Oxidative stress and its Haemodynamic Consequences in Chronic Kidney Disease

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Summary

Chronic kidney disease (CKD) is associated with increased cardiovascular risk in comparison with the general population. This can be observed even in the early stages of CKD, and rises in proportion to the degree of renal impairment. Not only is cardiovascular disease (CVD) more prevalent in CKD, but its nature differs too, with an excess of morbidity and mortality associated with congestive cardiac failure, arrhythmia and sudden death, as well as the accelerated atherosclerosis which is also observed. Conventional cardiovascular risk factors such as hypertension, dyslipidaemia, obesity, glycaemia and smoking, are highly prevalent amongst patients with CKD, although in many of these examples the interaction between risk factor and disease differs from that which exists in normal renal function. Nevertheless, the extent of CVD cannot be fully explained by these conventional risk factors, and non-conventional factors specific to CKD are now recognised to contribute to the burden of CVD.

Oxidative stress is a state characterised by excessive production of reactive oxygen species (ROS) and other radical species, a reduction in the capacity of antioxidant systems, and disturbance in normal redox homeostasis with depletion of protective vascular signalling molecules such as nitric oxide (NO). This results in oxidative damage to macromolecules such as lipids, proteins and DNA which can alter their functionality. Moreover, many enzymes are sensitive to redox regulation such that oxidative modification to cysteine thiol groups results in activation of signalling cascades which result in adverse cardiovascular effects such as vascular and endothelial dysfunction.

Endothelial dysfunction and oxidative stress are present in association with many conventional cardiovascular risk factors, and can be observed even prior to the development of overt, clinical, vascular pathology, suggesting that these phenomena represent the earliest stages of CVD.

In the presence of CKD, there is increased ROS production due to upregulated NADPH oxidase (NOX), increase in a circulating asymmetric dimethylarginine (ADMA), uncoupling of endothelial nitric oxide synthase (eNOS) as well as other mechanisms. There is also depletion in exogenous antioxidants such as ascorbic acid and tocopherol, and a reduction in activity of endogenous antioxidant systems regulated by the master gene regulator Nrf-2. In previous studies, circulating markers of oxidative stress have been shown to be increased

in CKD, together with a reduction in endothelial function in a stepwise fashion relating to the severity of renal impairment.

Not only is CVD linked to oxidative stress, but the progression of CKD itself is also in part dependent on redox sensitive mechanisms. For example, administration of the ROS scavenger tempol attenuates renal injury and reduces renal fibrosis seen on biopsy in a mouse model of CKD, whilst conversely, supplementation with the NOS inhibitor L-NAME causes proteinuria and renal impairment. Previous human studies examining the effect of antioxidant administration on vascular and renal function have been conflicting however. The work contained in this thesis therefore examines the effect of antioxidant administration on vascular and endothelial function in CKD.

Firstly, 30 patients with CKD stages 3 – 5, and 20 matched hypertensive controls were recruited. Participants with CKD had lower ascorbic acid, higher TAP and ADMA, together with higher augmentation index and pulse wave velocity. There was no difference in baseline flow mediated dilatation (FMD) between groups. Intravenous ascorbic acid increased TAP and O_2^-, and reduced central BP and augmentation index in both groups, and lowered ADMA in the CKD group only. No effect on FMD was observed

The effects of ascorbic acid on kidney function was then investigated, however this was hindered by the inherent drawbacks of existing methods of non-invasively measuring kidney function. Arterial spin labelling MRI is an emerging imaging technique which allows measurement of renal perfusion without administration of an exogenous contrast agent. The technique relies upon application of an inversion pulse to blood within the vasculature proximal to the kidneys, which magnetically labels protons allowing measurement upon transit to the kidney. At the outset of this project local experience using ASL MRI was limited and there ensued a prolonged pre-clinical phase of testing with the aim of optimising imaging strategy. A study was then designed to investigate the repeatability of ASL MRI in a group of 12 healthy volunteers with normal renal function. The measured T1 longitudinal relaxation times and ASL MRI perfusion values were in keeping with those found in the literature; T1 time was 1376 ms in the cortex and 1491 ms in the whole kidney ROI, whilst perfusion was 321 mL/min/100g in the cortex, and 228 mL/min/100g in the whole kidney ROI. There was good reproducibility demonstrated on Bland Altman analysis, with a CV_{ws} was 9.2% for cortical perfusion and 7.1% for whole kidney perfusion.

Subsequently, in a study of 17 patients with CKD and 24 healthy volunteers, the effects of ascorbic acid on renal perfusion was investigated. Although no change in renal perfusion was found following ascorbic acid, it was found that ASL MRI demonstrated significant differences between those with normal renal function and participants with CKD stages 3 – 5, with increased cortical and whole kidney T1, and reduced cortical and whole kidney perfusion. Interestingly, absolute perfusion showed a weak but significant correlation with progression of kidney disease over the preceding year.

Ascorbic acid was therefore shown to have a significant effect on vascular biology both in CKD and in those with normal renal function, and to reduce ADMA only in patients with CKD. ASL MRI has shown promise as a non-invasive investigation of renal function and as a biomarker to identify individuals at high risk of progressive renal impairment.

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Authors Declaration

The work presented in this thesis was that of the author and his supervisors, Dr Patrick Mark and Professor Christian Delles. All experimental work was carried out by the Author unless otherwise stated.

This thesis has been composed by myself and is a record of work performed by myself. It has not been previously submitted for a higher degree.

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List of Abbreviations

(adj)Aix	(Adjusted) Augmentation index
4D	Deutsche Diabetes Dialyse Studie
8-iso-PGF2α	8-iso-prostaglandin F2α
8-OHdG	8-hydroxy-2′-deoxyguanosine
ADC	Apparent diffusion coefficient
ADMA	Asymmetric dimethylarginine
AGE	Advanced glycation end products
ANOVA	Analysis of variance
Apo	Apolipoprotein
ARE	Antioxidant response element
ARICS	Atherosclerosis Risk in Communities Study
ASL	Arterial spin labelling
BH4	Tetrahydrobiopterin
BMI	Body mass index
BOLD	Blood oxygen level dependent
Cbfa-1	Core binding factor a1
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration

CM   Chylomicron

CMD   Corticomedullary differentiation

CPH   1-hydroxy-3-carboxy-2, 2, 5, 5-tetramethylpyrrolidine

CREATE   Cardiovascular Risk Reduction by Early Treatment with Epoetin Beta
                  study

CRP   C reactive protein

CT   Computed tomography

CVD   Cardiovascular disease

CVws   Coefficient of variance (within subject)

DCCT   Diabetes Control and Complications Trial

DDAH   Dimethylarginine dimethylaminohydrolase

DM   Diabetes mellitus

DTNB   5, 5'-dithiobis-2-nitrobenzoic acid

DWI   Diffusion weighted imaging

EC   Endothelial cell

ECG   Electrocardiogram

EDHF   Endothelium derived hyperpolarisation factor

EGF   Epithelial growth factor

eGFR   Estimated glomerular filtration rate

ELISA   Enzyme linked immunosorbent assay

eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ESRD	End stage renal disease
FA	Fractional anisotropy
$FADH_2$	Flavin adenine dinucleotide
FAIR	Flow-sensitive alternating inversion recovery
FGF	Fibroblast growth factor
FMD	Flow mediated dilatation
FMN	Flavin mononucleotide
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulphide
GSTA2	Glutathione S-transferase A2
GTN	Glyceryl trinitrate
HASTE	Half Fourier acquisition single shot turbo spin echo
$HbA1_c$	Haemoglobin $A1_c$
HD	Haemodialysis
HDL	High density lipoprotein
HOPE	Heart Outcomes Prevention Evaluation study

HPLC	High performance liquid chromatography
HTN	Hypertension
HV	Healthy volunteer
ICC	Intra-class correlation
KDIGO	Kidney Disease Improving Global Outcomes
KDOQI	Kidney Disease Outcomes Quality Initiative
KEAP-1	Kelch like-ECH-associated protein 1
LGE	Late gadolinium enhancement
L-NAME	N(G)-nitro-L-arginine methyl ester
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
M2VP	1-methyl-2-vinylpyridinium trifluoromethanesulfonate
MDRD	Modification of Diet in Renal Disease study
MOLLI	Modified Look Locker inversion recovery
MRFIT	Multiple Risk Factor Intervention Trial
MRI	Magnetic resonance imaging
mtROS	Mitochondrial reactive oxygen species
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide

NOX   Nicotinamide adenine dinucleotide phosphate oxidase

NQO1   NADPH: Quinone oxidoreductase 1

Nrf-2   Nuclear factor (erythroid-derived 2) related factor 2

$O2^-$ Superoxide anion

PAH   Para aminohippuric acid

PAI-1   Plasminogen activator inhibitor – 1

PCR   Protein to creatinine ratio

PD   Peritoneal dialysis

PDGF   Platelet derived growth factor

PRMT   Protein arginine methyltransferase

PTH   Parathyroid hormone

RAAS   Renin angiotensin aldosterone system

RALES   Randomised Aldactone Evaluation Study

RH-PAT   Reactive hyperaemia peripheral artery tonometry

ROI   Region(s) of interest

ROS   Reactive oxygen species

RRT   Renal replacement therapy

SDMA   Symmetric dimethylarginine

SHARP   Study of Heart and Renal Protection study

SHR	Spontaneously hypertensive rats
SNO	s-nitrosylation
SNR	Signal to noise ratio
SOD	Superoxide dismutase
SPC	Solid phase chromatography
SPE	Solid phase extraction
TAP	Total antioxidant potential
TBARS	Thiobarbituric acid reacting substances
TGFβ-1	Transforming growth factor beta – 1
TNFα	Tumour necrosis factor alpha
True-FISP	True fast imaging with steady state free precession
UK	United Kingdom
UKPDS	United Kingdom Prospective Diabetes study
US	United States
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

Chapter 1

Introduction

1.1 Cardiovascular disease and chronic kidney disease

1.1.1 Epidemiology of cardiovascular disease

There is extensive evidence that patients with chronic kidney disease (CKD) are at a significantly elevated risk of cardiovascular disease (CVD). Data from the United Kingdom (UK) renal registry show that cardiovascular disease is the cause of death in 22% of patients requiring renal replacement therapy (RRT) (Pruthi R, 2013), and moreover, this risk is not limited to patients with end stage renal disease (ESRD) requiring haemodialysis (HD), but applies even in the earliest stages of renal impairment, with the risk of cardiovascular complications rising in proportion to the degree of renal impairment (Culleton et al., 1999). In fact, for the vast majority of patients with CKD, CVD proves a greater burden than progression of their renal disease: most patients with CKD will die of a cardiovascular cause before requiring renal replacement therapy.

United States (US) registry data show an increased incidence of many manifestations of CVD in patients with CKD (US Renal Data System, 2011a). For example, the prevalence of atherosclerotic coronary artery disease was 42.9% in older CKD patients compared to 17.4% in the general population, and the prevalence of myocardial infarction was 10.3% compared to 2.3% (figure 1.1).

1.1.2 Chronic kidney disease and cardiovascular risk

There are a number of possible explanations for the increase in cardiovascular risk associated with CKD. Firstly, there are risk factors for CVD which are also common aetiological factors in CKD. Diabetes and hypertension (HTN) are the commonest causes of CKD in the developed world (US Renal Data System, 2011b) and also predispose to atherosclerotic coronary artery disease. CKD is also associated with advanced age and co-morbidity such that even when these cardiovascular risk factors are not implicated in the aetiology of CKD, such factors are highly prevalent. For example diabetes has a prevalence of 9.4%, and smoking of 14% in patients with CKD (Rao A, 2013). Patients with CKD have been notoriously undertreated in the past, with low prescribing rates of cardioprotective medications, and underutilisation of revascularisation (Herzog et al., 2007). Lastly, there may be specific aspects of CKD which endow cardiovascular risk due to circulating factors comprising the 'uraemic milieu'.
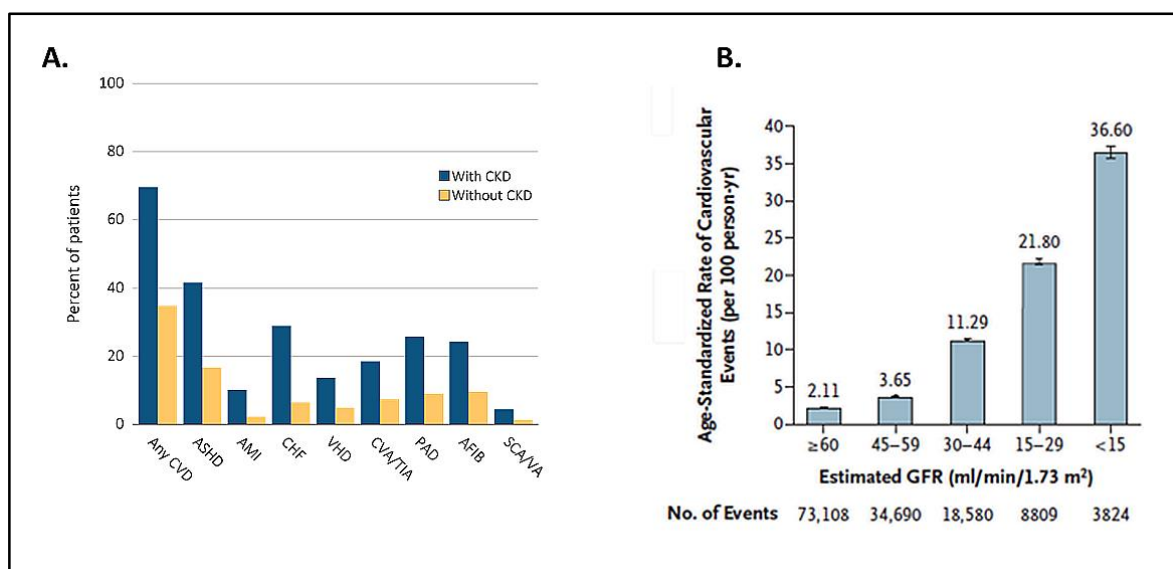
Figure 1.1 The prevalence and nature of cardiovascular disease in CKD.

Many manifestations of cardiovascular disease are increased in prevalence in CKD (A), in comparison with the general population. This risk is established early in CKD and rises in proportion to the degree of renal impairment (B). ASHD = Atherosclerotic heart disease, CHF = Congestive heart failure, VHD = Valvular heart disease, CVA = Cerebrovascular accident, TIA – transient ischaemic attack, PAD = Peripheral arterial disease, AFIB = Atrial fibrillation, SCA/VA = Sudden cardiac arrest/Ventricular arrhythmias. Adapted from US Renal Data System 2011a, and Go et al 2004.

There is evidence to support the hypothesis that uraemia itself is an independent cardiovascular risk factor. Go et al, showed an increase in cardiovascular events, hospitalisation, and mortality occurring in proportion to severity of renal impairment, even after adjustment for diabetes, hypertension, dyslipidaemia, sociodemographic characteristics and the presence or absence of prior CVD (Go et al., 2004). The adjusted hazard ratio for CVD was 2.0 in patients with an estimated glomerular filtration rate (eGFR) between 15 and 30 mL/min/1.73m2 and 3.4 with an eGFR below 15 mL/min/1.73m^2. Similarly, Manjunath et al found a linear relationship between eGFR and incidence of atherosclerotic cardiovascular events after adjustment for other cardiovascular risk factors, with a 7% increase in events for every 10ml/min fall in eGFR (Manjunath et al., 2003).

Patients with ESRD are subject to specific cardiovascular risks associated with the modality of renal replacement they receive. Those receiving HD are subject to acute cardiovascular complications such as cardiac stunning (McIntyre et al., 2008) and arrhythmia (Santoro et al., 2008), as well as longer term effects relating to chronic inflammation secondary to dialysis membrane bioincompatibility (Cheung, 1990), and vascular and infectious complications of vascular access, all of which increase cardiovascular mortality. Patients

receiving peritoneal dialysis (PD) may be at higher cardiovascular risk due to inflammation associated with repeated episodes of peritonitis, or due to retention of fluid and uraemic toxins associated with the lower clearance of PD. Whether either of HD or PD is associated with higher cardiovascular risk continues to be debated. For example whilst certain studies have found the highest arterial stiffness in PD patients (Covic et al., 2004), this has not been replicated in other studies (Chang et al., 2010). In a study of data derived from the Australia and New Zealand Dialysis and Transplant Registry, incident PD patients had lower mortality in the first 90 days of therapy, but higher mortality after 12 months (McDonald et al., 2009). Only PD patients who were younger than 60 years of age and without comorbidities enjoyed lower mortality up to the first 12 months of treatment, which reflects the findings of a retrospective cohort study of American patients commencing dialysis in 2003, in which PD patients had improved survival among subgroups aged below 65 years, without a history of CVD or diabetes (Weinhandl et al., 2010). In fact, HD was associated with lower mortality in patients with existing CVD upon starting dialysis. These conflicting results demonstrate that individual factors such as age, comorbidity and dialysis vintage, are at least as significant as dialysis modality in predicting cardiovascular risk.

