predictor of FMD (Table 7.4, Model 1). When either the presence of the metabolic syndrome or the number of the metabolic syndrome risk factors was added to the model, renal function still independently predicted FMD (Table 7.4, Models 2 & 3). However, when the risk factors for the metabolic syndrome were added individually to the model, renal function was substituted by SBP as an independent predictor of FMD (Table 7.4, Model 4).

Figure 7.3 Correlations between FMD and SBP, plasma glucose, waist circumference, and waist/hip ratio.



Key: Male: black square; female: white circle. Figures A and B: male: --- fitted line, female: — fitted line. Figures C and D: — fitted line for total.

Table 7.4 Multivariate analysis of renal function, conventional cardiovascular risk factors, and risk factors for the metabolic syndrome, as independent predictors of FMD.

	FMD			
Predictors	**Model 1**	**Model 2**	**Model 3**	**Model 4**
Estimated glomerular filtration rate	**0.02****	**0.02****	**0.02****	0.09
Age	-0.09	-0.09	-0.09	-0.05
Gender (male/ female)	0.12	0.12	0.12	0.06
Smoking status (yes/no)	-0.02	-0.02	-0.02	-0.03
The presence of MS (yes/no)	-	-0.07	-	-
Number of MS risk factors (0 to 5)	-	-	-0.16	-
Waist circumference	-	-	-	-0.11
Systolic blood pressure	-	-	-	**-0.06***
Diastolic blood pressure	-	-	-	0.10
Glucose	-	-	-	-0.05
Triglycerides	-	-	-	-0.06
HDL cholesterol	-	-	-	0.09
r^2	**0.04****	**0.04****	**0.04****	**0.09***

Key: The table gives standard regression coefficients (β values). *p < 0.01, **p < 0.05; FMD: flow-mediated dilatation; HDL: high density lipoprotein; MS: metabolic syndrome; r^2: multiple coefficient of determination.

7.4 Discussion

7.4.1 Main findings

In minimally co-morbid CKD patients with eGFR ranging from 8 to 154 ml/min/1.73m^2, I have previously shown in Chapter 5 that renal function is related to an increase in arterial stiffness and endothelial dysfunction, measured by CF-PWV and FMD, respectively. Here, I have further shown that, regardless of renal function, CKD patients with the metabolic syndrome have higher arterial stiffness compared to those without the metabolic syndrome. The presence of the metabolic syndrome and the number of risk factors for it independently predict arterial stiffness. Furthermore, when risk factors for the metabolic syndrome were added individually into multivariate analysis, waist circumference and SBP were determinants of CF-PWV, independent of renal function, age, gender, and smoking status.

7.4.2 Relationships and predictors of carotid-femoral pulse wave velocity

CKD patients recruited to this study were carefully chosen to have low co-morbidity. The prevalence of the metabolic syndrome in this CKD population is thus lower than previously reported in dialysis (Young *et al.*, 2007; Zhe *et al.*, 2008), hypertensive (Mule *et al.*, 2006; Schillaci *et al.*, 2005; Seo *et al.*, 2005), and diabetic patients (Martens *et al.*, 2008; Yokoyama *et al.*, 2007), but is comparable to healthy subjects (18% *versus* 10 - 19%) (Achimastos *et al.*, 2007; Ferreira *et al.*, 2007; Ferreira *et al.*, 2005; Li *et al.*, 2005). Despite this selection bias against the metabolic syndrome and its risk factors, there is an increase in arterial stiffness in CKD patients with the metabolic syndrome or risk factors for it, irrespective of renal function, and that these independently predict arterial stiffness when considered alongside conventional risk factors. This finding is in keeping with previous data from healthy subjects (Czernichow *et al.*, 2005; Ferreira *et al.*, 2007; Ferreira *et al.*, 2005; Kovaite *et al.*, 2007; Li *et al.*, 2005; Nakanishi *et al.*, 2003; van Popele *et al.*, 2000), hypertensive (Mule *et al.*, 2006; Schillaci *et al.*, 2005; Seo *et al.*, 2005; Vyssoulis *et al.*, 2008), and diabetes patients (Martens *et al.*, 2008; Yokoyama *et al.*, 2007).

Additionally, in this study, waist circumference predicts arterial stiffness, independent of renal function, age, BP, and gender, in CKD patients. This finding was previously shown only in hypertensive patients (Schillaci *et al.*, 2005). Obesity is associated with an increased risk of cardiovascular disease (Elsayed *et al.*, 2008) and with increased arterial stiffness (Orr *et al.*, 2008). This finding supports waist circumference, a marker of central obesity, as a better surrogate for arterial stiffness than BMI or other cholesterol subtypes such as LDL cholesterol and triglycerides in uraemic patients.

7.4.3 Relationships and predictors of flow-mediated dilatation

The finding that, regardless of renal function, CKD patients with the metabolic syndrome did not have significant impaired endothelial function was similar to data from studies in healthy subjects (Mattsson *et al.*, 2008; Wendelhag *et al.*, 2002), subjects at risk of developing diabetes mellitus (Ghiadoni *et al.*, 2008; Scuteri *et al.*, 2008) and those with peripheral artery disease (Golledge *et al.*, 2008), but not all (Kovaite *et al.*, 2007; Lind, 2008). In a study of ~1,000 elderly subjects including those with cardiovascular disease and diabetes where endothelial function was measured by both the invasive forearm technique and FMD (Lind, 2008), only the invasive forearm technique showed a reduction of endothelial function in the metabolic syndrome group. It is recognised that the invasive forearm technique measures endothelial function of the resistance artery while FMD measures endothelial function of the conduit artery. Thus, it is possible that the deterioration in endothelial function in the metabolic syndrome may occur predominantly in the resistance artery and, hence, by using the FMD technique, endothelial dysfunction was not detected in this study cohort. In addition, the lack of an association between a number of risk factors for the metabolic syndrome and FMD seen here is in contrast to a previous report in subjects at risk of developing diabetes mellitus (Ghiadoni *et al.*, 2008). However, it is possible that a genetic-linked to diabetes and insulin resistance state of the subjects may confound the findings of that study.

Of all the risk factors studied here, conventional and metabolic syndrome-related, only SBP predicts endothelial function in the final multivariate analysis. These results both confirm and contrast with previous data. In support of the current study, evidence has shown that BP is an independent predictor of endothelial function (Scuteri *et al.*, 2008). Additionally, a study reported that the presence of the metabolic syndrome was not a predictor of endothelial function (Kovaite *et al.*, 2007). By contrast, a study has shown that renal function predicts endothelial function in CKD patients (Annuk *et al.*, 2001). However, that study was performed in CKD patients with diabetes in which the nature of the disease itself may confound the finding and endothelial function was assessed using an invasive forearm technique which measured endothelial dysfunction in a different vascular bed compared to the FMD technique used in this study. The presence of the metabolic syndrome was also shown to predict endothelial function but this was studied in patients with peripheral arterial disease (Golledge *et al.*, 2008).

As previously shown in Chapters 5 and 6 that uraemia does not contribute to the increase in cardiovascular complications in CKD patients with low comorbidity. Yet, emerging cardiovascular risk factors including inflammation, oxidative stress and the imbalance between NO and ET-1 systems significantly contribute to these. The findings of Chapter 7 are in keeping with the hypotheses of the study. However, the finding that the presence of the metabolic syndrome and number of the metabolic syndrome risk factors are predictors of PWV should be interpreted with caution as SBP and DBP are in the criteria for a diagnosis of the metabolic syndrome. Nonethless, this study, again, confirms the role of BP in determining cardiovascular complications and importantly extends the type of emerging cardiovascular risk factors that determine PWV to obesity which could be targeted as another modifiable risk factor in reduction of cardiovascular disease in CKD.

Of note, an inverse association between DBP and CF-PWV and a positive association between DBP and FMD seen in the multivariate analysis of this study are surprising. However, these could be explained by an effect of pulse pressure which is positively associated with CF-PWV and inversely related to FMD since both SBP and DBP were entered into the analysis.

7.4.4 Limitations

There are several criteria for the diagnosis of the metabolic syndrome (Grundy *et al.*, 2004). As the patients of this study are non-diabetic, we cannot use the metabolic syndrome diagnosis criteria proposed by WHO and, therefore, the evaluation of an effect of insulin resistance to arterial stiffness and endothelial dysfunction is limited, and it may not be comparable with other studies using WHO criteria. Some of the medication taken by our patients, such as ACE inhibitors, ARBs, β-blockers, and statins, may have had effects on both arterial stiffness and endothelial dysfunction. However, all patients were stabilised on their therapy and did not receive their medication on the day of the study until after measurements had been made. The low number of smokers in this study may be responsible for the lack of expected relationships between smoking status and arterial stiffness and endothelial dysfunction. In addition, as this was a cross-sectional study, we cannot fully define the causal relationships behind the associations described.

7.4.5 Summary

In summary, the present study shows that, in the absence of cardiovascular disease and diabetes, irrespective of renal function, age, smoking status, and gender, CKD patients with the metabolic syndrome have increased arterial stiffness compared to those without the metabolic syndrome but there was no difference in endothelial function. Whilst BP remains the strongest determinant of both arterial stiffness and endothelial dysfunction, the presence of the metabolic syndrome or risk factors for it is also an important determinant of arterial stiffness in the CKD population. As these are not related to renal function, they provide a target for intervention to improve cardiovascular outcomes at all stages of CKD.

Chapter 8

Relationships of coronary artery calcification and fetuin-A to arterial stiffness and endothelial dysfunction in chronic kidney disease

8.1 Introduction

Arterial calcification is a common pathological process in CKD (Moe *et al.*, 2008; Salusky *et al.*, 2002). An increase in the calcification inducers - including hypercalcaemia, hyperphosphataemia, elevated calcium–phosphate products, increased PTH levels, and excessive treatment with vitamin D, - and a reduction in the calcification inhibitors such as fetuin-A contribute to this (Moe *et al.*, 2008). Arterial calcification reduces aortic and arterial elasticity, leading to an impairment of cardiovascular haemodynamics and, hence increase morbidity and mortality (Demer *et al.*, 2008; London *et al.*, 2005).

Arterial calcification occurs mainly in the aorta; however, it is also present in the coronary arteries (Dellegrottaglie *et al.*, 2005). Coronary artery calcification (CAC) quantified using computed tomography and presented as CAC score is commonly used as a technique for measuring arterial calcification (Dellegrottaglie *et al.*, 2005; Moe *et al.*, 2008). CAC scores are increased in ESRD (Kobayashi *et al.*, 2008; Kramer *et al.*, 2005; Porter *et al.*, 2007). Evidence suggests that the process of CAC starts before patients reach ESRD and the extent of CAC may substantially be related to a declining GFR (Piers *et al.*, 2009). Additionally, CAC is associated with arterial stiffness in ESRD (Haydar *et al.*, 2004; Nitta *et al.*, 2004; Schlieper *et al.*, 2009). A recent study also suggests the relationship between CAC and arterial stiffness in pre-dialysis CKD (Lemos *et al.*, 2007). Endothelial dysfunction is an initial step in the development of atherosclerotic plaque and thus may also play a crucial role in arterial calcification. Studies have shown the associations of endothelial dysfunction and CAC in patients with coronary artery disease (Huang *et al.*, 2005; Ramadan *et al.*, 2008) but there are no data in CKD.

Fetuin-A, a potent calcification inhibitor generated from hepatocytes, plays an important role in the pathogenesis of arterial calcification. Circulating fetuin-A concentrations are reduced in ESRD compared to those of healthy subjects (Mazzaferro *et al.*, 2007). A few studies have suggested a relationship of fetuin-A to arterial stiffness (Hermans *et al.*, 2006; Kuzniar *et al.*, 2008) and endothelial dysfunction (Caglar *et al.*, 2007) in ESRD. Data regarding arterial stiffness and

endothelial dysfunction in pre-dialysis CKD is limited. However, a recent study suggests a graded decrease of fetuin-A concentrations with declining GFR (Caglar *et al.*, 2008b).

8.1.1 Aims and hypotheses

The aims of this study were to assess the relationships of CAC and fetuin-A to arterial stiffness and endothelial dysfunction in a group of CKD patients with minimal co-morbidity from CKD stage 1 to pre-dialysis.

Hypotheses:

1. In this minimally co-morbid CKD group, the presence and extent of arterial calcification, measured using the CAC score, would increase as renal function declined while there would be a reduction in the circulating levels of the calcification inhibitor, fetuin-A.

2. The increase in CAC score and the reduction of fetuin-A concentrations would independently relate to arterial stiffness, measured using CF-PWV, and endothelial dysfunction, measured using FMD in this group of patients.

8.2 Methods

8.2.1 Subjects

From the subjects recruited for a study investigating the relationship of arterial stiffness and endothelial dysfunction to renal function (Chapter 5), 25 subjects were sub-recruited to this study. There were 5 subjects in each CKD stage 1 to 3 and 5 subjects in stage 4 and 5 combined. Five age-matched healthy subjects were also recruited as a control group. The inclusion and exclusion criteria were similar to the criteria described in Chapter 5.

8.2.2 Study design and protocol

This was a prospective, cross-sectional study. For the measurement of CF-PWV and FMD, subjects refrained from alcohol for at least 24 hours and caffeinated drinks, food and smoking for at least 12 hours before the study. Subjects were asked to withhold their current medication on the morning of the study. All CF-PWV and FMD measurements were performed in a quiet, temperature-controlled room. After 30 minutes of supine rest, participants underwent BP measurements, and then blood samples were taken for creatinine, glucose, total cholesterol, electrolytes, calcium, phosphate, PTH, and fetuin-A. After that, CF-PWV and FMD were measured. All techniques were performed as described in Chapter 2. Within a period of 1 month after CF-PWV and FMD measurements, each subject underwent 16-slice MDCT in order to quantify the presence and extent of CAC. The quantification of CAC score was performed as described in Chapter 2, section 2.7 (Figure 2.2 and Table 2.1).

8.2.3 Laboratory investigations

Serum fetuin-A concentrations were determined by a commercially available ELISA (BioVendor GmbH, Heidelberg, Germany), as previously described in Chapter 2, section 2.9.2.7.

8.2.4 End points

The end points were the CAC score and serum fetuin-A concentrations relating to GFR, CF-PWV, and FMD.

8.2.5 Data analyses

Descriptive data is given as mean ± SEM. The means of the subject characteristics between controls and CKD patients, and subjects with CAC and without CAC were compared using the unpaired Student's *t* test. Subjects were also divided into 4 groups according to the severity of CAC: group 1 (normal): CAC score = 0; group 2 (minimal): CAC score = 0 - 10; group 3 (mild): CAC score = 11 - 100; and group 4

(moderate): CAC score >100, according to the CAC cut points proposed by He, *et al* (He *et al.*, 2000). CF-PWV and FMD between 4 groups were compared by one-way analysis of variance (ANOVA). Correlation coefficients were analysed using the Pearson method. A significant level was p value < 0.05.

8.3 Results

8.3.1 Subject characteristics

Twenty five subjects were enrolled to the study. Table 8.1 presents the characteristics of the control and CKD patients as a whole. For patients, the duration of having CKD was 13±4 (range 1 - 55) years. As expected, patients had lower eGFR. Age, gender, BP, glucose, cholesterol, BMI and calcium-phosphate related parameters were similar between the patients and controls. The characteristics of each CKD patient including diagnosis and medication are summarised in Table 8.2. There were 2 patients on a vitamin D supplementation and 1 patient on calcium acetate.

Table 8.1 Subject characteristics.

	Controls	CKD patients
n	5	20
Male/Female (n)	4/1	15/5
Smoker/Non-smoker (n)	1/4	8/12
eGFR ($ml/min/1.73m^2$)*	98±8 (76-123)	61±7 (13-108)
Age (year)	49±3 (42-57)	48±2 (29-64)
Body mass index (kg/m^2)	29±4 (20-46)	29±1 (19-41)
Mean arterial pressure (mmHg)	89±6 (75-106)	88±2 (74-106)
Glucose (mmol/L)	5.8±0.2 (4.9-5.8)	4.8±0.2 (2.5-5.7)
Total cholesterol (mmol/L)	5.0±0.3 (4.2-5.8)	4.5±0.1 (3.7-5.8)
Calcium (mmol/L)	2.34±0.04 (2.25-2.46)	2.36±0.03 (2.07-2.57)
Phosphate (mmol/L)	1.99±0.95 (0.97-5.80)	1.19±0.08 (0.75-2.00)
Bicarbonate (mmol/L)	26±1 (22-28)	26±1 (19-30)
Parathyroid hormone (ng/L)	66±7 (78-89)	92±21 (17-323)

Key: Data presented as mean ± SEM (range); *$p < 0.01$ for the unpaired Student's *t* test; eGFR: estimated glomerular filtration rate.

Table 8.2 Individual CKD patient characteristics.

Patient	Age (year)	Gender	BP (mmHg)	eGFR (ml/min/1.73m^2)	Diagnosis	Medication
1	46	Female	109/67	96	Reflux nephropathy	None
2	54	Male	128/71	108	Unknown	Candesartan (8mg)
3	48	Female	129/87	99	Unknown	None
4	54	Male	98/66	92	TBM disease	None
5	54	Male	125/80	90	Unknown	Ramipril (10mg), Diltiazem (300mg), Doxazosin (4mg), Simvastatin (20mg)
6	52	Female	93/64	77	Reflux nephropathy	None
7	50	Male	118/77	79	FSGS	Lisinopril (5mg)
8	50	Male	143/87	61	Polycystic kidney disease	Atenolol (100mg), Enalapril (10mg), Nifedipine (30mg), Simvastatin (20mg), Allopurinol (200mg), Levothyroxine (200μg)

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Patient	Age (year)	Gender	BP (mmHg)	eGFR (ml/min/1.73m^2)	Diagnosis	Medication
9	47	Male	119/70	76	Glomerulonephritis	Propranolol (5mg), Simvastatin (20mg)
10	39	Female	102/70	79	Reflux nephropathy	Amoxycillin (750mg), Amitriptyline (25mg)
11	57	Male	127/85	33	Glomerulonephritis	Furosemide (20mg), Metoprolol (100mg), Enapapril (20mg), Nifedipine (30mg), Allopurinol(100mg)
12	40	Male	98/65	52	FSGS	Ramipril (10mg), Irbesartan (75mg), Simvastatin (20mg)
13	48	Male	115/86	56	IgA nephropathy	Metoprolol (50mg), Lisinopril (20mg), Omeprazole (20mg)
14	58	Male	98/66	55	IgA nephropathy	Ramipril (2.5mg), Simvastatin (20mg)
15	64	Male	119/82	54	Unknown	Lithium (400mg)

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Patient	**Age (year)**	**Gender**	**BP (mmHg)**	**eGFR (ml/min/1.73m^2)**	**Diagnosis**	**Medication**
16	51	Male	124/67	24	Membranous	Furosemide (750mg), Bendroflumethiazide (5mg), Lisinopril (40mg), Candesartan (12mg), Amlodipine (20mg), Atorvastatin (10mg), Alfacalcidol (0.25μg), Warfarin (1mg)
17	45	Male	118/73	28	Polycystic kidney disease	Bendroflumethiazide (2.5mg), Lisinopril (20mg), Amlodipine (10mg), Lansoprazole (30mg)
18	29	Male	108/67	23	IgA nephropathy	Metoprolol (100mg), Ramipril (2.5mg), Nifedipine (30mg)
19	43	Male	118/81	22	Reflux nephropathy	Bumetanide (1mg), Candesartan (2mg), Diltiazem (120mg), Atorvastatin (20mg), Alfacalcidol (0.25μg), Warfarin (1mg), Allopurinol (100mg)
20	39	Female	110/75	13	Unknown	Thiamine hydrochloride (300mg), Phosex (3g), Folic acid (5mg), Ferrous sulphate (400mg), Ranitidine (150mg)

Key: FSGS: focal segmental glomerulosclerosis; membranous: membranous glomerulopathy; TBM: thin basement membrane disease. Doses are total per day.

8.3.2 Coronary artery calcification score and fetuin-A

The overall mean CAC score was 24±14 (range 0-342). The CAC score of CKD patients was higher than that of the controls but this did not reach significance (25±17 (range 0-342) and 19±18 (range 0-89), respectively, $p = 0.9$). The overall mean fetuin-A concentrations were 0.4±0.0 (range 0.2-0.6) g/L. CKD patients and controls had comparable fetuin-A concentrations (0.4±0.0 (range 0.2-0.6) g/L and 0.4±0.1 (range 0.2-0.5) g/L, respectively, $p = 0.9$). There was no effect of gender on the CAC score and fetuin-A.

Neither CAC score nor fetuin-A correlated with eGFR ($r^2 = 0.03$ and 0.02, respectively, both $p > 0.05$). eGFR linearly correlated with potassium, bicarbonate, and PTH ($r^2 = 0.47$, 0.28, 0.41, respectively, all $p < 0.01$) but had no relationship to calcium. CAC score and fetuin- A did not correlate with each other ($r^2 = 0.00$, $p = 0.66$).

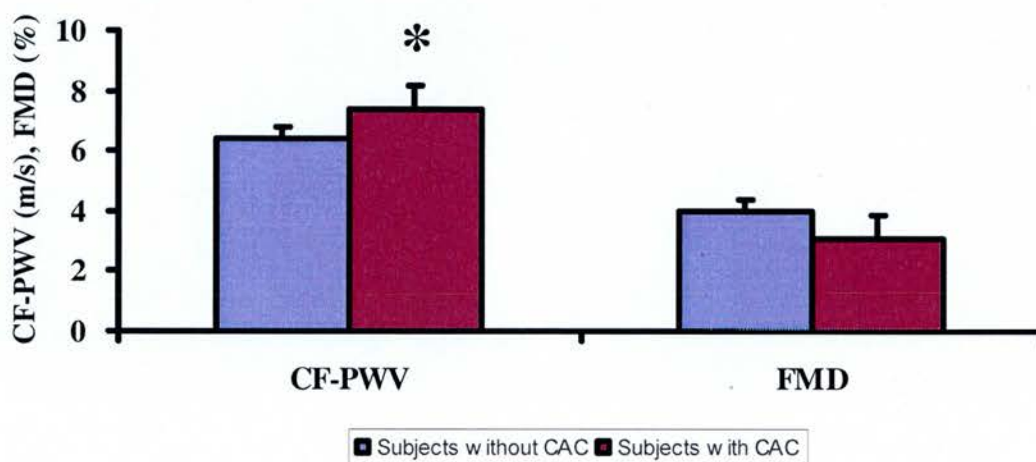
Subjects were classified as those with and without CAC (Table 8.3). Eleven subjects (2 controls and 9 CKD patients) had CAC score >0 giving an overall prevalence of CAC of 44%. The prevalence of CAC in the controls and CKD patients were 40% and 45%, respectively. The mean of the CAC score in subjects with CAC was 53±31 (range 0.2-342). Fetuin-A concentrations, renal function, or calcium-phosphate related parameters were not different between subjects with or without CAC (Table 8.3). CKD patients with CAC had CKD for a longer duration compared to patients without CAC but this was not significant (Table 8.3). Subjects with CAC had significantly higher CF-PWV compared to those without (Table 8.3, Figure 8.1). However, FMD and response to GTN were lower in subjects with CAC than those without CAC but these were not significant (Table 8.3, Figure 8.1).

Table 8.3 Demographic data, CAC score, fetuin-A, CF-PWV, and FMD of the subjects with and without CAC.

	Subjects with CAC	Subjects without CAC
n	11	14
Male/Female (n)	10/1	9/5
Smoker/Non-smoker (n)	3/8	6/8
eGFR (ml/min/1.73m^2)	60±9 (22-108)	75±8 (13-123)
Duration of CKD in patients (year)	20±7 (1-55)	7±3 (1-22)
Age (year)	49±1 (42-57)	48±2 (29-64)
Body mass index (kg/m^2)	31±2 (20-40)	28±2 (19-46)
Mean arterial pressure (mmHg)	85±2 (75-101)	91±3 (74-106)
Glucose (mmol/L)	5.1±0.1 (4.6-5.7)	4.6±0.2 (2.5-5.8)
Total cholesterol (mmol/L)	4.4±0.2 (3.7-5.7)	4.8±0.2 (3.7-5.8)
Calcium (mmol/L)	2.38±0.05 (2.07-2.57)	2.33±0.01 (2.25-2.41)
Phosphate (mmol/L)	1.20±0.09 (0.89-2.00)	1.46±0.35 (0.75-5.80)
Bicarbonate (mmol/L)	26±1 (19-29)	26±1 (19-30)
Parathyroid hormone (ng/L)	109±32 (17-323)	70±15 (23-247)
Coronary artery calcification	53.36±30.46 (0.20-342.40)	-
Fetuin-A (g/L)	0.34±0.04 (0.15-0.60)	0.36±0.03 (0.22-0.54)
CF-PWV (m/s)*	7.4±0.4 (5.9-9.8)	6.4±0.2 (5.3-8.2)
FMD (%)	3.12±0.51 (0.00-5.40)	3.97±0.76 (0.70-10.30)
GTN (%)	10.72±1.43 (3.00-20.40)	11.05±0.98 (6.70-17.80)

Key: Data presented as mean ± SEM (range); *p< 0.05 for the unpaired Student's *t* test; CF-PWV: carotid femoral pulse wave velocity; eGFR: estimated glomerular filtration rate; FMD: flow-mediated dilatation; GTN: endothelium-independent vasodilating response to glyceryl trinitrate.

Figure 8.1 CF-PWV and FMD in subjects with and without CAC.

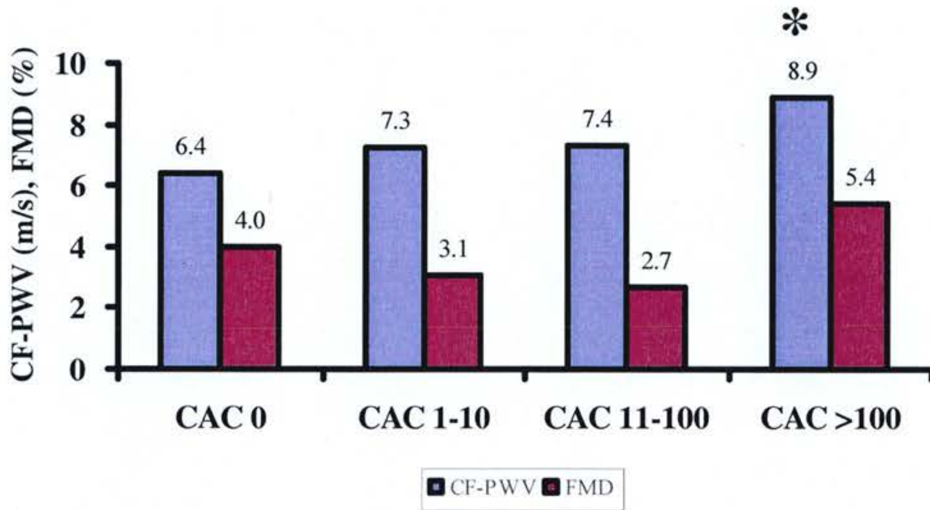


Key: *$p < 0.05$ for the unpaired Student's *t* test comparing subjects with and without CAC.

8.3.3 Relationships to carotid-femoral pulse wave velocity and flow-mediated dilatation

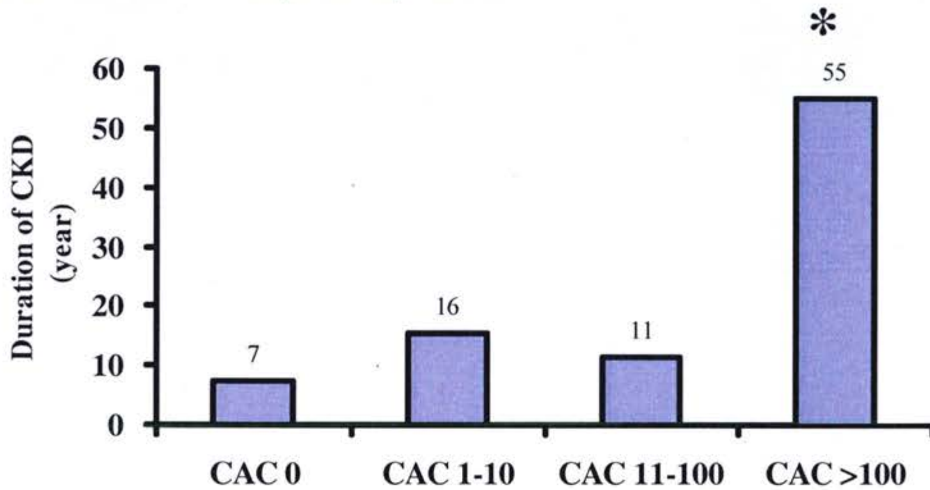
Dividing all subjects into 4 groups according to the severity of the CAC score, CF-PWV showed a trend of incrementally increased with the CAC score while FMD did not (Figure 8.2). In univariate analysis, the CAC score correlated to CF-PWV ($r^2 = 0.18$, $p < 0.05$) but not FMD. The CAC score also positively correlated with the duration of CKD ($r^2 = 0.53$, $p < 0.01$, Figure 8.3) but not with renal function, calcium, phosphate, bicarbonate, PTH, or conventional cardiovascular risk factors including age, gender, smoking status, BP, glucose, and cholesterol. The correlation between the CAC score and CF-PWV remains after adjusting for conventional cardiovascular risk factors, renal function, and calcium-phosphate parameter. By contrast, serum fetuin-A concentrations did not correlate with CF-PWV, FMD, renal function, conventional cardiovascular risk factors, or any uraemia-related parameters.

Figure 8.2 CF-PWV and FMD by severity of CAC.



Key: *$p < 0.05$ by the unpaired Student's *t* test compared to CAC score = 0. Group 1 (normal): CAC score = 0; group 2 (minimal): CAC score = 1 - 10; group 3 (mild): CAC score = 11 - 100; group 4 (moderate): CAC score >100.

Figure 8.3 Duration of CKD by severity of CAC.



Key: *$p < 0.01$ by the unpaired Student's *t* test compared to CAC score = 0. Group 1 (normal): CAC score = 0; group 2 (minimal): CAC score = 1 - 10; group 3 (mild): CAC score = 11 - 100; group 4 (moderate): CAC score >100.

8.4 Discussion

8.4.1 Main findings

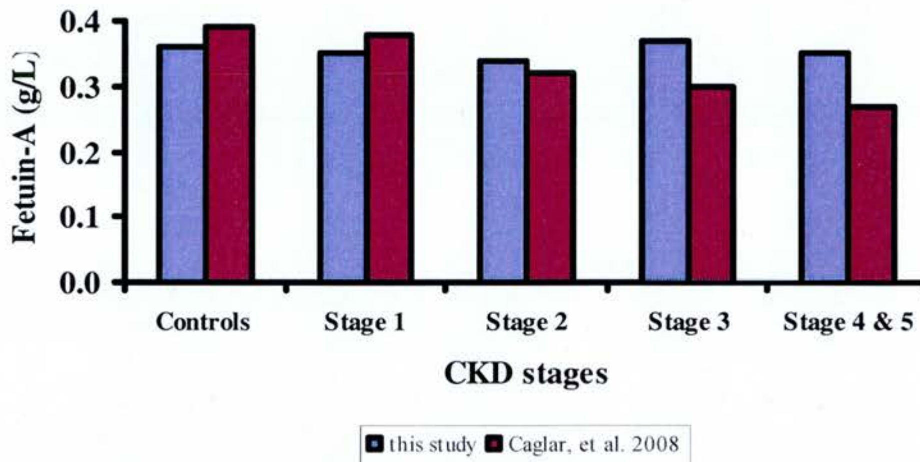
The current study shows that, irrespective of renal function, minimally co-morbid CKD patients who develop CAC have increased arterial stiffness and a trend of impaired endothelial function compared to those without CAC. In contrast, fetuin-A has no relationship to arterial stiffness and endothelial dysfunction in this group of patients.

The prevalence of CAC in this study is lower than in previous reports in dialysis and pre-dialysis patients (Adeney *et al.*, 2009; Barraclough *et al.*, 2008; Garland *et al.*, 2008; Lemos *et al.*, 2007; Mazzaferro *et al.*, 2007; Piers *et al.*, 2009; Porter *et al.*, 2007; Russo *et al.*, 2007). The extent of CAC seen here is also very low compared to previous data in pre-dialysis CKD with the same level of renal function (mean CAC score 53 *versus* 300 to 640) (Barraclough *et al.*, 2008; Garland *et al.*, 2008; Piers *et al.*, 2009; Porter *et al.*, 2007; Russo *et al.*, 2007). Most of the previous studies include patients with diabetes (Barraclough *et al.*, 2008; Garland *et al.*, 2008; Lemos *et al.*, 2007; Mazzaferro *et al.*, 2007; Porter *et al.*, 2007) or subjects with cardiovascular co-morbidity (Barraclough *et al.*, 2008; Lemos *et al.*, 2007; Mazzaferro *et al.*, 2007). All of these strongly reflect the influence of dialysis, diabetes, and cardiovascular co-morbidity on the development and progression of atherosclerosis and support the fact that CAC is a marker of increased atherosclerosis and cardiovascular risk.

The finding of no relationships of either CAC or fetuin-A to renal function is consistent with recent studies in pre-dialysis CKD (Garland *et al.*, 2008; Piers *et al.*, 2009) and patients with coronary artery disease (Ix *et al.*, 2006) but is in contrast to data in ESRD (Kobayashi *et al.*, 2008; Kramer *et al.*, 2005; Porter *et al.*, 2007). A study performed by Caglar, *et al.* has shown a graded decline in fetuin-A concentrations from CKD stage 1 to 5 (Caglar *et al.*, 2008b) which is different from the finding of the current study. Figure 8.4 depicts fetuin-A concentration of the two studies. As these two studies use a similar study design and technique for

quantifying fetuin-A concentrations, the only explanation of the difference is that the study by Caglar, *et al* has much larger sample size (241 subjects) compared to this study.

Figure 8.4 Comparing fetuin-A concentrations of this study to Caglar, *et al.* 2008.



The finding that CAC is highly associated with the duration of CKD is interesting. As this study was performed in patients with relatively low co-morbidity, this suggests that it takes time for uraemia on its own to develop atherosclerosis. The high prevalence of CAC seen in the literature is possible to be markedly influenced by co-morbidity. This is also supported by results from a study in CKD stages 3 to 5 that CAC is more associated with the presence of atherosclerosis than GFR (Piers *et al.*, 2009). One of the mechanisms of increased arterial calcification is a shift in the balance between calcification inducers/inhibitors towards an increased inducer side and reduced inhibitors. Here, although the levels of calcification inducers such as calcium-phosphate products were associated with GFR, the levels were not significantly increased compared to the healthy control group and thus the balance between calcification inducers/ inhibitors may not be altered.

Here, the association between the CAC score and CF-PWV is in keeping with reports in patients with diabetes mellitus (Tsuchiya *et al.*, 2004), those with coronary artery disease (Mitsutake *et al.*, 2007), middle-aged (Kullo *et al.*, 2006) and elderly subjects (van Popele *et al.*, 2006). It is also consistent with data in dialysis (Haydar *et al.*, 2004; Nitta *et al.*, 2004; Schlieper *et al.*, 2009) and pre-dialysis CKD (Lemos

et al., 2007). Additionally, in some of those studies, CAC is an independent predictor of arterial stiffness (Haydar *et al.*, 2004; Nitta *et al.*, 2004). The findings confirm the role for arterial calcification in the pathogenesis of arterial stiffening leading to haemodynamic disturbance in CKD patients.

No association between the CAC score and FMD was found in this study which is in contrast to previous data in patients with coronary artery disease, either symptomatic (Huang *et al.*, 2005) or asymptomatic (Ramadan *et al.*, 2008) but it is in keeping with data from a study in patients with erectile dysfunction (Kaiser *et al.*, 2004). Again, studies which show associations were confounded with diabetes and cardiovascular disease which are, themselves, related to both endothelial dysfunction and CAC.

The current study found no associations between fetuin-A and either CF-PWV or FMD. This is in contrast to studies in dialysis (Hermans *et al.*, 2006; Kuzniar *et al.*, 2008) and male subjects with normal renal function (Roos *et al.*, 2009), which show associations of fetuin-A to arterial stiffness, and studies in renal transplantation (Caglar *et al.*, 2007) and pre-dialysis (Caglar *et al.*, 2008b) which shows relationships of fetuin-A to endothelial dysfunction. The finding that the CAC score was not associated with fetuin-A concentration is in contrast to the theory that fetuin-A acts as a calcification inhibitor and its levels are reduced in accordance with increased CAC. However, in this study, subjects have no established cardiovascular disease and their extent of CAC seen here is also minor compared to those with co-morbidity. It is possible that fetuin-A would probably be more related to cardiovascular co-morbidity and, thus, be unaltered in this group of patients.

8.4.2 Limitations

The number of subjects studied was small and thus the study may not be sufficiently powered enough to show significant differences. At the time of starting the study, there was no study directly looking at the relationship of the CAC score and fetuin-A to arterial stiffness and endothelial dysfunction. Therefore, the calculation of the appropriate sample size was not possible. The number of 5 subjects per groups was chosen as the best fit in line with the possibility of recruiting patients when the study

started. Some of the medication taken by the patients, such as ACE inhibitors, ARBs, β-blockers, and statins may have had effects on both arterial stiffness and endothelial function. However, all patients were stabilised on their therapy and did not receive their medications on the day of the study until after measurements had been made. Other medication such as oral calcium supplements, vitamin D supplements, and calcium-based phosphate binders may also influence the levels of calcium/phosphate and the extent of CAC. But the majority of the subjects studied were not taking any of these forms of medication. In addition, because of the cross sectional design of this study, causal relationships behind the associations described cannot be fully defined.

8.4.3 Summary

Minimally co-morbid CKD patients have low prevalence and extent of CAC. Irrespective of renal function, CKD patients who develop CAC have increased arterial stiffness. Fetuin-A was not related to either arterial stiffness or endothelial dysfunction in this group of patients. A larger study is required to confirm these associations.

Chapter 9

Blood pressure independent reduction in proteinuria and arterial stiffness after acute endothelin-A receptor antagonism in chronic kidney disease

9.1 Introduction

Hypertension is a frequent finding in CKD patients and its prevalence increases as CKD progresses (Anonymous, 2002a). Despite advances in antihypertensive treatment, the majority of CKD patients fail to reach target BP (Peralta *et al.*, 2005). Proteinuria is a common feature of CKD and is independently associated with an adverse renal outcome (Jafar *et al.*, 2003). ACE inhibitors and ARBs are anti-hypertensive agents which are thought to reduce proteinuria to a greater extent than accounted for by BP lowering alone and are regarded as the main treatment for proteinuria in CKD (Jafar *et al.*, 2003). Yet, many CKD patients have significant residual proteinuria despite optimal treatment (Ruggenenti *et al.*, 2008).

CKD is strongly associated with cardiovascular disease (Parfrey *et al.*, 1999; Sarnak *et al.*, 2000; Stack *et al.*, 2001). Hypertension (McCullough *et al.*, 2007), proteinuria (Hillege *et al.*, 2002), arterial stiffness (Blacher *et al.*, 1999a) and endothelial dysfunction (Endemann *et al.*, 2004) are important contributors to cardiovascular risk in CKD. It is important to search for newer treatments in CKD that will not only lower BP and proteinuria beyond the levels achieved with standard therapies but will also have favourable effects on arterial stiffness and endothelial function and thus offer longer term cardiovascular and renal protection.

ET-1 is an endogenous vasoconstrictor produced within the vasculature. In CKD, ET-1 is involved in the development and progression of renal injury. ET-1 exerts its action via ET_A and ET_B receptors, with the principal effects in CKD being ET_A receptor mediated. Evidence has shown that selective ET_A receptor antagonism, but not mixed $ET_{A/B}$ antagonism, reduces BP, increase renal blood flow, and reduces the effective filtration fraction (FF) in CKD patients (Goddard *et al.*, 2004b). However, this was a small study with only 8 subjects included and 6 of them were treated with ACE inhibitors which may also reduce BP and confound the finding. Additionally, ET-1 is associated with arterial stiffness and endothelial dysfunction in diabetes (Karakitsos *et al.*, 2007), ischaemic heart disease (Heintz *et al.*, 1993) and dialysis patients (Dammers *et al.*, 2005; Demuth *et al.*, 1998). Data regarding the effects of

ET antagonism on arterial stiffness and endothelial dysfunction in pre-dialysis CKD is limited.

9.1.1 Aims and hypotheses

Based on the previous work (Goddard *et al.*, 2004b), the effects of selective ET_A antagonism, BQ123, on proteinuria, arterial stiffness and endothelial dysfunction were studied in non-diabetic CKD patients who are not on dialysis.

Hypotheses:

1. Selective ET_A antagonism would reduce proteinuria and arterial stiffness and endothelial dysfunction.

2. These effects would be greater than those achieved with BP reduction alone. Importantly, as the synergism between ACE inhibitors and selective ET_A receptor antagonism was previously shown (Goddard *et al.*, 2004a). I anticipated that these effects might occur on top of the benefits associated with standard treatment with ACE inhibitors and/or ARBs.

9.2 Methods

9.2.1 Subjects

Twenty two patients, aged 18-70 years with stable proteinuric CKD, were recruited into the studies. Patients who were on dialysis or with a renal transplant were excluded. Patients with significant co-morbidity, including diabetes mellitus, heart disease, lung disease, and peripheral vascular disease were excluded. Patients with vasculitis and systemic inflammatory disease were also excluded to avoid other influence on vascular reactivity. All patients were treated with ACE inhibitors and/or ARBs. Doses of one or both drugs were titrated to the maximum tolerated. All medication was unchanged over a 3-month period prior to the studies.

9.2.2 Drugs

BQ123 (Clinalfa AG), a selective ET_A receptor antagonist, was infused at 1000 nmol/min for 15 minutes. This dose was selected from previous studies as being ET_A selective based on plasma BQ123 concentration and relating these to the binding constant for the ET_A receptor, as well as by a lack of rise in plasma ET-1 concentration after its infusion (Goddard *et al.*, 2004b). BQ123 was dissolved in 15ml of 0.9% physiological saline (Baxter Healthcare Ltd) and infused intravenously at a constant rate of 1 ml/min. A saline vehicle was administered as a placebo.

Nifedipine 10 mg (Adalat, Bayer) was used as an active control and was administered orally at the same time as BQ123 or saline was started in its respective phase. Nifedipine was selected as an active control because of the similarity of its antihypertensive profiles and effects on renal haemodynamics to BQ123. Moreover, it is a clinically tolerable agent which is also used as a standard treatment in CKD patients (de Leeuw *et al.*, 2004).

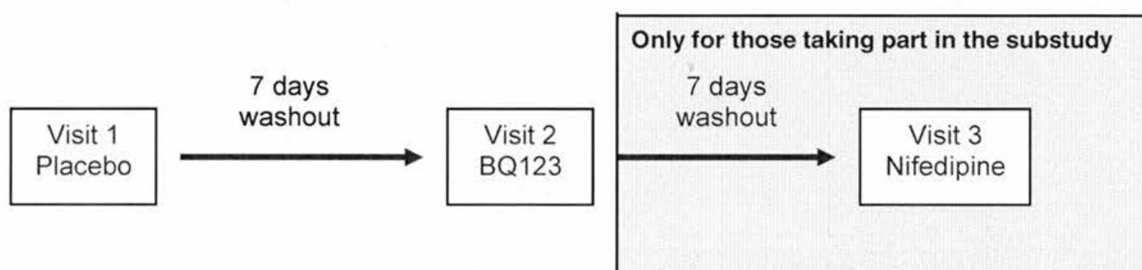
PAH (Clinalfa) and inutest (Fresenius Pharma) for the measurements of renal plasma flow and GFR, respectively, were dissolved in 5% dextrose (Baxter) and administered as a bolus loading dose of 0.4g of PAH and 3.5g of inutest in 100ml of dextrose over 15 minutes, along with a maintenance infusion of 6.6g/l of PAH and 10g/l of inutest at a rate of 2 ml/min. For subjects with a calculated GFR <50 ml/minor <30 ml/min, doses of PAH and inutest were reduced by one third and two third, respectively (see Chapter 2, section).

9.2.3 Study design and protocol

This was a randomised, double blind, placebo-controlled study. As previous studies with BQ123 have shown a reduction in BP in CKD patients and this BP reduction may contribute to changes in arterial stiffness and protein reduction, nifedipine (10 mg) was used as an open-label active control in a substudy of 10 subjects. All of the subjects attended for 2 visits, receiving placebo and BQ123 in a randomised order with those taking part in the substudy (nifedipine, 10 mg) randomly chosen and attended for 3 randomised visits. Each study visit was separated by at least a 7-day

washout period to ensure complete washout of the study drugs. This washout period was selected based on the fact that the haemodynamic changes of BQ123 return to the baseline after 4 hours (Goddard *et al.*, 2004b) and the half-life of nifedipine is ~2 hours. An example of a randomisation of a studied drug in one patient is given in Figure 9.1.

Figure 9.1 An example of a randomisation of a study in one patient.



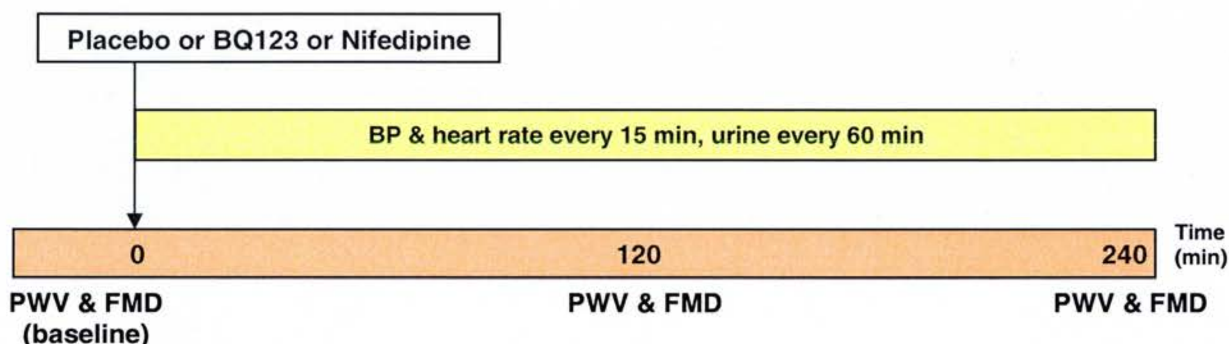
For the duration of the study, subjects were asked to adhere to their usual salt intake. All subjects abstained from alcohol, nicotine and caffeine-containing products for 24 hours. Subjects had a light breakfast before attending on each study day. All studies were conducted in a quiet, temperature-controlled room (22-24°C) with the subject recumbent throughout, except when voiding urine. All patients completed a 24-urine collection for quantification of proteinuria on each study day to assess equivalent baseline urinary protein leak between study phases. Patients continued taking their normal medications up to and including each study day with the exception of diuretics which they omitted that morning.

On the study day, a cannula was inserted into an antecubital vein in each arm. Diuresis was induced by administrating 500ml of 5% dextrose intravenously over 30 minutes through the left arm cannula. Then, maintenance infusion of PAH and inutest and 5% dextrose at 180 ml/hr continued throughout the study. After a 2-hour equilibration period, baseline measurements were made over 1 hour. BQ123 or a placebo was administered through the cannula on the right arm or with nifedipine was given orally, followed by 4 hours of further measurements (Figure 9.2). BP, cardiac output - as measured by cardiac index (CI), and heart rate were recorded

every 15 minutes throughout the study using a well-validated non-invasive automated technique (Thomas, 1992). Urine was collected every 60 minutes by spontaneous voiding.

At the midpoint of each collection period, blood samples were taken from the right antecubital cannula for PAH, inulin, sodium, and haematocrit. At 0, 30, 60, 120, and 240 minutes, additional blood samples were taken for the measurement of plasma ET-1. CF-PWV and FMD were measured immediately before drug administration and at 2 and 4 hours after dosing. CF-PWV and FMD measurements were performed as described in Chapter 2.

Figure 9.2 Study protocol.



9.2.4 Laboratory investigations

At pre-specified time points, venous blood was collected into EDTA tubes (Sarstedt) for the measurement of PAH, inulin, haematocrit, and plasma ET-1 and into plain tubes (Sarstedt) for the measurement of serum sodium. Additionally, 20ml aliquots of urine from each voiding were collected into plain tubes for the measurement of urinary PAH, inulin, sodium, and protein.

Haematocrit was measure on whole blood using a Coulter counter. All of other blood samples were centrifuged immediately at 1000*g* at 4°C for 20 minutes. Plasma and urine samples were then stored in plain tubes at -80°C. PAH and inulin were determined by a HPLC method (Chan *et al.*, 1988) and a spectrophotometry after hydrolysis to fructose (Schreiner, 1950), respectively. Plasma and urine sodium

concentrations were measured using an ion selective electrode. Urine protein was measured using a colorimetric method with pyrogallol red (Watanabe *et al.*, 1986). Plasma ET-1 was determined by radioimmunoassay as described in Chapter 2.

9.2.5 End points

The co-primary end points were reductions in BP and proteinuria. The secondary end points were a reduction in arterial stiffness and an improvement in endothelial function.

9.2.6 Data analyses

BP at each time point was calculated as the mean of two recordings and was represented by SBP, DBP, and MAP. Bioimpedence data at each time point were calculated as the mean of 4 recordings, each the average of 15 consecutive heartbeats. Data were corrected for body surface area to give CI ($L/min/m^2$), for direct comparison between subjects. Systemic vascular resistance index (SVRI) was calculated as SVRI = MAP / CI ($dynes/s/m^2/cm^5$). Effective renal plasma flow (ERPF) and GFR were calculated from PAH and inulin clearance, respectively. Effective renal blood flow (ERBF) was calculated as ERBF = ERPF / (1 – haematocrit). Effective renal vascular resistance (ERVR) was calculated as ERVR = MAP / ERBF. FF was calculated as FF = (GFR / ERPF) x 100. Urinary protein excretion was calculated as urinary protein x urinary flow rate. Likewise, urinary sodium excretion was calculated as urinary sodium x urinary flow rate.

The number of subjects required to show a significant difference in BP and ERVR was based on a previous study in CKD patients using the same dose of BQ123 (Goddard *et al.*, 2004b). Haemodynamic and urine results are presented as mean ± SEM change from baseline for drug and placebo and placebo corrected change from baseline for the results of the substudy. Statistical analysis was performed on untransformed data. Three comparisons of interest were pre-identified as placebo *versus* BQ123, placebo *versus* nifedipine, and BQ123 *versus* nifedipine. Responses were examined by repeated-measures ANOVA, and Bonferroni's correction was

used to assess significance at specific time points. Statistical significance was taken as $p < 0.05$.

9.3 Results

9.3.1 Subject characteristics

A total of 22 subjects completed the placebo and BQ123 phases of the study. Ten of them completed the 3-phase substudy. No adverse events were observed. Individual subject characteristics and baseline demographic data are presented in Tables 9.1 and 9.2.

Table 9.1 Subject characteristics.

Subject	Diagnosis	Creatinine (μmol/L)	BP (mmHg)	ACE inhibitor	ARB
1	IgAN	136	131/81	Ramipril 2.5mg	-
2	Membranous	78	114/63	Ramipril 10mg	Candesartan 4mg
3	FSGS	328	107/70	Lisinopril 40mg	Valsartan 80mg
4	FSGS	154	122/79	Ramipril 10mg	Candesartan 4mg
5	IgAN	131	129/67	Lisinopril 40mg	Candesartan 4mg
6	IgAN	89	111/69	Ramipril 2.5mg	-
7	IgAN	159	120/79	Ramipril 5mg	-
8	FSGS	487	158/84	Lisinopril 20mg	-
9	FSGS	266	116/68	Ramipril 10mg	-
10	Membranous	90	120/69	Enalapril 40mg	Losartan 50mg
11	Membranous	208	146/95	Enalapril 40mg	-

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Subject	Diagnosis	Creatinine (μmol/L)	BP (mmHg)	ACE inhibitor	ARB
12	IgAN	167	142/75	Ramipril 10mg	Candesartan 32mg
13	FSGS	181	123/68	Lisinopril 40mg	-
14	IgAN	118	108/67	Lisinopril 40mg	Candesartan 16mg
15	FSGS	332	145/83	Ramipril 10mg	Valsartan 160mg
16	IgAN	195	138/75	Lisinopril 40mg	Candesartan 16mg
17	IgAN	100	142/79	Ramipril 10mg	-
18	Membranous	282	142/70	Ramipril 10mg	-
19	Membranous	159	133/82	Lisinopril 40mg	-
20	FSGS	129	128/80	Lisinopril 40mg	Valsartan 80mg
21	IgAN	158	127/76	Lisinopril 40mg	Valsartan 160mg
22	FSGS	343	123/68	Ramipril 10mg	Candesartan 8mg

Key: FSGS: focal segmental glomerulosclerosis; IgAN: IgA nephropathy; membranous: membranous glomeruloropathy. Doses are total per day.

Table 9.2 Subject baseline data for the main and substudy.

	Main study	Substudy
n	22	10
Male/Female (n)	20/2	10/0
Age (year)	46±3 (29-69)	45±11 (29-64)
Body mass index (kg/m^2)	28±1 (20-37)	30±6 (21-37)
Systolic blood pressure (mmHg)	128±3 (99-167)	128±3 (108-142)
Diastolic blood pressure (mmHg)	75±2 (63-95)	76±1 (67-84)
Mean arterial pressure (mmHg)	92±2 (80-103)	93±2 (84-101)
Heart rate (bpm)	56±2 (38-75)	57±2 (42-66)
SVRI (dynes/s/m^2/cm^5)	3360±230 (1800-5510)	3530±230 (1980-5510)
Cardiac index (L/min/m^2)	3.0±0.2 (1.8-4.7)	2.9±0.2 (1.8-4.4)
Glomerular filtration rate (ml/min)	43±5 (12-99)	43±7 (15-99)
ERBF (ml/min)	1810±233 (106-4632)	1968±390 (530-4632)
ERVR (mmHg/min/ml)	11.5±4.4 (2.0±107.8)	7.1±1.5(2.0-18.2)
UNaV (μEq/min)	197±21 (27-392)	193±28 (95-392)
Urine protein excretion (μg/min)	1570±371 (165-8616)	1520±577 (109-8616)
Plasma endothelin-1 (pg/ml)	5.7±0.3 (3.6-10.5)	6.7±0.5 (3.8-8.7)
CF-PWV (m/s)	7.5±4.4 (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)

Key: Values are given as mean of baseline pretreatment periods over the 2 or 3 study days ± SEM. CF-PWV: carotid-femoral pulse wave velocity; ERBF: effective renal blood flow; ERVR: effective renal vascular resistance; FMD: flow-mediated dilatation; SVRI: systemic vascular resistance index; UNaV: urinary sodium excretion.