Patients with kidney transplants have improved outcomes compared to HD and PD patients, although are a self-selecting group who are sufficiently fit for surgery. Nevertheless the immunosuppressive regimes required to avoid rejection can induce diabetes, dyslipidaemia, and hypertension (Gillis et al., 2014b).

1.1.3 The nature of cardiovascular disease in CKD

CVD in normal renal function is typically manifest as atherosclerotic coronary artery disease, with infarction, ischaemic cardiomyopathy, and arrhythmia typically following as secondary phenomenon. As has been discussed, coronary artery disease is highly prevalent in CKD, but is characterised by medial calcification rather than the lipid laden atherosclerotic plaques seen in typical coronary artery disease. Furthermore, heart failure and arrhythmia are equal in importance to coronary artery disease as causes of cardiovascular morbidity CKD. Data from the US registry showed that the prevalence of congestive cardiac failure was 28.8% in CKD compared to 6.4% in the general population, whilst the prevalence of atrial fibrillation was 24.4% compared to 9.6%, and cardiac arrest and ventricular arrhythmia 4.5% compared to 1.3% (United States Renal Data System, 2011c).

Cardiovascular mortality has also been shown to be only partly related to the degree of atherosclerosis in CKD. In a recent study of 1541 patients referred for a computed tomography (CT) coronary angiogram, patients with renal impairment had a higher cardiovascular event rate, and higher rates of both occlusive and non-occlusive coronary disease (Bittencourt et al., 2015). Two thirds of cardiovascular events in CKD however, were secondary to a non-coronary cause, such as sudden death, arrhythmic death, or death from heart failure. This was reflected in a retrospective longitudinal study of patients with CKD 3 – 5, which found a negative correlation between eGFR and risk of cardiac death, but no association between degree of coronary artery disease and risk of cardiac death (Muntner et al., 2002). Altogether, this demonstrates that patients with CKD are at risk not only of accelerated coronary artery disease, but also a broad range of other cardiovascular morbidity.

1.2 Conventional cardiovascular risk factors

The conventional cardiovascular risk factors applicable to the general population such as dyslipidaemia, hypertension, obesity, diabetes and smoking, are equally relevant to individuals with CKD. In many cases however, the interaction between risk factor and disease differs in CKD in comparison with the general population, reflecting the differences in cardiovascular pathology in this group. This discrepancy may explain the differences in response to conventional preventative strategies, as is discussed below.

1.2.1 Dyslipidaemia

In individuals with normal renal function, there is a well-accepted relationship between total cholesterol and low density lipoprotein (LDL) cholesterol and cardiovascular risk (Lewington et al., 2007). Statin therapy effectively lowers LDL cholesterol and there is evidence that this is efficacious in the primary prevention of CVD. In a recent Cochrane review including 18 randomised controlled trials and over 56,000 patients, statin therapy was associated with a risk ratio of 0.75 for combined fatal and non-fatal coronary artery events and a risk of 0.62 for revascularisation events (Taylor et al., 2013). A meta-analysis confirmed the benefits of statin therapy even in individuals with low cardiovascular risk, with a relative risk of 0.90 for those with a less than 20% 10 year risk of CVD and 0.83 for those with less than 10% 10 year risk of CVD (Tonelli et al., 2011).

One might expect the benefits of lipid lowering treatment to extend to patients with CKD, especially given the higher event rate and cardiovascular risk associated with renal impairment, however there are demonstrable differences in the epidemiology and

pathophysiology of dyslipidaemia in CKD that have historically resulted in controversy regarding the role of lipid lowering therapy. Additionally, as previously discussed, much of the cardiovascular burden in CKD relates to non-atherosclerotic disease which might diminish the benefits of statin treatment.

In contrast to the general population, low total cholesterol levels are associated with increased all cause and cardiovascular mortality in haemodialysis patients, a phenomenon termed "reverse epidemiology" (Iseki et al., 2002) and this U-shaped relationship has also been observed in patients with CKD not requiring dialysis (Kovesdy et al., 2007). It is possible that low cholesterol in this context is a marker of inflammation and malnutrition, which is often associated with CKD and confers poor outcome. Supporting this hypothesis, in a study of 986 patients with CKD not on dialysis, the increase in mortality associated with low cholesterol was abrogated when adjusted for markers of inflammation and malnutrition (Kovesdy et al., 2007). In another study of 1167 dialysis patients, those with the lowest total serum cholesterol had the highest mortality, whilst those with the highest cholesterol had highest cardiovascular event rate. Only in those with a normal serum albumin was total cholesterol a significant predictor of mortality (Iseki et al., 2002). Together, these findings suggest that low cholesterol is an epiphenomenon of malnutrition and the mortality risk associated with this exceeds that associated with hypercholesterolaemia.

Given these differences, it is not surprising to find that the profile of dyslipidaemia is quite different in CKD compared to the general population albeit varying according to individual factors such as modality of renal replacement therapy, use of immunosuppressive therapy, the presence of proteinuria and the nephrotic syndrome, and the severity of renal impairment.

LDL cholesterol is often normal or low, and there is a greater abundance of modified LDL types which are often highly atherogenic. Oxidised LDL (ox-LDL), for example, is elevated tenfold in CKD in comparison to normal controls, in part due to reduced potency of HDL associated antioxidant enzymes (Samouilidou et al., 2012). Lipoprotein(a) (Lp(a)) is another modified form of LDL which is avidly taken up by atherosclerotic plaques and as such is a strong predictor of CVD (Enas et al., 2006). Lp(a) levels are largely genetically determined by the size of the inherited apo(a) isoform (Lackner et al., 1991), but presence of renal impairment (Kronenberg et al., 2000), degree of proteinuria, hypertrigylceridaemia and inflammation also increase Lp(a) (Uhlig et al., 2005). With reduced glomerular filtration, there is delayed catabolism of triglyceride rich lipoproteins due to reduced expression of vascular lipoprotein lipase (LPL) together with accumulation of the LPL inhibitors

apolipoprotein CIII (apo CIII) and apo CI. *Ex vivo* catabolism of very low density lipoprotein (VLDL) and chylomicrons (CM) derived from CKD patients is also reduced, suggesting that inherent changes to lipoproteins themselves also contributes to triglyceride accumulation. High density lipoprotein (HDL) is a negative predictor of CVD, exerting a protective effect by removal of cholesterol from lipid laden macrophages, by activating endothelial nitric oxide synthase (eNOS), and by exerting an antioxidant effect on oxidised LDL (Assmann and Gotto, 2004). Both the quantity and functionality of HDL is reduced in CKD and this is associated with adverse outcome (Baragetti et al., 2013).

Early interventional studies failed to show any benefit of statins in CKD. For example in the Deutsche Diabetes Dialyse Studie (4D) of atorvastatin in dialysis patients, despite a lowering of LDL cholesterol by 42% there was no reduction in the primary endpoint (a composite of death from cardiac causes, nonfatal myocardial infarction, and stroke), although there was a reduction in the rate of all combined cardiac events (Wanner et al., 2005). Since then however, the largest study of lipid lowering in renal impairment was conducted in the Study of Heart and Renal Protection (SHARP) study, of 9270 non dialysis CKD patients. After a median follow up period of 4.9 years, those randomised to the intervention of simvastatin and ezetimibe enjoyed a 17% reduction in major atherosclerotic events (Baigent et al., 2011). These findings were consolidated in a recent meta-analysis involving 38 studies and 37,274 patients, which showed a 20% reduction in cardiovascular events in CKD patients not requiring dialysis (Palmer et al., 2014).

Despite the many differences in lipid pathophysiology in patients with CKD, there is now good evidence for the efficacy of lipid lowering therapy in CKD and this is reflected by the Kidney Disease Improving Global Outcomes (KDIGO) guidelines for lipid management. Statin therapy is recommended in individuals with CKD older than 50 years of age with eGFR < 60 mL/min/1.73m^2 or with significant proteinuria, and in individuals aged 18 – 49 years where there is an additional vascular risk factor including prior coronary artery disease or ischaemic stroke, diabetes mellitus, or estimated 10 year risk of CVD of > 10% (Kidney Disease Improving Global Outcomes, 2013).

1.2.2 Hypertension

In the general population, controlling HTN reduces cardiovascular and cerebrovascular event rate (Amery et al., 1985) and most studies have shown a linear relationship between blood pressure and cardiovascular risk (National Institute for Health and Care Excellence,

2011), with a 25% reduction in CVD following a 10 mmHg reduction in systolic blood pressure.

HTN is common in patients with CKD, occurring as an aetiological factor, a consequence of kidney disease, or both. The prevalence of HTN in CKD is around 80% but varies according to body mass index (BMI), ethnicity, and age, as it does in the general population (Buckalew et al., 1996). Factors affecting the prevalence of HTN in CKD include degree of renal impairment, with an inverse relationship seen between GFR and blood pressure, and cause of CKD, with prevalence varying between 93% in renovascular disease, and 54% in glomerulonephritis (Ridao et al., 2001). Traditionally, hypertension was thought to be due to reduced renal sodium clearance (Tedla et al., 2011); now other mechanisms are thought to play an additional role including activation of the renin-angiotensin-aldosterone system, the sympathetic nervous system, and the endothelin system (Dhaun et al., 2006).

There is a wealth of evidence to show the efficacy of treating HTN in CKD, both for reducing cardiovascular risk and progression of renal impairment. After 16 years of follow up in the Multiple Risk Factor Intervention Trial (MRFIT) of 332,544 men there was increased progression to ESRD in individuals with higher blood pressure, with an the incidence of ESRD of 5.43 per 100,000 person years in those with systolic blood pressure under 117 mmHg, compared to 32.37 per 100,000 person years when systolic blood pressure was over 140 mmHg (Klag et al., 1997). Sub group analysis of the Modification of Diet in Renal Disease (MDRD) Study showed slower decline and reduced proteinuria in those with effectively treated HTN (Peterson et al., 1995). In addition to this, a recent meta-analysis found that lowering blood pressure reduced CV risk to a similar extent in those with and without CKD (Ninomiya et al., 2013), with a reduction in major cardiovascular events of 17% for every 5 mmHg reduction in blood pressure. Whilst inhibitors of the renin angiotensin system have been shown to be the most efficacious anti-hypertensive to reduce proteinuria and halt decline in renal function, no particular anti-hypertensive has been shown to be more effective at reducing cardiovascular risk.

KDIGO guidelines recommend lowering blood pressure to below 140/90 mmHg in patients with CKD, and below 130/80 in patients with albuminuria more than 30 mg in 24 hours (Kidney Disease Improving Global Outcomes, 2013). There is evidence however, that excessive blood pressure lowering may be harmful, such that the target under which blood pressure should be lowered is a point of ongoing debate. Some studies have shown higher mortality associated with blood pressure below 130/70 mmHg (Kovesdy et al., 2013),

however low blood pressure in this context may represent hypotension associated with severe cardiac disease rather than overzealous blood pressure lowering on the part of clinicians. Other studies have shown adverse renal outcomes with blood pressure higher or lower than the range 130-139/60-79 mmHg (Sim et al., 2014), with the suggestion that very low blood pressure may compromise renal perfusion and hasten the progression of CKD. Further research into the ideal target for CKD populations is needed, and development of biomarkers of renal 'stress' or 'injury' may help clinicians to define optimal blood pressure targets and allow an individualised approach management in this groups of patients.

1.2.3 Obesity and indolence

The existence of a specific obesity related glomerulopathy has been known since Weisinger's description in 1974, characterised histologically by focal and segmental glomerulosclerosis (FSGS), and clinically by heavy proteinuria and progressive renal impairment (Weisinger et al., 1974). There are important renal sequelae of obesity aside from this specific pathology however, due to the numerous haemodynamic and immunomodulatory effects of adiposity. As such, obesity has been shown to independently increase the risk of new onset kidney disease (Fox et al., 2004), as well as increase the risk of progression of CKD to ESRD up to 7 fold (Hsu et al., 2006). Obesity is therefore an important reversible cardiovascular risk factor as well as a contributing factor to the establishment and progression of CKD.

Renal haemodynamics are altered in obesity with increased glomerular filtration and glomerular hypertension, leading to reduced nephron density and foot process effacement (Chen et al., 2006). Mediators of these haemodynamic effects include a potentiated renin angiotensin system, enhanced aldosterone secretion, plasminogen activator inhibitor – 1 (PAI-1), and adiponectin (Camici et al., 2012). Adipose tissue contains an abundance of immune cells such as macrophages, lymphocytes and eosinophils and these cells are increased in number and activity in obesity (Xu et al., 2003). Release of inflammatory mediators such as tumour necrosis factor alpha (TNFα) and interleukin (IL) – 6 contributes to glomerulopathy and oxidative stress, and ultimately to the insulin resistance, HTN, and left ventricular hypertrophy are associated with obesity and collectively termed the metabolic syndrome. Obesity is also associated with development of secondary hyperparathyroidism in patients with CKD which itself predisposes to CVD (Kovesdy et al., 2007).

There remains however, an 'obesity paradox', in that although increased BMI predisposes to CVD and worsening of CKD, there is evidence of a U-shaped relationship (Flegal et al., 2013) between BMI and mortality in a range of conditions, including CKD, congestive cardiac failure (Kalantar-Zadeh et al., 2004) and acute illness (Landi et al., 2000). Data from the Atherosclerosis Risk in Communities Study (ARIC) showed that obesity is associated with metabolic syndrome both in patients with and without CKD, but an association between obesity and mortality existed only in the non CKD group (Flegal et al., 2013). This phenomenon has been described as "competing risks" with the adverse effects of obesity competing with the protective effects of adequate nutrition and muscle mass, and the relative importance of each differing according to clinical circumstances. This is effect is especially marked in patients with ESRD (Abbott et al., 2004), where the benefits of having adequate nutritional reserve when facing the catabolic challenge of haemodialysis outweigh the negative effects of adiposity, such that low body weight patients have worse outcome

This leaves clinicians in a quandary when managing CKD and cardiovascular risk in patients with renal impairment; do the benefits of weight loss in terms of blood pressure, insulin sensitivity, and dyslipidaemia, outweigh the potential harms of aggravating protein calorie malnutrition in obese patients with CKD? A recent meta-analysis of weight loss regimes comprising a mixture of calorie restriction, exercise, and bariatric surgery showed effective reduction in proteinuria(Afshinnia et al., 2010). Similarly, a systematic review found improvements in BMI, systolic blood pressure and proteinuria associated with both surgical and non-surgical weight loss interventions (Palmer et al., 2014). For patients with CKD, no significant change in GFR was noted over a 7.4 month follow up however no control group was available for comparison, to determine if this represents a 'positive' result. Another recent study showed sustained weight loss achieved with a multi-disciplinary approach comprising diet, exercise, and orlistat (MacLaughlin et al., 2010). There was less deterioration in renal function in the weight loss treatment group, but the study was not powered to detect this. KDIGO guidelines simply advise maintaining healthy weight and undergoing regular physical activity without making a specific recommendation for individuals who are already obese. Further research is required into the effect of weight loss and physical activity of cardiovascular and mortality outcomes in patients with CKD.

1.2.4 Glycaemia

Diabetes mellitus (DM) is common in the CKD population and is an important and treatable cardiovascular risk factor in this group. Data derived from the UK registry of patients

commencing RRT show that diabetes is an aetiological factor in 25.4% of patients, and present as a co morbid disease in 9.4% (Rao A, 2013). In the general population diabetes and is associated with a 55% increase in the risk of myocardial infarction (The Health and Social Care InformationThe Health and Social Care Information Centre, 2012). The presence of kidney disease, defined as either reduced GFR or the presence of albuminuria, has a synergistic effect with diabetes, such that patients with both conditions experience a grossly elevated cardiovascular risk. In the Heart Outcomes Prevention Evaluation (HOPE) study, participants with diabetes and kidney disease had a relative risk of 1.97 for cardiovascular events in comparison to those with diabetes but normal kidneys (Gerstein et al., 2001). Similarly a systematic review of 11 cohort studies showed an odds ratio of 2.0 for CVD in those with microalbuminuria and type 2 diabetes, in comparison to those with type 2 diabetes without microalbuminuria (Dinneen and Gerstein, 1997).

Current Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines suggest treating diabetes aiming for a target haemoglobin A_{1c} (HbA$_{1c}$) less than 7% in order to prevent or delay microvascular complications such as diabetic nephropathy (KDOQI, 2012). Early evidence for the efficacy of such intensive glycaemic control was derived from the Diabetes Control and Complications Trial (DCCT) (DCCT Research Group, 1993) and the United Kingdom Prospective Diabetes Study (UKPDS) (UKPDS Group, 1998). More recently, the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial showed a 21% reduction in prevention or progression of albuminuria with intensive glycaemic control (Patel et al., 2008).

Nevertheless, caution must be exercised when aiming for such low glycaemic targets in CKD, given that renal impairment limits excretion of endogenous insulin and prescribed exogenous insulin and oral hypoglycaemic agents, resulting in an increased risk of hypoglycaemic events. Indeed, KDOQI guidelines suggest aiming for a higher HbA$_{1c}$ target in patients at risk of hypoglycaemia or in patients with co morbidities. ACCORD and ADVANCE both showed an increase in hypoglycaemic events when aiming for HbA$_{1c}$ below 7%, and mortality was higher in the intensively treated cohort of the ACCORD study. Furthermore, subgroup analysis of the ACCORD study showed that intensive glucose lowering resulted in 41% higher cardiovascular mortality in patients with CKD (Papademetriou et al., 2014). This is in keeping with an earlier retrospective study which showed the familiar U-shaped association between HbA$_{1c}$ and mortality as has been observed with other previously described biological parameters (Shurraw et al., 2011).

Therefore, whilst there is a desire for intensive glycaemic control in CKD both to prevent ESRD as well as ameliorate the cardiovascular risk associated with worsening uraemia, this must be tempered by the risk of hypoglycaemia. An individualised approach to diabetic management in CKD is to be advised.

1.2.5 Smoking

In the general population, smoking is an important reversible cardiovascular risk factor and is also implicated in the aetiology of a number of malignancies. In CKD, cigarette smoking has been shown to hasten progression of renal impairment in CKD (Hallan and Orth, 2011), and is also an independent risk factor for CVD (Jungers et al., 1997).

1.3 Non-conventional cardiovascular risk factors

As has been discussed, conventional cardiovascular risk factors are highly prevalent in CKD, even if the interaction between risk factor and outcome differs from the general population. In addition to this, a number of non-conventional risk factors have been identified which are specific to CKD.

1.3.1 CKD mineral and bone disorder

Regulation of bone biochemistry occurs via an interplay of a number of circulating factors and their target receptors in the gut, kidney, bone and parathyroid gland. In CKD these are invariably deranged, with the range of abnormalities observed collectively termed CKD mineral and bone disorder. Urinary excretion of phosphate is compromised by falling glomerular filtration rate and in response there is inducement of fibroblast growth factor – 23 (FGF-23) secretion by osteocytes (Larsson et al., 2003). Circulating FGF-23 encourages phosphate excretion in the urine, via activation of a specific FGF receptor and its essential co receptor Klotho (Urakawa et al., 2006). Gut absorption of phosphate is also reduced, with the effect that serum phosphate is maintained within a normal range (Razzaque and Lanske, 2007). Renal disease also impairs the secondary hydroxylation of vitamin D into its active form such that without treatment, there is effective vitamin D deficiency resulting in secondary hyperparathyroidism and ultimately leading to tertiary hyperparathyroidism, characterised by autonomous production of parathyroid hormone (PTH). Vitamin D deficiency is also exacerbated by FGF-23, which inhibits expression of 25-hydroxyvitamin D 1α-hydroxylase.

In a retrospective study of 6730 patients with CKD, there was a 35% increase in risk of myocardial infarction, and 23% increase in risk of death for every 1 mg/dL (0.323 mmol/L) increment of serum phosphate (Kestenbaum et al., 2005). Furthermore, even in normal renal function, higher serum phosphate is associated with increased cardiovascular risk (Dhingra et al., 2007), and with poor outcome in congestive cardiac failure (Ess et al., 2013). Higher levels of FGF-23 are also associated with increased all-cause mortality and cardiovascular risk, independently of renal function and serum phosphate. First demonstrated in incident dialysis patients, where those with the highest quartile of FGF-23 had a 20% increase in risk of death (Gutierrez et al., 2008), this was then observed in patients with CKD. Seiler et al found that CKD patients with higher FGF-23 were more than twice as likely to suffer a composite outcome of a vascular event or death (Seiler et al., 2010). As with serum phosphate there is now evidence linking FGF-23 with poor outcome even in normal renal function, with higher FGF-23 associated with greater left ventricular mass, higher coronary artery calcium score, and higher incident heart failure and coronary artery events (Kestenbaum et al., 2014).

Disturbance in phosphate homeostasis is associated with a number of mechanisms which are responsible for this cardiovascular risk, including vascular calcification (Scialla et al., 2013), left ventricular hypertrophy (Yamamoto et al., 2013) (Patel et al., 2009), accelerated atherosclerosis (Foley et al., 2009) and endothelial dysfunction (Yilmaz et al., 2010).

There has understandably been great interest in the modification of phosphate handling in CKD as a means to lower cardiovascular risk, typically by restricting dietary intake or by pharmacological reduction of its gastrointestinal absorption. In a randomised controlled prospective study, phosphate restriction led to a 33.5% reduction in FGF-23, and a 12% reduction in serum phosphate (Di Iorio et al., 2012). Similarly, phosphate binders have been shown to reduce serum phosphate and FGF-23 (Covic et al., 2013), especially in combination with dietary restriction (Isakova et al., 2013). There is however, little evidence of a beneficial effect of either approach on surrogate cardiovascular outcome measures (Seifert et al., 2013), with one study in fact showing an increase in vascular calcification following treatment with a range of phosphate binders, in comparison to placebo (Block et al., 2012).A randomised, double blinded, placebo controlled trial investigating the effect of phosphate lowering on cardiovascular end points is yet to be carried out.

1.3.2 Anaemia

Anaemia is common in patients with CKD due to reduced red cell lifespan and production. Kidney fibroblasts normally respond to a falling haemoglobin by increasing erythropoietin production, which causes increased erythrocyte production in the bone marrow. In CKD this mechanism is impaired and a compensatory increase in erythropoietin secretion does not occur. Given the association of CKD with inflammation, either due to uraemia, the nature of the underlying kidney disease, or due to co-morbid conditions, anaemia can also arise due to the erythropoietin unresponsiveness and abnormal iron handling which occurs in anaemia of chronic disease (Babitt and Lin, 2012).

Prior to the advent of recombinant erythropoietin, patients with ESRD often suffered severe anaemia, which was associated with a range of adverse clinical outcomes including left ventricular hypertrophy, blood transfusion dependency, and fatigue. One study found that for every 10 g/L reduction in haemoglobin, there was a 5% increase in risk of mortality, and 8% increase in risk of (Locatelli et al., 2004). In the same study, even in patients with CKD not requiring dialysis who have a tendency to suffer less severe anaemia, there was a similar association between anaemia and morbidity and mortality. Similarly, in a prospective cohort study of Canadian patients with mild to moderate CKD, the likelihood of worsening left ventricular hypertrophy increased by 32% for every 5 g/L reduction in haemoglobin (Levin et al., 2006). A retrospective study of 5885 patients with CKD found that those with a haemoglobin less than 105 g/L were at significantly greater risk of cardiovascular events, death and progression to end stage renal disease (Thorp et al., 2009).

Renal anaemia can now be effectively treated with the use of recombinant erythropoietin stimulating agents (ESA) and intravenous iron preparations. With the advent of ESA, there was optimism that cardiovascular outcomes could be substantially improved in CKD and dialysis patients due to sustained normalisation of haemoglobin levels. This optimism has since diminished, after a number of clinical trials failed to demonstrate improved outcomes in a variety of renal scenarios such as pre dialysis CKD, dialysis, and transplant populations. It is now felt that the cardiovascular toxicity of these medications matches the morbidity associated with anaemia.

In the Cardiovascular Risk Reduction by Early Treatment with Epoetin Beta (CREATE) study, 603 patients were randomly assigned to a haemoglobin target of either 130 to 150 g/L or 105 to 130 g/L (Drüeke et al., 2006). No significant difference was seen in cardiovascular

event rate, mortality, nor quality of life. In the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) study, patients were randomised to either darbepoetin therapy with a target haemoglobin of 130 g/L, or to placebo, with darbepoetin rescue therapy given if haemoglobin fell to less than 90 g/L (Pfeffer et al., 2009). The treatment arm showed a significant reduction in blood transfusions, and a modest improvement in fatigue score, at the cost of a nearly doubled risk of stroke.

The data suggests therefore, that the risks of normalising haematocrit, or the higher dose of ESA or iron required to achieve this, at least exceeds the cardiovascular risks associated with anaemia. Certainly, ESA have a range of undesirable biological side effects which could cause cardiovascular harm, including upregulation of endothelin-1 production (Briet et al., 2013), calcification of vascular smooth muscle cells (VSMC) (Won et al., 2014), hypersensitivity of angiotensin II signalling(Yamakado et al., 1991), and an increase in circulating asymmetric dimethylarginine (ADMA) (Scalera et al., 2005). Likewise, intravenous administration of iron can overwhelm the bodies typical iron sequestering mechanisms, allowing the reaction of poorly bound iron with hydrogen peroxide in the Fenton reaction, producing large quantities of ROS and inducing oxidative stress (Anraku et al., 2004).

A retrospective study of 12733 dialysis patients found that higher ESA dosage was associated with worse outcome, with higher rates of mortality and hospitalisation in patients treated with more than 20000 units/week of epoetin (Servilla et al., 2009). Similarly, a meta-regression analysis of 31 studies and 12956 patients found that high ESA dose was associated with increased cardiovascular events and mortality independent of haemoglobin concentration (Koulouridis et al., 2013). In contrast, another study found that deleterious effects of ESA and iron therapy occur only at high haematocrit levels. A retrospective analysis of Medicare haemodialysis patients allocated 269717 subjects into quartiles based on haematocrit levels (Brookhart et al., 2010). In those with haematocrit less than 0.33 intensive use of iron and ESA was associated with reduced mortality, whilst in those with haematocrit greater than 0.33, higher use of iron and ESA was associated with increased mortality. In the pre dialysis population, Jing et al found a modest reduction in mortality in CKD patients when a low haemoglobin was targeted (around 100 g/L), whilst no difference in outcome was observed with any haemoglobin target in dialysis patients (Jing et al., 2012).

A balance must be struck therefore, between maintenance of haemoglobin at a level sufficient to reduce need for blood transfusion and the cardiovascular sequelae of profound

anaemia, without excessive use of potentially harmful ESA or iron preparations, and without over compensation to a hazardous level of haematocrit. As such, Renal Association guidelines recommend a target haemoglobin of 100 – 120 g/L in ESA treated patients with CKD (Renal Association, 2010).

1.3.3 Uraemic cardiomyopathy

Left ventricular hypertrophy is a pathological outcome resulting from a number of mechanisms associated with renal impairment, and confers increased risk of adverse cardiovascular outcome. As discussed earlier, in contrast to the normal population CVD in CKD more often manifest as sudden death, arrhythmia, and heart failure than atherosclerotic coronary artery disease and myocardial infarction, and left ventricular hypertrophy predisposes to these events. Furthermore, the pathology of cardiomyopathy is different in CKD compared to the general population.

Early echocardiographic studies into the nature of uraemic cardiomyopathy described a high prevalence of left ventricular (LV) hypertrophy and dilatation with LV mass and cavity size proving independent predictors of mortality. With the increased availability of cardiac magnetic resonance imaging (CMR), uraemic cardiomyopathy has been somewhat re-described accounting for the over estimation of LV mass which occurs using echocardiography (Stewart et al., 1999).

In a prospective study of 134 patients with ESRD receiving maintenance haemodialysis, Mark et al redefined the characteristics of uraemic cardiomyopathy using gadolinium enhanced CMR (Mark et al., 2006). 28.4% of patients had evidence of myocardial fibrosis demonstrated by late enhancement following administration of gadolinium contrast. Half of these had subendocardial fibrosis, typically caused by myocardial ischaemia, associated with LV hypertrophy and dilatation, systolic dysfunction, and occurred in patients with conventional risk factors for CVD. In the other half, a different pattern of cardiovascular abnormalities was observed, comprising more diffuse myocardial fibrosis, associated with LV hypertrophy but not dilatation nor systolic dysfunction, and occurring in the absence of conventional cardiovascular risk factors.

Uraemic cardiomyopathy in patients with non-dialysis CKD is less well described. Park et al performed a cross sectional analysis of 3487 participants of the CRIC study who had undergone echocardiographic measurement of LV mass, and estimation of eGFR using

cystatin C (Park et al., 2012). Renal function and LV mass were negatively correlated, and after adjustment for conventional cardiovascular risk factors, albuminuria, haemoglobin, and bone biochemistry, the prevalence of left ventricular hypertrophy (LVH)was elevated 1.4-fold in those with eGFR below 44 mL/min/1.73m^2, and 2.2-fold in those with eGFR below 30 mL/min/1.73m^2.

Edwards et al evaluated LV geometry and associations with aortic stiffness using CMR in CKD stages 2 and 3 (Edwards et al., 2008). Even at these early stages of renal impairment, abnormalities of LV geometry were observed, with significantly higher LV mass than controls, and up to a third of patients exhibiting LVH. Furthermore, aortic compliance measured using CMR was increased, as was arterial and ventricular stiffness measured using echocardiography, albeit with preserved ventricular-arterial coupling. The data suggest that LV mass and contractility are increased in response to arterial stiffness in order to maintain ventricular-arterial coupling and optimise LV function, at the cost of development of LVH and increased LV stiffness.

A number of other mechanisms have also been identified as risk factors for the development of uraemic cardiomyopathy. In a cross sectional observational study of 70 patients with renal impairment and 30 patients with HTN and normal renal function, McQuarrie et al found that urinary mineralocorticoid excretion was an independent predictor of LV mass in CKD but not in HTN (McQuarrie et al., 2011). Further evidence for the role of aldosterone in development of uraemic cardiomyopathy was derived from the CRIB-2 randomised control study in which spironolactone was shown to improve markers of LV systolic and diastolic function in 112 patients with early CKD (Edwards et al., 2010).

Uraemic cardiomyopathy is therefore a common clinical occurrence in CKD and occurs as a result of a number of pathophysiological processes, ultimately leading to a different pathology to the typical ischaemic cardiomyopathy observed in patients with normal renal function.

1.4 Oxidative stress

1.4.1 Oxidative stress

Oxidative stress refers to a state where there is disturbance of physiological redox homeostasis in favour of an excess of oxidative free radicals and their derivatives. Reactive oxygen species (ROS) act as signalling molecules when present in physiological quantities.

In excess however, ROS and derived oxidative species such as reactive carbonyl (RCS) and nitrogen species (RNS), cause reduced bioavailability of the anti-atherogenic vascular signalling molecule nitric oxide (NO) and activation of pro-atherogenic redox sensitive signalling cascades.

In contrast to other cardiovascular risk factors which are manifest as a disturbance in single molecules or physiological axes, oxidative stress is a nebulous term characterised by the excess of free radicals, evidence of oxidant damage to macromolecules such as lipids, proteins and DNA, and physiological effects of this such as endothelial and arterial dysfunction, accelerated atherosclerosis, and increased cardiovascular risk.

1.4.2 Sources of reactive oxygen species

ROS have a number of intracellular sources, with the relative contribution of each varying between cells and dependent on the quantity of various stimulatory factors. ROS are not the only important radical species: NO reacts with O_2^- to produce peroxynitrite and other RNS which are more stable than highly reactive ROS, exerting oxidant effect distant from the site at which they are generated. Ultimately most processes which use oxygen as substrate produce ROS either by design or as a by-product, and the various sources of ROS are discussed below.

1.4.2.1 Mitochondria

Mitochondrial ROS (mtROS) are produced in vast quantities during oxidative phosphorylation, the essential cellular process by which ATP is generated by the electron transfer chain, a string of protein complexes residing on the inner mitochondrial membrane. Molecular oxygen is reduced to water by electrons donated by nicotine adenine dinucleotide (NADH) at complex I and flavin adenine dinucleotide (FADH2) at complex II. Meanwhile, protons are pumped into the intramembrane space to create a membrane potential resulting in a proton motive force which is harnessed by the enzyme ATP synthase in complex 5 to drive the synthesis of ATP from ADP and phosphate (Murphy, 2009).

Not all oxygen is reduced to water during oxidative phosphorylation; it is estimated that 0.2% to 2% is reduced to O_2^- (Madamanchi and Runge, 2007). O_2^- leak occurs in complex I, towards the mitochondrial matrix, and in complex II, towards both the intermembrane space and the mitochondrial matrix, where it is quickly dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) 1 and 2. Although O_2^- has a short half-life, within the matrix

radical-radical interaction with NO can occur to form RNS. H_2O_2 is produced in large quantities in the intermembrane space, and is sufficiently stable to migrate outwith the mitochondrion and participate in cell signalling cascades elsewhere in the cell.

A number of mitochondrial scavenger systems exist to eliminate ROS. As described, H_2O_2 is produced from O_2^- by SOD1 in the intermembrane space, and mitochondrion specific SOD2 in the matrix (Okado-Matsumoto and Fridovich, 2001). H_2O_2 is then reduced to water by glutathione peroxidase (GPx), resulting in oxidation of glutathione (GSH) in the process. H_2O_2 is also reduced by the enzyme catalase, but this is only present in cardiac mitochondria (Radi et al., 1991). Peroxiredoxins are antioxidant enzymes with high affinity for H_2O_2. Overexpression of peroxiredoxin-3 prevents left ventricular hypertrophy and failure following myocardial infarction (Rhee et al., 2005) suggesting a role in cellular protection from excessive mtROS which occurs during ischaemia. Thioredoxins are another family of small proteins relevant to mtROS scavenging; thioredoxin-2 reduces mitochondrial permeability to ROS thereby preventing downstream effects of mtROS (He et al., 2008).