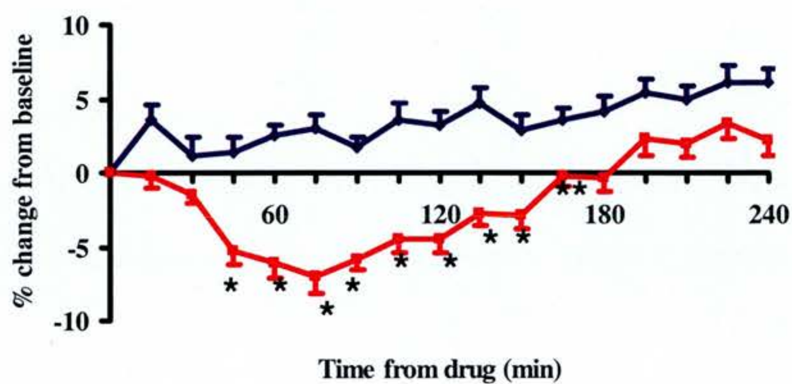
9.3.2 Main study

9.3.2.1 Systemic haemodynamics

Placebo was associated with an increased in SBP (127.6±3.3 *versus* 136.6±3.4 mmHg, $p < 0.01$), DBP (75.9±1.7 *versus* 79.9±2.2 mmHg, $p < 0.01$), and MAP (93.1±2.1 *versus* 98.8±2.4 mmHg, $p < 0.01$) and SVRI (3360±230 *versus* 3670±290 dynes/s/m^2/cm^5) from baseline to the end of the study (Figures 9.3-9.4 A & B). BQ123 reduced SBP, DBP, MAP, and SVRI from baseline (-14.2±3.0, -6.8±0.8, -9.2±1.2 mmHg, and -610±100 dynes/s/m^2/cm^5, respectively, all $p < 0.01$ compared to placebo), with the peak effects at 75 minutes after drug administration (Figures 9.3A and 9.4A). BQ123 increased CI (0.3±0.1 L/min/m^2, $p < 0.05$ *versus* placebo). There were no significant differences in the heart rate between placebo and BQ123 throughout the study (see Appendix 3 for full systemic haemodynamic data).

Figure 9.3 Changes in MAP after ET_A receptor antagonism.

A



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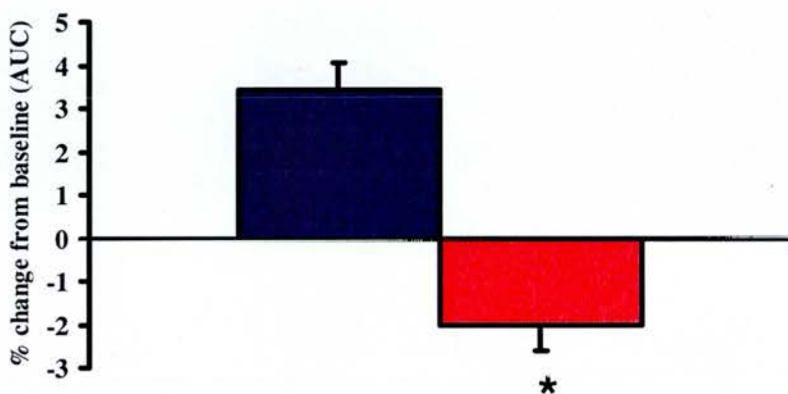
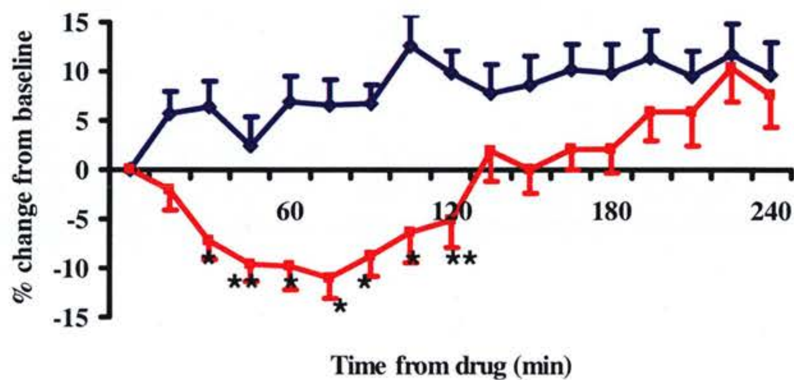


Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *p < 0.01 *versus* placebo; **p < 0.05 *versus* placebo.

Figure 9.4 Changes in systemic vascular resistance index after ET_A receptor antagonism.

A



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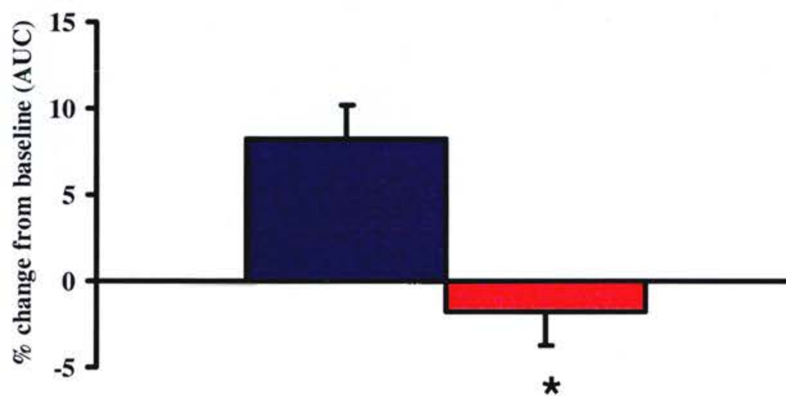


Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *p < 0.01 *versus* placebo; **p < 0.05 *versus* placebo.

9.3.2.2 Arterial stiffness and endothelial dysfunction

Placebo increased CF-PWV (7.5±0.4 *versus* 7.8±0.4 m/s, p < 0.01) whereas BQ123 reduced CF-PWV (-0.8±0.1 m/s, p < 0.01 *versus* placebo) (Table 9.3, Figures 9.5 A & B). There were no differences in FMD of the brachial artery between placebo and BQ123 for the whole period of the study (Table 9.3, Figures 9.6 A & B). Placebo and BQ123 both increased FMD from baseline but these did not reach significant (4.4±0.6 *versus* 5.1±0.7 %, p = 0.08 and 4.4±0.5 *versus* 5.5±0.8 %, p = 0.06, respectively).

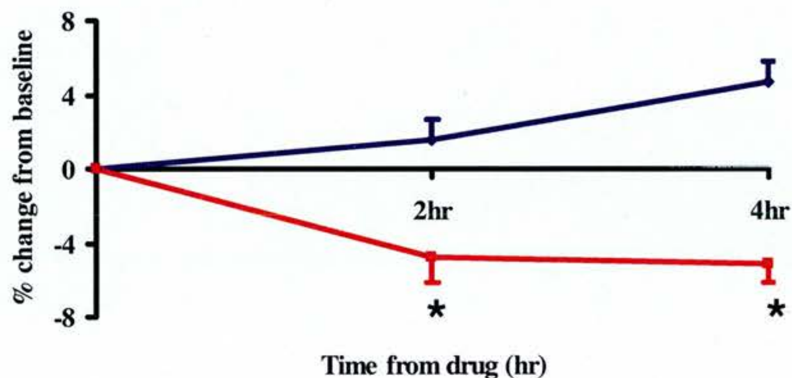
Table 9.3 Changes in CF-PWV and FMD after ET_A receptor antagonism and placebo in 22 subjects of the main study.

	Placebo			BQ123		
Time from drug	**0hr**	**2hr**	**4hr**	**0hr**	**2hr**	**4hr**
CF-PWV						
CF-PWV (m/s)	7.5±0.4	7.6±0.4	7.8±0.4	7.3±0.4	7.0±0.4	7.0±0.3
Absolute Δ CF-PWV (m/s)	na	0.1±0.1	0.3±0.1	na	-0.3±0.1	-0.4±0.1
% Δ CF-PWV	na	1.6±1.1%	4.7±1.0%	na	-4.8±1.5%	-5.1±1.0%
FMD						
FMD (%)	4.4±0.6	4.9±0.6	5.1±0.7	4.4±0.5	4.1±0.6	5.5±0.8
Absolute Δ FMD (%)	na	0.5±0.3	0.6±0.3	na	-0.3±0.4	1.1±0.5
% Δ FMD	na	33±20%	37±22%	na	44±52%	17±11%

Key: Values are mean ± SEM; absolute Δ: absolute change from baseline; % Δ: percentage change from baseline. CF-PWV: carotid-femoral pulse wave velocity; FMD: flow-mediated dilatation.

Figure 9.5 Changes in CF-PWV after ET_A receptor antagonism.

A



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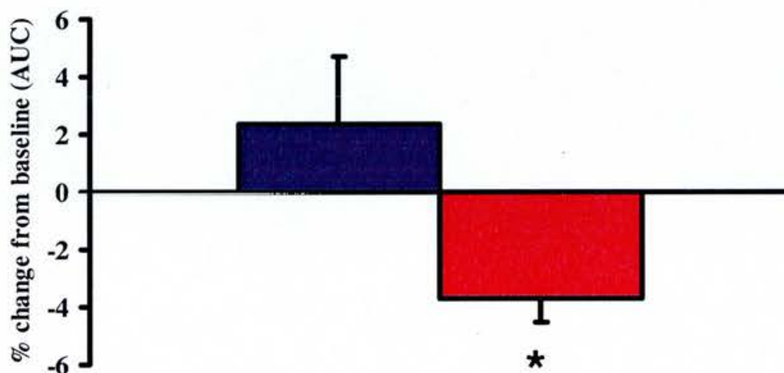

Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *$p < 0.01$ *versus* placebo; **$p < 0.05$ *versus* placebo.

Figure 9.6 Changes in FMD after ET_A receptor antagonism.

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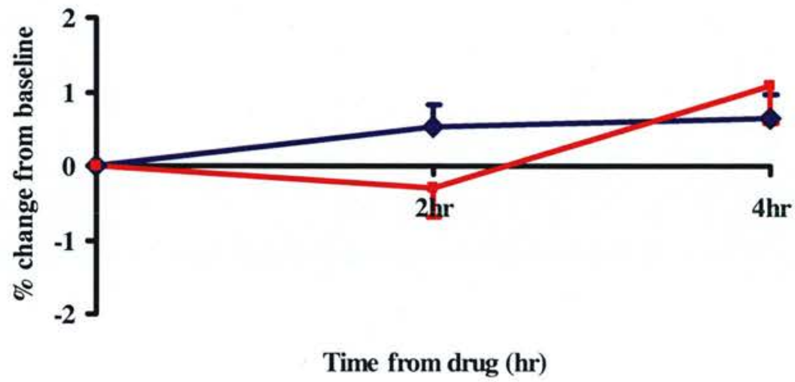

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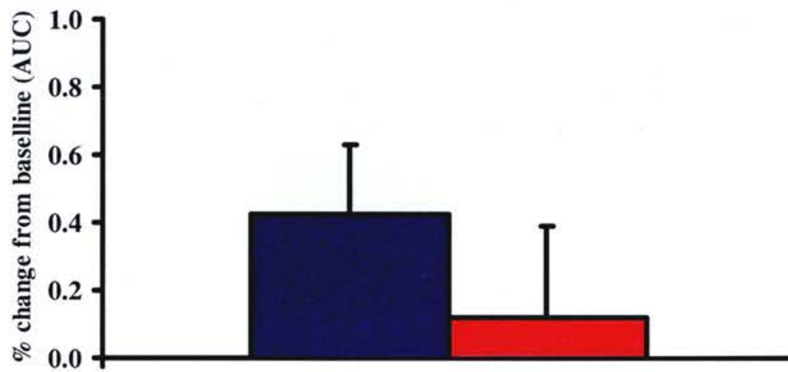

Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *$p < 0.01$ *versus* placebo; **$p < 0.05$ *versus* placebo.

9.3.2.3 Renal haemodynamics

Placebo was associated with a gradual reduction in ERBF (1810±233 *versus* 1454±181 ml/min, $p < 0.01$) and an increase in ERVR (11.5±4.4 *versus* 12.8±3.8 mmHg/min/ml, $p < 0.05$) to the study end (Table 9.4, Figures 9.7 & 9.8). By contrast, BQ123 produced an increase in ERBF (365±104 ml/min, $p < 0.01$ *versus* placebo, Table 9.4, Figure 9.7) and a reduction in ERVR (-3.0±0.9 mmHg/min/ml, $p < 0.01$ *versus* placebo, Table 9.4, Figure 9.8). There were no significant changes in GFR with either placebo or BQ123.

Table 9.4 Changes in renal haemodynamics after ET_A receptor antagonism and placebo in 22 subjects of the main study.

	Placebo					BQ123				
Time from drug	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**
ERBF										
ERBF (ml/min)	1810±233	1667±206	1645±211	1538±180	1454±186	1967±235	1945±224	1970±249	1832±219	1643±186
Absolute Δ ERBF (ml/min)	na	-143±41	-165±46	-272±61	-356±65	na	176±85	201±95	63±65	-126±91
% Δ ERBF	na	-5.3±2.2%	-7.1±2.7%	-10.0±3.0%	-15.8±4.0%	na	9.5±3.1%	16.7±4.7%	9.4±4.3%	5.7±8.7%
ERVR										
ERVR (mmHg/min/ml)	11.5±4.4	11.3±3.6	11.6±3.7	11.7±3.5	12.8±3.8	14.5±6.6	11.3±4.9	11.6±5.1	12.5±5.3	12.8±4.6
Absolute Δ ERVR (mmHg/min/ml)	na	-0.2±0.7	0.1±0.7	0.1±0.9	1.3±0.7	na	-3.2±1.5	-2.9±1.3	-0.2±1.1	-1.6±1.8
% Δ ERVR	na	9.9±2.2%	14.5±3.4%	20.8±4.5%	30.3±4.9%	na	-13.9±2.3%	-14.1±3.9%	-5.8±3.3%	6.4±5.4%

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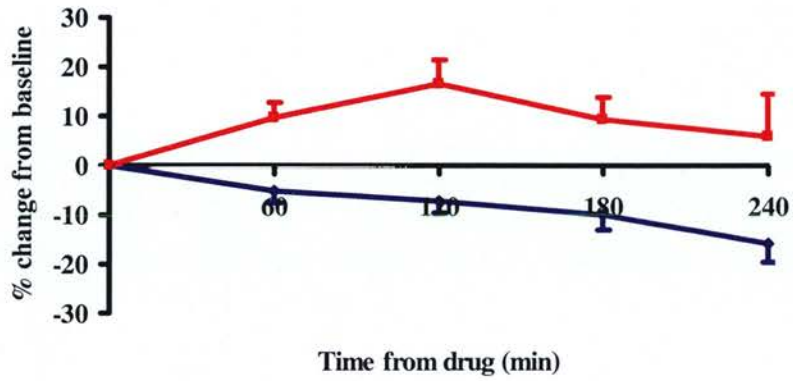
	Placebo					BQ123				
Time from drug	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**
GFR										
GFR (ml/min)	43.0±4.8	47.1±5.7	42.8±4.7	43.1±4.8	41.1±4,4	43.0±4.7	44.5±5.0	46.6±6.0	46.9±5.6	43.1±5,1
Absolute Δ GFR (ml/min)	na	4.0±1.5	-0.2±0.9	0.1±1.4	-1.9±1.6	na	1.5±1.9	3.6±2.1	3.9±1.7	0.0±1.3
% Δ GFR	na	7.4±3.0%	-0.1±2.6%	0.8±3.1%	-2.7±4.4%	na	-0.7±2.5%	7.6±3.6%	7.6±3.6%	-3.1±3.4%
FF										
FF (%)	4.7±0.7	5.1±0.7	5.0±0.7	4.8±0.5	5.2±0.7	43.0±4.7	4.6±0.6	4.4±0.6	5.0±0.6	4.6 ±0.4
Absolute Δ FF (ml/min)	na	0.4±0.2	0.3±0.2	0.1±0.2	0.8±0.2	na	-0.5±0.2	-0.7±0.3	-0.1±0.3	-0.5±0.4
% Δ FF	na	14.5±3.6%	12.4±4.1%	15.4±2.3%	20.1±5.0%	na	-6.6±3.5%	-5.7±5.6%	4.9±5.1%	-0.2±5.6%

Key: Values are mean±SEM; absolute Δ: absolute change from baseline; % Δ: percentage change from baseline. ERBR: effective renal blood flow; ERVR: effective renal vascular resistance; FF: filtration fraction; GFR: glomerular filtration rate.

Figure 9.7 Changes in effective renal blood flow after ET_A receptor antagonism.

A



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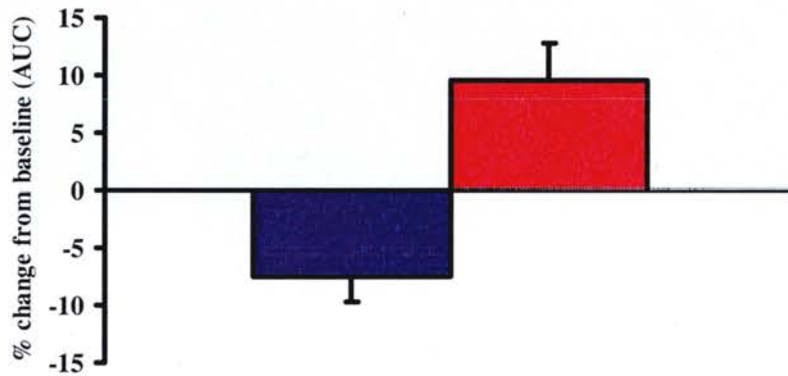
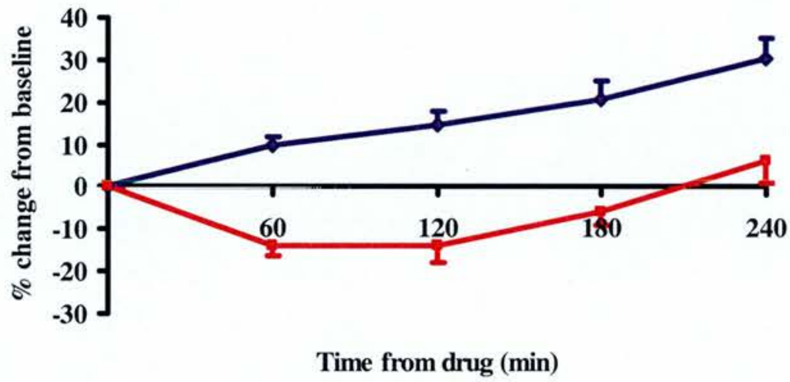


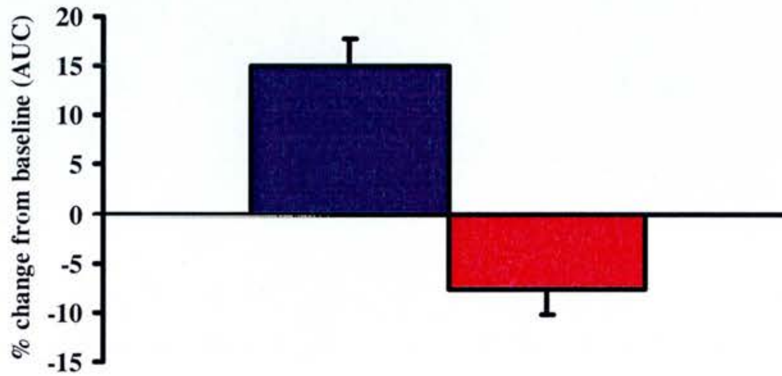
Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *$p < 0.01$ *versus* placebo; **$p < 0.05$ *versus* placebo.

Figure 9.8 Changes in effective renal vascular resistance after ET_A receptor antagonism.

A



B



Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *p < 0.01 *versus* placebo; **p < 0.05 *versus* placebo.

9.3.2.4 Urinary sodium and protein excretion

BQ123 produced a marked natriuresis with a maximum increase of 36±15 μmol/min ($p < 0.05$ *versus* placebo, Table 9.5, Figures 9.9 A & B). There was no change in potassium excretion. BQ123 also led to a sustained reduction in proteinuria throughout the time course of the study with a maximum reduction of 496±141 μg/min ($p < 0.01$ *versus* placebo, Table 9.5, Figures 9.10 A & B), equivalent to a reduction in protein leak of ≈30%. The size of this effect related to baseline urinary protein excretion and was seen across all levels of GFR. Subjects with higher baseline urinary protein excretion had a greater reduction ($r^2 = 0.78$, $p < 0.05$, Figure 9.10C).

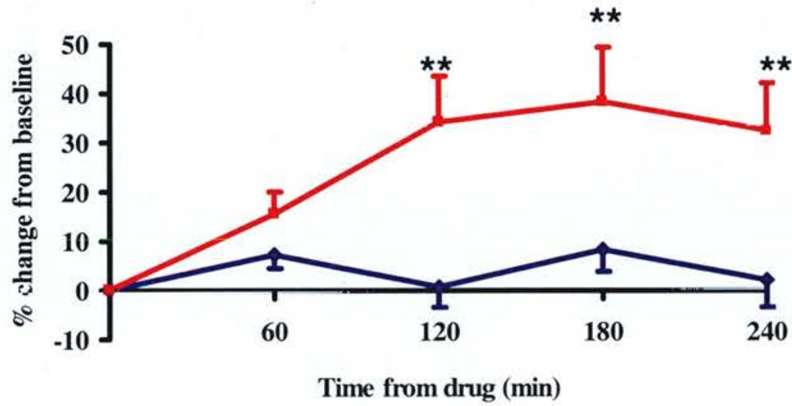
Table 9.5 Changes in urinary sodium and protein excretion after ET_A receptor antagonism and placebo in 22 subjects of the main study.

	Placebo					BQ123				
Time from drug	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**
UNaV										
UNaV (μmol/min)	202±20	213±20	196±18	206±18	192±16	170±25	188±21	198±22	195±19	196±22
Absolute Δ UNaV (μmol/min)	na	11±5	-6±9	4±10	-10±11	na	18±9	29±17	25±13	26±19
% Δ UNaV	na	7±3%	1±4%	8±5%	2±6%	na	16±4%	34±9%	38±11%	32±10%
Urinary protein excretion										
Urinary protein excretion (μg/min)	1382±255	1492±295	1402±271	1266±214	1297±228	1758±442	1501±399	1319±306	1263±307	1386±372
Absolute Δ urinary protein excretion (μg/min)	na	110±50	20±36	-116±59	-85±50	na	-257±120	-439±152	-495±141	-372±127
% Δ urinary protein excretion	na	4±4%	2±4%	-3±5%	1±5%	na	-16±4%	-22±4%	-25±4%	-24±3%

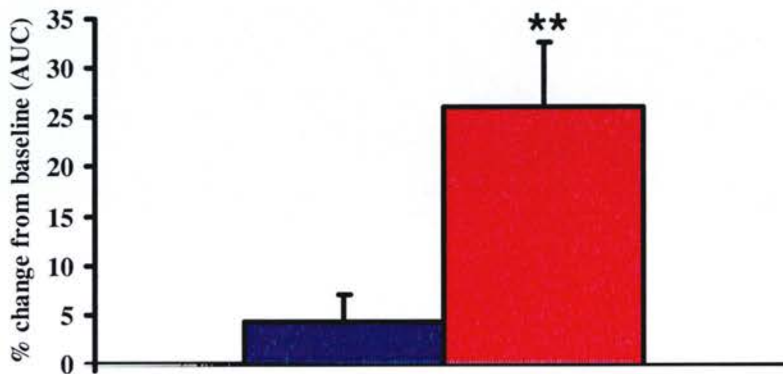
Key: Values are mean±SEM; absolute Δ: absolute change from baseline; % Δ: percentage change from baseline. UNaV: urinary sodium excretion.

Figure 9.9 Changes in urinary sodium excretion after ET_A receptor antagonism.

A



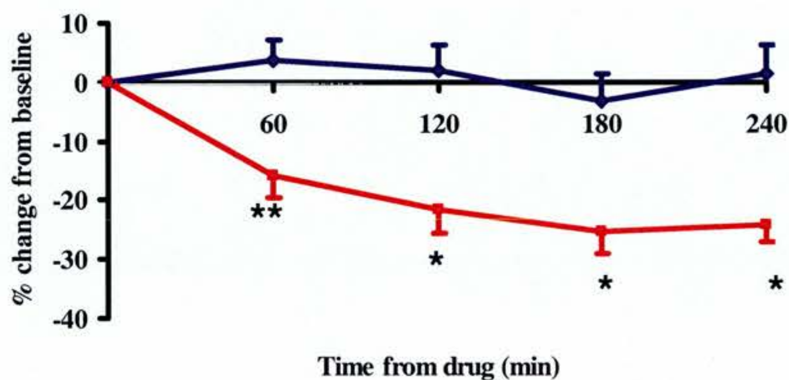
B



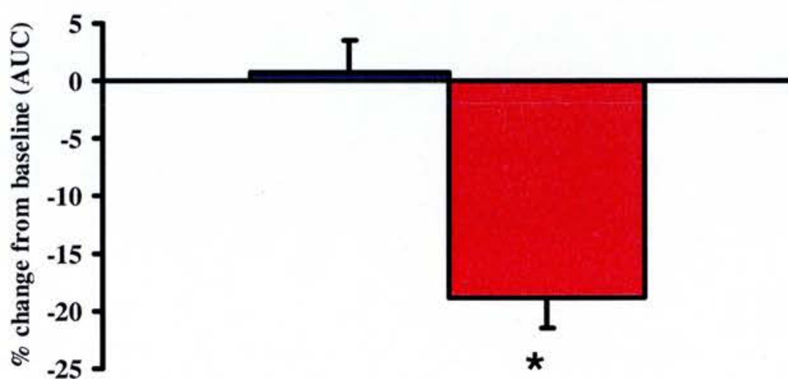
Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *$p < 0.01$ *versus* placebo; **$p < 0.05$ *versus* placebo.

Figure 9.10 Changes in urinary protein excretion after ET_A receptor antagonism.

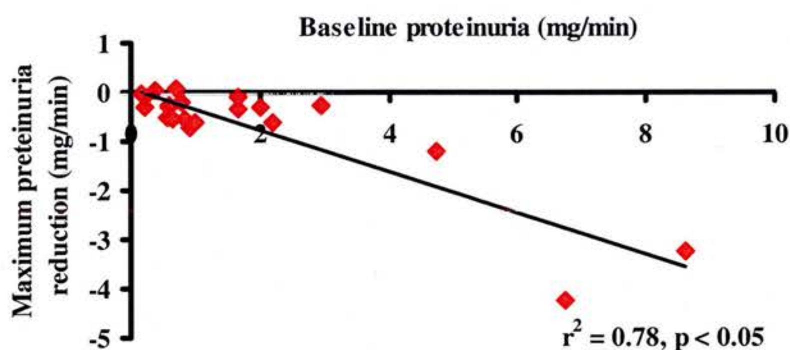
A



B



C



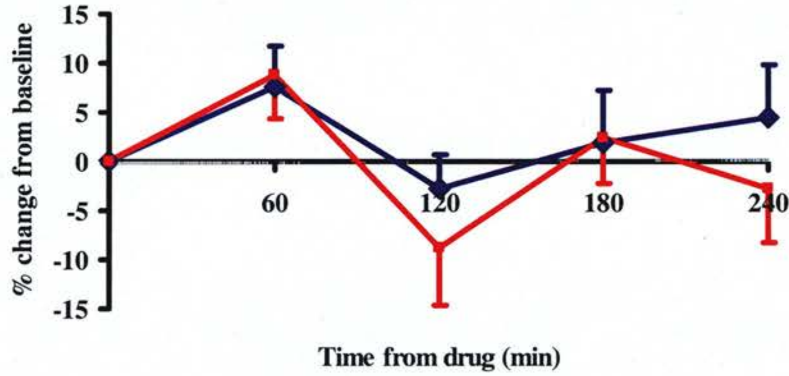
Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *p < 0.01 *versus* placebo; **p < 0.05 *versus* placebo.

9.3.2.5 Plasma endothelin-1

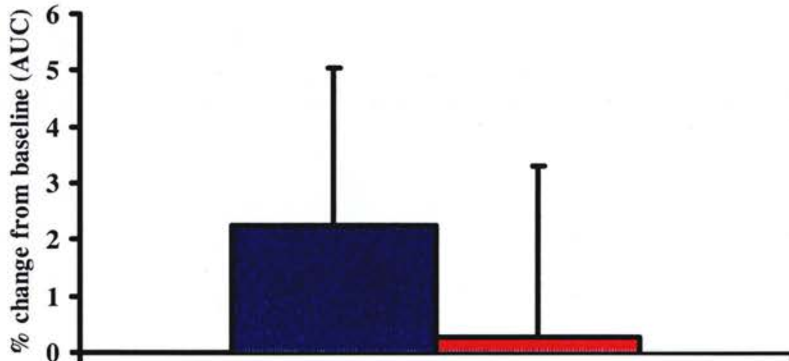
There were no changes in plasma ET-1 concentrations with either placebo or BQ123 (Table 9.6, Figure 9.11).