Conversely, a number of local and systemic factors increase mtROS. There is a linear relationship between cellular oxygen concentration, and mtROS production, albeit with a paradoxical rise in the setting of profound hypoxia (Guzy and Schumacker, 2006). There is also an association between mitochondrial membrane potential and mtROS production, with an increase in O_2^- occurring at extremes of both high and low values (Toime and Brand, 2010). ROS itself increases mtROS production (Zorov et al., 2014), in a process termed "ROS induced ROS". The mechanism behind this is poorly understood, but may occur through the protein p66[Shc], found in the mitochondrial intermembrane space. p66[Shc] exists in an active and inactive state, and can be activated by oxidants such as oxidised LDL and inactivated by antioxidants such as GSH. When activated p66[Sch] forms a complex with cytochrome c and transfers electrons to molecular oxygen to directly form O_2^- (Nemoto et al., 2006). Other factors which increase mtROS production include and transcription factors such as p53 and NFκB (Szczepanek et al., 2012). Angiotensin II also increases mtROS production via protein kinase C, resulting in depletion of mitochondrial GSH, and an increase in cellular H_2O_2 and peroxynitrite (Doughan et al., 2008).

1.4.2.2 NADPH oxidase

The NADPH oxidase (NOX) family of enzymes are membrane bound proteins which have the principle biological function of generating ROS by the transfer of electrons to molecular

oxygen. There are seven subtypes of NOX which are expressed variably in a diverse range of cell types. NOX2 is the prototypical NADPH oxidase enzyme, expressed in phagocytes, typically existing in the membrane of intracellular vesicles in association with p22^{phox}. Upon phagocyte stimulation, p22^{phox} interacts with phosphorylated p47^{phox} and other cytoplasmic subunits, resulting in the active NOX2 enzyme complex which binds with the plasma membrane, or phagosome membrane. NOX2 transfers electrons from its donor, NADPH, to molecular oxygen, producing ROS as part of anti-microbial defences (Babior et al., 2002).

The other NOX isoforms share a number of features with NOX2, including their enzymatic activity, subunit structure, and transmembrane location. Several of the NOX isoforms are dependent on the same cytosolic subunits for activation, with p22^{phox} associating with NOX1, NOX3 and NOX4, as well as NOX2 (Ambasta et al., 2004).

All isoforms are widely distributed. In the vasculature, there is expression of NOX1, NOX4, and even NOX2 by endothelial cells (ECs) and VSMC (Brandes and Kreuzer, 2005). In the kidney there is high expression of NOX1, NOX4, and NOX5 in the cortex and by renal tubular cells (Gill and Wilcox, 2006). NOX expression and activity is induced by signalling molecules such as angiotensin II, and transcription factors such as TGFβ-1, TNFα and platelet derived growth factor (PDGF) (Manea et al., 2015). Expression of obligate subunits also represents a means of NOX regulation, and expression of p22^{phox} and others is induced by angiotensin II.

Evidence for the involvement of NOX in CVD can be found in patients with chronic granulomatous disease, who have an inherited mutation in genes encoding the components of NOX2. These individuals have early mortality due to impaired innate immunity, and also have increased production of pro-inflammatory mediators and recurrent infection. Despite the increased prevalence of vascular risk factors and presence of chronic inflammation, these patients have better endothelial function than matched controls, and reduced atherosclerotic burden, concomitant with reduced NOX2 activity and reduced levels of downstream markers of oxidative stress such as isoprostanes and oxidised LDL (Sibley et al., 2014). Furthermore, several NOX isoforms are inhibited by statins, a mechanism by which part of the beneficial effect of therapy might be mediated. Although NOX4 is most highly expressed in the vasculature, there is greatest evidence for the role of NOX1 in vascular disease. For example, mice which are NOX1 deficient have a lower systemic blood pressure (Gavazzi et al., 2006), and a blunted hypertensive response to angiotensin II (Matsuno et al., 2005). A similar effect is observed in p47^{phox} deficiency, such that it has been postulated that NOX1 in association

with the p47^{phox} subunit is responsible for ROS dependent hypertension (Landmesser et al., 2002).

Renal NOX expression has also been implicated in hypertension and CVD. For example, salt loaded rats were found to have increased expression of NOX2 and p47^{phox} in the renal cortex, and increased markers of oxidative stress, even in the presence of renin angiotensin inhibition (Kitiyakara et al., 2003), suggesting a direct link between sodium intake and renal ROS production. NOX are also highly expressed in the renal cortex of spontaneously hypertensive (Chabrashvili et al., 2002), and 5/6 nephrectomised rats. In a human study using laser Doppler flowmetry as a marker of endothelial function, NO dependent vasodilatation was reduced in CKD but improved by tempol, and by the NADPH oxidase inhibitor apocynin (DuPont et al., 2014). Taken together, the data suggests the increased expression and activity of a number of NOX isoforms in patients with renal disease and their involvement in endothelial dysfunction.

1.4.2.3 Xanthine oxidase

Xanthine oxidoreductase (XOR) is part of the molybdoflavin enzyme family, and exists interchangeably as xanthine oxidase (XO) and xanthine dehydrogenase (XDH). *In vivo*, most XOR exists as XDH, but conversion can occur due to oxidation of thiol groups (Hille and Nishino, 1995). XOR reacts with many substrates, but its classical role is purine degradation, catalysing the conversion of hypoxanthine to xanthine, and xanthine to uric acid. XOR is widely distributed, and in mammals is found in highest amounts in the liver and the small intestine, but is also expressed within ECs and cardiac myocytes, both in the cytoplasm and on the cell membrane. Basal expression is low, but is upregulated by many cell specific transcription factors including IFN-γ, TNF-α, IL-1, IL-6, glucocorticoids and hypoxia (Hoidal et al., 1997).

During oxidation of hypoxanthine or xanthine, electrons are donated first to the molybdenum site, then transferred via the FAD$^+$ site, to reduce either molecular oxygen or NAD$^+$ (Xia et al., 1999). XDH preferentially reduces NAD$^+$, aside from in settings of NAD$^+$ deficiency, which can occur in the setting of inflammation. Reduction of molecular oxygen results in production of O_2^- and H_2O_2. In the past much focus has been on O_2^- production by XOR, but more recent research has suggested that of the two forms of ROS, H_2O_2 is produced in far greater quantities (Kelley et al., 2010).

It is also increasingly understood that XOR exists within a complex redox system; for example XOR can also catalyse the formation of NO from its degradation products, especially in hypoxic conditions when NOS is inhibited (Millar et al., 1998). Furthermore, urate has both oxidant and antioxidant properties. On the one hand, several oxidants are scavenged and neutralised by urate, and XOR, and therefore ROS production, is also inhibited by urate. Conversely, urate has pro-oxidant effects, forming radicals with other oxidants including O_2^- and peroxynitrite, and inducing ROS production by NOX (Hayden and Tyagi, 2004). Indeed, whilst uric acid is thought to be an important antioxidant in blood, hyperuricaemia induces hypertension in rats (Sanchez-Lozada et al., 2005), and causes endothelial cell dysfunction in culture (Choi et al., 2014).

Nevertheless, there is evidence that XOR activity is involved in several cardiovascular conditions in humans, and that its inhibition may improve outcomes. Epidemiological evidence links hyperuricaemia with the presence of target organ damage in hypertension (Viazzi et al., 2005), and with poor outcomes in heart failure (Hamaguchi et al., 2011). In such patients, XOR activity is increased and is negatively correlated with endothelial function. A number of studies have investigated the use of the XOR inhibitor allopurinol and its metabolite oxypurinol, in a variety of cardiovascular settings including ischaemia-reperfusion injury, acute and chronic heart failure, and coronary artery disease. Allopurinol was shown to reduce oxidative stress and myocardial events following primary percutaneous coronary intervention in a study of 40 patients suffering ST segment elevation myocardial infarction (Rentoukas et al., 2010). In another study of chronic heart failure, allopurinol improved endothelial dependent vasodilatation in a dose dependent manner (George et al., 2006). A recent meta-analysis of XOR inhibition in CVD including 40 studies, found marked heterogeneity in outcome measures, with focus on surrogate markers rather than clinical end points (Higgins et al., 2012).

The association between oxidative stress and progression of CKD will be discussed in greater depth later in this chapter, however an abundance of data links ROS with pathophysiological processes associated with progression of CKD. Accordingly, various modulators of ROS production have been studied to determine if these may slow the decline of renal function, including inhibitors of XOR. A recent systematic review found 19 randomised control trials investigating the effect of allopurinol on CKD progression (Kanji et al., 2015), with meta-analysis finding in favour of allopurinol with a difference in eGFR of 3.2 ml/min.1.73m^2, as well as a reduction in blood pressure.

Although XOR is therefore an important source of ROS, further research is therefore needed to clarify the role of XOR inhibition in ameliorating the pathological sequelae of oxidative stress.

1.4.2.4 Uncoupled endothelial nitric oxide synthase

All three isoforms of nitric oxide synthase (NOS) produce NO by a 5 electron oxidation of a guanidine group of its substrate, L-arginine. NOS activity is reliant on molecular oxygen and NADPH as co-substrates, and tetrahydrobiopterin (BH_4), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) as cofactors of the reaction. NO production requires electron transfer from NADPH bound at the C-terminal, to a haem moiety at the N-terminal, via the flavin groups. Another electron is also probably donated by BH_4. At the haem group, molecular oxygen is reduced and activated, and used for the oxidation of L-arginine, firstly to hydroxyl-L-arginine, and then to L-citrulline and NO (Förstermann and Sessa, 2012). Endothelial nitric oxide synthase (eNOS) is the principle isoform of NOS within the cardiovascular system, present in ECs, cardiac myocytes, renal tubular epithelial cells and platelets. Under physiological conditions, eNOS constitutively produces vascular NO, which is involved in vasodilatation, angiogenesis, platelet and inflammatory cell activation, and other aspects of vascular function.

In certain circumstances, the reduction of molecular oxygen and haem becomes 'uncoupled' with the production of NO, such that O_2^- is produced instead. When this occurs, uncoupled eNOS contributes to oxidative stress by reduced production of NO, and increased production of O_2^- and its downstream products such as H_2O_2 and peroxynitrite (Forstermann et al., 1994). There is evidence that eNOS uncoupling is involved in oxidative stress and endothelial dysfunction in the presence of a range of typical cardiovascular risk factors including dyslipidaemia (Stroes et al., 1997), hypertension (Higashi et al., 2002) and diabetes (Heitzer et al., 2000).

A number of mechanisms are thought to be involved with eNOS uncoupling. Depletion of the essential cofactor BH_4 is one putative mechanism behind eNOS uncoupling, suggested by the fact that NO production by eNOS correlates with intracellular concentrations of BH4 (Werner-Felmayer et al., 1993), and that supplementation with BH_4 increases NO production (Pieper, 1997). BH_4 concentration is dependent on the balance between production by and degradation, as well as the degree of 'recycling' between BH_4 and its oxidised metabolites. Factors which influence this include the presence of ROS and derivatives; for example,

peroxynitrite causes oxidation of BH_4 to BH_3 and BH_2, which have no cofactor activity with eNOS (Kuzkaya et al., 2003). In a mouse model of accelerated atherosclerosis, treatment with peroxynitrite results in strikingly depleted BH_4 concentrations, and increased ROS production in a manner dependent on eNOS expression (Laursen et al., 2001).

L-arginine deficiency is another mechanism by which eNOS uncoupling can occur. That said, the normal plasma concentration of L-arginine is around 100 µmol/L, whilst the level below which NO production is significantly reduced is around 3 µmol/L, such that one might not expect L-arginine deficiency to play a role in eNOS uncoupling *in vivo* (Closs et al., 2000). Nevertheless, L-arginine supplementation has been shown to increase NO production, and improve endothelial function in hypertension and dyslipidaemia (Imaizumi et al., 1992).

Asymmetric dimethyl-L-arginine (ADMA) is a circulating inhibitor of eNOS, and can also cause eNOS uncoupling at high levels. The enzymes involved in both production and degradation of ADMA are redox sensitive such that ADMA levels are elevated in oxidative stress. Furthermore, ADMA is excreted by the kidneys and is elevated in renal impairment. ADMA is considered separately in section 1.4.2.5.

Finally, redox modification of eNOS can also lead to uncoupling. In conditions of oxidative stress, s-glutathionylation of thiol groups occurs and can alter enzyme function. S-glutathionylation of cysteine residues within the reductase domain results in eNOS uncoupling. Arteries from spontaneously hypertensive rats (SHR) show eNOS s-glutathionylation, associated with reduced NO and increased ROS production. This is associated with reduced endothelial dependent vasodilatation, and is ameliorated by reversal of s-glutathionylation by thiol specific reducing agents (Chen et al., 2010).

Therefore, a number of mechanisms contribute to eNOS uncoupling, and given that renal impairment is associated with increased serum ADMA, oxidative stress, and antioxidant deficiency, it is likely that these mechanisms contribute to ROS production in CKD. Evidence for this comes from the fact that 5/6 nephrectomised rats showed reduced oxidative stress, and increased endothelial dependent vasodilatation when supplemented with BH_4 and L-arginine; supplementation with both of these showed an additive effect (Arellano-Mendoza et al., 2011).

1.4.2.5 ADMA

ADMA, and its stereo-isomer symmetric dimethylarginine (SDMA), are by-products of the methylation of arginine residues on cellular proteins, a common translational modification resulting from the action of protein arginine methyltransferases (PRMT). PRMT-1 is found in a number of cells in the cardiovascular system, and symmetrically methylates arginine to produce ADMA which is released from cells via specific transporters. Methylated arginines are eliminated by urinary excretion and, in the case of ADMA, metabolised by dimethylarginine dimethylaminohydrolase (DDAH). In renal impairment, plasma ADMA levels are elevated due to reduced urinary excretion, as well as by inhibition of DDAH by hyperhomocysteinaemia (Tarnow et al., 2004). In states of oxidative stress, PRMT-1 is upregulated, and DDAH downregulated, which is another mechanism by which ADMA is increased in CKD. ADMA inhibits all isoforms of NOS, and also causes eNOS uncoupling resulting in O_2^- production, and is therefore an important mediator of oxidative stress (Sydow and Munzel, 2003).

ADMA has been identified as a marker of endothelial dysfunction and cardiovascular outcomes. Cardiovascular mortality and event rate has been shown to be correlated to plasma ADMA concentration in a number of settings including atrial fibrillation (Xia et al., 2008) and after coronary angioplasty (Cavusoglu et al., 2009). Furthermore, ADMA has been found to be associated with cardiovascular event rate in chronic kidney disease, as well as in haemodialysis patients (Zoccali et al., 2001) and those with kidney transplants (Frenay et al., 2015). For example, in a study of 820 patients with stage 3 and 4 CKD, a one standard deviation increment in serum ADMA was associated with a 19% increase in cardiovascular mortality (Lu et al., 2011).

1.4.3 Regulation of oxidative stress by antioxidant systems

Antioxidants are a diverse array of substances which neutralise oxidants, and/or prevent oxidation of downstream macromolecules. Antioxidants can be classified by whether they act in a non-specific ROS scavenging manner, or whether they act in a substrate specific manner. They can also be categorised by whether they are lipid or water soluble; the former being important in preventing oxidation of cell membrane components, whilst the latter regulating the redox state of plasma and cytoplasm. Similar to ROS production, the predominance of any particular antioxidant varies between cell type and intracellular locations.

1.4.3.1 Enzymatic antioxidants

The major enzymatic antioxidants include SOD, catalase, peroxiredoxin, and glutathione peroxidase. The three SOD isoforms catalyse the breakdown of O_2^- to H_2O_2 and O_2, and are present in high quantities around cellular locations of ROS production, such as the mitochondria. In addition to mitochondrial SOD, the other SOD isoforms include extracellular SOD, which is produced and secreted by vascular smooth muscle cells and binds to components of the extracellular matrix to regulate interstitial redox status (Wassmann et al., 2004).

Reduced glutathione plays an essential role in intracellular redox regulation by providing reducing potential for many cellular processes. Several isoforms of the enzyme glutathione peroxidase (GPx) are found in abundance in cytoplasm. GPx-1 reduces H_2O_2, whilst oxidised lipids are the preferred substrate of GPx-4. GSH is oxidised to glutathione disulphide (GSSG) in the process, which can be recycled to GSH by the enzyme glutathione reductase, with NADPH acting as the electron donor (Sindhu et al., 2005).

Thioredoxins are a ubiquitous groups of small ubiquitous proteins which are important intracellular antioxidants. Thioredoxins contain reduced cysteine residues which allow enzymatic repair of oxidative damage to cellular proteins, by reducing thiol disulphide bonds which develop in the context of oxidative. The enzyme thioredoxin reductase reduces thioredoxins in order to recycle them for their participation these reactions (Ebrahimian and Touyz, 2008). Overexpression of thioredoxins increases the lifespan of transgenic mice (Yoshida et al., 2005), and reduces the development of diabetic nephropathy in a mouse model of diabetes (Hamada et al., 2007).

1.4.3.2 Non-enzymatic antioxidants

Non enzymatic antioxidants are generally low molecular weight proteins which remove ROS by undergoing oxidation themselves. These include ascorbic acid (vitamin C), vitamin E, glutathione, bilirubin and urate.

Ascorbic acid is a six carbon ketolactone, which acts as an obligate cofactor for a number of enzymes. Humans, like all primates, cannot synthesize ascorbic acid such that we rely on dietary intake of ascorbic acid to prevent vitamin C deficiency. The current recommended daily intake of ascorbic acid is 90 – 100mg per day in non-smoking men and women (Carr and Frei, 1999), based on the amount required to prevent overt clinical features of scurvy,

the prototypical vitamin C deficiency syndrome. Ascorbic acid acts as a reducing agent to scavenge ROS, and also acts as an antioxidant by stabilising BH_4, facilitating its action as a co-substrate for NO formation (Abudu et al., 2004). Conversely, ascorbic acid also has pro-oxidant properties, producing ROS by reaction with iron. Of the group of eight tocopherols collectively termed "vitamin E", α-tocopherol is the most biologically active, whilst γ-tocopherol is the commonest in the typical Western diet. Vitamin E is a lipophilic antioxidant, particularly prevalent in the cell membrane (Ando and Tappel, 1985).

Glutathione is a cysteine containing peptide and has a number of antioxidant actions typically exerted intracellularly, where it is found at high concentration. The active thiol group of the cysteine residues donate electrons to scavenge ROS and maintain NADPH in its reduced form (Masella et al., 2005). Additionally, reduced glutathione maintains ascorbic acid in its reduced form in order to facilitate its antioxidant effect. Oxidised glutathione is termed glutathione disulphide, and is reduced by glutathione reductase and NADPH; the ratio of glutathione to glutathione disulphide is often measured as a marker of redox state.

1.4.3.3 Antioxidant regulation

Key to regulation of antioxidant enzyme transcription is the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) – antioxidant response element (ARE) signalling pathway. The Nrf-2 transcription factor is a master regulator of antioxidant activity, and regulates transcription through binding with ARE promotor regions present on several antioxidant genes (Jaiswal, 2004). Whilst there is constitutive activity of Nrf-2, this is limited by its ubiquitin dependent degradation. Nrf-2 is kept within the cytoplasm by Kelch-like ECH-associated protein 1 (Keap-1) which facilitates its ubiquitination. Interaction between Nrf-2 and Keap-1 can be impaired directly by oxidation of Keap-1 thiol groups, or indirectly via Nrf-2 phosphorylation by specific protein kinases; both of these mechanisms reduce Nrf-2 ubiquitination and allow translocation to the nucleus where binding to ARE can occur. ARE was first discovered on the NADPH:quinone oxidoreductase 1 (NQO1) gene, but it is now known that Nrf-2 upregulates the transcription of many genes, including thioredoxin reductase, SOD, glutathione reductase, and many others, including those involved in GSH synthesis (Ma, 2013).

1.5 Endothelial dysfunction

1.5.1 Endothelial dysfunction and cardiovascular disease

The endothelium is the single cell luminal lining of the vascular system and releases a variety of factors which play an important role in regulation of VSMC tone and proliferation, inflammatory cell chemotaxis and activation, and platelet activation and aggregation. In particular, NO produced by ECs diffuses freely into VSMC where it activates guanylate cyclase and causes endothelium dependent vasodilatation (figure 1.2) (Ignarro, 1990), although vasodilatation is also mediated by other vascular signalling molecules including endothelium derived hyperpolarising factor (EDHF) (Félétou and Vanhoutte, 2006).
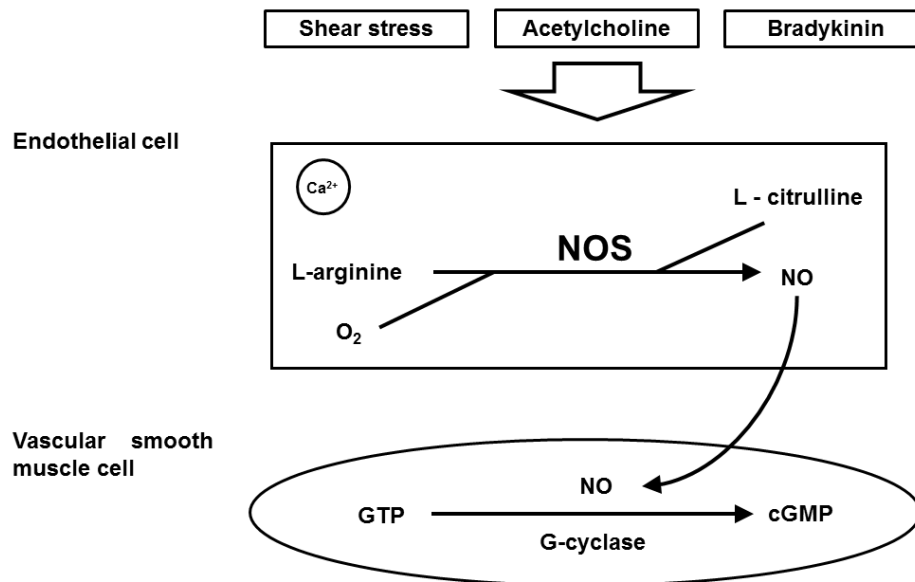
Figure 1.2 VSMC relaxation in response to NO

NO is generated from by NOS also utilising molecular oxygen whilst producing the by-product L-citrulline. NO is freely diffusible through cell membranes, and moves from the endothelial cell to the vascular smooth muscle cell where it activates G-cyclase. The cGMP which is produced activates protein kinases which ultimately lead to release of intracellular calcium and smooth muscle relaxation. NO = Nitric oxide, NOS = Nitric oxide synthase, G-cyclase = Guanylyl cyclase, cGMP = cyclic guanosine monophosphate.

NO also has cyclic guanine monophosphate (cGMP) independent effects, exerted via s-nitrosylation (SNO) of protein cysteine residues on signalling molecules, which reduces their biological activity (Lima et al., 2010). In many cases, such as the SNO of mitogen-activated protein kinase phosphatase 7 (MPK7), NFκB and tissue factor, this has anti-atheromatous effects (Marshall and Stamler, 2001). Oxidative stress is associated with reduced

bioavailability of NO by a number of mechanisms already discussed: inhibition and uncoupling of eNOS, depletion of NO by its reaction with ROS to form peroxynitrite, and an increase in circulating ADMA. Thus, oxidative stress is associated with reduced bioavailability of favourable signalling molecules such as NO, together with alteration of signalling cascades which promote proliferation and inflammation, which ultimately predisposes to CVD.

Data from the Multi Ethnic Study of Atherosclerosis demonstrated that the presence of endothelial dysfunction predicts future cardiovascular events in patients without a history of vascular disease, even after adjustment for conventional cardiovascular risk factors (Yeboah et al., 2009b). Endothelial dysfunction has also been observed in the presence of many cardiovascular risk factors including hypertension (Panza et al., 1993, Panza et al., 1990), hypercholesterolaemia (Kawano et al., 2002), diabetes (Nitenberg et al., 1993) and smoking (Heitzer et al., 1996). Endothelial function is reduced in the presence of established CVD and in proportion to its severity (Davignon and Ganz, 2004); for example, flow mediated dilatation (FMD) is 10.2% in patients without coronary artery disease, 7.7% in patients with single vessel disease, 5.2% in two vessel disease and 2.0% in triple vessel disease (Kaku et al., 1998). Taken together, it has been postulated that endothelial dysfunction represents the earliest stage of the development of atherosclerosis and CVD, as well as a final common mechanism by which conventional cardiovascular risk factors exert pathophysiological effect.

1.5.2 Endothelial dysfunction and chronic kidney disease

As has already been described, a number of factors can disrupt redox homeostasis via dysregulation of the various sources of ROS and antioxidant systems, including angiotensin II, oxidised LDL, and ADMA. It is therefore unsurprising that endothelial dysfunction has been observed in CKD, which is characterised by inflammation, activation of the renin angiotensin aldosterone system (RAAS), and an increase in circulating NOS inhibitors such as ADMA.

Exposure of ECs in culture to uraemic serum results in switching to a pro-atherogenic phenotype. For example, a proteomic analysis showed increased expression of proteins relating to inflammation and oxidative stress, such as proteasome components, superoxide dismutase and glutathione peroxidase, which probably occurs via NFκB signalling (Carbo et al., 2008). Similarly, rabbit aortic ECs cultured in uraemic serum show increased NFκB

nuclear translocation and DNA binding, reduced NO production and increased TNFα production (Feng et al., 2011). In some studies uraemic serum was shown to increase EC proliferation (Monroy et al., 2015, Serradell et al., 2003) whereas in other studies reduced proliferation, and rather apoptosis was observed (García-Jérez et al., 2015). The effect may depend on the concentration of uraemic serum, with proliferation seen at lower concentration, and apoptosis at higher concentrations; or to draw a clinical correlation, the effect of uraemia on the vascular endothelium may depend on the severity of renal disease. Patients with chronic kidney disease also show reduced differentiation of endothelial progenitor cells (Goligorsky et al., 2010), which may reflect reduced capacity for vascular repair.

In wire myography studies, isolated small resistance vessels derived from animal models or human tissue are mounted onto a myograph and exposed to various vasoactive substances in order to measure reactivity. Resistance vessels from patients with CKD show reduced vasodilatation to acetylcholine, but maintained vasodilatation to sodium nitroprusside, suggestive of impaired endothelial dependent vasodilatation. Application of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) to control vessels results in a vasodilator profile similar to the CKD vessels, confirming that the reduced NO bioavailability is involved in the mechanism of endothelial dysfunction (Morris et al., 2001).

These *ex vivo* results are reflected by the results of *in vivo* vascular function studies such as FMD and venous occlusion plethysmography. For example, in one study of 105 patients with CKD, FMD was reduced to 3.8% compared to 5.7% in controls (Dogra et al., 2006). In another study of 304 patients with CKD not on dialysis, FMD was reduced in a stepwise manner with increasing severity of renal impairment (Yilmaz et al., 2011). Similarly, endothelial function measured using plethysmography was shown to be reduced in CKD; brachial artery blood flow showed a lesser response to metacholine infusion in CKD as compared to controls, whilst response to nitroprusside infusion was the same in both groups (Morris et al., 2000).

1.5.3 Causes of endothelial dysfunction in chronic kidney disease

There is therefore evidence to show that uraemic serum is damaging to ECs in culture that explanted vessels from patients with CKD have an impaired vasodilator response, and that patients with CKD have endothelial dependent vasodilatation. There is also evidence that this effect is mediated by oxidative stress.

1.5.3.1 Upregulated sources of ROS

Du Pont et al, measured cutaneous vascular function in response to a number of vasoactive substances in patients with CKD compared to healthy controls (DuPont et al., 2014). They found reduced NO dependent vasodilatation, which was improved by administration of the SOD mimetic tempol, and the NOX inhibitor apocynin, suggesting that the vascular dysfunction associated with CKD is at least in part mediated by NADPH oxidase derived ROS.

In a study examining the determinants of endothelial dysfunction, CKD was associated with reduced endothelial function, with correlation between eGFR and FMD (Yilmaz et al., 2006), and increased biomarkers of oxidative stress. On multivariate analysis, ADMA and oxidised LDL cholesterol were significant predictors of endothelial dysfunction. Other studies have found endothelium dependent vasodilatation to be positively correlated to total antioxidant activity and serum glutathione concentration, and negatively associated with oxidised glutathione (Annuk et al., 2001). Khaira et al found that FMD was positively associated with total antioxidant capacity and vitamin C, and to be negatively associated with the presence of oxidative end products such as thiobarbituric acid reactive substances (TBARS) (Khaira et al., 2011).

1.5.3.2 The renin angiotensin system

Angiotensin II impairs endothelial function by a number of mechanisms. Firstly, it causes tyrosine phosphorylation of eNOS, impairing NO production and impairing endothelium dependent vasodilatation (Loot et al., 2009). Secondly, it acts via angiotensin II type 1 (AT1) receptors and mitogen activated protein (MAP) kinase signalling to increase arginase activity which depletes L-arginine availability (Shatanawi et al., 2015). Lastly, it upregulates expression of the potent vasoconstrictor endothelin-1 (Hong et al., 2004). The RAAS is strongly activated in CKD, and pharmacological inhibition has been shown to improve endothelial function in other groups such as patients with coronary artery disease (Anderson et al., 2000) or hypertension (Goto et al., 2000). One study showed improvement in FMD after treatment with the ACE inhibitor Ramipril (Yilmaz et al., 2009). Similarly, aldosterone production is increased in CKD, and aldosterone has been shown to increase ROS production by NOX both in endothelial cells (Chrissobolis et al., 2014) and renal mesangial cells (Miyata et al., 2005). The role of mineralocorticoid antagonism in preventing complications of CKD is a subject of ongoing research.

1.5.3.3 Uraemic toxins

Uraemic retention toxins are water soluble or protein bound substances in blood which are increased in concentration by renal impairment. Given the levels of these toxins correlate inversely with GFR, it can prove difficult to elucidate an independent association with cardiovascular risk and outcome. Indoxyl sulfate is a protein bound uraemic toxin which is produced by the liver during tryptophan metabolism, and has recently been shown to independently predict CVD and mortality in CKD patients (Lin et al., 2012). Endothelial cells cultured in indoxyl sulfate show increased ROS production which is ameliorated by NOX inhibition, and show markedly reduced levels of the antioxidant glutathione (Dou et al., 2007). Indoxyl sulfate has also been associated with progressive renal impairment, and reduces renal Nrf-2 expression via NFκB signalling (Bolati et al., 2013). Other uraemic retention toxins implicated in causing endothelial dysfunction include *p*-cresyl sulfate and malondialdehyde (Watanabe et al., 2015).

1.5.3.4 ADMA in chronic kidney disease

As described earlier, ADMA is a circulating inhibitor of NO production and causes eNOS uncoupling, and its concentration in serum is inversely correlated to eGFR. ADMA reduces NO production by competing with the substrate L-arginine and also by other mechanisms, such as inhibition of eNOS phosphorylation at the Ser1177 residue (Kajimoto et al., 2012). ECs cultured in high concentrations of ADMA show impaired NO production higher affinity to mononuclear cells (Böger et al., 2000), whilst vessels exposed to ADMA show reduced endothelium dependent vasodilatation (Cardounel et al., 2007). In patients with CKD, ADMA has been shown to be an independent predictor of endothelial function, and for every 0.1 µmol/L increase in ADMA, there is a 37% increase in a composite outcome of all-cause mortality, myocardial infarction and stroke (Tarnow et al., 2004). Indeed, even in healthy individuals with normal renal function, a negative correlation has been documented between ADMA and FMD (Ardigo et al., 2007). Moreover, baseline ADMA predicted FMD measured at six years of follow up (Furuki et al., 2008).

1.5.3.5 Inflammation

CKD is a state of low grade inflammation, characterised by an elevation in many inflammatory chemokines and activation of immune cells. For example, a number of studies have shown elevations in C reactive protein (CRP), IL-6, TNFα, and a number of adhesion molecules (Panichi et al., 2001, Lee et al., 2015). There is also activation of immune cells,

especially M1 macrophage (Li et al., 2015) and polymorphonuclear leukocytes (Sela et al., 2005).

Inflammation is highly associated with oxidative stress; many inflammatory pathways are activated by disturbed redox signalling, whilst ROS are also produced as an end result of many inflammatory processes. Activation of the immune system in CKD may in part be caused by increased activity of NFκB, which regulates expression of a number of inflammatory mediators (Li and Karin, 1999), and NFκB is upregulated by ROS and glutathione depletion (Morgan and Liu, 2011). NFκB also reduces Nrf-2 expression thereby reducing antioxidant capacity.

A cross sectional analysis of patients with CKD 3 – 5 found elevations in inflammatory markers even in moderate renal disease, which was associated with platelet activation and increased von Willebrand factor, a circulating marker of endothelial damage (Landray et al., 2004).

1.5.3.6 Reduced antioxidant capacity

In addition to these pro-oxidant processes which are augmented in CKD, endothelial dysfunction also arises from impaired antioxidant capacity. Firstly, there is a reduction in exogenous antioxidants: in a study of 58 patients with CKD, there was reduction in the serum concentration of vitamin C which correlated with eGFR, and this was associated with reduced FMD (Takahashi et al., 2011). Some studies have shown reduced vitamin E in patients with CKD, whilst others have found this only in patients receiving haemodialysis. Other non-enzymatic antioxidants such as bilirubin are also reduced, and total antioxidant capacity, a measure of extracellular non enzymatic reducing potential, has been shown to be reduced in proportion to severity of renal impairment. The levels of many enzymatic antioxidants are also reduced in CKD, including SOD, GPx, catalase, and paraoxonase-1 (Johnson-Davis et al., 2011). Furthermore, regulation of antioxidant gene expression has also been demonstrated in CKD (Atamer et al., 2008). In an animal model of renal impairment, Kim et al, showed increased lipid peroxidation, glutathione depletion, mononuclear cell activation and increased NFκB signalling in 5/6 nephrectomised rats (Kim and Vaziri, 2010). This was associated with a reduction in Nrf-2, and increase in the repressor protein Keap-1, with resultant down regulation of Nrf-2 target genes such as catalase, SOD, glutathione peroxidase and NQO1.