Figure 9.11 Changes in plasma ET-1 concentrations after ET_A receptor antagonism.

A



B



Key: Blue line/block: placebo; red line/block: BQ123. A presents mean percentage of change from baseline ± SEM. B presents mean area under curve (AUC) of the percentage of change from baseline ± SEM. *$p < 0.01$ *versus* placebo; **$p < 0.05$ *versus* placebo.

Table 9.6 Changes in BP, heart rate, and plasma ET-1 after ET_A receptor antagonism and placebo in 22 subjects of the main study.

	Placebo					BQ123				
Time from drug	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**	**0hr**	**1hr**	**2hr**	**3hr**	**4hr**
ET-1										
Plasma ET-1 (pg/ml)	5.7±0.3	5.9±0.3	5.4±0.3	5.7±0.3	5.7±0.3	5.9±0.3	6.3±0.4	5.5±0.2	5.9±0.3	5.5±0.2
Absolute Δ plasma ET-1 (pg/ml)	na	0.3±0.2	-0.2±0.2	0.0±0.3	0.1±0.4	na	0.4±0.3	-0.7±0.3	0.0±0.3	-0.4±0.3
% Δ plasma ET-1	na	7.6±4.1%	-2.7±3.4%	1.9±5.3%	4.5±5.4%	na	8.8±4.5%	-8.8±5.8%	2.5±4.7%	-2.7±5.6%

Key: Values are mean±SEM; absolute Δ: absolute change from baseline; % Δ: percentage change from baseline. ET-1: endothelin-1.

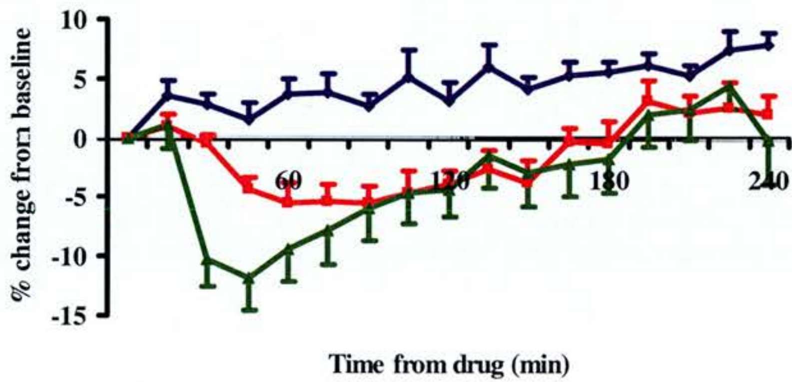
9.3.3 Substudy with nifedipine 10mg

BQ123 and nifedipine led to a similar reduction in MAP (Figures 9.12 A-C). Nifedipine caused an initial increase in heart rate (10 bpm from baseline) that returned to baseline within 60 minutes. Despite the consistent change in MAP, BQ123 reduced CF-PWV to a greater extent than did nifedipine (at 4 hours post dose, absolute reduction in CF-PWV from baseline, -0.6±0.0 *versus* -0.3±0.1 m/s, Figures 9.13 A-C). BQ123 and nifedipine had no significant effects on FMD (Figures 9.14 A-C).

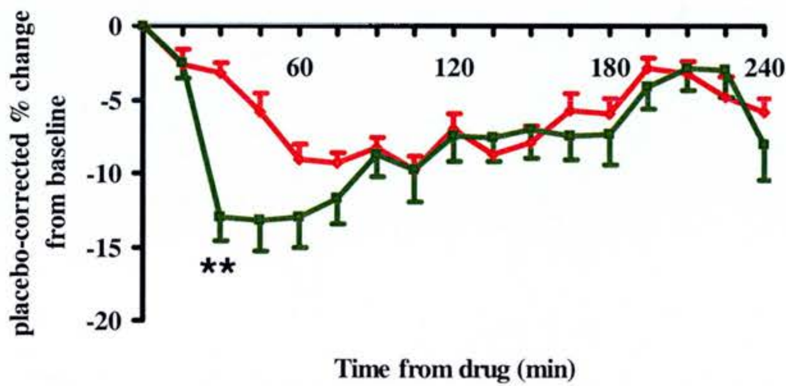
BQ123 and nifedipine also increased ERBF to a similar degree and led to a similar level of natriuresis (at maximum, BQ123: 64±37 μmol/min; nifedipine: 37±34 μmol/min). In contrast to BQ123, nifedipine was associated with a gradual increase in urinary protein excretion (at maximum, 190±142 μg/min, $p < 0.01$ *versus* placebo) while BQ123 produced a consistent reduction in proteinuria (Figures 9.15 A-C). See Appendix 4 for full results of the substudy.

Figure 9.12 Changes in MAP after ET_A receptor antagonism and nifedipine 10mg.

A



B



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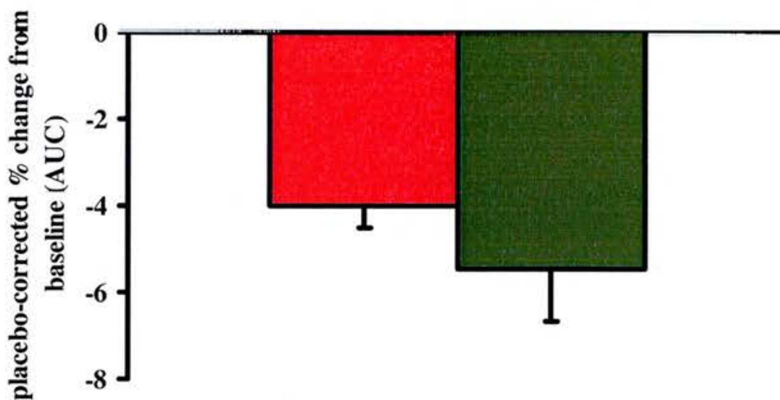
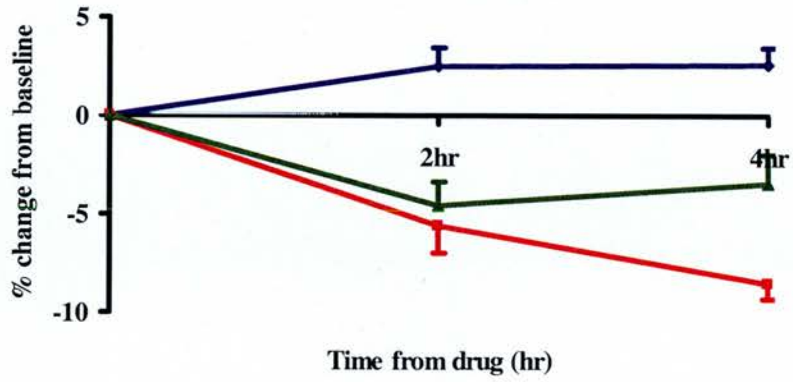


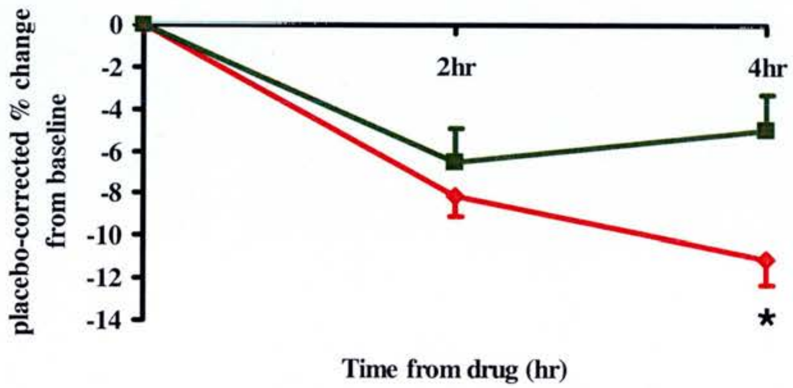
Key: Blue line: placebo; green line/block: nifedipine; red line/block: BQ123; *p < 0.01 *versus* nifedipine; **p < 0.05 *versus* nifedipine.

Figure 9.13 Changes in CF-PWV after ET_A receptor antagonism and nifedipine 10mg.

A



B



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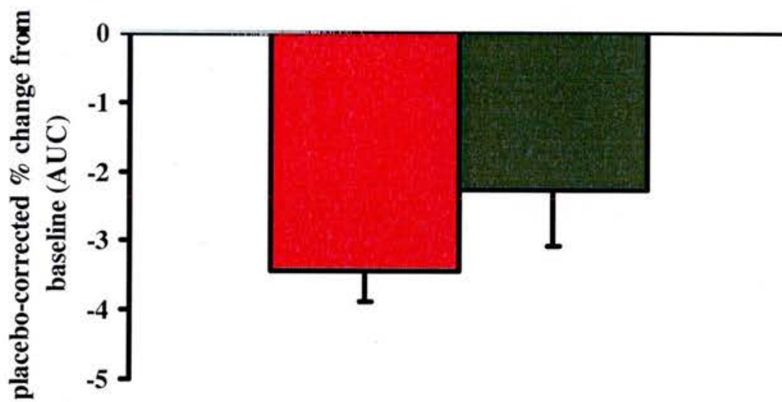
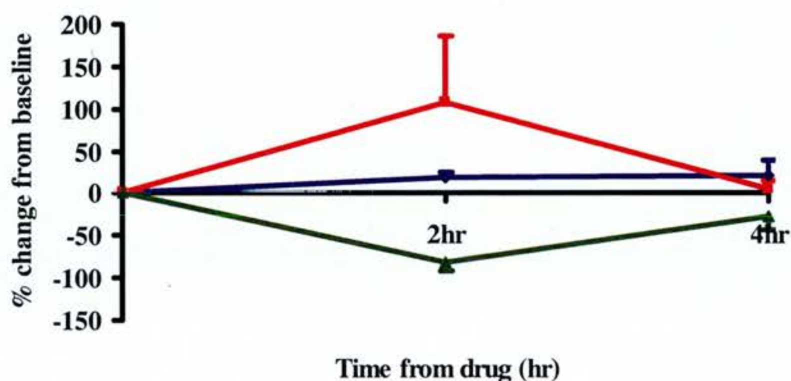


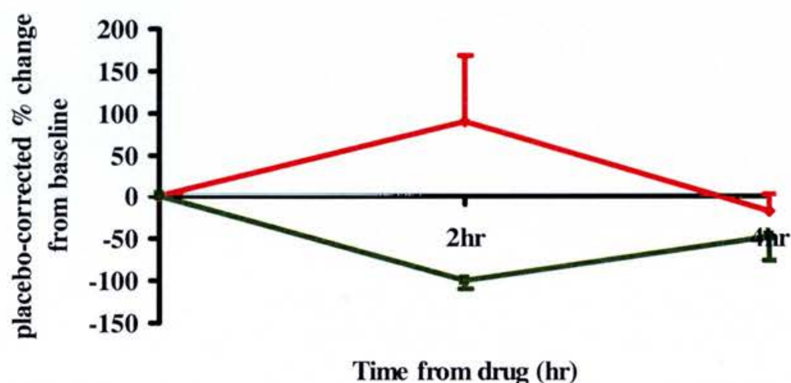
Key: Blue line: placebo; green line/block: nifedipine; red line/block: BQ123; *p < 0.01 *versus* nifedipine; **p < 0.05 *versus* nifedipine.

Figure 9.14 Changes in FMD after ET_A receptor antagonism and nifedipine 10mg.

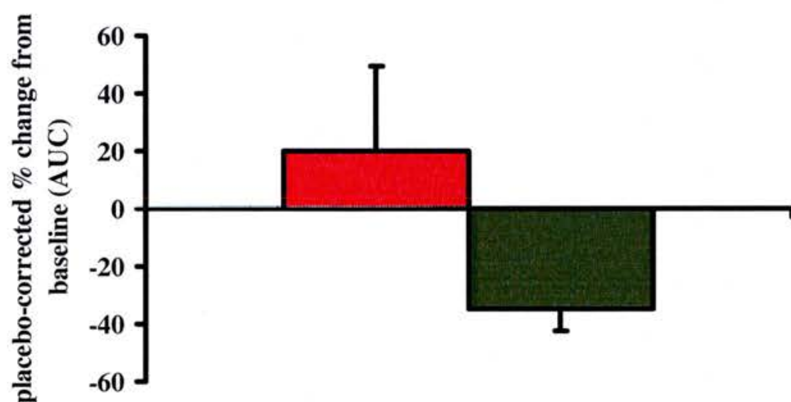
A



B



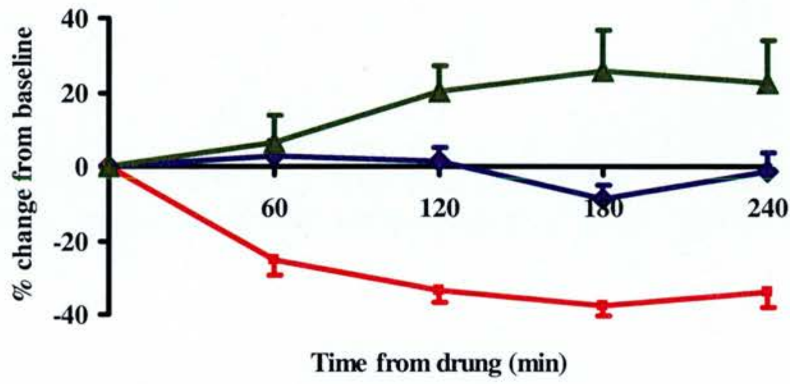
C



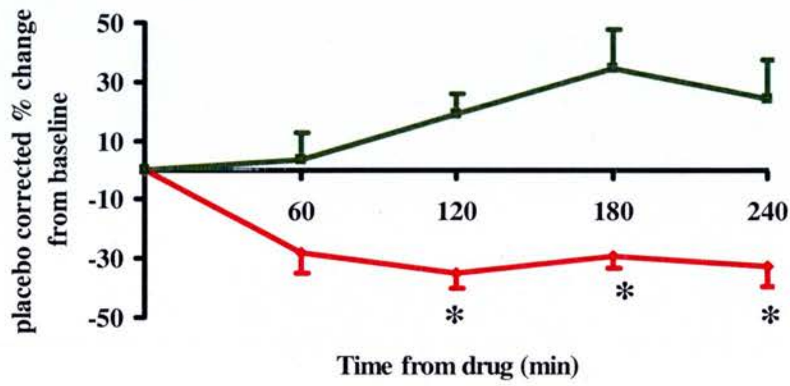
Key: Blue line: placebo; green line/block: nifedipine; red line/block: BQ123; *p < 0.01 *versus* nifedipine; **p < 0.05 *versus* nifedipine.

Figure 9.15 Changes in urinary protein excretion after ET_A receptor antagonism and nifedipine 10mg.

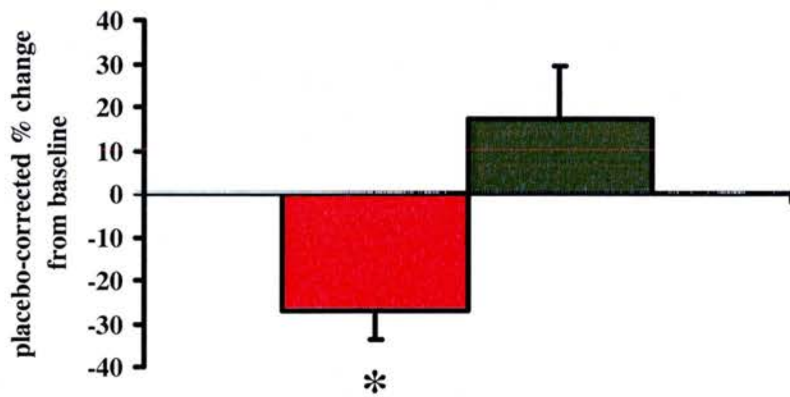
A



B



C



Key: Blue line: placebo; green line/block: nifedipine; red line/block: BQ123; *p < 0.01 *versus* nifedipine; **p < 0.05 *versus* nifedipine.

9.4 Discussion

9.4.1 Main findings

Selective ET_A receptor antagonism reduces BP, proteinuria, and arterial stiffness in CKD patients with varying degrees of proteinuric nephropathy. These effects are seen on top of the maximally tolerated dose of ACE inhibitors and ARBs. Importantly, the reductions in proteinuria and CF-PWV are greater than those seen with nifedipine, an alternative way to achieve a similar of BP reduction.

9.4.2 Effects of BQ123 on blood pressure

The findings of this study confirm the role of ET-1 in maintaining vascular tone in CKD, mediated mainly through the ET_A receptor (Dhaun *et al.*, 2006; Hand *et al.*, 1999). A few studies, using selective ET_A and mixed $ET_{A/B}$ receptor antagonists, have shown BP reduction with ET receptor antagonists in untreated hypertensive patients (Krum *et al.*, 1998; Nakov *et al.*, 2002). Here, the reduction of BP is less than that in a previous study in hypertensive CKD patients with a similar range of renal function (% change from baseline in MAP -7±1 *versus* -13±2 %, respectively) (Goddard *et al.*, 2004b). The BP reduction seen in the current study confirms that ET receptor antagonism may provide a novel strategy to lower BP to a greater extent than existing treatments, such as renin-angiotensin blockers, in CKD patients whose BP control is often difficult.

9.4.3 Effects of BQ123 on carotid-femoral pulse wave velocity and flow-mediated dilatation

ET_A receptor antagonism with BQ123 also reduced CF-PWV compared with placebo. The improvement of CF-PWV seen in the current study is likely in part due to reduction in BP caused by BQ123. However, although when the BP reduction with BQ123 was similar to that with nifedipine, the reductions in CF-PWV were significantly greater with BQ123. It is important to note that, although nifedipine

caused an expected increase in heart rate, which may have a minor impact on PWV (Lantelme *et al.*, 2002), heart rate had returned to baseline within 60 minutes after administration of nifedipine and before the measurement of PWV. Overall, this result suggests that the favourable effects of ET_A receptor antagonism on arterial stiffness are independent of BP.

Epidemiological evidence suggests that PWV is an independent risk factor for cardiovascular morbidity and mortality (Blacher *et al.*, 1999a; Laurent *et al.*, 2001). In renal patients, the survival rate is better for patients whose PWV declined in response to a BP-lowering treatment (Guerin *et al.*, 2001). Several studies have shown that BP lowering agents such as ACE inhibitors and ARBs could reduce PWV independent of BP reduction. This was reported in hypertensive (Williams *et al.*, 2006), diabetes (Karalliedde *et al.*, 2008), and ESRD patients (Ichihara *et al.*, 2005; London *et al.*, 1996). Data from the present study suggests that ET_A receptor antagonist may reduce arterial stiffness even further in CKD patients who are already on ACE inhibitors and/or ARBs treatment. Importantly, this reduction in arterial stiffness from ET_A receptor antagonist is BP-independent.

Although it has recently been shown that ACE inhibitors and ARBs could improve endothelial function both in hypertension (Benndorf *et al.*, 2007; Morimoto *et al.*, 2008) and proteinuric CKD (Yilmaz *et al.*, 2007), this effect was not seen with BQ213 in our study. In contrast to effects seen on arterial stiffness, the effects of ET_A receptor antagonism on endothelial function, measured by the brachial artery FMD, were not striking, even though there was a trend of improvement in FMD after BQ123. This may be explained by the fact that the effects of altering endothelial function may take longer to develop. Moreover, this finding is in contrast to previous data that showed an improvement in an endothelium-dependent vasodilatation in patients with coronary artery disease (Bohm *et al.*, 2002; Halcox *et al.*, 2001) but is consistent with two recent reports in patients with systemic sclerosis (Sfikakis *et al.*, 2007) and insulin resistance (Shemyakin *et al.*, 2006). Instead, these two studies have shown that endothelial function was improved by mixed $ET_{A/B}$ receptor antagonists. Interestingly, in the study of coronary artery disease patients, the effects of mixed $ET_{A/B}$ receptor antagonists on endothelium-dependent vasodilatation occur on top of ACE inhibitor treatment (Bohm *et al.*, 2005).

9.4.4 Effects of BQ123 on renal haemodynamics

BQ123 increased renal blood flow in association with a reduction in renal vascular resistance, suggesting that ET-1 is involved in the increased renovascular tone in CKD and its effect is mediated through ET_A receptor. There were no significant changes in GFR but a fall in FF, as seen in previous studies (Dhaun *et al.*, 2007a; Goddard *et al.*, 2004b), was observed, suggesting that ET-1 induces and ETA receptor-mediated vasoconstriction. This effect of ET-1 is likely to act efferent arteriole but does not exclude an effect on mesangial cells and the filtration coefficient.

9.4.5 Effects of BQ123 on urinary protein and sodium excretion

Consistent with a reduction in FF, BQ123 produced a sustained reduction in urinary protein excretion. Similar to the reduction in CF-PWV, the reduction in proteinuria continued even when the BP-lowering effect of BQ123 has waned and BP has returned to baseline, suggesting that this reduction in proteinuria was BP-independent. Nifedipine, as a control drug, was closely matched both the decrease in BP and the increase in renal blood flow seen with BQ123. Nifedipine acts mainly on the afferent arteriole and therefore, as expected, it produced increases in both GFR and proteinuria throughout the study period (see Appendix 4). As BQ123 had little effect on GFR and substantially reduced FF and proteinuria over the same time scale as the increase in renal blood flow, these suggests that the effects are not just occurred on the afferent arteriole but also seen at the efferent arteriole, similar to, and on top of, that seen with ACE inhibitors.

In the current study, across the range of GFR, the reduction in proteinuria was related to baseline proteinuria, with subjects with a higher level of baseline urinary protein excretion had greater reductions. This effect is similar to the effects seen with ACE inhibitors (Jafar *et al.*, 2003).

A reduction in proteinuria is linked to reducing risk of CKD progression (Jafar *et al.*, 2003) and consequence cardiovascular disease (Tonelli *et al.*, 2006). Despite maximum renin-angiotensin system blockade, many proteinuric CKD patients still have significant residual proteinuria (Ruggenenti *et al.*, 2008). In this study, all of the subjects were established on treatment with ACE inhibitors, with the majority also taking ARBs. The ET and renin-angiotensin systems were known to interact (Rossi *et al.*, 1999), and a synergistic effect between these two systems, in terms of systemic haemodynamics, was demonstrated in humans (Dhaun *et al.*, 2007b; Goddard *et al.*, 2004a). Results of the current study suggest that ET_A receptor antagonism can produce a further reduction in proteinuria by ≈30% on top of that achieved with optimal treatment with inhibitors of renin-angiotensin system. Thus, if maintained longer term, ET_A receptor antagonism should reduce both disease progression and cardiovascular morbidity and mortality in CKD patients.

BQ123 also produced significant natriuresis which is likely due to the increase in renal blood flow seen with ET_A receptor antagonism. In fact, nifedipine which caused a similar change in renal haemodynamics also caused increased urinary sodium excretion. Additionally, all of the subjects showed a net diuresis even with placebo. Altogether 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.

9.4.6 Limitations

As an acute study, the beneficial effects of ET_A receptor antagonism on BP, proteinuria, and arterial stiffness seen here have to be confirmed in a longer-term study with a range of antihypertensive agents. The range of CKD patients enrolled on this study is relatively homogenous, comprising pre-dialysis patients with minimal co-morbidity. Further work is justified in other CKD populations such as those with diabetes, vasculitis, or renal vascular disease. In addition, this is a crossover design study, which may affect data interpretation and is not ideal for a longer-term study in a large group of patients.

9.4.7 Summary

ET_A receptor antagonism lowers BP, reduces proteinuria, and improves arterial stiffening on top of standard BP lowering treatment in CKD patients with a moderate reduction of renal function. These data supports a role for selective ET_A receptor antagonism as a novel therapeutic approach in controlling hypertension and proteinuria, and reducing arterial stiffness in CKD patients. These effects should be confirmed in larger and longer-term studies.

Chapter 10

Conclusions and future work

10.1 Conclusions

The series of prospective, cross-sectional studies presented in this thesis explore the degree of arterial stiffness and endothelial dysfunction in relatively low-risk CKD patients without diabetes or cardiovascular co-morbidity, across a wide range of renal function from normal GFR to pre-dialysis. The contribution of conventional and different types of emerging cardiovascular disease risk factors to arterial stiffness and endothelial dysfunction in this group of patients was also assessed. Additionally, an interventional study examining the effects of selective ET_A receptor antagonism on arterial stiffness and endothelial dysfunction in the same group of patients was performed.

10.1.1 Reproducibility of carotid-femoral pulse wave velocity, central augmentation index, and flow-mediated dilatation

This study confirms the reproducibility of non-invasive techniques used to assess arterial stiffness (PWV and AIx) and endothelial dysfunction (FMD).

PWV and AIx, assessed using the SphymoCor system, are two of the most widely used non-invasive techniques for measuring arterial stiffness (Oliver *et al.*, 2003b). Brachial artery FMD is a widely used non-invasive measure of endothelial-dependent vasomotor function of a conduit artery (Corretti *et al.*, 2002). These techniques were previously shown to be reproducible (Bots *et al.*, 2005; Donald *et al.*, 2006; Simova *et al.*, 2008; Wilkinson *et al.*, 1998). However, these techniques still carry some limitations. The quality of the pressure waveforms at the femoral site or the carotid site may affect the calculation of PWV and AIx. PWV calculation can also be affected by the inaccuracy of the measurement of the distance between the two recording sites. Additionally, one of the most important limitations of FMD is the variability in the analysis of an ultrasound image. These limitations could occur either within one observer by doing repeated measurements or between two separate observers.

The studies in healthy subjects and CKD patients confirm that CF-PWV, AIx, and FMD are reproducible, which is consistent with previous studies. In fact, intra- and inter- observer reproducibility of both CF-PWV and FMD, which are the main techniques used throughout this thesis are better than others (Donald *et al.*, 2008; Frimodt-Moller *et al.*, 2008; Hardie *et al.*, 1997; Simova *et al.*, 2008; Wilkinson *et al.*, 1998), confirming the validity of the assessment of arterial stiffness and endothelial function in this thesis. In addition, the training time for the investigators to achieve this high level of reproducibility is within ~4 weeks.

10.1.2 Plasma endothelin-1 and fractional excretion of endothelin-1 in chronic kidney disease

This study shows that in a large cohort of CKD patients without dialysis or established cardiovascular co-morbidity, plasma ET-1 increases linearly as renal function declines, whereas FeET-1 increases exponentially.

The increase in levels of plasma ET-1 in this study are comparable to previous studies in pre-dialysis CKD (Cottone *et al.*, 2009; Dammers *et al.*, 2005; Goddard *et al.*, 2007; Vlachojannis *et al.*, 1997). As data regarding the correlation of increased plasma ET-1 and renal function in the literature are not consistent (Cottone *et al.*, 2009; Dammers *et al.*, 2005; Goddard *et al.*, 2007; Vlachojannis *et al.*, 1997; Warrens *et al.*, 1990), this study has confirmed that plasma ET-1 has an inverse linear correlation with GFR. The increase in plasma ET-1 concentrations seen in this study could either be from increased renal ET-1 production or reduced renal clearance of ET-1.

By contrast, FeET-1 exponentially increases as GFR declines, strengthening the concept of two independent ET-1 systems in the vasculature and the kidneys (Benigni *et al.*, 1991; Brooks *et al.*, 1991; Wesson, 2001). In fact, as the clearance of circulating ET-1 is mainly through the pulmonary system, the exponential increase of FeET-1 seen here suggests that the increased plasma ET-1 concentrations in CKD would probably be caused by increased renal ET-1 production rather than reduced renal clearance of ET-1. However, as the study used spot urine for the measurement

of urinary ET-1 and the calculation of FeET-1, this should be confirmed in a clearance study.