1.6 Arterial function and stiffness

In addition to the deleterious alterations in EC function, changes to vascular structure also occur in CKD which bestow cardiovascular risk. This vascular remodelling is caused by a number of pathophysiological processes associated with uraemia, many of which are common to endothelial dysfunction, and are also related to oxidative stress.

1.6.1 Structure and function of the arterial system

The role of the vasculature is to provide continuous blood flow to end organs which require constant perfusion in order to avoid tissue ischaemia and dysfunction. Vascular function therefore involves conversion of pulsatile blood flow occurring during each cardiac cycle into a constant flow, and facilitating transit of blood to peripheral organs and tissues.

The aorta and large arteries are termed conduit arteries and are extremely compliant, which accommodates a large volume of blood which is ejected during systole. This elasticity generates potential kinetic energy which is released during diastole when the recoil of the aorta causes forward propulsion of blood. This results in a cushioning effect which dampens peak systolic pressure and augments diastolic pressure, which is thought to protect the end organ microcirculation from high pressure and maintain coronary blood flow during diastole. Small arteries are less distensible due to differences in the composition of their extracellular matrix and are termed resistance arteries (Westerhof et al., 2009).

The arterial wall has viscoelastic properties which results from the nature of its underlying components, including VSMC, endothelial cells, and extracellular matrix. The relative proportions of the major scaffolding proteins, collagen and elastin, are especially important in determining vascular compliance, and alterations to the normal cycle of degradation and production of these can result in arterial stiffness.

Arterial stiffness is therefore characterised histologically by disordered endothelial cells, increased collagen, damaged elastin and infiltration by inflammatory cells, pathologically by increased intima to media thickness, hypertrophied and proliferated VSMC, and vascular calcification, and clinically by systolic hypertension, and increased pulse pressure. These changes can be measured non-invasively as described in section 1.9.3, by characteristic abnormalities in the arterial waveform, and by an increase in the pulse wave velocity (PWV).

1.6.2 Alterations in the extracellular matrix

Matrix metalloproteinases (MMP) are a group of endopeptidases involved in the turnover of elastin and collagen which are important in the determining the elasticity of the arterial wall (Hadler-Olsen et al., 2011). Excessive MMP activity results in uncoiled, ineffective collagen and degraded elastin and evidence exists which implicates MMP, especially MMP-2 and MMP-9, in the development of arterial stiffness (Yasmin et al., 2005). MMP-2 has been found to be upregulated in arteries of patients undergoing renal transplantation, with expression negatively correlated with contractility and endothelial dependent relaxation. Pharmacological inhibitors of MMP such as doxycycline improve arterial stiffness (Chung et al., 2008) and prevent aortic dissection in Marfanoid mice (Xiong et al., 2008), demonstrating the important of the endopeptidases to normal vascular structure and repair. The expression of MMP by inflammatory and endothelial cells is up regulated by cytokines and ROS. For example, ROS signalling through MAP kinase results in upregulation of MMP-1, and oxidative inhibition of tyrosine phosphatases results in downregulation of MMP inhibitors (Kar et al., 2010).

Advanced glycation end products (AGE) result from the non-enzymatic reaction between cellular proteins and glucose via the Maillard reaction (McNulty et al., 2007) which occurs in a time dependent manner relative to serum glucose concentration. AGE result in excessive and abnormal cross linking of collagen peptides, which results in a stiff and inflexible extra cellular matrix, and also cause collagen cross linking which inhibits its proteolysis, serving to perpetuate these undesirable collagen molecules within the vascular matrix (Semba et al., 2009). AGE also stimulate production of inflammatory cytokines and reactive oxygen species by interaction with immunoglobulin superfamily receptors, establishing an inflammatory milieu ideal for promotion of arterial stiffness and endothelial dysfunction.

1.6.3 Endothelial and vascular smooth muscle cell dysfunction

VSMC tone is determined both inherently, by interaction with endothelial cells, and due to circulating factors such as angiotensin II. Hypertension, by increasing intraluminal pressure, results in mechanosensitive changes to calcium signalling within VSMC which increases muscle tone. Hypertension is therefore strongly associated with stiffness, both as a cause and a consequence. ECs are involved in regulating VSMC tone through a number of signalling molecules including NO and EDHF. The association between EC and VSMC function was investigated in a study of 89 healthy volunteers (McEniery et al., 2006). FMD was inversely associated with PWA, PWV, and central BP, which remained statistically significant after

adjustment for confounders, demonstrating the relationship between endothelial function and arterial stiffness.

1.6.4 Effect of systemic factors on arterial function and stiffness

Local and systemic activation of the RAAS is another contributor to arterial function and remodelling. VSMC tone is increased by circulating factors such as angiotensin II, endothelin and ROS, which are released locally by endothelial cells and can act in a paracrine fashion to reduce distensibility of the vascular wall. Angiotensin II also causes VSMC hypertrophy, proliferation, and conversion to an inflammatory phenotype. When angiotensin II is applied directly to VSMC, there is an increase in IL-6 production and NFκB activation, in a dose dependent manner which is abrogated by inhibition of ROS production (Kranzhofer et al., 1999). Remodelling is also mediated by angiotensin II via increased production of MMP (Takagishi et al., 1995) and collagen by VSMC (Mifune et al., 2000). These effects on arterial stiffness can be ameliorated by pharmacological RAAS blockade, and indeed this might account for some of the survival benefit of this treatment. In a study of 150 patients receiving maintenance haemodialysis, ACE inhibitors reduced cardiovascular and all-cause mortality in a blood pressure independent manner; those patients whose PWV was reduced by therapy were significantly more likely to have a survival benefit (Guerin et al., 2001).

Aldosterone has also been implicated in the development of arterial stiffness. In normotensive rats, supplementation with angiotensin II caused arterial thickening, impaired endothelium dependent vasodilation, together with activation of NADPH oxidase and an increase in TBARs (Virdis et al., 2002). These effects were corrected by spironolactone, suggesting that aldosterone is a cause of vascular remodelling, mediated in part by endothelium dysfunction and oxidative stress. A randomised controlled trial of spironolactone in patients with stages 2 and 3 CKD found a significant reduction in arterial stiffness and left ventricular mass index (Edwards et al., 2009). The Randomised Aldactone Evaluation Study (RALES) was a large multi-centre randomised control trial which demonstrated the benefit of mineralocorticoid inhibition in heart failure. Subgroup analysis from the RALES study showed that patients with high levels of procollagen peptides had a higher risk of mortality, and these markers reduced during spironolactone therapy (Zannad et al., 2000); indeed those with higher markers of vascular fibrosis were more likely to benefit from mineralocorticoid inhibition. All things considered, these results suggest that aldosterone is involved in arterial remodelling via activation of pro-fibrotic pathways.

1.6.5 Vascular calcification

The phenomenon of vascular calcification in renal impairment is readily appreciated by viewing an abdominal or pelvic X-ray of a patient receiving maintenance haemodialysis, where the radio-opaque tramlines of calcified vessels are clearly visible. Vascular calcification is characterised by deposition of calcium phosphate within vessels, and is now understood to be an active, cell mediated process, whereby VSMC convert to an osteoblast like phenotype, releasing matrix and inflammatory chemokines. Two types of vascular calcification exist: intimal calcification is a focal process associated with atherosclerosis, and results in localised inflammation and formation of occlusive plaques. Medial calcification is a diffuse process, typically characterised by mineralisation within the medial layer of conduit vessels. The latter, historically termed Mönckeberg's arteriosclerosis, is common in CKD. The two processes can coexist in the same patient, and indeed the same vessel, and both are associated with cardiovascular mortality. In a study of patients with pre dialysis CKD, vascular calcification was assessed by CT of the abdominal aorta and superficial femoral artery, and found to be associated with poor renal function and higher PWV (Toussaint et al., 2008).

A number of elements of the uraemic milieu have been shown to promote vascular calcification. Disturbance in calcium and phosphate metabolism which occurs in CKD may cause calcification directly, by deposition within vessel walls, but also activates pro-calcific mechanisms in VSMC. Phosphate is transported into VSMC by sodium dependent phosphate co-transporter, Pit-1, and causes osteogenic differentiation by activation of transcription factors such as core binding factor a1 (cbfa-1) (Steitz et al., 2001). Uraemic serum also causes VSMC osteoblastic differentiation independently of phosphate concentration however, and this might be mediated through oxidative stress. For example, H_2O_2 was shown to induce an osteogenic phenotype in VSMC, signalling via Runx2, Msx2-wnt, and NOX (Byon et al., 2008). Indeed, even the calcific effects of hyperphosphataemia are mediated in part through redox signalling. In a model of uraemic vascular calcification using bovine aortic smooth muscle cells, calcium deposition was abrogated by inhibition of mtROS production (Zhao et al., 2011). Amongst others, indoxyl sulfate is one specific uraemic toxin which has been shown to induce arterial calcification and expression of osteogenic proteins.

Calcium based phosphate binders have historically been used in renal impairment to reduce serum phosphate concentration in order to manage CKD bone mineral disease. There is a

concern however, that iatrogenic calcium loading may contribute to the vascular calcification seen in these patients. As with phosphate, calcium causes calcification of VSMC *in vitro*, and studies of non-calcium containing phosphate binders have shown a reduction in vascular calcification compared with calcium based therapies. As yet however there is no evidence showing improved cardiovascular outcomes associated with a calcium-free approach to phosphate lowering. There is a similar controversy regarding the role of vitamin D replacement. Vitamin D deficiency is common in CKD and induces hyperparathyroidism which is highly associated with cardiovascular outcome and arterial stiffness. Conversely, some studies have found no association between vitamin D levels and vascular calcification (Barreto et al., 2009), and it has been shown that high doses of 1-25-vitamin D can induce osteoblastic differentiation of VSMC.

As well as these biochemical, hormonal, and iatrogenic elements contributing to vascular calcification in CKD, there is deficiency of a number of protective mechanisms. Circulating regulators of extracellular calcium such as fetuin A and matrix GLA protein are reduced in CKD (Luo et al., 1997), and patients with the lowest levels of these inhibitors have worse cardiovascular outcomes. Pyrophosphate depletion causes massive arterial calcification in mice, and levels of this inhibitor are reduced by HD.

1.6.6 Arterial stiffness and chronic kidney disease

In summary then, CKD is associated with a number of processes which cause vascular calcification, VSMC proliferation and dysfunction, and alterations to the extracellular matrix which induce arterial stiffness. Patients with renal impairment suffer accelerated age associated arterial stiffening, and those patients with demonstrably higher arterial stiffness suffer worsened cardiovascular outcomes.

1.7 Oxidative stress and endothelial dysfunction in the kidney

The discussion so far has focused on oxidative stress as a mediator of CVD, however redox signalling has a role in the regulation of kidney function in normal physiology, and there is evidence that oxidative stress plays a role in the pathophysiology of renal impairment, and is an important mediator of progressive CKD.

1.7.1 Redox signalling in renal physiology

Redox signalling plays a role in several aspects of renal function, and as such many of the regulatory components of redox physiology have been identified in the kidney. For example, all three isoforms of NOS are highly expressed, including eNOS which is expressed within the renal vasculature, especially in the glomerulus. Neuronal NOS is expressed by macula densa cells and is involved in tubuloglomerular feedback. Inducible NOS is expressed at high levels even under basal conditions, especially within the interstitium, although its transcription is further enhanced by a number of factors including lipopolysaccharide, hyperglycaemia and hypertension. Several NOX isoforms are also highly expressed, especially NOX-4 which is abundantly produced in the renal cortex.

Infusion of L-NAME into rats with normal renal function causes afferent and efferent arteriolar vasoconstriction, reduced filtration fraction, reduced medullary blood flow and systemic hypertension (Zatz and de Nucci, 1991). Many of the effects of local and systemic RAAS are also mediated through redox signalling. For example, renal afferent arterioles infused with angiotensin II show impaired endothelium mediated dilatation, together with increased NOX mediated generation of O_2^- and an increase in excretion of peroxidated lipids (Tojo et al., 2002). Angiotensin II infusion also results in upregulation of transcription factors such as NFκB, and cytokines which disrupt normal redox homeostasis. Co-infusion with tempol ameliorates this, and prevents isoprostane excretion (Ding et al., 2015).

There is also evidence of redox signalling involvement in the tubuloglomerular feedback mechanism, by which macular densa cells sense sodium delivery to the distal tubule and regulate glomerular pressure. Accordingly, in response to increased tubular sodium delivery, vasoconstriction is induced primarily in the afferent arteriole, resulting in reduced glomerular pressure and a reduction in single nephron GFR. Macula densa cells show high expression of NOX and neuronal NOS. Microperfusion of L-NAME into macular densa cells enhances tubuloglomerular feedback leading to increased afferent arteriolar vasoconstriction and further reduced glomerular pressure (Welch et al., 2000). This effect tends to occur only during salt delivery, and is absent when tubular sodium is reduced by loop diuretic, suggesting that NO is involved in regulation of tubuloglomerular feedback only during salt loading.

1.7.2 Progression of chronic kidney disease

CKD has a tendency to worsen despite treatment of blood pressure and any other reversible or aetiological factors, and there is evidence that common pathological mechanisms are responsible for this irrespective of the original renal insult. The rate of progression of CKD is extremely variable, relating to clinical parameters such as blood pressure, proteinuria, age, and the presence of various comorbidities. It was shown decades ago, however, that progression of renal impairment shows stronger correlation with degree of tubulointerstitial atrophy and fibrosis than the extent of glomerular disease (Schainuck et al., 1970) . It is thought that tubular atrophy is a final common mechanism by which CKD progresses to ESRD, due to inflammation, infiltration by fibroblasts and mesenchymal to fibroblast transition of resident epithelial cells, ultimately leading to tubulointerstitial scarring and fibrosis (Kuncio et al., 1991).

For example, it was shown using a rat remnant kidney model that progressive renal impairment was more closely associated with dissociation between remaining glomeruli and associated tubules, than with sclerosis of remaining glomeruli (Gandhi et al., 1998). Similarly, in scoring systems used to measure likelihood progression to ESRD in many glomerular diseases including IgA nephropathy, the degree of tubulointerstitial atrophy on kidney biopsy is a significant predictor (Roberts et al., 2009). A number of mechanisms have been suggested by which glomerular damage can be transmitted to the interstitium. Periglomerular tubules may be obliterated by proliferative changes in the glomerulus, whilst leakage of filtrate out of the glomerulus may deposit toxic compounds and proteins around tubular epithelial cells. This in turn can lead to protein reabsorption which causes release of lyosomal enzymes and ROS production. Additionally, injured tubular epithelial cells produce cytokines and transcription factors which recruit inflammatory cells and cause apoptosis of normal glomerular and tubular cells (Chevalier and Forbes, 2008).

Dysfunction of the renal microvasculature is an important component of the tubulointerstitial injury which drives CKD, such that tissue hypoxia and ischaemia is a characteristic feature of CKD. Bohle et al showed peritubular capillary loss in a range of human glomerular and tubulointerstitial diseases, which was associated with the degree of renal impairment (Bohle et al., 1996). The degree of peritubular capillary rarefaction is correlated with degree of tubulointerstitial fibrosis, glomerulosclerosis, and degree of renal impairment (Choi et al.). Microvascular dysfunction may also result from RAAS induced vasoconstriction, and vessel occlusion by inflammatory infiltrate.

1.7.3 Oxidative stress in the progression of chronic kidney disease

Given the role of redox signalling in vascular function, one may postulate that oxidative stress may have a pathophysiological role in progressive CKD, and this has demonstrated in studies using agents which manipulate redox homeostasis.

For example, Baylis et al showed that chronic supplementation of L-NAME to rats with normal renal function caused proteinuria, renal impairment, and glomerular damage (Baylis et al., 1992). Furthermore, Ding et al showed that tempol ameliorated renal impairment in 5/6 nephrectomised mice, reducing NFκB expression, TGF-β induced fibrosis, and tubular atrophy (Ding et al., 2015). Similarly, the serine protease inhibitor camostat mesilate attenuated renal impairment and tubulointerstitial fibrosis in a mouse model of CKD, partly by reducing expression of NOX components and scavenging hydroxyl radicals (Ueda et al., 2015). Enhanced signalling via epithelial growth factor (EGF) has been shown to be one mechanism by which a number of pro-fibrotic pathways are activated, and EGF inhibition has been shown to ameliorate renal fibrosis and progressive renal impairment. EGF is activated by a number of stimuli including MAP kinases, which are themselves activated by ROS. Rhyu et al implicated ROS activated MAP kinase pathways in the renal fibrosis which occurs in a rat model or allograft nephropathy; at least part of the attenuating effects of ROS inhibition may occur through these mechanisms (Rhyu et al., 2005).

1.8 Vascular effects of antioxidants

1.8.1 Antioxidants and cardiovascular mortality in the general population

Due to the contribution of oxidative stress to endothelial dysfunction, arterial stiffness and therefore cardiovascular risk, there has been great interest in the therapeutic use of antioxidants.