10.1.3 Relationships of renal function to arterial stiffness and endothelial dysfunction in chronic kidney disease

This study shows that in minimally co-morbid CKD patients, arterial stiffness, measured by CF-PWV, increases incrementally as renal function declines whereas endothelial dysfunction, measured by FMD of the brachial artery, is only a feature of late stage CKD. However, renal function is not an independent determinant of either CF-PWV or FMD in this group of patients.

Previous studies have shown associations between PWV and GFR in patients with dialysis or cardiovascular co-morbidity (Briet *et al.*, 2006; Lacy *et al.*, 2006; Stancanelli *et al.*, 2007; Wang *et al.*, 2005). This study confirms that even in relatively 'clean' CKD patients, increased arterial stiffness still occurs but it is not mainly driven by uraemia. The fact that FMD is only reduced in late stage CKD is intriguing and is different from previous data (Caglar *et al.*, 2008b; Yilmaz *et al.*, 2006; Yilmaz *et al.*, 2008a). This result suggests that uraemia itself has a minor impact on endothelial function and it takes time for uraemic patients by themselves to develop endothelial dysfunction.

In this study, increased arterial stiffness and endothelial dysfunction are mainly driven by age and BP. Ageing is an unavoidable physiological phenomenon. BP is a factor that could be controlled and all CKD patients in this study have a good control of BP. Therefore, apart from these two well known cardiovascular risk factors, there must be some other factors which can be regarded as emerging cardiovascular risk factors that contribute to vascular complications in CKD. One of these could be ET-1 as shown in Chapter 4 that plasma ET-1 concentrations are inversely associated with GFR. Additionally, as a vasoconstrictor ET-1 counter balance with a vasodilating NO. Hence, an increase in an endogenous NO inhibitor, ADMA, may be the other risk factor. Additionally, vascular complications are highly linked to inflammation

and oxidative stress; therefore, these two factors may be involved. The metabolic syndrome as a cluster of metabolic abnormalities and risk factors for cardiovascular disease and arterial calcification as a part of the pathophysiology of arterial stiffness may also contribute. All of these are worth exploring and are assessed in the further studies of this thesis.

10.1.4 Relationships of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction in chronic kidney disease

This study shows that in CKD patients without diabetes or established co-morbidity, BP is still the major determinant of arterial stiffness and endothelial dysfunction. Additionally, hsCRP and ADMA also independently predict arterial stiffness, and 8-Isop and ET-1 independently predict endothelial dysfunction.

This data confirms the finding of Chapter 5 that uraemia itself has a minor role in increased arterial stiffness and endothelial dysfunction and BP is still the strongest factor contributing to arterial stiffness and endothelial dysfunction in this group of patients. This, therefore, highlights the importance of a tight BP control in CKD. As the majority of CKD patients still fail to achieve a target BP level (Chobanian *et al.*, 2003; Mancia *et al.*, 2007), this may explain the very high risk of cardiovascular morbidity and mortality seen in the CKD population.

This study further stresses the important roles of inflammation, oxidative stress and NO/ET imbalance as determinants of vascular complications in CKD. The measure of these emerging risk factors, either alone or in combination may help identify subjects with a higher risk of cardiovascular disease. These results also indicate that interventional studies to examine the influence of these emerging risk factors on arterial stiffness and endothelial dysfunction in CKD, using potential therapeutic agents such as ET antagonism are of great interest.

10.1.5 Risk factors for the metabolic syndrome independently predict arterial stiffness and endothelial dysfunction in chronic kidney disease

This study shows that in minimally co-morbid CKD patients, regardless of renal function, CKD patients with the metabolic syndrome have higher arterial stiffness compared to those without. The presence of the metabolic syndrome and the number of risk factors for it independently determines arterial stiffness. Importantly, when risk factors for the metabolic syndrome are considered individually, waist circumference and SBP were independent determinants of CF-PWV.

This study, again, confirms the minimal role of uraemia on arterial stiffness and endothelial dysfunction. This study also confirmed that metabolic syndrome, either by itself or the number of risk factors for it, influences arterial stiffness but has a lower impact on endothelial dysfunction. BP, as one of the risk factors for the metabolic syndrome is, once again, the strongest determinant of arterial stiffness and endothelial dysfunction.

Interestingly, waist circumference also independently contributes to arterial stiffness, confirming the association of obesity and increased risk of cardiovascular morbidity (Elsayed *et al.*, 2008). This finding is very important because weight control, leading to a reduction of waist circumference, is one of the modifiable risk factors that could be performed by the patients themselves without using any medication. Evidence suggests that risk of cardiovascular morbidity and mortality is reduced with a weight reduction (Lavie *et al.*, 2009).

Although not independent predictors of either arterial stiffness or endothelial dysfunction in this study, glucose and cholesterol, as parts of the metabolic syndrome, could still be potential risk factors to be modified. Hyperglycaemia and dyslipidemia are linked to atherosclerosis (Kanter *et al.*, 2007). A tight control of them would improve the metabolic syndrome status of the patients and reduce the number of risk factors for the metabolic syndrome, leading to a reduction of arterial stiffness and thus cardiovascular complications.

10.1.6 Relationships of arterial calcification to arterial stiffness and endothelial dysfunction in chronic kidney disease

This study shows that, irrespective of renal function, minimally co-morbid CKD patients who develop CAC have increased arterial stiffness and a trend of impaired endothelial function compared to those without. In contrast, fetuin-A has no relationships to arterial stiffness and endothelial dysfunction in this group of patients.

The prevalence and extent of CAC in this study is very low compared to previous data (Barraclough *et al.*, 2008; Garland *et al.*, 2008; Piers *et al.*, 2009; Porter *et al.*, 2007; Russo *et al.*, 2007), which is consistent with the fact that these are patients with a low-co-morbidity. Although the extent of CAC is low, this study still suggests the role of arterial calcification in the pathophysiology of arterial stiffness. With a small sample size, the non-associations seen in this study have to be confirmed in a larger study.

10.1.7 Blood pressure-independent reduction in proteinuria and arterial stiffness after acute endothelin-A receptor antagonism in chronic kidney disease

This study shows that, irrespective of renal function, selective ET_A receptor antagonism reduces BP, proteinuria, and arterial stiffness in CKD patients without diabetes or established cardiovascular co-morbidity. These effects are seen on top of the maximally tolerated dose of ACE inhibitors and ARBs. Importantly, the reductions in arterial stiffness are greater than those seen with nifedipine, an alternative method of BP reduction.

This interventional study confirms the role of ET-1 in maintaining vascular tone in CKD and suggests that ET receptor antagonism may be a novel strategy to lower BP in CKD patients whose BP is often difficult to control.

Importantly, this study also confirms the contribution of ET-1 system on increased arterial stiffness as shown in Chapter 6 and suggests that this is mediated through the ET_A receptor in pre-dialysis. The reduction of arterial stiffness seen in this study is independent of a BP reduction and occurs on top of standard hypertensive treatment with ACE inhibitors or ARBs. Since PWV is an independent predictor for cardiovascular morbidity and mortality (Blacher *et al.*, 1999a; Laurent *et al.*, 2001), an improvement of PWV shown here provides evidence that selective ET_A receptor antagonism may be a therapeutic agent that not only controls hypertension but also reduces arterial stiffness and thus improves cardiovascular risk in CKD.

Of course, as an acute study, these findings have to be confirmed in a longer-term study with a wider range of CKD patients and antihypertensive agents.

10.2 Future work

The observations in this thesis raise further questions to be answered and areas to be explored.

10.2.1 The relationships of renal function to arterial stiffness and endothelial dysfunction in diabetic nephropathy

As the studies in this thesis show that, in a group of relatively low co-morbid CKD, uraemia has a minor role in arterial stiffness and endothelial dysfunction. A study in matched- diabetic nephropathy patients would be of interest. The hypotheses are that the degree of arterial stiffness and endothelial dysfunction would be higher and the starting point of developing arterial stiffness and endothelial dysfunction would be earlier in diabetic nephropathy compared to data presented in this thesis.

10.2.2 The relationships of emerging cardiovascular risk factors to arterial stiffness and endothelial dysfunction in diabetic nephropathy

Again, to elucidate the role of inflammation, oxidative stress and NO/ET imbalance, it is important to study these risk factors in matched diabetic nephropathy. The hypotheses are that the prevalence of inflammation, oxidative stress and NO/ET imbalance would be higher and the associations between these risk factors and arterial stiffness and endothelial dysfunction would be stronger than in those studied in this thesis.

10.2.3 Modification of the metabolic syndrome risk factors reduces arterial stiffness and endothelial dysfunction in CKD

As shown in this thesis that the metabolic syndrome itself, the number of risk factors for it, or its individual risk factors are independently linked to arterial stiffness and endothelial dysfunction, it is important to investigate whether the modification of these risk factors for the metabolic syndrome would improve arterial stiffness and endothelial dysfunction and thus reduce cardiovascular risk in CKD. It would be interesting to perform the studies on various groups of renal patients such as low co-morbid CKD, diabetic nephropathy, and those on dialysis to be able to the compare the beneficial effects.

10.2.4 The relationship of cardiovascular calcification and the role of calcium/phosphate-binding agents to arterial stiffness and endothelial dysfunction

Although the sample size of the study assessing the role of cardiovascular calcification on arterial stiffness and endothelial dysfunction is relatively small, it still shows that CAC is independently linked to arterial stiffness in CKD. This is

worth confirming in a larger-scale study. If the relationship persists, an interventional study with calcium/phosphate-binding agents such as selevamer on reducing calcium load status (Chertow *et al.*, 2002) and improving arterial stiffness and endothelial dysfunction in pre-dialysis would be of great interest.

10.2.5 Chronic ET_A receptor blockade in chronic kidney disease

Given the reduction of proteinuria and arterial stiffness shown in the acute study of this thesis, this needs to be confirmed in chronic studies. The attention would be focussed on examining to the role of ET_A receptor antagonism in slowing the rate of renal progression and improving proteinuria, arterial stiffness or endothelial dysfunction and studies should also be carried out on various CKD populations such as patients with diabetes, vasculitis, or renal vascular disease.

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Appendices

Appendix 1: Full subject characteristics for studies in Chapters 5 to 7 by CKD stage

	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Demographic						
N	23	28	30	30	20	7
Male/female (n)	13/10	17/11	18/12	22/8	16/4	4/3
Smoker/non-smoker (n)	2/21	9/19	5/25	6/24	4/16	0/7
Creatinine (μmol/L)*	78±13	77±13	102± 21	177±5	364±105	632±181
Estimated glomerular filtration rate (ml/min/1.73m^2)*	97±19	108±17	77±9	45±9	23±4	12±3
Age (year)	47±8	43±11	49±9	51±10	45±9	51±12
Weight (kg)	77±15	86±16	84±16	81±16	82±18	71±22
Height (m)	1.71±0.12	1.72±0.09	1.71±0.12	1.68±0.08	1.73±0.10	1.68±0.13
Body mass index (kg/m^2)	26±6	29±5	28±4	28±6	28±5	25±7
Waist circumference (cm)	90±12	96±12	96±14	98±14	95±18	91±20
Hip circumference (cm)	106±11	110±10	108±7	108±11	105±9	100±9
Waist/hip ratio	0.84±0.07	0.87±0.07	0.89±0.10	0.90±0.07	0.91±0.11	0.90±0.12
Haemodynamics						
Brachial systolic blood pressure (mmHg)**	113±16	114±15	116±13	120±12	121±12	132±16
Central systolic blood pressure (mmHg)**	103±17	104±15	108±13	111±14	111±12	123±18
Brachial diastolic blood pressure (mmHg)	71±10	71±10	74±9	76±9	76±7	76±7
Central diastolic blood pressure (mmHg)	72±9	73±10	76±9	76±9	77±7	77±7

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	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Brachial mean arterial pressure (mmHg)	85±12	86±11	88±10	90±10	91±8	95±8
Central mean arterial pressure (mmHg)	86±12	87±12	90±10	91±10	91±8	95±9
Brachial pulse pressure (mmHg)*	42±9	43±8	41±7	45±10	45±9	56±17
Central pulse pressure (mmHg)*	32±10	32±7	33±7	35±9	35±10	47±18
Heart rate (bpm)	58±7	63±8	62±10	60±8	58±9	63±13
Left ventricular hypertrophy (n (%))	0 (0)	0 (0)	0 (0)	2 (7)	2 (10)	1 (14)
Plasma levels						
Haemoglobin (g/L)*	140±77	142±10	142±15	131±18	120±12	114±16
Urea (mmol/L)*	5.0±0.9	5.3±1.4	6.2±2.0	11.4±4.4	19.7±6.2	28.0±3.1
Sodium (mmol/L)	140±2	140±2	140±2	140±2	141±2	141±3
Potassium (mmol/L)*	4.1±0.3	4.1±0.3	4.3±0.3	4.4±0.5	4.7±0.4	5.2±0.4
Bicarbonate (mmol/L)*	27±2	26±2	26±2	25±3	23±3	21±2
Albumin (g/L)**	43±3	42±3	41±3	40±4	38±10	40±3
Calcium (mmol/L)	2.29±0.07	2.27±0.08	2.29±0.10	2.32±0.11	2.25±0.15	2.33±0.13
Phosphate (mmol/L)*	1.09±0.15	1.05±0.13	1.10±0.15	1.17±0.19	1.42±0.27	1.95±0.50
Folate (μg/L)	9.2±4.3	8.5±3.1	9.8±4.3	8.1±3.1	9.1±3.9	9.1±6.5
Parathyroid hormone (ng/L)*	50±13	54±25	64±71	117±102	227±158	434±348
Glucose (mmol/L)	5.0±0.5	5.0±0.5	5.0±0.6	5.1±0.4	5.1±0.5	4.8±0.7
Haemoglobin A_1C (% total Hb)	5.4±0.4	5.5±0.4	5.7±0.4	5.7±0.3	5.5±0.4	5.6±0.4
Insulin (mU/L)	7.3±4.9	11.7±9.6	11.6±7.2	10.7±13.3	8.9±4.5	8.5±3.5
Insulin resistance	1.5±0.8	2.8±2.5	2.6±1.8	2.5±3.8	1.9±1.1	2.0±1.2
Urate (mmol/L)*	0.31±0.10	0.34±0.09	0.38±0.09	0.46±0.10	0.49±0.10	0.53±0.08

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	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Total cholesterol (mmol/L)	4.7±0.9	4.9±0.8	4.7±1.0	4.8±0.8	4.5±0.8	4.4±0.8
Triglycerides (mmol/L)	1.1±0.5	1.2±0.7	1.7±1.2	1.4±0.9	1.6±0.6	1.8±0.8
HDL cholesterol (mmol/L)	1.4±0.4	1.3±0.3	1.2±0.4	1.3±0.3	1.1±0.4	1.3±0.5
LDL cholesterol (mmol/L)	4.6±0.7	4.5±0.9	4.4±0.8	4.2±0.7	4.1±0.8	4.3±0.9
Cholesterol/HDL ratio	3.6±0.9	3.9±1.3	4.2±1.3	3.5±0.8	4.2±1.3	4.1±1.4
Vitamin A (μmol/L)*	2.1±0.4	2.0±0.6	2.7±0.7	3.5±0.8	4.5±0.9	5.2±1.5
Vitamin B1 (ng/g Hb)	425±98	462±72	453±122	460±125	482±159	425±139
Vitamin B2 (nmol/L)	232±36	367±82	355±70	383±87	347±61	315±46
Vitamin B6 (μmol/L)*	61±31	40±18	48±30	34±14	56±39	52±28
Vitamin B12 (ng/L)*	387±89	404±126	401±177	368±108	515±176	578±240
Vitamin C (μmol/L)	41±22	34±20	27±20	28±32	21±19	21±11
Vitamin E (μmol/L)	29±6	28±8	32±7	29±5	29±8	32±4
Copper (μmol/L)	16±3	15±4	17±5	17±3	16±3	17±6
Manganese (nmol/L)**	180±86	166±61	171±70	170±78	123±56	99±30
Selenium (μmol/L)*	1.01±0.14	0.88±0.15	0.92±0.18	0.79±0.18	0.87±0.26	0.86±0.25
Zinc (μmol/L)	13±2	12±2	12±2	13±3	11±2	12±3
Urine levels						
Albumin/creatinine ratio (mg/mmol)*	0.3±0.7	3.3±6.4	27.3±43.3	62.2±110.7	94.5±106.5	131.9±79.4
Medication						
∝**))%(ν(σρεκχολβ–	na	2 (7)	6 (20)	2 (7)	5 (25)	3 (43)
Angiotensin converting enzyme inhibitors (n (%))*	na	6 (21)	15 (50)	22 (73)	8 (40)	4 (57)
Angiotensin receptor blockers (n (%))**	na	2 (7)	3 (10)	7 (23)	6 (30)	0 (0)

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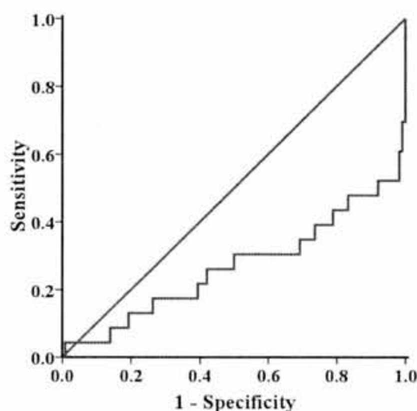
	Control	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Aspirin (n (%))	na	1 (4)	3 (10)	5 (17)	0 (0)	0 (0)
β*)%(ν(σρεκχολβ–	na	2 (7)	9 (30)	8 (27)	8 (40)	3 (43)
Calcium channel blockers (n (%))*	na	2 (7)	5 (17)	13 (43)	14 (70)	5 (71)
Diuretics (n (%))*	na	1 (4)	1 (7)	9 (30)	9 (4)	2 (27)
Statins (n (%))*	na	3 (11)	7 (23)	14 (47)	6 (30)	3 (43)

Key: *$p < 0.01$, **$p < 0.05$ for one-way ANOVA by CKD stage.

Appendix 2: ROC curve for eGFR



Area under the curve

Test result variable(s): eGFR

Area	Standard Error (a)	Asymptotic Significant (b)	Asymptotic 95% Confidence Interval	
			Upper Bound	Lower Bound
0.268	0.070	0.000	0.131	0.405

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Coordinates of the curve

Test result variable(s): eGFR

The positive actual state is FMD ≤1.5%

Positive if greater than or equal to	Sensitivity	1 - Specificity
6.70	1.000	1.000
7.95	0.957	1.000
9.93	0.913	1.000
11.75	0.870	1.000
12.26	0.826	1.000
13.21	0.783	1.000
14.12	0.739	1.000
15.12	0.696	1.000
16.40	0.696	0.991
17.16	0.652	0.991
17.63	0.609	0.991
18.41	0.609	0.982
20.15	0.565	0.982
21.58	0.522	0.982
21.70	0.522	0.974

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Positive if greater than or equal to	Sensitivity	1 - Specificity
21.87	0.522	0.965
22.37	0.522	0.956
23.05	0.522	0.947
24.15	0.522	0.939
25.31	0.522	0.930
25.85	0.522	0.921
26.25	0.478	0.921
26.87	0.478	0.912
27.30	0.478	0.904
27.56	0.478	0.895
27.83	0.478	0.886
28.45	0.478	0.877
30.92	0.478	0.868
32.88	0.478	0.860
33.10	0.478	0.851
33.32	0.478	0.842
33.80	0.478	0.833
34.34	0.435	0.833
34.88	0.435	0.825
36.08	0.435	0.816
38.07	0.435	0.807
39.27	0.435	0.798
40.11	0.435	0.789
41.33	0.391	0.789
41.79	0.391	0.781
43.07	0.391	0.772
44.39	0.391	0.763
45.12	0.391	0.754
46.26	0.391	0.746
47.51	0.391	0.737
49.29	0.348	0.737
50.38	0.348	0.728
51.09	0.348	0.719
52.12	0.348	0.711
52.83	0.348	0.702
53.29	0.348	0.693
53.94	0.304	0.693
55.51	0.304	0.684
56.87	0.304	0.675
57.29	0.304	0.667
57.94	0.304	0.658
58.84	0.304	0.649
60.16	0.304	0.640
61.45	0.304	0.632
63.27	0.304	0.623
65.19	0.304	0.614
66.03	0.304	0.605
67.59	0.304	0.596
68.81	0.304	0.588
69.06	0.304	0.579
69.30	0.304	0.570

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Positive if greater than or equal to	Sensitivity	1 - Specificity
69.81	0.304	0.561
70.48	0.304	0.553
70.98	0.304	0.544
71.77	0.304	0.535
72.80	0.304	0.526
74.05	0.304	0.518
75.32	0.304	0.509
76.00	0.304	0.500
76.25	0.261	0.500
76.43	0.261	0.491
76.66	0.261	0.482
76.86	0.261	0.474
78.53	0.261	0.465
80.41	0.261	0.456
80.90	0.261	0.447
81.52	0.261	0.439
82.22	0.261	0.430
82.63	0.261	0.421
82.79	0.217	0.421
83.81	0.217	0.412
84.91	0.217	0.404
85.17	0.217	0.395
85.60	0.174	0.395
86.62	0.174	0.386
87.43	0.174	0.377
87.52	0.174	0.368
88.00	0.174	0.360
88.78	0.174	0.351
89.33	0.174	0.342
89.74	0.174	0.333
89.96	0.174	0.325
90.11	0.174	0.316
90.24	0.174	0.307
90.40	0.174	0.298
90.74	0.174	0.289
91.00	0.174	0.281
91.64	0.174	0.272
92.43	0.174	0.263
94.47	0.130	0.263
96.80	0.130	0.254
97.95	0.130	0.246
98.84	0.130	0.237
99.42	0.130	0.228
100.04	0.130	0.219
100.56	0.130	0.211
100.91	0.130	0.202
102.19	0.130	0.193
103.37	0.087	0.193
104.95	0.087	0.184
106.54	0.087	0.175
107.07	0.087	0.167

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Positive if greater than or equal to	Sensitivity	1 - Specificity
107.67	0.087	0.158
108.00	0.087	0.149
108.60	0.087	0.140
109.68	0.043	0.140
110.72	0.043	0.132
111.28	0.043	0.123
112.08	0.043	0.114
113.24	0.043	0.105
113.83	0.043	0.096
114.23	0.043	0.088
115.14	0.043	0.079
116.26	0.043	0.070
118.41	0.043	0.061
120.14	0.043	0.053
122.33	0.043	0.044
124.80	0.043	0.035
128.91	0.043	0.026
136.61	0.043	0.018
143.81	0.043	0.009
150.55	0.000	0.009
155.13	0.000	0.000

Appendix 3: Full systemic haemodynamic data of the main study in Chapter 9

Placebo

Placebo

Time from drug (min)	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
MAP																	
MAP (mmHg)	93.1±2.1	96.3±1.9	93.9±1.9	94.1±1.7	95.4±2.1	95.7±2.0	94.6±2.0	96.2±2.0	96.1±2.2	97.4±2.2	95.7±2.1	96.3±2.1	96.8±2.0	98.0±2.0	97.7±2.2	98.8±2.4	98.8±2.4
Absolute Δ MAP	na	3.1±0.9	0.8±1.2	1.0±1.0	2.2±0.7	2.6±0.8	1.4±0.7	3.1±1.1	2.9±0.8	4.3±0.9	2.6±0.9	3.2±0.8	3.6±1.0	4.9±0.8	4.5±0.8	5.6±1.0	5.7±0.9
% Δ MAP	na	3.6±1.0	1.2±1.2	1.4±1.1	2.5±0.7	2.9±1.0	1.7±0.8	3.5±1.1	3.2±0.9	4.7±1.0	2.9±1.0	3.6±0.8	4.1±1.0	5.4±0.9	5.0±0.9	6.1±1.1	6.1±1.0
SVRI																	
SVRI (dynes/s/m2/cm5)	33.6±2.3	35.4±2.5	34.9±2.0	33.9±2.2	35.8±2.5	35.3±2.3	35.9±2.6	37.5±2.7	36.9±2.7	35.6±2.3	36.4±2.7	36.7±2.5	36.6±2.6	37.1±2.6	35.9±2.0	37.3±2.7	36.7±2.9
Absolute Δ SVRI	na	1.8±0.8	1.3±0.8	0.3±1.1	2.2±0.9	1.6±1.0	2.3±0.8	3.9±1.3	3.2±1.0	2.0±1.1	2.8±1.2	3.1±1.0	3.0±1.1	3.4±1.1	2.2±0.9	3.7±1.2	3.1±1.4
% Δ SVRI	na	5.7±2.2	6.3±2.7	2.4±2.9	6.9±2.7	6.5±2.7	6.8±1.8	12.6±3.0	9.9±2.3	7.8±3.0	8.6±2.9	10.3±2.5	9.9±2.8	11.3±2.9	9.5±2.6	11.8±3.1	9.6±3.2
CI																	
CI (L/min)	3.0±0.2	3.0±0.2	2.9±0.2	3.0±0.2	2.9±0.2	2.9±0.2	2.9±0.2	2.8±0.2	2.9±0.2	3.0±0.2	2.9±0.2	2.9±0.2	2.9±0.2	2.9±0.2	2.9±0.1	2.9±0.2	3.0±0.2
Absolute Δ CI	na	0.0±0.1	-0.2±0.1	0.0±0.1	-0.1±0.1	-0.1±0.1	-0.2±0.0	-0.2±0.1	-0.2±0.1	-0.1±0.1	-0.1±0.1	-0.2±0.1	-0.2±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1
% Δ CI	na	-1.5±2.1	-4.5±1.6	0.0±2.5	-3.4±1.9	-2.7±2.0	-4.6±1.7	-6.9±2.9	-5.5±2.2	-1.2±3.5	-4.2±2.6	-5.2±2.6	-4.2±2.6	-4.3±2.5	-3.4±2.1	-4.0±2.7	-1.6±3.2
Heart rate																	
Heart rate (bpm)	56±2	56±2	56±2	57±2	57±2	56±2	56±2	55±2	56±2	56±2	57±2	56±2	57±2	56±2	57±2	56±2	58±2
Absolute Δ Heart rate	na	-0.5±0.7	-0.9±0.6	0.8±0.9	0.9±0.8	-0.7±0.7	-0.1±0.7	-0.1±0.6	0.0±0.9	-0.4±0.6	0.5±0.9	-0.2±0.8	0.1±1.0	-0.3±0.9	0.4±0.8	-0.3±0.7	1.8±0.9
% Δ Heart rate	na	-0.6±1.2	-1.2±0.9	1.6±1.5	1.9±1.3	-0.8±1.2	0.2±1.2	-1.5±1.2	0.5±1.6	-0.3±1.1	1.2±1.6	0.1±1.5	0.7±1.6	-0.2±1.6	1.1±1.3	-0.4±1.2	3.2±1.6

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline; CI: cardiac index; MAP: mean arterial pressure; SVRI: systemic vascular resistance index.