Interest in the role of antioxidants in the prevention of CVD began in the 20th century when epidemiological data arose that showed reduced mortality in individuals with a high intake of fresh fruit and vegetables. In a retrospective study of regional variations of fruit and vegetable intake in the United Kingdom, Acheson et al found reduced cardiovascular mortality associated with a high intake of fresh fruit (Acheson and Williams, 1983), and in an analysis of the National Health and Nutrition Examination Survey I (NHANES I) cohort,

the relationship between reported ascorbic acid consumption and cardiovascular mortality in the United States in the 1970s was studied (Enstrom et al., 1992); standardised mortality rates were reduced to 0.41 – 0.78 in men and 0.55 – 0.99 in women. Early prospective cohort studies seemed to confirm these results, with a study of 11,178 participants from the Established Populations for Epidemiologic Studies of the Elderly cohort finding a relative risk of 0.58 for cardiovascular events associated with vitamin E supplementation (Losonczy et al., 1996).

Later observational data was more equivocal. For example in the Health Professionals Follow-up study of 39,910 male health professionals in the United States there was no significant reduction in cardiovascular death associated with high ascorbic acid intake, but there was a relative risk of 0.63 associated with high intake of α-tocopherol (Rimm et al., 1993), with the results echoed in the Multinational Monitoring of Trends and Determinants in CVD (MONICA) study (Tuomilehto et al., 1987). In the Rotterdam study, the rate of MI in previously healthy individuals was inversely correlated with beta-carotene intake, but there was no association with vitamin C and E intake (Klipstein-Grobusch et al., 1999). In NHANES I there was a reduction in all-cause mortality in individuals with the highest ascorbic acid intake, whilst in NHANES II there was increased mortality in men with the highest ascorbic acid intake and no association in women (Enstrom et al., 1992). In trials which used self-reported dietary intake to dichotomise patients into high and low intake, a small but statistically significant protective effect was found for α-tocopherol (OR 0.74; 95% confidence interval 0.66 – 0.83) and ascorbic acid (OR 0.89; 95% CI 0.79 – 0.99), but not β-carotene (OR 0.88; 95% CI 0.77 – 1.01). When plasma or serum levels were measured β-carotene (OR 0.46; 95% CI 0.37 – 0.58) and ascorbic acid (OR 0.58; 95% CI 0.47 – 0.72) reduced cardiovascular events but α-tocopherol did not (Asplund, 2002). Aside from anything else, this shows confounding effect that dietary self-reporting can have on study outcome.

Furthermore, the protective effects of antioxidant vitamins have not been confirmed in interventional studies, with a number of randomised control trials carried out with equivocal results. One meta-analysis showed that vitamin E supplementation did not reduce mortality, cardiovascular death or cerebrovascular accidents in comparison with control treatment, whilst in fact β-carotene supplementation resulted in a small increase in all-cause mortality (OR 1.07; 95% CI 1.02 – 1.11) and cardiovascular death (OR 1.1; 95% CI 1.03- 1.17) (Vivekananthan et al., 2003). Another meta-analysis found no reduction in the incidence of

CVD associated with β-carotene, α-tocopherol, or ascorbic acid supplementation. Another systematic review and meta-analysis including nearly a quarter of a million patients suggested that β-carotene, vitamin A and vitamin E supplementation may increase all-cause mortality, and ascorbic acid supplementation had no significant effect (Vivekananthan et al., 2003).

There may be a number of reasons behind this discrepancy. Bias may be incurred in observational studies due to unreliable self-reporting of nutritional intake, underreported supplement intake, and the association of high vitamin intake with 'healthy lifestyle bundle', including exercise and other beneficial habits, which is not replicated merely by supplementation with single or multi vitamins. Intake of fresh fruit and vegetables, which represents the major dietary source of antioxidant vitamins, may also provide less well recognised dietary antioxidants, or other beneficial substances, which would also be absent from a supplement intervention.

However, individuals with a greater burden of oxidative stress may show greater response to antioxidants. A more rewarding approach may be to target individuals with high levels of oxidative stress who would also be expected to have a greater incidence of cardiovascular events, and represent a cohort where a treatment effect may easier to demonstrate over the short follow up period involved in a clinical trial. For that reason, patients with CKD represent an ideal cohort to target with antioxidant therapy.

1.8.2 Antioxidant therapy in chronic kidney disease

In animal models of CKD, there is some benefit to antioxidant supplementation in terms of biomarkers of oxidative stress as well as cardiovascular outcomes. Antioxidant (5000 units/kg α-tocopherol) fortified feed given to 5/6 nephrectomised mice improves their nitric oxide bioavailability, nitric oxide synthase expression and blood pressure (Maranon et al., 2013). Similarly, Dahl salt sensitive rats fed on a high salt plus N-acetyl cysteine diet had an enhanced glutathione system and attenuated markers of renal inflammation in comparison to a high salt diet alone (Tian et al., 2006).

There is some evidence that antioxidant therapy is efficacious in patients with end stage renal disease receiving haemodialysis, with various studies demonstrating a reduction in markers of oxidative stress, improvements in blood pressure and iron handling, but with only limited evidence to suggest improvements in cardiovascular end points. For example, The

Secondary Prevention with Antioxidants of CVD in End-stage renal disease (SPACE) trial was a randomised control trial of vitamin E supplementation in 196 haemodialysis patients with a history of CVD (Boaz et al., 2000). The intervention comprised of 800 IU α-tocopherol per day for 500 days. Patients assigned to the treatment arm had a markedly reduced rate of composite cardiovascular events (RR 0.46; 0.27 – 0.78), myocardial infarction (RR 0.3; 0.11 – 0.78) compared to placebo, but no reduction in overall mortality. Similarly, in another study of 134 haemodialysis patients, 1.2g N-acetylcysteine per day was found to reduce a composite cardiovascular event rate by 40% (Tepel et al., 2003). Daily supplementation with 500mg ascorbic acid was found to increase serum ascorbate concentration, aggravate hyperoxalaemia, and have no significant effect on cardiovascular outcome in an older study of haemodialysis patients (Ono, 1989).

Data regarding the use of antioxidants in haemodialysis patients was recently reviewed systematically, and marked heterogeneity in intervention, dosage, and outcome measure were, reducing the applicability of meta-analytical statistics (Coombes and Fassett, 2012). Only three of 56 studies examined an effect on clinical outcomes, with the remainder investigating effect on oxidative stress biomarkers. In most of the 53 studies included, most found that antioxidant supplementation reduced oxidative stress. Most conclusive was the evidence for α-tocopherol and N-acetyl cysteine. Ascorbic acid supplementation was found to increase serum ascorbate levels, and to decrease oxidative stress in 4 of the 11 studies in which it was included.

Several criticisms of these studies have limited the adoption of such interventions into routine clinical practice: the number of patients involved was small and few of the studies examined changes in clinical end points, whilst the remainder examined an array of oxidative markers too heterogeneous to allow meta-analysis. The SPACE trial has been criticised for not including a control group comprised of healthy age matched participants, in order to confirm that oxidative stress is greater in the dialysis cohort and to allow a demonstration of the mechanism of action of α-tocopherol. The same criticism can be made of the other two interventional studies examining changes to cardiovascular end points; in neither was it an inclusion criterion that patients must have a demonstrable increase in oxidative stress in comparison to a group of healthy controls.

Equivocal results have also been found in interventional studies of antioxidant therapy in patients with CKD not requiring dialysis. Oral N-acetyl cysteine supplementation was found not to reduce proteinuria, plasma homocysteine levels, or systolic blood pressure in 20

individuals with renal impairment and albuminuria (Renke et al., 2010). The effect of 8 weeks of vitamin E supplementation was studied in a pilot study of eight patients and six controls (Saran et al., 2003). Whilst concentrations of ADMA were reduced, no significant reductions in F2-isoprostanes were noted. A prospective double blinded randomised control trial investigated the use of an antioxidant cocktail (mixed tocopherols and alpha lipoic acid) to reduce markers of oxidative stress in moderate renal impairment (CKD stages 3 and 4) (Ramos et al., 2011). No reduction in F2-isoprostanes, protein thiols, CRP, or IL-6 was observed after 2 months of treatment in comparison to placebo.

A number of studies have investigated the use of ascorbic acid on ameliorating endothelial dysfunction and oxidative stress. Cross et al used plethysmography to measure the forearm dilator response to acetylcholine, and measured the dilator response to shear stress in brachial and radial arteries, before and after intra-arterial infusion of ascorbic acid in 33 patients with CKD (Cross et al., 2003). An increase in the dilator response to acetylcholine of resistance vessels was seen after ascorbic acid, which was abrogated by NOS inhibition. No increase in FMD was observed in the brachial or radial arteries. Similarly, whilst 2g oral ascorbic acid was found to increase FMD in haemodialysis patients, no improvement in endothelial function was observed in patients with pre dialysis CKD. Conversely, in a study of 13 patients with kidney transplants, and a mean serum creatinine of 126 µmol/L, oral ascorbic acid was shown to reduce markers of oxidative stress and improve endothelium dependent vasodilatation (Williams et al., 2001).

A Cochrane systematic review recently investigated the utility of antioxidant therapy in patients with CKD and ESRD (Jun et al., 2012). With regard to patients not receiving dialysis, no benefit was observed in a meta-analysis of studies investigating cardiovascular outcomes, however there were a limited number of studies included, and none of which used ascorbic acid used as the intervention. Further investigation into the use of antioxidants as therapy in CKD is sorely needed.

1.8.3 Agonists of endogenous antioxidant systems

Recently, novel therapeutics have been developed which manipulate intrinsic antioxidant systems such as Nrf-2. Bardoxolone methyl is a first in class semi-synthetic triterpenoid, which binds to Keap-1 cysteine residues, preventing its association with the transcription factor Nrf-2, allowing its translocation to the nucleus where it up regulates several antioxidant genes. In phase 1 studies, initially designed to investigate an anti-tumour effect,

bardoxolone was shown to increase eGFR, as well as increase antioxidant enzyme NQO1 mRNA in peripheral monocytes (Hong et al., 2012). The phase 2 Bardoxolone Methyl Treatment: Renal Function in CKD/Type 2 Diabetes (BEAM) study investigated the change in eGFR after 24 – 52 weeks of bardoxolone treatment in 227 patients with diabetic kidney disease (Pergola et al., 2011). In the 75 mg dose group, eGFR improved by 11 mL/min/1.73m^2 compared to placebo, with the main adverse effects comprising muscle pains, liver enzyme disturbances, hypomagnesaemia and GI upset. There was therefore some excitement regarding the phase 3 Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Mellitus: the Occurrence of Renal Events (BEACON) study (de Zeeuw et al., 2013), which sadly evaporated when it was terminated early by the independent safety monitoring committee due to increased cardiovascular event rate in the treatment arm. In the bardoxolone arm 96 patients were hospitalised or died from heart failure, compared to 55 in the placebo group (HR 1.83, 95% CI 1.32 – 2.55), whilst no difference was seen in the primary end point of progression to ESRD. Interestingly, eGFR was increased in the treatment arm, at the expense of an increase in albumin to creatinine ratio and blood pressure. Explanations for this unexpected outcome include diastolic cardiac dysfunction occurring secondary to selective inhibition of endothelin-1 A receptors, or endothelial dysfunction and arrhythmia occurring secondary to hypomagnesaemia, another side effect of the medication.

The role of Nrf-2 agonists like bardoxolone, and more broadly of other modulators of endogenous antioxidant systems, remains uncertain.

1.8.4 Adverse effects of antioxidant therapy

Despite the lack of clarity as to the benefit of antioxidant supplementation, one might postulate that this therapeutic intervention may be recommended purely on the basis of an absence of harm. Certain evidence has accumulated over the decades of antioxidant research that this may not be the case, with a number of prospective trials demonstrating a potentially harmful effect.

For example both the Heart Outcomes Prevention Evaluation – The Ongoing Outcomes (HOPE-TOO) study (Lonn et al., 2005), and the GISSI-Prevenzione study (Marchioli, 1999), demonstrated a higher risk of heart failure associated with α-tocopherol. Whilst HOPE-TOO found no increase in overall mortality in the group receiving 400 IU/day α-tocopherol, there was a significant increase in incidence heart failure and concomitantly no benefit in terms of

cardiovascular or malignant outcomes. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) trial (Albanes et al., 1995) found that α-tocopherol supplementation 75 IU/day increased mortality from haemorrhagic strokes, although with no overall increase in mortality; β-carotene surprisingly increased the incidence of lung cancer, ischaemic heart disease, and overall mortality. Subgroup analysis of ATBC found that hypertensives receiving α-tocopherol also had a higher incidence of subarachnoid haemorrhage than controls. Systematic review of the data found increased mortality associated with beta carotene, vitamin E and vitamin A.

Ascorbic acid also has some associated dangers. *In vitro*, ascorbate undergoes Fenton reaction with transition metal ions which results in the production of hydroxyl radicals, suggesting that ascorbic may have pro-oxidant properties in certain circumstances. It remains unclear, however, if this is an important source of oxidants *in vivo*, given that the concentration of transition metal ions is kept low by binding proteins such as transferrin and caeruloplasmin. Some have suggested that Fenton chemistry does occur *in vivo*, but rather than act in a deleterious manner causing oxidative stress, the H_2O_2 produced might act in a beneficial manner as a vasodilating endothelium dependent hyperpolarising factor. Garry et al, found that both ascorbate and BH_4 caused NO independent relaxation of rabbit iliac artery rings that was abolished by the addition of catalase, which removes H_2O_2 (Garry et al., 2009). This suggests that even pro-oxidant effects of ascorbic acid (and BH_4) might enhance vascular function through the EDHF effect by generating H_2O_2. This highlights the complex nature of redox homeostasis in the EC endothelial cell, and demonstrates the importance of NO independent pathways in VSMC function.

Oxalic acid is the terminal metabolite of ascorbate which is excreted in the urine and therefore ascorbic acid represents an oxalate load which will possibly increase serum or urinary concentrations of oxalate. High dose supplementation is therefore may not be desirable in the CKD population who already have a tendency towards high serum oxalate concentrations. There are case reports of nephrocalcinosis and systemic oxalosis occurring after ascorbic acid supplementation, but this has tended to occur at extreme dosages over several years (Yaich et al., 2014). Other studies have shown an increase in oxalate levels in CKD patients supplemented with ascorbic acid, but with no adverse outcomes occurring as a result.

1.9 Measuring oxidative stress and vascular function

The half-life of ROS is very short, and in the case of O_2^-, measured in milliseconds, which renders their measurement in biological samples extremely challenging. Additionally, ROS are produced by a number of sources, and the relative contribution of these to total production varies between cell types and tissues. Finally, as has been discussed, as well as an increase in ROS production, oxidative stress is manifest by reduction in beneficial vascular signalling molecules such as NO, and by disturbance in redox signalling. As such, it may be more useful to measure the downstream products of oxidative stress such as the oxidation products of lipids, proteins, and other molecules, which have a longer half-life and are more widely distributed. Similarly, it may be more meaningful to measure the pathophysiological sequelae of oxidative stress, such as the effects on endothelial and arterial function.

1.9.1 Biomarkers of oxidative stress

1.9.1.1 Characteristics of a useful biomarker

The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu and Tavel, 2010). Biomarkers are often used as secondary endpoints or surrogate markers of clinical outcomes in interventional studies, especially when a change in clinical outcome is difficult to measure because of the sample size or duration of study required, or because an invasive or harmful investigation would be required. A useful biomarker should therefore have a strong association with clinically relevant outcomes. In the case of CKD this could include mortality, cardiovascular events, or worsening of renal function. A corollary of this is that a change in the risk of an outcome following an intervention or any other clinical development should be reflected by a change in the biomarker.

1.9.1.2 Biomarkers of oxidative stress

Oxidative stress is therefore a broad term encompassing alterations in signalling cascades as well as an increase in short lived radical species which are produced in a regionalised manner, and oxidative damage to downstream macromolecules. As such, a variety of methods have been used in the measurement of oxidative stress, and typically a panel of biomarkers is used in clinical studies. There remains no standardisation of the panel of

biomarkers which should be used however, and no single measure widely accepted to represent oxidative stress. The merits of different biomarkers of oxidative stress are discussed below.