BQ123

BQ123

Time from drug (min)	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
MAP																	
MAP (mmHg)	92.4±1.5	92.2±1.6	91.0±1.4	87.5±1.6	86.7±1.6	85.8±1.6	86.9±1.4	88.2±1.6	88.1±1.3	89.7±1.5	89.7±1.5	92.1±1.4	91.9±1.4	94.3±1.5	94.0±1.4	95.4±1.5	94.2±1.4
Absolute Δ MAP	na	-0.2±0.7	-1.4±0.6	-4.9±0.8	-5.7±1.0	-6.6±1.1	-5.5±0.7	-4.2±0.9	-4.3±0.9	-2.6±0.7	-2.7±0.9	-0.2±0.7	-0.5±0.8	2.0±1.0	1.7±0.8	3.0±1.0	1.8±0.9
% Δ MAP	na	-0.2±0.8	-1.5±0.6	-5.3±0.9	-6.1±1.0	-7.0±1.1	-5.9±0.7	-4.5±0.9	-4.5±0.9	-2.8±0.7	-2.8±0.9	-0.2±0.8	-0.4±0.9	2.3±1.1	1.9±0.9	3.4±1.1	2.2±1.0
SVRI																	
SVRI (dynes/s/m2/cm5)	32.9±2.1	31.9±1.9	30.0±1.8	29.6±2.0	29.8±2.4	29.1±2.0	29.7±2.0	30.3±2.2	30.5±1.8	33.2±2.3	32.3±1.9	33.2±2.0	33.2±2.1	34.3±2.2	33.7±1.9	35.6±2.3	34.6±2.2
Absolute Δ SVRI	na	-1.0±0.8	-2.8±0.8	-3.2±0.8	-3.0±1.0	-3.8±0.9	-3.1±0.8	-2.6±1.5	-2.4±1.2	0.3±1.3	-0.5±1.1	0.3±0.7	0.3±1.0	1.4±1.3	0.9±1.4	2.7±1.4	1.8±1.4
% Δ SVRI	na	-2.1±1.9	-7.3±1.8	-9.7±1.8	-9.9±2.4	-11.0±2.0	-8.9±2.1	-6.5±3.0	-5.2±2.7	1.9±3.1	0.1±2.5	2.1±2.1	2.1±2.5	5.8±2.8	5.8±3.4	10.4±3.5	7.5±3.2
CI																	
CI (L/min)	3.1±0.2	3.1±0.2	3.2±0.2	3.2±0.2	3.2±0.2	3.2±0.2	3.2±0.2	3.2±0.2	3.1±0.2	3.0±0.2	3.0±0.2	3.0±0.2	3.0±0.2	3.0±0.2	3.0±0.1	2.9±0.1	2.9±0.2
Absolute Δ CI	na	0.0±0.1	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.0±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.2±0.1	-0.2±0.1
% Δ CI	na	1.5±1.7	5.7±1.4	4.2±1.3	4.2±2.1	4.1±1.9	3.1±2.1	4.1±4.4	1.2±2.6	-4.0±2.5	-2.7±2.3	-2.6±1.9	-2.4±2.1	-3.0±2.4	-2.1±3.7	-5.1±3.3	-3.7±3.6
Heart rate																	
Heart rate (bpm)	57±2	58±2	59±2	59±2	59±2	58±2	58±2	57±2	56±2	57±2	57±2	58±2	58±2	58±2	57±2	56±2	57±2
Absolute Δ Heart rate	na	0.5±0.7	1.6±0.5	2.4±0.5	2.2±0.5	1.1±0.5	0.9±0.7	0.2±0.5	-0.8±0.4	-0.1±0.5	0.4±0.6	1.1±0.5	1.3±0.6	0.5±0.5	0.0±0.5	-1.0±0.6	0.2±0.6
% Δ Heart rate	na	0.9±1.3	2.8±0.9	4.2±0.8	3.8±0.9	2.0±0.9	1.4±1.3	0.2±0.8	-1.4±0.6	-0.2±0.8	0.4±1.0	2.2±0.8	2.3±1.0	1.0±0.9	0.0±0.9	-1.8±1.1	0.1±1.1

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline; CI: cardiac index; MAP: mean arterial pressure; SVRI: systemic vascular resistance index.

Appendix 4: Full data of the substudy in Chapter 9

Placebo

Placebo

Time from drug (min)	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
Systemic haemodynamic																	
MAP																	
MAP (mmHg)	92.6±2.1	96.0±2.7	95.2±2.3	94.0±2.8	96.0±2.8	96.2±2.6	95.1±2.5	97.3±2.9	95.5±3.2	98.3±3.2	96.4±2.5	97.6±2.8	97.8±2.6	98.3±2.9	97.4±2.6	99.4±2.8	99.9±3.0
Absolute Δ MAP	na	3.4±1.1	2.6±0.8	1.4±1.3	3.4±1.3	3.6±1.5	2.5±1.0	4.7±2.1	2.9±1.6	5.7±1.7	3.9±0.9	5.0±1.0	5.2±0.8	5.7±1.0	4.9±0.9	6.9±1.5	7.4±1.0
% Δ MAP	na	3.6±1.2	2.8±10.9	1.5±1.4	3.6±1.4	3.9±1.6	2.7±1.0	5.2±2.2	3.1±1.7	6.0±1.9	4.1±0.9	5.3±1.1	5.6±0.9	6.1±1.0	5.2±0.9	7.4±1.6	7.9±1.0
SVRI																	
SVRI (dynes/s/m2/cm5)	35.3±2.3	36.7±2.5	37.1±2.1	36.2±2.6	36.9±2.6	37.3±2.7	37.0±2.7	40.5±3.0	38.4±3.0	38.6±2.6	38.6±2.7	38.8±2.4	37.7±2.5	38.2±2.6	37.7±2.0	40.8±2.7	39.7±3.1
Absolute Δ SVRI	na	1.4±0.7	1.8±0.7	0.9±0.7	1.6±0.6	2.0±1.0	1.7±1.1	5.2±1.3	3.1±1.1	3.3±0.9	3.3±1.0	3.5±0.8	2.4±1.0	2.9±1.0	2.4±0.7	5.5±1.0	4.4±1.4
% Δ SVRI	na	4.0±2.0	6.5±2.1	1.4±2.0	3.9±1.5	5.7±2.7	4.6±2.3	15.0±2.7	7.2±2.3	9.6±2.7	9.2±2.5	10.6±2.1	7.8±2.8	8.3±2.8	8.4±2.0	16.5±2.8	12.0±3.0
CI																	
CI (L/min)	2.9±0.2	2.9±0.2	2.7±0.1	2.9±0.2	2.9±0.2	2.8±0.2	2.8±0.2	2.6±0.2	2.8±0.2	2.8±0.2	2.7±0.2	2.7±0.2	2.8±0.2	2.8±0.2	2.8±0.1	2.6±0.2	2.8±0.2
Absolute Δ CI	na	0.0±0.1	-0.1±0.1	0.1±0.1	0.0±0.0	0.0±0.1	0.0±0.0	-0.2±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	-0.1±0.1	0.0±0.1	0.0±0.1	-0.1±0.1	-0.2±0.1	-0.1±0.1
% Δ CI	na	0.2±1.9	-2.9±1.7	0.7±2.0	-0.2±1.1	-0.8±2.0	-1.0±2.0	-7.6±2.5	-3.3±1.8	-2.4±2.0	-3.9±1.9	-4.1±2.0	-0.7±2.6	-1.0±2.3	-2.5±1.7	-6.9±2.2	-2.4±2.5
Heart rate																	
Heart rate (bpm)	57±1	58±2	57±1	58±2	59±1	57±1	56±1	56±1	56±1	57±2	58±2	58±1	59±2	57±2	57±2	56±1	58±2
Absolute Δ Heart rate	na	0.6±0.8	0.0±0.4	0.8±1.0	1.3±0.9	-0.6±0.8	-1.2±0.6	-1.5±0.7	-1.9±1.0	-0.8±0.7	0.1±1.1	0.1±1.1	1.4±1.1	-0.2±1.0	-0.2±0.9	-1.8±0.7	0.9±0.9
% Δ Heart rate	na	1.1±1.3	0.0±0.7	1.6±1.8	2.6±1.6	-0.8±1.4	-1.9±1.1	-2.5±1.2	-3.1±1.6	-1.2±1.3	0.4±1.8	0.5±1.8	2.7±1.9	-0.2±1.7	-0.4±1.5	-3.0±1.1	1.5±1.6

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline; CI: cardiac index; MAP: mean arterial pressure; SVRI: systemic vascular resistance index.

Placebo

Time from drug (min)	0	60	120	180	240
Arterial stiffness and endothelial function					
CF-PWV					
CF-PWV (m/s)	7.5±0.4		7.7±0.4		7.7±0.4
Absolute Δ CF-PWV	na		0.2±0.1		0.2±0.1
% Δ CF-PWV	na		2.5±0.9		2.6±0.9
FMD					
FMD (%)	3.7±0.6		4.3±0.7		4.1±0.6
Absolute Δ FMD	na		0.6±0.2		0.4±0.2
% Δ FMD	na		18.3±5.8		21.4±17.5
Renal haemodynamic					
ERBF					
ERBF (ml/min)	2112±264	1837±215	1908±233	1804±189	1726±206
Absolute Δ ERBF	na	-275±55	-204±63	-308±86	-386±70
% Δ ERBF	na	-11±1	-7±2	-9±3	-16±2
ERVR					
ERVR (mmHg/min/ml)	6.3±0.9	7.1±0.9	6.8±0.8	7.0±0.9	7.9±1.0
Absolute Δ ERVR	na	0.9±0.1	0.5±0.2	0.7±0.2	1.6±0.3
% Δ ERVR	na	16.7±1.5	12.9±3.3	18.6±3.7	30.5±3.8
GFR					
GFR (ml/min)	44±5	47±5	45±5	45±5	46±4
Absolute Δ GFR	na	3.2±1.1	1.3±0.9	0.9±1.5	1.5±1.9
% Δ GFR	na	7.3±2.6	4.6±2.6	2.4±3.5	6.8±4.4
FF					
FF (%)	3.7±0.5	4.2±0.4	3.9±0.4	3.8±0.3	4.2±0.4
Absolute Δ FF	na	0.5±0.1	0.3±0.1	0.1±0.3	0.7±0.2
% Δ FF	na	21.3±6.7	14.9±8.5	16.9±10.1	30.4±11.5
Urinary sodium and protein excretion					
UNaV					
UNaV (mmol/min)	225±43	236±48	235±42	235±41	215±33
Absolute Δ UnaV	na	11±10	10±15	10±24	-10±24
% Δ UNaV	na	3±6	5±9	8±9	1±9
Proteinuria					
Proteinuria (mg/min)	1320±281	1423±327	1397±318	1196±247	1201±237
Absolute Δ proteinuria	na	103±65	77±53	-124±75	-119±72
% Δ proteunuria	na	3±4	1±4	-9±4	-1±5
Plasma ET-1					
ET-1					
Plasma ET-1 (pg/ml)	6.3±0.3	6.4±0.3	5.9±0.3	5.9±0.3	6.1±0.3
Absolute Δ plasma ET-1	na	0.1±0.2	-0.4±0.3	-0.4±0.4	-0.2±0.5
% Δ plasma ET-1	na	3.0±3.6	-5.1±3.8	-4.7±6.4	1.7±7.3

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline. CF-PWV: carotid-femoral pulse wave velocity; ERBF: effective renal blood flow; ERVR: effective renal vascular resistance; ET-1: endothelin-1; FF: filtration fraction; FMD: flow-mediated dilatation; GFR: glomerular filtration rate; UNaV: urinary sodium excretion.

BQ123

BQ123

Time from drug (min)	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
Systemic haemodynamic																	
MAP																	
MAP (mmHg)	93.3±2.1	94.2±2.6	92.9±2.3	89.2±2.2	88.1±2.3	88.3±2.7	88.2±2.6	89.1±3.2	89.6±2.2	90.8±2.8	89.8±3.0	92.9±2.5	92.8±2.5	96.2±2.7	95.2±2.5	95.6±2.5	95.1±2.2
Absolute Δ MAP	na	0.9±0.9	-0.4±0.7	-4.1±1.0	-5.2±1.6	-5.0±1.4	-5.1±1.3	-4.1±1.7	-3.7±1.1	-2.5±1.5	-3.4±1.7	-0.4±1.2	-0.5±1.7	2.9±1.6	1.9±1.5	2.3±2.0	1.8±1.5
% Δ MAP	na	0.9±1.0	-0.4±0.7	-4.4±1.0	-5.5±1.7	-5.4±1.5	-5.5±1.4	-4.6±1.8	4.0±1.2	-2.7±1.6	-3.8±1.9	-0.4±1.3	-0.5±1.8	3.2±1.7	2.0±1.5	2.6±2.1	2.0±1.6
SVRI																	
SVRI (dynes/s/m2/cm5)	35.2±2.1	34.2±2.0	33.2±2.0	33.0±2.3	33.9±2.9	32.8±2.4	32.7±2.3	34.4±2.6	33.6±2.0	36.8±2.7	35.2±2.2	35.5±2.2	36.9±2.4	38.0±2.5	37.2±2.2	39.0±2.7	37.9±2.4
Absolute Δ SVRI	na	-0.9±0.6	-1.9±0.3	-2.1±0.3	-1.2±1.0	-2.3±0.6	-2.5±0.8	-0.8±0.9	-1.5±0.7	1.7±1.2	0.1±0.7	0.4±0.9	1.8±1.0	2.9±0.6	2.2±0.9	3.9±1.0	2.8±0.7
% Δ SVRI	na	-2.0±1.9	-5.5±0.8	-7.0±1.1	-5.9±2.2	-7.8±2.0	-7.8±2.2	-3.5±2.4	-4.3±2.0	3.4±3.3	-0.2±1.9	0.8±2.5	4.4±2.7	7.4±1.6	6.3±2.4	10.4±2.7	7.5±2.0
CI																	
CI (L/min)	2.8±0.1	2.9±0.1	3.0±0.1	2.9±0.2	2.9±0.2	2.9±0.2	2.9±0.2	2.8±0.2	2.9±0.2	2.7±0.2	2.7±0.2	2.8±0.2	2.7±0.2	2.7±0.2	2.7±0.1	2.7±0.1	2.7±0.1
Absolute Δ CI	na	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	0.0±0.1	0.0±0.1	-0.1±0.1	-0.1±0.1	0.0±0.1	-0.1±0.1	-0.1±0.0	-0.1±0.1	-0.2±0.1	-0.1±0.0
% Δ CI	na	3.6±2.2	5.1±0.7	2.8±1.0	0.9±1.8	3.2±2.2	3.4±2.5	-0.4±2.1	0.9±2.0	-4.8±2.1	-3.2±1.8	-0.2±2.4	-4.0±2.0	-3.9±1.3	-3.3±2.1	-6.5±1.9	-4.9±1.5
Heart rate																	
Heart rate (bpm)	57±2	58±2	60±2	59±2	59±2	59±2	58±2	57±2	57±2	58±2	58±2	59±2	59±2	57±2	58±2	56±2	58±2
Absolute Δ Heart rate	na	0.7±0.7	2.5±0.5	2.2±0.4	2.0±0.6	1.4±0.5	1.3±0.6	-0.1±0.4	-0.4±0.3	0.9±0.6	0.6±0.6	1.9±0.5	1.4±0.7	0.3±0.5	0.5±0.6	-0.7±0.5	0.5±0.8
% Δ Heart rate	na	1.1±1.2	4.2±0.8	3.7±0.7	3.2±1.0	2.2±0.9	2.0±1.0	-0.4±0.8	-0.7±0.6	1.4±1.0	0.7±1.0	3.3±1.0	2.4±1.2	0.3±0.8	0.7±0.9	-1.5±0.9	0.6±1.3

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline; CI: cardiac index; MAP: mean arterial pressure; SVRI: systemic vascular resistance inde

BQ123

Time from drug (min)	0	60	120	180	240
Arterial stiffness and endothelial function					
CF-PWV					
CF-PWV (m/s)	7.3±0.4		6.9±0.4		6.7±0.4
Absolute Δ CF-PWV	na		-0.4±0.1		-0.6±0.0
% Δ CF-PWV	na		-5.6±1.4		-8.5±0.8
FMD					
FMD (%)	4.4±0.6		4.0±0.6		4.6±0.6
Absolute Δ FMD	na		-0.4±0.4		0.2±0.4
% Δ FMD	na		107.8±77.3		4.3±10.4
Renal haemodynamic					
ERBF					
ERBF (ml/min)	2015±236	2162±234	2231±242	2055±233	1888±208
Absolute Δ ERBF	na	147±47	215±100	39±49	-127±84
% Δ ERBF	na	10±3	15±5	3±2	-4±3
ERVR					
ERVR (mmHg/min/ml)	7.1±1.0	5.9±0.8	5.9±0.8	6.8±1.0	7.5±1.1
Absolute Δ ERVR	na	-1.2±0.2	-1.2±0.2	-0.2±0.1	0.4±0.2
% Δ ERVR	na	-14.5±2.2	-14.4±3.2	-3.6±2.3	7.3±3.7
GFR					
GFR (ml/min)	42±4	40±3	42±4	42±4	41±4
Absolute Δ GFR	na	-2.2±1.5	0.0±1.5	-0.4±1.3	-1.1±1.4
% Δ GFR	na	-0.1±2.9	-0.9±3.6	-0.9±3.7	-4.0±3.1
FF					
FF (%)	4.3±0.5	3.5±0.3	3.3±0.3	3.7±0.3	4.0±0.4
Absolute Δ FF	na	-0.8±0.3	-1.0±0.4	-0.6±0.3	-0.3±0.3
% Δ FF	na	-12.4±3.3	-13.5±9.5	-6.6±8.7	-1.6±9.9
Urinary sodium and protein excretion					
UNaV					
UNaV (mmol/min)	175±33	200±32	222±45	212±37	229±47
Absolute Δ UnaV	na	25±9	47±17	37±11	54±23
% Δ UNaV	na	20±8	28±11	25±10	31±10
Proteinuria					
Proteinuria (mg/min)	2148±600	1696±538	1318±370	1347±398	1478±480
Absolute Δ proteinuria	na	-452±205	-830±245	-800±234	-670±210
% Δ proteunuria	na	-25±4	-34±3	-38±3	-34±4
Plasma ET-1					
ET-1					
Plasma ET-1 (pg/ml)	6.7±0.3	7.0±0.4	5.8±0.2	6.5±0.4	5.6±0.2
Absolute Δ plasma ET-1	na	0.2±0.3	-1.0±0.3	-0.2±0.4	-1.1±0.3
% Δ plasma ET-1	na	3.5±4.5	-10.4±4.7	-1.6±5.5	-11.3±6.5

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline. CF-PWV: carotid-femoral pulse wave velocity; ERBF: effective renal blood flow; ERVR: effective renal vascular resistance; ET-1: endothelin-1; FF: filtration fraction; FMD: flow-mediated dilatation; GFR: glomerular filtration rate; UNaV: urinary sodium excretion.

Nifedipine

Nifedipine

Time from drug (min)	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
Systemic haemodynamic																	
MAP																	
MAP (mmHg)	92.7±2.5	93.7±3.3	83.2±3.3	81.7±3.3	83.7±2.7	85.3±3.1	87.0±3.2	88.3±3.0	88.4±2.5	91.3±3.5	89.9±3.3	90.6±3.5	90.8±2.9	94.4±3.3	94.8±3.3	96.6±2.6	92.4±4.1
Absolute Δ MAP	na	1.1±0.8	-9.4±2.2	-11.0±2.7	-8.9±2.5	-7.4±2.6	-5.6±2.4	-4.4±2.4	-4.2±2.1	-1.4±2.9	-2.8±2.6	-2.0±2.5	-1.9±2.5	1.7±2.5	2.2±2.3	5.6±1.0	5.7±0.9
% Δ MAP	na	1.1±2.0	-10.2±2.3	-11.8±2.9	-9.4±2.6	-7.8±2.9	-6.0±2.6	-4.6±2.6	-4.4±2.3	-1.5±2.7	-2.9±2.9	-2.2±2.8	-1.9±2.9	2.0±2.8	2.4±2.6	4.4±2.1	-0.2±3.7
SVRI																	
SVRI (dynes/s/m2/cm5)	34.4±2.4	34.4±2.6	24.6±2.4	22.9±2.0	25.9±2.0	28.2±2.3	29.8±2.3	32.3±2.8	33.7±3.0	34.7±2.7	33.9±2.4	35.4±2.6	34.3±2.3	38.4±2.5	35.7±2.2	36.9±2.4	34.9±2.5
Absolute Δ SVRI	na	0.0±0.6	-9.8±1.0	-11.5±1.2	-8.5±0.9	-6.2±0.7	-4.6±0.7	-2.1±1.1	-0.7±1.1	0.3±0.9	-0.5±1.0	1.0±0.7	-0.1±1.0	4.0±1.2	1.3±0.6	2.5±0.7	0.5±0.8
% Δ SVRI	na	0.0±0.6	-9.8±1.0	-11.5±1.2	-8.5±0.9	-6.2±0.7	-4.6±0.7	-2.1±1.1	-0.7±1.1	0.3±0.9	-0.5±1.0	1.0±0.7	-0.1±1.0	4.0±1.2	1.3±0.6	2.5±0.7	0.5±0.8
CI																	
CI (L/min)	2.9±0.2	3.0±0.2	4.0±0.3	4.0±0.3	3.6±0.2	3.4±0.2	3.2±0.2	3.0±0.2	3.0±0.2	2.9±0.2	2.9±0.2	2.8±0.2	2.9±0.2	2.6±0.1	2.8±0.1	2.8±0.2	2.9±0.2
Absolute Δ CI	na	0.1±0.0	1.1±0.2	1.1±0.1	0.6±0.1	0.4±0.1	0.3±0.0	0.1±0.1	0.0±0.1	0.0±0.0	-0.1±0.0	-0.1±0.0	-0.1±0.1	-0.3±0.1	-0.1±0.0	-0.1±0.1	0.0±0.1
% Δ CI	na	-1.5±2.1	-4.5±1.6	0.0±2.5	-3.4±1.9	-2.7±2.0	-4.6±1.7	-6.9±2.9	-5.5±2.2	-1.2±3.5	-4.2±2.6	-5.2±2.6	-4.2±2.6	-4.3±2.5	-3.4±2.1	-4.0±2.7	-1.6±3.2
Heart rate																	
Heart rate (bpm)	58±2	59±2	69±3	69±2	66±2	63±2	61±2	60±2	61±2	59±2	57±2	59±2	59±2	58±2	58±2	58±2	58±2
Absolute Δ Heart rate	na	0.7±1.2	10.6±2.0	10.2±1.1	7.4±0.7	4.7±0.8	2.9±0.8	1.4±0.8	2.1±0.9	0.4±0.5	-1.3±0.7	0.6±0.9	1.0±0.9	-0.4±0.6	-0.3±0.8	0.1±0.8	0.1±0.9
% Δ Heart rate	na	1.5±1.9	18.0±3.3	17.5±1.9	12.7±1.3	8.1±1.3	5.0±1.3	2.3±1.3	3.6±1.6	0.7±0.7	-2.3±1.2	1.3±1.6	1.6±1.5	-0.7±1.1	-0.2±1.1	-0.1±1.4	0.5±1.5

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline; CI: cardiac index; MAP: mean arterial pressure; SVRI: systemic vascular resistance index.

Nifedipine

Time from drug (min)	0	60	120	180	240
Arterial stiffness and endothelial function					
CF-PWV					
CF-PWV (m/s)	7.4±0.3		7.0±0.3		7.1±0.3
Absolute Δ CF-PWV	na		-0.4±0.1		-0.3±0.1
% Δ CF-PWV	na		-4.6±1.2		-3.4±1.4
FMD					
FMD (%)	5.1±0.7		1.6±3.1		3.1±0.6
Absolute Δ FMD	na		-3.5±0.5		-2.1±0.4
% Δ FMD	na		-81.2±10.1		-26.7±17.5
Renal haemodynamic					
ERBF					
ERBF (ml/min)	1776±240	1880±262	1984±233	1851±250	1893±275
Absolute Δ ERBF	na	104±61	208±81	76±66	117±95
% Δ ERBF	na	4±4	17±6	7±4	5±5
ERVR					
ERVR (mmHg/min/ml)	7.8±1.0	7.0±1.0	6.7±0.9	7.2±0.8	7.6±1.0
Absolute Δ ERVR	na	-0.9±0.5	-1.2±0.5	-0.7±0.5	-0.2±0.4
% Δ ERVR	na	-12.1±4.3	-13.4±4.9	-5.5±3.8	-1.6±4.7
GFR					
GFR (ml/min)	43±5	43±5	45±6	50±6	47±6
Absolute Δ GFR	na	0.2±2.3	2.8±1.8	7.9±2.2	4.4±1.7
% Δ GFR	na	5.4±5.4	8.6±3.5	16.7±3.8	9.7±3.8
FF					
FF (%)	4.0±0.4	4.2±0.4	3.9±0.3	4.5±0.4	4.4±0.4
Absolute Δ FF	na	0.2±0.3	-0.1±0.2	0.4±0.2	0.4±0.2
% Δ FF	na	7.1±10.2	-0.4±11.6	13.9±10.7	9.0±10.5
Urinary sodium and protein excretion					
UNaV					
UNaV (mmol/min)	180±27	193±45	240±60	215±37	207±46
Absolute Δ UnaV	na	14±23	60±33	35±20	27±24
% Δ UNaV	na	4±13	27±15	21±14	11±13
Proteinuria					
Proteinuria (mg/min)	1092±213	1178±237	1262±237	1291±279	1283±287
Absolute Δ proteinuria	na	86±73	170±77	199±96	190±142
% Δ proteunuria	na	6±7	20±7	26±11	23±11
Plasma ET-1					
ET-1					
Plasma ET-1 (pg/ml)	6.9±0.4	8.4±0.9	6.3±0.2	6.0±0.3	6.5±0.4
Absolute Δ plasma ET-1	na	1.4±1.0	-0.6±0.5	-1.0±0.4	-0.4±0.5
% Δ plasma ET-1	na	29.9±18.6	-3.2±5.9	-11.0±3.4	-2.1±5.4

Key: Values are mean±SEM; absolute Δ: absolute change from baseline;% Δ: percentage change from baseline. CF-PWV: carotid-femoral pulse wave velocity; ERBF: effective renal blood flow; ERVR: effective renal vascular resistance; ET-1: endothelin-1; FF: filtration fraction; FMD: flow-mediated dilatation; GFR: glomerular filtration rate; UNaV: urinary sodium excretion.

Appendix 5: Publications

Neeraj Dhaun, Pajaree Lilitkarntakul, Iain M. MacIntyre, Eline Muilwijk, Neil R. Johnston, David C. Kluth, David J. Webb and Jane Goddard
Am J Physiol Renal Physiol 296:1477-1483, 2009. First published Mar 11, 2009;
doi:10.1152/ajprenal.90713.2008

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Urinary endothelin-1 in chronic kidney disease and as a marker of disease activity in lupus nephritis

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Dhaun N, Lilitkarntakul P, MacIntyre IM, Muilwijk E, Johnston NR, Kluth DC, Webb DJ, Goddard J. Urinary endothelin-1 in chronic kidney disease and as a marker of disease activity in lupus nephritis. *Am J Physiol Renal Physiol* 296: F1477–F1483, 2009. First published March 11, 2009; doi:10.1152/ajprenal.90713.2008.—Chronic inflammation contributes to the development and progression of chronic kidney disease (CKD). Identifying renal inflammation early is important. There are currently no specific markers of renal inflammation. Endothelin-1 (ET-1) is 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. In a prospective study, plasma and urinary ET-1 were measured in 132 subjects with CKD stages 1 to 5, and fractional excretion of ET-1 (FeET-1) was 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, 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 done before and after 6 mo of treatment. 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%, 10.0 ± 3.0 pg/μmol, both $P < 0.001$), and correlated with CRP, and 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% ($P < 0.01$). 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 while measured renal function is still normal.

systemic lupus erythematosus; renal inflammation; biomarker

CHRONIC KIDNEY DISEASE (CKD) is common, affecting 6–11% of the population in the developed world (23). Chronic inflammation is a major contributor to the development and progression of CKD (21). Current treatments for inflammatory CKD include immunosuppressive therapy, which is often associated with significant side effects (18). 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 (15). 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 a 21-amino acid peptide implicated in the development and progression of CKD (10). It is produced both within the vasculature and the kidney (10). ET-1 is the most potent endogenous vasoconstrictor (39). In addition, both within the kidney and elsewhere, ET-1 has a number of other major effects including cell proliferation (32), inflammation (30), and fibrosis (17). Although plasma ET-1 levels are not a reliable measure of vascular ET-1 production, owing to its predominantly abluminal release (40), urinary ET-1 excretion is independent of plasma ET-1 concentrations (13, 31) and is well-correlated with renal ET-1 production (4, 38). A few small studies showed a rise in plasma (20) and urine (26) ET-1 in severe CKD, and our group previously demonstrated increases in plasma and urinary ET-1 concentrations in eight subjects with noninflammatory renal disease, across a range of glomerular filtration rates (GFR) (13). 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 hypothesized 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 noninflammatory, mild, and more florid inflammatory renal diseases, respectively.

METHODS

This was a prospective, cross-sectional study approved by the Multi-centre Research Ethics Committee for Scotland. It was performed between June 2005 and October 2007 in accordance with the Declaration of Helsinki, and with the written informed consent of all participants.

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Subjects. CKD subjects were recruited from the Nephrology outpatient clinic at the Royal Infirmary of Edinburgh. For *study 1*, the inclusion criteria were male or female CKD patients, 18–65 yr old with a blood pressure (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 hematuria 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 comorbidity. 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.

Estimation of GFR. GFR was calculated using the Cockcroft and Gault equation as an estimate of creatinine clearance: GFR = [140 − age (yr)] × weight (kg) × (0.85 if female)/(72 × serum creatinine) (6). GFR was further corrected by body surface area (BSA): BSA = [71.84 × weight (kg)$^{0.425}$ × height (cm)$^{0.725}$]/10,000 as defined by Du Bois and Du Bois (11).

Plasma and urine ET-1 assessment. For plasma ET-1, 10 ml of venous blood were collected into an EDTA tube and centrifuged immediately at 2,500 *g* for 20 min at 4°C. For urine ET-1, a 20-ml aliquot of urine was collected into plain tubes with 2.5 ml of 50% acetic acid. Samples were stored at −80°C until analysis. After extraction (29), ET-1 was determined by radioimmunoassay (7). The mean recovery of ET-1, from extraction to assay, was >90% for both plasma and urine. The intra- and interassay 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.

Study protocol. Subjects refrained from alcohol for at least 24 h and caffeinated drinks, food, and smoking for at least 12 h before the study. All studies were conducted in a quiet, temperature-controlled room. Following a brief medical inquiry 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, with an appropriate sized cuff, using a validated oscillometric sphygmomanometer, the Omron HEM-705CP (25). Following this, blood was taken for analysis and urine was collected and tested for presence of blood and/or protein.

Data and statistical analysis. Data were stored and analyzed in Microsoft Excel (version 11.3.7, Microsoft). Fractional excretion of ET-1 (FeET-1) was calculated by [(urine ET-1/plasma ET-1 × plasma creatinine/urine creatinine) × 100]%. The D'Agostino and Pearson omnibus test was used to evaluate the distribution characteristic of the data. Means were compared by one-way ANOVA, Kruskal-Wallis test, unpaired Student's *t*-test, and Mann-Whitney *U*-test where appropriate. Correlation coefficients were calculated using the Pearson method. To measure the sensitivity and specificity for FeET-1 at different values, a conventional receiver-operator curve (ROC) curve was generated using subjects with IgAN and microhematuria 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. Descriptive data are given as means ± SD.

RESULTS

Study 1. One hundred forty two 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), noninflammatory glomerular disease (n = 8), obstructive nephropathy (n = 5), TBM (n = 2), cystinuria (n = 2), Alport disease (n = 1), and medullary cystic kidney disease (n = 1). Twenty-five CKD subjects had no known cause for their renal disease. GFR ranged from 8 to 154 ml·min^{-1}·1.73 m^{-2}. Baseline characteristics of all study subjects are shown in Table 1.

There was a negative linear correlation between GFR and plasma ET-1 (Table 2 and Fig. 1*A*, r^2 = 0.22, P < 0.001). FeET-1 increased exponentially as GFR declined (Table 2 and Fig. 1*B*, 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). Similarly, although there was no impact of gender on plasma ET-1 (r = −0.08, P = 0.346), FeET-1 was higher in males than in females (3.0 ± 3.3 vs. 2.0 ± 2.2%, r = −0.167, P < 0.05). There was no relationship between GFR and serum CRP, consistent with our subjects comprising of a noninflammatory cohort of CKD patients.

Study 2. One hundred fourteen subjects took part in *study 2*: healthy volunteers (HV; n = 29), TBM (n = 8), IgAN (n = 22), microscopic hematuria of presumed glomerular origin (MH; n = 35), SLE with nephritis (n = 10), and RA (n = 10). All subjects with TBM, IgAN, and lupus nephritis had biopsy-proven renal diagnoses. Of those with SLE, four had type IV

Table 1. *Demographic data for non-CKD and CKD subjects in study 1*

	Non-CKD Subjects (n = 27)	CKD Subjects (n = 115)	P Value
Age, yr	48±9 (32–64)	47±10 (23–65)	ns
Sex, male/female	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*, mg/dl	0.88±0.15 (0.62–1.11)	2.20±1.86 (0.62–9.33)	P < 0.001
eGFR, ml·min^{-1}·1.73 m^{-2}	94±18 (68–131)	63±35 (8–154)	P < 0.001
Cholesterol†, mg/dl	193±31 (131–255)	178±35 (116–317)	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

Values are means ± SD (range). 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; ns, not significant. *To convert to μmol/l, multiply by 88.4. †To convert to mmol/l, multiply by 0.0259.

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Table 2. *Plasma ET-1 and FeET-1 for subjects in study 1 at different eGFR*

	eGFR			
	<30 ml·min⁻¹·m⁻²	30–60 ml·min⁻¹·m⁻²	>60 ml·min⁻¹·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

Values are means ± SD (range).

lupus nephritis on renal biopsy, two type V, and four both types IV and V (37). These subjects were studied before, and 6 mo 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 GFR ranging from 61 to 153 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$. A cohort of subjects with RA was 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 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$. Subject characteristics are shown in Table 3.

All groups of subjects had similar plasma ET-1 concentrations (Fig. 2*A*; HV: 5.3 ± 1.8, TBM: 4.4 ± 0.8, IgAN: 5.1 ± 1.0, MH: 4.5 ± 0.7, SLE: 4.7 ± 0.9, RA: 4.6 ± 0.9 pg/ml). However, both FeET-1 and urinary ET-1:creatinine ratio were significantly higher in lupus nephritis subjects compared with HV and all other renal groups (Fig. 2, *C* and *D*; SLE:FeET-1 7.7 ± 2.7%, urinary ET-1:creatinine ratio 10.0 ± 3.0 pg/μmol, $P < 0.05$ vs. TBM and $P < 0.001$ vs. HV, IgAN, and MH, for both). Both FeET-1 and urinary ET-1:creatinine ratio were similar between HV, TBM, IgAN, and MH.

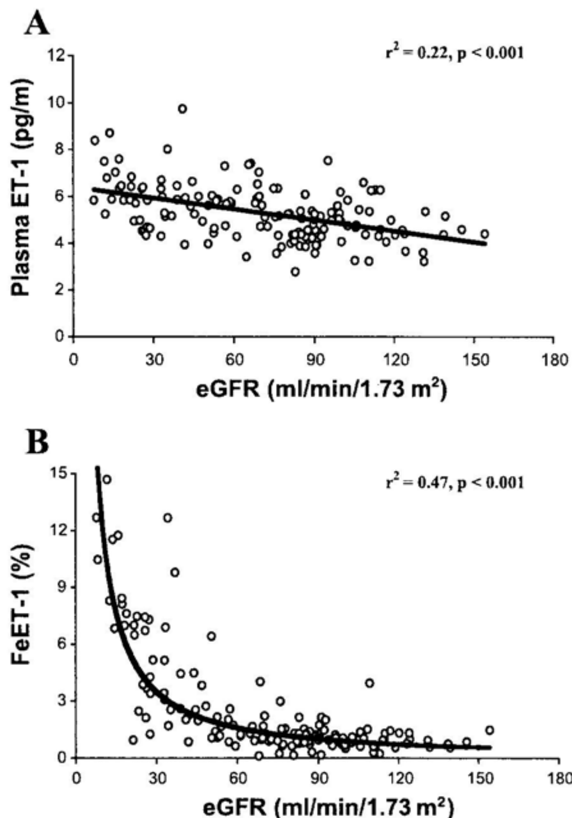

Fig. 1. Scatter plots for estimated glomerular filtration rate (eGFR; $ml \cdot min^{-1} \cdot 1.73\ m^2$) and plasma endothelin-1 (ET-1; pg/ml; *A*), $r^2 = 0.22$, $P < 0.001$, and fractional urinary excretion of ET-1 (FeET-1; %; *B*), $r^2 = 0.47$, $P < 0.001$.

CRP concentrations followed a similar pattern (Fig. 2*B*; SLE: 63 ± 15 mg/l, $P < 0.001$ vs. HV and other renal subjects). Despite similar CRP concentrations to subjects with RA (Fig. 2*B*; RA: 61 ± 16 mg/l), subjects with lupus nephritis had a significantly higher FeET-1 and urinary ET-1:creatinine ratio (Fig. 2, *C* and *D*; SLE vs. RA: FeET-1 7.7 ± 2.7 vs. 3.7 ± 2.1%, urinary ET-1:creatinine ratio 10.0 ± 3.0 vs. 2.1 ± 1.3 pg/μmol, $P < 0.01$ for both). 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.

For FeET-1, the area under the ROC was 1.0 (curve not shown). Table 4 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.

For subjects with SLE and nephritis, FeET-1 fell significantly following treatment (Fig. 3; 7.7 ± 2.7 vs. 3.6 ± 1.4%, $P < 0.01$). Effects of treatment on other disease markers are shown in Table 5.

DISCUSSION

Consistent with our previous findings in a limited number of subjects (8 CKD and 8 healthy controls) (13), we 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 also showed 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, whereas there is little impact of inflammation on plasma ET-1. Finally, 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 showed increased plasma ET-1 concentrations in predialysis (8, 20) and dialysis-requiring (9, 20) CKD patients. Our results are in keeping with these studies. However, we also demonstrated that plasma ET-1 increases linearly as renal function declines, across a wide range of GFRs and in a relatively homogeneous, noninflammatory CKD population. This is likely to be largely due to reduced renal filtration of ET-1 and thus renal clearance from the circulation. Impor-

Table 3. *Demographic data for study 2 subjects*

	HV (n = 29)	TBM (n = 8)	IgAN (n = 22)	MH (n = 35)	SLE (n = 10)	RA (n = 10)
Age, yr	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 (male/female)	12/17	3/5	17/5	14/21	4/6	3/7
BMI kg/m²	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[a] mg/dl	0.87±0.14 (0.62−1.05)	0.83±0.11 (0.68−0.98)	1.04±0.26 (0.55−1.30)	0.92±0.18 (0.55−1.33)	0.94±0.24 (0.67−1.43)	0.92±0.10 (0.81−1.14)
eGFR ml·min⁻¹·1.73 m⁻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[b] mg/dl	193±35 (147−282)	205±42 (127−228)	178±27 (139−228)	201±42 (124−340)	189±31 (143−236)	193±19 (166−232)
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)

Values are means ± SD (range). HV, healthy volunteers; TBM, subjects with thin basement membrane disease; IgAN, immunoglobulin A nephropathy; MH, microhematuria of presumed glomerular origin; SLE, systemic lupus erythematosus with nephritis; RA, rheumatoid arthritis. For CRP, $P < 0.001$ for SLE vs. all renal groups. For ACR, $P < 0.05$ for TBM vs. SLE, $P < 0.01$ for HV vs. IgAN and for MH vs. SLE, $P < 0.001$ for HV vs. SLE, and $P < 0.05$ for RA vs. SLE. [a]To convert to μmol/l, multiply by 88.4. [b]To convert to mmol/l, multiply by 0.0259.

tantly, we also showed an exponential rise in FeET-1 as GFR declines. It is well established that a number of renal cell types are able to synthesize ET-1 (10, 19, 33) and that ET-1 is an important regulator of renal function in CKD (10, 14). It has been shown in animal models that plasma ET-1 does not account for urinary ET-1 (4) and that renal cortical interstitial ET-1 levels correlate with urinary ET-1 excretion (38). 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. Our results also show a positive correlation between BP and FeET-1 and increasing BP, as GFR falls, may well be one of the stimuli for renal ET-1 production. Furthermore, men had a higher FeET-1 than women suggesting that gender may influence renal ET-1 production and so explain the gender bias of some renal diseases. ET-1 is proinflammatory and its upregulation in CKD may contribute to disease progression (10). Thus, antagonizing the effects of ET-1 may offer therapeutic benefits in patients with CKD, and this is supported by preclinical and clinical data (1, 5, 14).

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 noninflammatory condition, and in those with IgAN nephropathy, associated with mild renal inflammation, were no different to those in HV. 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. Although a urinary biomarker of disease activity would be ideal, being noninvasive 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 whether 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 HV and those with other renal diagnoses does support its use as a discriminatory test in the clinical management of lupus nephritis.

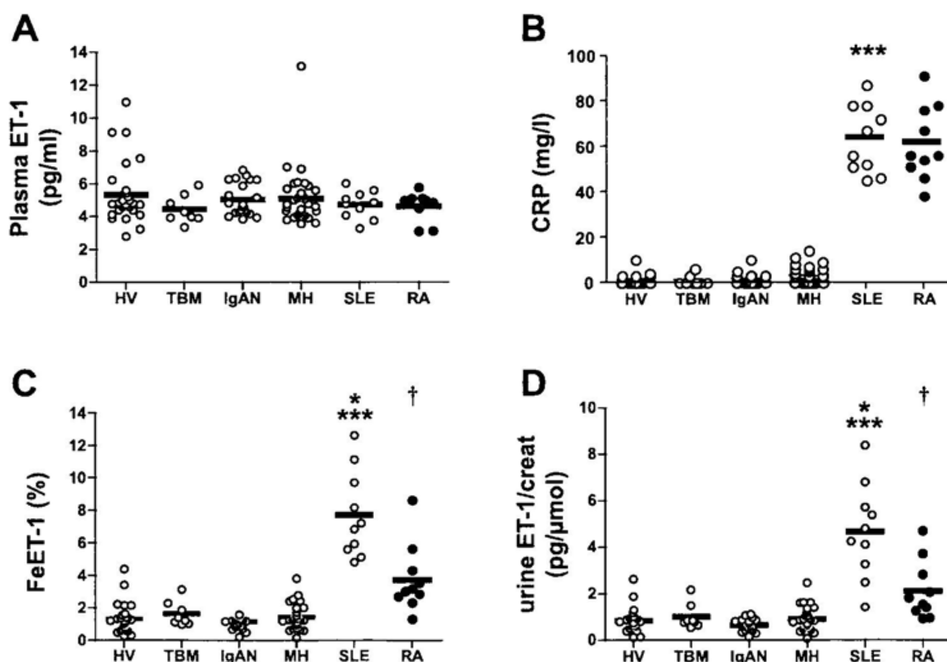

Fig. 2. Plasma ET-1 (pg/ml; *A*), serum C-reactive protein (CRP; mg/l; *B*), FeET-1 (%; *C*), and urinary ET-1:creatinine ratio (pg/μmol; *D*) in healthy volunteers (HV) and subjects with thin basement membrane disease (TBM), immunoglobulin A nephropathy (IgAN), microhematuria of presumed glomerular origin (MH), systemic lupus erythematosus with nephritis (SLE), and rheumatoid arthritis (RA). *A-D*: horizontal black bars show mean value. *B*: $***P < 0.001$ for SLE vs. all groups except RA, for which P = not significant (ns). *C* and *D*: $*P < 0.05$ for SLE vs. TBM. $***P < 0.001$ for SLE vs. all other groups. †$P < 0.01$ for SLE vs. RA.

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Table 4. *FeET-1 test characteristics for renal subjects at different cutoff values*

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

CI, confidence interval.

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. It is important to note that we used the Cockcroft and Gault equation as our estimate of GFR. Although imperfect as this will not detect more subtle loss of renal function, in particular renal reserve, its use is preferable in the clinic setting where renal clearance studies are impractical.

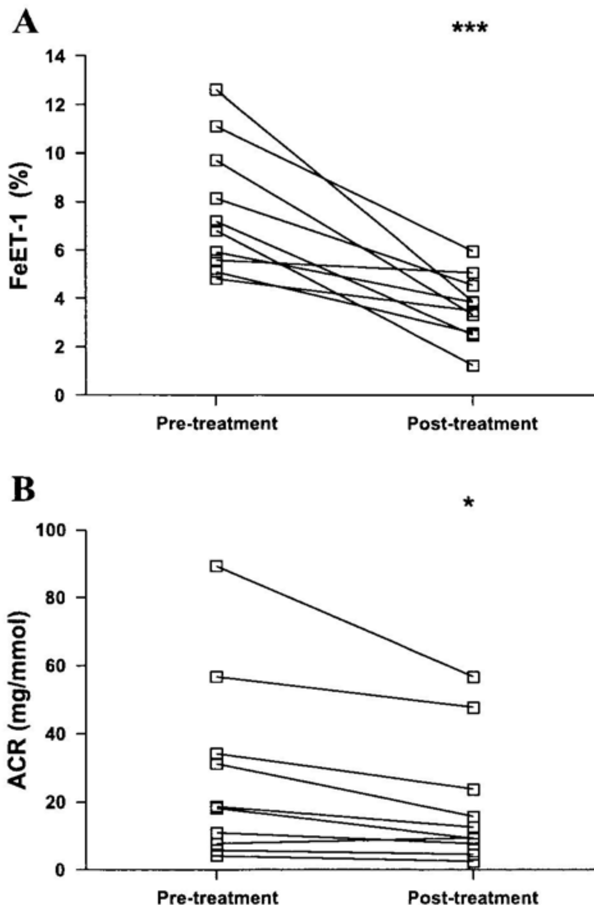

Fig. 3. FeET-1 (%; *A*) and albumin:creatinine ratio (ACR; *B*) in patients with SLE with nephritis pre- and posttreatment. *$P < 0.05$ and ***$P < 0.001$.

Table 5. *CRP, ACR, dsDNA, and complement levels in subjects with lupus nephritis pre- and posttreatment*

	Pretreatment (n = 10)	Posttreatment (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	$P < 0.05$

Values are means ± SD. Normal ranges for double-strand DNA (dsDNA) and complement are shown in brackets.

FeET-1 and CRP were both significantly elevated in subjects with lupus nephritis compared with HV 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 RA, 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 RA had a lower FeET-1 than those with lupus nephritis, they had a greater FeET-1 than HV 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 (34, 42).

The current clinical study was designed to look at a preselected group of patients: those referred to the renal clinic on the basis of an abnormal urinalysis (hematuria ± 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 RA 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 (28), 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 (2, 16) despite other evidence of systemic inflammation (12, 22), 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 mo, 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 hematuria, and/or reduction in proteinuria (7 of 10). Other immunological markers of disease activity (double-strand 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 recognize 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-mo 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 noninflammatory 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 noninflammatory 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 showed both microalbuminuria (35) and nephrotic range proteinuria (36) associated with urinary ET-1 albeit in different cohorts of patients. Certainly, both in vitro (24) and in vivo (3) experiments showed 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 remain 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 (41). In a recent study by Pitashny et al. (27), 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 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.

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Neeraj Dhaun, Iain M. MacIntyre, Vanessa Melville, Pajaree Lilitkarntakul, Neil R. Johnston, Jane Goddard and David J. Webb

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Blood Pressure–Independent Reduction in Proteinuria and Arterial Stiffness After Acute Endothelin-A Receptor Antagonism in Chronic Kidney Disease

Neeraj Dhaun, Iain M. MacIntyre, Vanessa Melville, Pajaree Lilitkarntakul, Neil R. Johnston, Jane Goddard, David J. Webb

Abstract—Endothelin 1 is implicated in the development and progression of chronic kidney disease and associated cardiovascular disease. We, therefore, studied the effects of selective endothelin-A receptor antagonism with BQ-123 on key independent surrogate markers of cardiovascular risk (blood pressure, proteinuria and renal hemodynamics, arterial stiffness, and endothelial function) in patients with nondiabetic chronic kidney disease. In a double-blind, randomized crossover study, 22 subjects with proteinuric chronic kidney disease received, on 2 separate occasions, placebo or BQ-123. Ten of these subjects also received nifedipine (10 mg) as an active control for the antihypertensive effect of BQ-123. Blood pressure, pulse wave velocity, flow-mediated dilation, renal blood flow, and glomerular filtration rate were monitored after drug dosing. BQ-123 reduced blood pressure (mean arterial pressure: $-7\pm1\%$; $P<0.001$ versus placebo) and increased renal blood flow ($17\pm4\%$; $P<0.01$ versus placebo). Glomerular filtration rate remained unchanged. Proteinuria ($-26\pm4\%$; $P<0.01$ versus placebo) and pulse wave velocity ($-5\pm1\%$; $P<0.001$ versus placebo) fell after BQ-123, but flow-mediated dilation did not change. Nifedipine matched the blood pressure and renal blood flow changes seen with BQ-123. Nevertheless, BQ-123 reduced proteinuria ($-38\pm3\%$ versus $26\pm11\%$; $P<0.001$) and pulse wave velocity ($-9\pm1\%$ versus $-3\pm1\%$; $P<0.001$) to a greater extent than nifedipine. Selective endothelin-A receptor antagonism reduced blood pressure, proteinuria, and arterial stiffness on top of standard treatment in renal patients. Furthermore, these studies suggest that the reduction in proteinuria and arterial stiffness is partly independent of blood pressure. If maintained longer term, selective endothelin-A receptor antagonism may confer cardiovascular and renal benefits in patients with chronic kidney disease. **(*Hypertension*. 2009;54:113-119.)**

Key Words: endothelin ■ blood pressure ■ arterial stiffness ■ proteinuria ■ chronic kidney disease

Chronic kidney disease (CKD) is common, affecting 6% to 11% of the population in the developed world.[1] Hypertension is a frequent finding in patients with CKD,[2] and its prevalence increases as CKD progresses.[3] Despite treatment with multiple antihypertensive agents, the majority of CKD patients fail to reach target blood pressure (BP).[4] Proteinuria is a common feature of CKD and is independently associated with an adverse renal outcome.[5] Current treatments for proteinuria focus on BP reduction,[5] ideally using angiotensin-converting enzyme (ACE) inhibitors[6] and angiotensin receptor blockers,[7] both of which are thought to reduce proteinuria to a greater extent than accounted for by BP-lowering alone.[5] Nevertheless, many CKD patients have significant residual proteinuria despite optimal treatment.[8]

CKD is also strongly associated with incident cardiovascular disease (CVD).[9] Hypertension[10] and proteinuria[11] make an important contribution to CVD risk in CKD, as do arterial stiffness[12] and endothelial dysfunction.[13] 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 will also have favorable effects on arterial stiffness and endothelial dysfunction and so offer longer term cardiovascular and renal protection.

Endothelin (ET) 1 is the most potent endogenous vasoconstrictor produced within the vasculature. It is implicated in both the development and progression of CKD.[14] The effects of ET-1 are mediated via 2 receptors, the ET_A and ET_B receptors, with the major pathological effects in CKD being ET_A receptor mediated.[14] However, there are currently few human studies.[15,16] ET-1 also contributes to arterial stiffness[17] and endothelial dysfunction[18] in patients with CVD; however, there are no similar studies in CKD patients.

Our group has shown previously that selective ET_A receptor antagonism, but not mixed $ET_{A/B}$ antagonism, reduces BP, increases renal blood flow, and reduces the effective filtration fraction in patients with CKD.[15] However, this was a small

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study (n=8) where only 2 of the subjects had overt proteinuria (>300 mg/d), and only 6 were treated with an ACE inhibitor. Thus, based on this work, we hypothesized that, in patients with nondiabetic proteinuric CKD, selective ET_A receptor antagonism would reduce proteinuria and arterial stiffness and improve endothelial dysfunction and that these effects would be greater than those achieved with BP reduction alone. Because we have previously demonstrated a synergism between ACE inhibitors and selective ET_A receptor antagonism in health,[19] we anticipated that these effects might be evident on top of standard treatment with ACE inhibitors and/or angiotensin receptor blockers.

Methods

Subjects

This was a randomized, double-blind, placebo-controlled study (please also see the online data supplement available at http://hyper.ahajournals.org). Because previous studies with BQ-123 have shown a reduction in BP in CKD patients, and because BP reduction may contribute to changes in arterial stiffness, protein excretion, and natriuresis, nifedipine was used as an open-label active control in a substudy. All of the subjects attended for 2 visits, receiving placebo and BQ-123 in a randomized order, with those taking part in the substudy (nifedipine, 10 mg) randomly chosen and attending for 3 randomized visits. Because previous studies with the same dose of BQ-123 have demonstrated that hemodynamic changes return to baseline after 4 hours,[15] and the half-life of nifedipine is ≈2 hours, each visit was separated by ≥7 days to ensure complete washout of the study drugs.

Twenty-two patients with stable proteinuric CKD were recruited into the studies, which were performed between May 2006 and December 2007 in the University of Edinburgh Clinical Research Centre with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles outlined in the Declaration of Helsinki.

Drugs

BQ-123 (Clinalfa AG), a selective ET_A receptor antagonist, was infused at 1000 nmol/min for 15 minutes. This dose was selected from previous studies as being ET_A selective based on plasma BQ-123 concentrations and relating these to the binding constant for the ET_A receptor, as well as by a lack of rise in plasma ET-1 concentration after its infusion.[15] The drug was dissolved in physiological saline (15 mL of 0.9%, Baxter Healthcare Ltd) and infused IV at a constant rate of 1 mL/min. Saline vehicle was administered as a placebo.

Nifedipine (10 mg; Adalat, Bayer) was used as an active control and administered orally at the same time that BQ-123 or saline was started in its respective phase. 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 hemodynamics, and to be a clinically tolerable agent that is also a standard treatment in CKD patients.[20] Although we investigated other agents, nifedipine was the only drug to fulfill all of these criteria.

Para-aminohippurate sodium (PAH; Clinalfa) and inutest (Fresenius Pharma) were dissolved in 5% dextrose (Baxter) and administered as a bolus loading dose of 0.4 g of PAH and 3.5 g of inutest in 100 mL of dextrose over 15 minutes, along with a maintenance infusion of 6.6 g/L of PAH and 10 g/L of inutest at a rate of 2 mL/min. For subjects with a calculated glomerular filtration rate (GFR) <50 mL/min or <30 mL/min, doses of PAH and inutest were reduced by one third and two thirds, respectively.

Assays

At prespecified time points, venous blood was collected into EDTA tubes (Sarstedt) for the measurement of PAH, inulin, hematocrit, and plasma ET-1 and into plain tubes (Sarstedt) for the measurement of serum sodium. In addition, 20-mL aliquots of urine from each voiding were collected into plain tubes for the measurement of urinary PAH, inulin, sodium, and protein.

Hematocrit was measured on whole blood using a Coulter counter. All of the other blood samples were centrifuged immediately at 1000*g* at 4°C for 20 minutes, and plasma and urine were stored in plain tubes at −80°C. Inulin was determined by spectrophotometry after hydrolysis to fructose[21] and PAH by high-performance liquid chromatography.[22] Plasma and urine sodium concentrations were measured by using an ion selective electrode and urine protein by using a colorimetric method with pyrogallol red.[23] After extraction, ET-1 was determined by radioimmunoassay.[24]

Assessment of Arterial Stiffness and Endothelial Function

Pulse wave velocity (PWV) was used as a measure of arterial stiffness,[25] using the SphygmoCor system (SphygmoCor Mx version 6.31, AtCor Medical), in which a high-fidelity micromanometer (SPC-301, Millar Instruments) was used to determine carotid-femoral PWV. Flow-mediated dilation (FMD) was used to assess endothelium-dependent vasomotor function.[26] 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 minutes of forearm ischemia.

Study Protocol

On each study day, a cannula was inserted into an antecubital vein in each arm (please also see the data supplement). Diuresis was induced by administering 500 mL of 5% dextrose IV over 30 minutes through the left arm cannula. Thereafter, maintenance infusions of PAH and inutest and 5% dextrose at 180 mL/h continued throughout the study. After a 2-hour equilibration period, baseline measurements were made over 1 hour. Drug or placebo was then administered through the right antecubital cannula or with nifedipine given orally, followed by 4 hours of further measurement. BP, cardiac output (as measured by cardiac index [CI]), and heart rate were recorded throughout the study by well-validated noninvasive automated techniques[27,28] every 15 minutes, and urine was collected every 60 minutes by spontaneous voiding.

At the midpoint of each collection period, blood was sampled from the right antecubital cannula for PAH, inulin, sodium, and hematocrit. At 0, 30, 60, 120, and 240 minutes, additional samples were taken for the measurement of plasma ET-1. PWV and FMD were measured immediately before drug and again at 2 and 4 hours after dosing.

Data Analysis

Data were stored and analyzed in Microsoft Excel (version 11.3.7, Microsoft Ltd). BP at each time point was calculated as the mean of 2 recordings and was represented by systolic BP, diastolic BP, and mean arterial pressure (MAP; diastolic BP+1/3 pulse pressure). Bioimpedance data at each time point were calculated as the mean of 4 recordings, each the average of 15 consecutive heartbeats. Data were corrected for body surface area to give CI, for direct comparison between subjects. Systemic vascular resistance index (SVRI) was calculated by dividing MAP by CI and was expressed in dynes per second per meter squared per centimeter^5. GFR and effective renal plasma flow were calculated from inulin and PAH clearances, respectively. Effective renal blood flow (ERBF) was calculated by dividing the effective renal plasma flow by (1−hematocrit) and effective renal vascular resistance (ERVR) by dividing MAP by ERBF. Urinary protein and sodium excretion (UNaV) were calculated as (urinary protein×urinary flow rate) and (urinary sodium×urinary flow rate), respectively.

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Table. Baseline Data for Main Study and Substudy

Parameter	Main Study	Substudy
Systolic BP, mm Hg	128±3 (99 to 167)	128±3 (108 to 142)
Diastolic BP, mm Hg	75±2 (63 to 95)	76±1 (67 to 84)
MAP, mm Hg	92±2 (80 to 103)	93±2 (84 to 101)
SVRI, dyne/s per m^2 per cm^5	3360±230 (1800 to 5510)	3530±230 (1980 to 5510)
CI, L/min[1] per m^2	3.0±0.2 (1.8 to 4.7)	2.9±0.2 (1.8 to 4.4)
Heart rate, bpm	56±2 (38 to 75)	57±2 (42 to 66)
PWV, m/s	7.5±0.4 (5.5 to 12.2)	7.4±0.5 (5.7 to 10.5)
FMD, %	4.4±0.6 (0.6 to 12.7)	4.4±0.9 (0.1 to 8.2)
ERBF, mL/min	1810±233 (106 to 4632)	1968±390 (530 to 4632)
ERVR, mm Hg/min[1] per mL[1]	11.5±4.4 (2.0 to 107.8)	7.1±1.5 (2.0 to 18.2)
GFR, mL/min per 1.73 m^2	43±5 (12 to 99)	43±7 (15 to 99)
UNaV, μEq/min	197±21 (27 to 392)	193±28 (95 to 392)
Urinary protein excretion, μg/min	1570±371 (165 to 8616)	1520±577 (109 to 8616)
Plasma ET-1 pg/mL	5.7±0.3 (3.6 to 10.5)	6.7±0.5 (3.8 to 8.7)

Values are given as means of baseline pretreatment periods over the 2 or 3 study days±SEM (range).

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.[15] The coprimary end points were reductions in BP and proteinuria, with the secondary end points being a reduction in arterial stiffness and improvement in endothelial function. Baseline hemodynamic data were calculated as the mean of the 2 time points that immediately preceded administration of the study drug. For urine data, only 1 baseline measurement was used immediately before drug dosing. Hemodynamic and urine results are expressed as mean±SEM change from baseline for drug and placebo and placebo-corrected change from baseline for substudy results. Statistical analysis was performed on untransformed data. Three comparisons of interest were preidentified as placebo versus BQ-123, placebo versus nifedipine, and BQ-123 versus nifedipine. Responses were examined by repeated-measures ANOVA, and Bonferroni's correction was used to assess significance at specific time points. Statistical significance was taken at the 5% level.

Results

All 22 of the CKD patients completed the placebo and BQ-123 phases of the study without adverse events. All of the subjects had similar baseline urinary protein leaks on each study day. Patient diagnoses were IgA nephropathy (n=9), membranous glomerulopathy (n=5), and focal segmental glomerulosclerosis (n=8). For individual subject characteristics and overall demographic data, please see Tables S1 and S2. Subject baseline parameters are shown in the Table.

Main Study

Systemic Hemodynamics

Placebo was associated with increases in systolic BP (127.6±3.3 versus 136.6±3.4 mm Hg; $P<0.001$), diastolic BP (75.9±1.7 versus 79.9±2.2 mm Hg; $P<0.001$), MAP (93.1±2.1 versus 98.8±2.4 mm Hg; $P<0.001$), and SVRI (3360±230 versus 3670±290 dyne/s per m^2 per cm^5; $P<0.05$) from baseline to study end (Figure 1A and 1B). BQ-123 led to a reduction in systolic BP (−14.2±3.0 mm Hg; $P<0.001$ versus placebo), diastolic BP (−6.8±0.8 mm Hg; $P<0.001$ versus placebo), MAP (−9.2±1.2 mm Hg; $P<0.001$ versus placebo), and SVRI (−610±100 dyne/s per m^2/cm^5; $P<0.001$ versus placebo), with the peak effects at 75 minutes after drug administration. BQ-123 also increased CI (0.3±0.1 L/min per m^2; $P<0.05$ versus placebo). There were no significant differences in the heart rate between placebo and BQ-123 throughout the time course of the study.

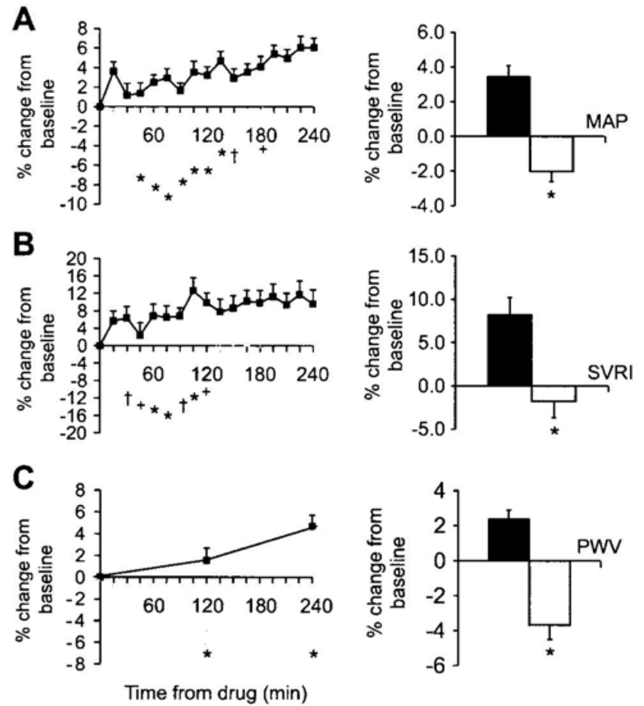

Figure 1. Systemic hemodynamics and arterial stiffness after ET_A receptor antagonism. Values are given as mean percentage of change from baseline±SEM (left) and mean area under curve (AUC) of the percentage of change from baseline±SEM (right). Black line/block, placebo; gray line/block, BQ-123. $+P<0.05$ vs placebo, $†P<0.01$ vs placebo, $*P<0.001$ vs placebo (ANOVA plus Bonferroni correction for significance at specific time points).

Arterial Stiffness and Endothelial Function

Although PWV increased after placebo (7.5±0.4 versus 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; $P<0.001$ versus placebo; Figure 1C). With regard to endothelial function, there were no differences in brachial artery FMD response between BQ-123 (4.4±0.5% versus 5.5±0.8%; $P=0.056$) and placebo (4.4±0.6% versus 5.1±0.7%; $P=0.082$).

Renal Hemodynamics

Administration of placebo was associated with a gradual reduction in ERBF (1810±233 versus 1454±181 mL/min; $P<0.001$) and an increase in ERVR (11.5±4.4 versus 12.8±3.8 mm Hg/min per mL; $P<0.05$) to the study end (Figure 2A and 2B). In contrast, BQ-123 produced a striking increase in ERBF (365±104 mL/min; $P<0.01$ versus placebo) and a reduction in ERVR (−3.0±0.9 mm Hg/min per mL; $P<0.01$ versus placebo). There were no significant

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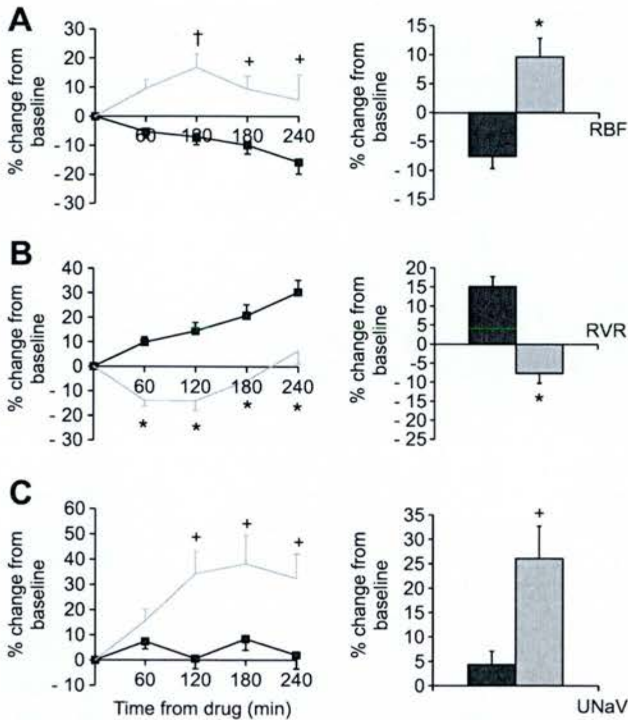

Figure 2. Renal hemodynamics and UNaV after ET_A receptor antagonism. RBF indicates renal blood flow; RVR, renal vascular resistance; UNaV, sodium clearance. Legend as for Figure 1.

changes in GFRs with either placebo or BQ-123 (maximal change from baseline: 2% for placebo and 3% for BQ-123).

Urinary Sodium and Protein Excretion

Placebo had little effect on UNaV, whereas BQ-123 produced a marked natriuresis with a maximum increase of 36±15 μmol/min ($P<0.05$ versus placebo; Figures 2C and 3). There was no change in potassium excretion.

Although 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 −496±141 μg/min ($P<0.01$ versus placebo), a reduction in protein leak of ≈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.

Substudy: Nifedipine (10 mg)

Ten of the 22 subjects took part in a fully randomized 3-way crossover substudy, which included placebo, BQ-123, and nifedipine. Subject baseline parameters are shown in Table 1. All 10 of the subjects completed the 3 phases of the substudy without adverse events.

BQ-123 and nifedipine produced a similar reduction in MAP (please see Figure S1). Nifedipine caused an initial increase in heart rate (mean increase of 10 bpm from baseline) that returned to baseline by 60 minutes. Despite the consistent change in MAP, BQ-123 reduced arterial stiffness to a greater extent than did nifedipine (PWV: −0.6±0.0 versus −0.3±0.1 m/s; $P<0.001$; please see Figure S1). BQ-123 and nifedipine also increased ERBF to a similar degree (please see Figure S1) and produced a similar natriuresis (at maximum, BQ-123: 64±37 μmol/min; nifedipine: 37±34 μmol/min). By contrast to BQ-123, nifedipine was associated with a gradual increase in urinary protein excretion (at maximum: 190±142 μg/min; $P<0.01$ versus placebo; please see Figure S1), whereas BQ123 produced a consistent reduction in proteinuria.

Discussion

We have demonstrated 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 maximally tolerated treatment with ACE inhibitors and angiotensin receptor blockers. Importantly, the reductions in proteinuria and arterial stiffness are greater than those found with an alternative method of BP reduction. These findings suggest a potential role for ET_A receptor antagonism in conferring longer-term cardiovascular and renal benefits 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. There are only 2 studies of the longer-term antihypertensive effects of ET receptor antagonism. These suggest that both selective ET_A[29] and mixed $ET_{A/B}$ antagonists[30] are effective at reducing BP, but neither

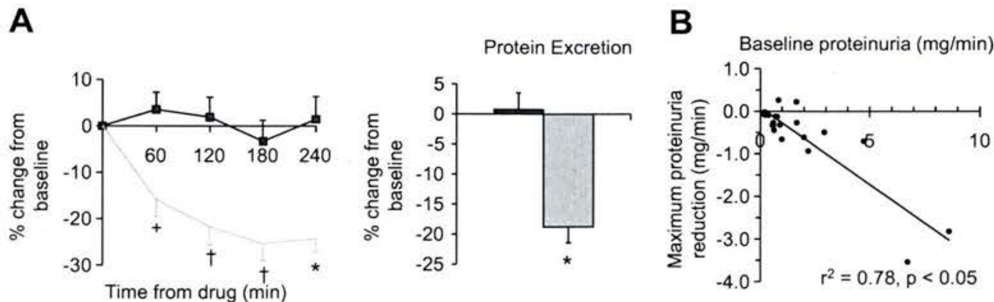

Figure 3. Protein excretion after ET_A receptor antagonism. Values are given as mean percentage of change from baseline±SEM (left), and mean area under curve (AUC) of the percentage of change from baseline±SEM (right). Black line/block, placebo; gray line/block, BQ-123. +$P<0.05$ vs placebo, †$P<0.01$ vs placebo, *$P<0.001$ vs placebo (ANOVA plus Bonferroni correction for significance at specific time points). B, Effect of baseline protein excretion on maximal proteinuria reduction (milligrams per minute) with BQ-123 ($r^2=0.78$; $P<0.05$). This remains significant ($r^2=0.29$; $P<0.05$) when the 2 outliers are removed.

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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 with placebo. This is likely to be attributed largely to the reduction in BP seen with BQ-123.[25] 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,[31] this had returned to baseline within an hour of the 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 and should be confirmed in longer-term studies with a range of BP-lowering agents.

There are a few clinical trials demonstrating that differential lowering of PWV with medical treatment results in different cardiovascular or renal outcomes,[32,33] but the importance of such studies is underscored by epidemiological data suggesting that PWV is an independent risk factor for CVD morbidity and mortality.[12,34] Karalliedde et al[35] recently showed a BP-independent reduction in PWV with valsartan compared with amlodipine in patients with type 2 diabetes mellitus and proteinuria. Our current data suggest that ET_A receptor antagonism may reduce arterial stiffness even further in patients already established on blockers of the renin-angiotensin system and, similarly, in a BP-independent manner. However, the effects of ET_A receptor antagonism on endothelial function, as assessed by FMD, were less impressive, although there was a trend toward improvement. It may well be that these effects take longer to develop.

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,[15,36] see a fall in the 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 the filtration coefficient. 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 a BP-independent effect. Our control drug, nifedipine, closely matched both the decrease in BP and the increase in renal blood flow seen with BQ-123. Nifedipine acts predominantly at the afferent arteriole, and, so, as expected, it produced steady increases in both GFR (7 mL/min at maximum; data not shown) and proteinuria throughout the study period. Because 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 an action not just at the afferent arteriole but with potential preferential dilation at the efferent arteriole, similar to, and on top of, that seen with ACE inhibitors.

Although the acute effects on proteinuria described here are likely to be largely explained by systemic and renal hemodynamic changes, it is important to consider other longer-term targets for selective ET_A receptor antagonism, eg, the podocyte, which has been implicated in the development of proteinuria.[37] Indeed, a recent study by Wenzel et al[16] showed a reduction in macroalbuminuria in subjects with diabetic nephropathy after 12-week dosing with the ET receptor antagonist avosentan. Interestingly, the authors observed no change in BP, supporting a BP-independent mechanism for the proteinuria reduction seen.

In the current study, the reduction in proteinuria 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.[5] Proteinuria reduction is important both for reducing risk of CKD progression[5] and for consequent CVD.[38] Despite maximum achievable renin-angiotensin system blockade, many patients with proteinuric CKD have significant residual proteinuria.[8] Importantly, in this study, all of the subjects were established on treatment with ACE inhibitors, with the majority also taking angiotensin receptor blockers. The ET and renin-angiotensin systems are known to interact,[39] and a synergistic effect, in terms of systemic hemodynamics, has been demonstrated between ET_A receptor antagonism and both ACE inhibition[19] and angiotensin II type 1 receptor antagonism[40] in humans. Our data suggest that 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. This is likely to be mainly attributable to the increase in renal blood flow seen with ET_A receptor antagonism. Indeed, nifedipine, which caused a similar change in renal hemodynamics, also caused natriuresis. In addition, all of the subjects showed a net diuresis, even with placebo. 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.

As a limitation, ours is an acute study, and it will be important to confirm that these effects are maintained longer term, as they are in patients with essential hypertension and pulmonary arterial hypertension.[41] Indeed, the acute studies in these areas predicted the beneficial chronic effects. In addition, we studied a relatively homogeneous CKD population, and further work is needed in a broader population of patients with CKD, including those with diabetes mellitus, vasculitis, and renal vascular disease. Finally, the crossover study design may affect data interpretation and is not ideal for longer-term studies in a larger cohort of subjects.

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Perspectives

The current data support a role for selective ET_A receptor antagonism as a novel and worthwhile therapeutic target in CKD to lower BP, arterial stiffness, and proteinuria on top of standard treatment, and on this basis, larger and longer-term studies in both diabetic and nondiabetic CKD are justified. Furthermore, with the availability now of both selective ET_A and mixed $ET_{A/B}$ receptor antagonists, a comparison of their renal effects would be of great interest. Indeed, whereas in pulmonary arterial hypertension the studies to date suggest both selective ET_A antagonism and mixed $ET_{A/B}$ antagonism to be of benefit, the current data support selective ET_A receptor antagonism over mixed blockade in CKD. There have been no major studies as yet in subjects on maximal renin-angiotensin system blockade, across a wide range of GFRs, or looking at natriuresis. Additional studies taking these clinically relevant factors into consideration are now needed. Furthermore, the effects of longer-term treatment on renal hemodynamics remain unclear.

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Disclosures

N.D., I.M.M., J.G., and D.J.W. have all received grants from Pfizer. N.D. and J.G. have had salary payments from Pfizer. J.G. and D.J.W. have acted as consultants to Pfizer and Speedel. The remaining authors report no conflicts.

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Online Supplement

Blood pressure independent reduction in proteinuria & arterial stiffness following acute endothelin-A receptor antagonism in chronic kidney disease

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Running title: Endothelin antagonism & CKD

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Supplementary methods

Subjects

Patients with significant co-morbidity, including diabetes mellitus, heart or lung disease, and peripheral vascular disease were excluded. All patients were treated with ACE inhibitors and/or ARBs for their proteinuria. 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. 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

For the duration of the study, subjects were asked to adhere to their usual salt intake. 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 performed in a quiet, temperature-controlled room at 22 to 24°C with the subject recumbent throughout, except when voiding urine. All patients completed a 24-hour urine collection for quantification of proteinuria on each study day to assess equivalent baseline urinary protein leak between study phases. Patients continued taking their normal medications up to and including each study day with the exception of diuretics, which they omitted that morning.

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Supplemental table / figure legends

Table S1: Individual subject characeristics. *To convert to μmol/l, multiply by 88.4. Doses are total per day. IgAN: immunoglobulin A nephropathy; FSGS: focal & segmental glomerulosclerosis; Membranous: membranous glomerulopathy; EPO: erythropoietin.

Table S2: Subject demographic data for main and sub-study. Values are mean ± SEM (range). *To convert to μmol/l, multiply by 88.4. †To convert to mmol/l, multiply by 0.0259.

Figure S1: Systemic and renal hemodynamics, arterial stiffness and protein excretion after ET_A receptor antagonism and nifedipine 10mg. MAP: mean arterial pressure; RBF: renal blood flow; PWV: pulse wave velocity. 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).

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Table S1: Individual subject characteristics

Subject	Diagnosis	Creatinine (mg/dl)*	Proteinuria (g/d)	BP	ACE inhibitor	ARB	Other drugs
1	IgAN	1.54	3.30	131/81	Ramipril 2.5mg		
2	Membranous	0.88	1.58	114/63	Ramipril 10mg	Candesartan 4mg	Atorvastatin 20mg Furosemide 40mg
3	FSGS	2.70	0.57	107/70	Lisinopril 40mg	Valsartan 80mg	Allopurinol 200mg Atorvastatin 20mg Fluoxetine 20mg Furosemide 40mg
4	FSGS	1.74	2.44	122/79	Ramipril 10mg	Candesartan 4mg	Simvastatin 80mg Zopiclone 12.5mg
5	IgAN	1.48	2.28	129/67	Lisinopril 40mg	Candesartan 4mg	Atorvastatin 20mg
6	IgAN	1.01	0.38	111/64	Ramipril 2.5mg		
7	IgAN	1.80	0.60	120/79	Ramipril 5mg		
8	FSGS	5.51	3.40	158/84	Lisinopril 20mg		Amlodipine 5mg Atenolol 50mg Atorvastatin 20mg Bendroflumethazide 2.5mg Omeprazole 20mg Sodium bicarbonate 3g
9	FSGS	3.01	8.72	116/68	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	1.02	0.69	120/69	Enalapril 40mg	Losartan 50mg	Amlodipine 5mg
11	Membranous	2.35	0.67	146/95	Enalapril 40mg		Allopurinol 200mg Amlodipine 5mg Furosemide 40mg Metoprolol 100mg

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12	IgAN	1.89	5.18	142/75	Ramipril 10mg	Candesartan 32mg	Aspirin 75mg Atorvastatin 40mg
13	FSGS	2.05	0.61	123/68	Lisinopril 40mg		Aspirin 75mg Atenolol 50mg Simvastatin 80mg Amlodipine 5mg
14	IgAN	1.33	0.77	108/67	Lisinopril 40mg	Candesartan 16mg	Aspirin 75mg Rosuvastatin 40mg
15	FSGS	3.75	1.33	145/83	Ramipril 10mg	Valsartan 160mg	Amlodipine 5mg Allopurinol 200mg
16	IgAN	2.21	8.02	138/75	Lisinopril 40mg	Candesartan 16mg	Amlodipine 5mg Atorvastatin 20mg Furosemide 40mg
17	IgAN	1.13	2.61	142/79	Ramipril 10mg		
18	Membranous	3.19	0.41	142/70	Ramipril 10mg		Amlodipine 5mg Atenolol 50mg
19	Membranous	1.80	1.46	133/82	Lisinopril 40mg		Metoprolol 100mg Omeprazole 20mg
20	FSGS	1.46	7.86	128/80	Lisinopril 40mg	Valsartan 80mg	Atorvastatin 20mg
21	IgAN	1.79	2.95	127/76	Lisinopril 40mg	Valsartan 160mg	Atenolol 50mg Allopurinol 200mg Atorvastatin 20mg
22	FSGS	3.88	2.60	123/68	Ramipril 10mg	Candesartan 8mg	

*To convert to μmol/l, multiply by 88.4.

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Table S2: Subject demographic data for main and sub-study

Demographic	Main study	Sub-study
Age, y	46 ± 3 (29 – 69)	45 ± 11 (29 – 64)
Body mass index, kg/m^2	28 ± 1 (20 – 37)	30 ± 6 (21 – 37)
SBP, mmHg	128 ± 3 (107 – 158)	131 ± 3 (108 – 145)
DBP, mmHg	75 ± 2 (63 – 95)	75 ± 2 (67 – 83)
MAP, mmHg	93 ± 2 (75 - 118)	93 ± 2 (84 – 101)
Creatinine*, mg/dl	2.16 ± 0.33 (0.88 – 5.51)	2.26 ± 1.00 (1.13 – 3.88)
Cholesterol†, mg/dl	170 ± 8 (127 – 228)	166 ± 35 (127 – 228)
Urinary sodium excretion, mEq/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)

*To convert to μmol/l, multiply by 88.4. †To convert to mmol/l, multiply by 0.0259.

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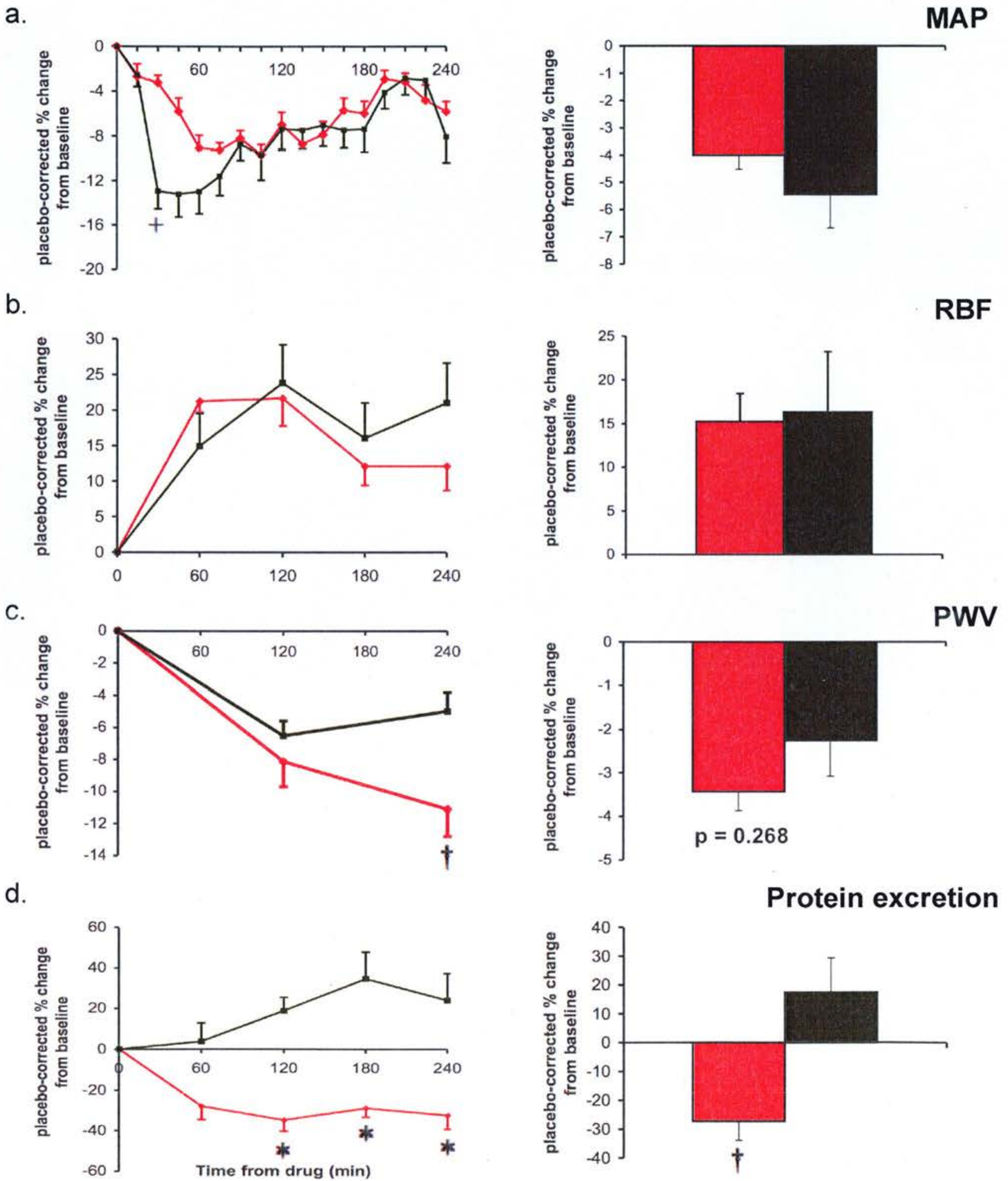

Figure S1. Systemic and renal hemodynamics, arterial stiffness and protein excretion after ET_A receptor antagonism and nifedipine 10mg

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