1.9.1.3 Measurement of oxidative damage

Lipids have numerous double bonds which render them especially susceptible to oxidative modification. Arachidonic acid is a polyunsaturated lipid present in cell membrane bound phospholipids. Prostaglandins, involved in immune signalling, are produced from arachidonic acid by cleavage from membrane phospholipids by phospholipase, and subsequent metabolism by cyclooxygenase. Isoprostanes are prostaglandin like compounds which are produced by radical induced lipid peroxidation of arachidonic acid, independently of cyclooxygenase enzymes (Morrow et al., 1990). F2-isoprostanes are a group of chemicals produced in this manner and named according to their chemical similarity to prostaglandin-F2α. They are formed in situ on the cell membrane, and cleaved by phospholipases to be released into the circulation. Concentration of F2-isoprostanes are not affected by the lipid content of the diet, nor confounded by hepatic or renal dysfunction, which improves their specificity as biomarkers of oxidative stress (Morrow, 2005). As well acting as a marker, isoprostanes may act as secondary messengers, exerting some of the deleterious effects of radical damage; isoprostanes reduce GFR and renal blood flow in rats, and cause vasoconstriction in other vascular beds including in the pulmonary and cerebral circulations (Takahashi et al., 1992). Isoprostanes also stimulate proliferation of endothelial cells (Yura et al., 1999), cardiac myocytes (Kunapuli et al., 1998), and cause platelet activation (Davì et al., 1999), all of which may contribute to processes underlying atherosclerosis and CVD. A number of studies have linked isoprostane levels and cardiovascular outcomes. For example, in a cross sectional study of 2850 patients those with the highest quartile of F2-isoprostanes had a 24% greater likelihood of having detectable coronary artery disease on cardiac CT, even after adjustment for conventional cardiovascular risk factors (Gross et al., 2005). Similarly, in a cohort study of 108 patients who had suffered a myocardial infarction, levels of F2-isoprostane had predictive value for estimating 30 day cardiovascular events (LeLeiko et al., 2009).

Another product of lipid peroxidation which has been studied for its role as a marker and indeed mediator of oxidant stress is malondialdehyde. Malondialdehyde is produced by degradation of polyunsaturated lipids by free radicals, and is elevated in the context of cardiovascular risk factors such as diabetes and smoking, and in the presence of coronary

artery disease (Ayala et al., 2014). Malondialdehyde may be measured directly using ELISA (Bevan et al., 2003), or by the colorimetric TBARS assay (Meagher and FitzGerald, 2000), which is somewhat less specific but more suitable to high throughput analysis. Much like isoprostanes, as well as being a marker of oxidative stress, malondialdehyde also acts as a mediator of oxidative damage; DNA damage can occur by its reaction with deoxyadenosine and deoxyguanosine, which forms potentially mutagenic DNA adducts (Dedon et al., 1998). Additionally, malondialdehyde reacts strongly with lysine residues, and these aldehyde lysine adducts have been found in apolipoprotein B present on the surface of oxidised LDL (Vanderyse et al., 1992). It has been postulated that this may be a mechanism which facilitates the interaction between oxidised LDL and macrophage, resulting in foam cells and the beginnings of atherosclerosis. In a study of 634 patients with coronary artery disease, patients with malondialdehyde in the highest quartiles had relative risk of 3.3 for major cardiovascular events over three year period, independently of cardiovascular risk factors and markers of inflammation (Walter et al., 2004). Similarly, in a study of 71 patients receiving maintenance haemodialysis for ESRD, malondialdehyde was an independent predictor of mortality (Scott et al., 2003). In another study of 76 dialysis patients, malondialdehyde was a predictor of prevalent CVD (Boaz et al., 1999). Conversely, in a study of 94 patients with CKD stages 3 – 5, whilst malondialdehyde rose progressively in proportion to renal impairment, it was not a predictor of outcome, nor of arterial stiffness or calcification (Terrier-Lenglet et al., 2011). These differences could be accounted for by methodological differences (for example measuring TBARS compared to direct measurement), by changes in malondialdehyde binding which occurs in haemodialysis patients (De Vecchi et al., 2009), or due to differences in the pathophysiology of CVD between the two populations.

In states of oxidative stress, radical mediated DNA modifications occur which result in a number of oxidised end products. In a rat model which entails using carbon tetrachloride poisoning to induce oxidative stress, 8-hydroxy-2′-deoxyguanosine (8-OHdG) was elevated in a dose and time dependent manner, confirming its use as a biomarker of oxidative stress (Kadiiska et al., 2005). Urinary 8-OHdG has commonly been measured as a marker of DNA oxidation, although this marker is neither specific nor sensitive. For example 8-OHdG does not represent all DNA oxidation products, and may represent oxidation of the dinucleotide pool prior to its incorporation into DNA

1.9.1.4 Measurement of antioxidant capacity

As discussed earlier, antioxidants may be enzymatic or non-enzymatic. The total antioxidant potential (TAP) of a biological sample can be measured which represents its non-enzymatic reducing potential, which results from the concentrations of ascorbic acid, tocopherol, urate, and bilirubin, amongst others. Several studies have shown a surprising increase in TAP in patients with CKD: in patients receiving haemodialysis, TAP is elevated in comparison to controls but falls following a dialysis session (Jackson et al., 1995), and in patients with CKD, TAP is unexpectedly higher than healthy volunteers (Karamouzis et al., 2008). This result is possibly due to accumulation of urate, the reducing effect of which is measured in the assay. As discussed earlier however, urate has both pro and antioxidant capacity *in vivo* and the increase in urate and other unmeasured solutes could compensate for other antioxidants which are reduced in CKD. The role of TAP in evaluating overall redox state is unclear, but has some merit as a marker of serum antioxidant status. Additionally, levels of the major antioxidant vitamins in blood can be measured, but it should be considered that the levels of ascorbic acid and α-tocopherol in plasma may not represent total body pools and utilisation.

The major enzymatic antioxidants which have been used as biomarkers of antioxidant capacity are glutathione peroxidase and SOD. Glutathione peroxidase is an important enzymatic antioxidant which catalyses conversion of hydrogen peroxide to water, converting the essential cofactor glutathione (GSH) to its oxidised form glutathione disulphide (GSSG) in the process. The ratio of GSH to GSSG has been used as a marker of the availability of GSH to participate in this reaction and has therefore been used as a biomarker of antioxidant capacity. Whilst, glutathione (GSH) shows correlation with eGFR and is reduced in moderate and severe CKD, GSSG remains stable regardless of the severity of renal impairment (Ceballos-Picot et al., 1996). GSH:GSSG ratio is therefore reduced in CKD and accordingly, there is a reduction in both plasma and erythrocyte glutathione peroxidase activity associated with this. In patients with CKD GSH:GSSG ratio correlates with FMD demonstrating the effect of reduced antioxidant capacity on endothelial function (Annuk et al., 2001).

1.9.1.5 Direct measurement of ROS and other radicals

As already described, direct measurement of ROS proves challenging for a number of reasons. Firstly, ROS production is highly compartmentalised, such that an increase in ROS from a particular source might not be reflected by an increase in total ROS levels within a

biological sample. Additionally, ROS are reactive and have a short half-life which render them difficult to measure. Finally, the complexity of redox biology is such that measurement of ROS alone might not even be desirable, as measurement of the downstream effects of ROS such as macromolecule oxidation and endothelial dysfunction may prove more meaningful. That said, there are a number of methods by which ROS can be measured, each of which have advantages and disadvantages specific to the radical being measured and the nature of the biological sample in which it is contained.

ROS can be measured by chemiluminescence techniques, whereby the luminescent probe is activated upon oxidation by O_2^-. Luminol and lucigenin are permeable to the cell membrane and therefore measure both intra- and extracellular quantities of ROS. Fluorescent probes can also be used, some of which have the advantage of being able to be rendered cell membrane impermeable, such that precise intracellular sources of ROS can be investigated. For example, dihydroethidium is permeable through the cell membrane whereupon it reacts with ROS to form an impermeable fluorophore which can be measured by flow cytometry or fluorescence microscopy (Wardman, 2007).

Electron paramagnetic resonance (EPR) spectroscopy is another method which directly measures ROS, or indeed any other radical species (Spasojevic, 2011). Spin is an inherent property of electrons, which produces an intrinsic magnetic moment. Most oxygen molecules have paired electrons but O_2^- and other radicals have an unpaired electron which creates a magnetic moment which can therefore interact with a magnetic field. When exposed to a magnetic field, an unpaired electron will align itself either parallel or antiparallel to the field, such that unpaired electrons will have one of two possible energy states depending on the direction of its spin. Electrons can move between the two states by either absorbing or releasing a photon of energy. When another magnetic field of varying strength is applied the energy difference between the two states increases until it matches the energy of the microwave radiation being applied. At this point electrons can transition between energy states, and because most electrons begin in the lower energy state, a net absorption of energy will occur, and this absorption can be measured by a spectroscopic detector. Certain spectral parameters such as the g-factor are specific for a given radical species, and the signal intensity of the spectra is dependent on concentration, which allows identification and measurement of individual radicals. In the case of short lived radical species such as ROS, a "spin trap" is used, which is a compound which fixes the radical group in a stable condition whilst evoking a characteristic spectroscopy tracing. The benefit

of EPR is that specific radical species can be measured at very low concentrations; the disadvantage being the cost of the equipment and the expertise required.

1.9.2 Measurement of endothelial function

1.9.2.1 Flow mediated dilatation

It was shown in 1980 by Furchgott et al that stripping rabbit aorta of endothelial cells abrogated the vasodilator effects of acetylcholine, confirming the obligatory role of the vascular endothelium in mediating this response (Furchgott and Zawadzki, 1980). Later, it was shown that nitric oxide synthase (NOS) inhibitors also diminished vascular smooth muscle cell response to acetylcholine, confirming nitric oxide (NO) as the endothelial derived factor responsible for this (figure 1.2) (Moncada et al., 1988). It had also been known that the vasodilator response to shear flow was endothelium dependent (Pohl et al., 1986), and thought to be mediated through NO via a number of flow sensitive cation channels on the endothelial cell surface (Cooke et al., 1991, Lansman et al., 1987). In 1994, it was proposed by Celermajer and Deanfield that measurement of the vasomotor response of peripheral conduit arteries to shear stress could be measured as a marker of NO bioavailability and endothelial function (Celermajer et al., 1992). Since then there has been further elucidation of the physiology of FMD and its clinical significance.

1.9.2.2 Physiology of flow mediated dilatation

Several intra and extracellular components of endothelial cells are involved in converting shear stress to a chemical signal directed towards vascular smooth muscle cells, in a process termed mechanotransduction, and which results in both rapid changes in vascular tone and longer term changes to vascular modelling.

Wherever there is flow, a force is exerted onto the luminal surface at a vector perpendicular to its direction. On the cell membrane, shear stress results in opening of specific potassium channels resulting in cell membrane hyperpolarisation and calcium influx (Cooke et al., 1991). Tyrosine kinase receptors such as vascular endothelium growth factor receptor 2 (VEGFR2), are also directly activated due to changes membrane fluidity caused by shear stress (Jin et al., 2003). Caveolae are small lipid lined invaginations on the cell membrane which are rich in proteins involved in cell signalling. Shear stress induces release of ATP and eNOS into the endothelial cell cytoplasm either directly, and/or indirectly due to calcium influx associated with shear sensitive ion channels, resulting in activation of signalling cascades, tyrosine kinase receptors, and an increase in NO production (Isshiki et al., 1998).

Extracellular components are also involved in mechanotransduction. For example shear stress directly results in re-organisation of intercellular junction proteins, such as platelet endothelial cell adhesion molecule and vascular endothelial cadherin, which leads to integrin activation and subsequent activation of downstream signalling cascades (Tzima et al., 2005). Additionally, physical deformation of the cytoskeleton results in signalling through membrane associated ion channels, via association with focal adhesion molecules, and also by its association with the nuclear membrane which may represent a mechanism by which shear stress results in longer term changes in arterial modelling via changes in gene expression (Alenghat and Ingber, 2002). A number of signalling cascades are initiated thereafter by the manifold mechanotransduction sensor sites, including MAP kinase, NFκB, focal adhesion kinase, and protein kinase C (Johnson et al., 2011).

The next step is to link these signalling cascades with vascular smooth muscle cell function, by release of endothelium derived vasodilators, including NO but also including other vasoactive factors such as prostaglandins and the EDHF. NO is produced at a basal rate by endothelial cells but production is increased in response to shear stress as evidenced by the alterations that can be induced with the use of nitric oxide synthase inhibitors. For example, a meta-analysis of 20 studies found that during conventional FMD, namely that induced by distal arterial occlusion for 5 minutes, infusion of the nitric oxide synthase (NOS) inhibitor N(G)monomethyl-L-arginine (L-NMMA) reduced brachial artery dilatation from 6.5% to 0.9% (Green et al., 2014). eNOS activity is regulated by a number of mechanisms. Firstly under basal conditions eNOS is primarily reserved within caveolae however a number of stimuli, including shear stress, can induce eNOS release into the cytoplasm where it is able to produce NO (Mineo and Shaul, 2012). Secondly, eNOS activity is calcium sensitive and calcium influx occurs via a number of mechanisms during FMD as previously discussed. Finally, eNOS activity is regulated by phosphorylation by kinases such as Akt (Dimmeler et al., 1999) and protein kinase A (Boo et al., 2002) which are activated by FMD induced pathways. Shear stress also affects gene transcription, generally inducing an anti-atherogenic phenotype, with increased expression of genes affecting vasodilators, antioxidant systems, and antithrombotic substances, and reduced expression of adhesion molecules and inflammatory cytokines (Himburg et al., 2007). Expression of eNOS is also increased through activation of NFκB (Davis et al., 2004). In this manner shear stress results in longer term changes to arterial structure through activation of transcription factors and alterations in gene expressing, as well as inducing shorter term changes in vascular function through direct activation of vasodilator pathways.

The dilator response is not entirely NO dependent, however. It has previously been shown that dilatation following 15 minutes of vessel occlusion is not attenuated by L-NMMA (Mullen et al., 2001). Similarly, when arterial occlusion is proximal to the site where the diameter is measured (Doshi et al., 2001), or when hyperaemia is provoked by an ischaemic hand grip manoeuvre rather than vessel occlusion (Agewall et al., 2002), FMD is NO independent. These observations have the implication that from a physiological perspective, factors aside from NO are involved in the endothelial response to shear stress. Furthermore, from an investigational perspective, the other implication is that attention must be paid to FMD methodology if a purely endothelium derived NO response is to be measured.

1.9.2.3 Clinical significance of flow mediated dilatation

The use of FMD as a surrogate marker of CVD relies on the principle that endothelial function is a systemic property whereby dysfunction measured at a peripheral site correlates with that systemically, including the coronary vasculature. Close correlation has been shown between brachial and coronary artery dilatation following hyperaemia induced by infusion of adenosine triphosphate (ATP) (r= 0.78, p < 0.001) (Takase et al., 1998), which supports this assumption. As such, measurement of FMD at a site amenable to non-invasive assessment provides a means by which to detect and quantify the early stages of atherogenesis.

The original Celermajer and Deanfield study demonstrated reduced vasodilatation in children and adults with vascular risk factors in comparison to healthy controls, and many studies since then have shown reduced FMD associated with other conventional risk factors. FMD measured in individuals with type 1 diabetes was reduced to $5.0 \pm 3.7\%$ in comparison to $9.3 \pm 3.8\%$ in controls, and was proportional to duration of diabetes and LDL cholesterol levels (Clarkson et al., 1996). Furthermore, lowering of LDL cholesterol either by apheresis (Tamai et al., 1997) or by HMG CoA reductase inhibitors (Stroes et al.) has been shown to improve endothelial function. In cigarette smokers, FMD was shown to be absent, or reduced to $4 \pm 3.9\%$ compared to $10 \pm 3.3\%$ in controls, with FMD reduced in a dose dependent fashion according to quantity and duration of smoking (Celermajer et al., 1993). Ageing has also been shown to associate with reduced endothelial function; in men FMD was preserved until the age of 40 years, after which there was a decline of 0.21% per year, whilst in women FMD was preserved until 50 years, and declined by 0.49% thereafter (Celermajer et al., 1994).

As well as acting as a marker of burgeoning vascular disease, there is evidence for a prognostic significance of FMD in certain populations. A number of studies have shown a relationship between reduced FMD and future cardiovascular events or deterioration of disease in the context of coronary artery disease (Kitta et al., 2009), congestive cardiac failure (Meyer et al., 2005), and following coronary intervention (Patti et al., 2005) (where FMD predicts in stent restenosis). In contrast, the evidence supporting the prognostic significance in healthy individuals at low risk of CVD is equivocal. One study of 2264 post-menopausal women without CVD found reduced FMD was independently associated with future events (Rossi et al., 2008), whilst another study found that FMD did not provide predictive power over established predictive models such as the Framingham risk score (Yeboah et al., 2009a).

Kidney disease has also been shown to lower brachial artery reactivity, with FMD reduced from $5.7 \pm 0.6\%$ in healthy volunteers to $3.8 \pm 0.3\%$ in patients with CKD (Dogra et al., 2006). FMD is reduced in a stepwise fashion in relation to the degree renal impairment (Yilmaz et al., 2006), with $8.97 \pm 1.36\%$ dilatation in controls compared to $6.71 \pm 0.38\%$ in CKD stage 3 and $5.97 \pm 0.52\%$ in stage 4. More recently, FMD has been shown to be an independent predictor of cardiovascular outcome in CKD (Yilmaz et al., 2011), with a near 50% reduction in fatal or non-fatal events for each 1% increase in FMD.

1.9.2.4 Other methods of measuring endothelial function

Other techniques have been used to measure endothelial function, which can be classified according to whether they are invasive or non-invasive, and by which circulatory bed which they examine.

Coronary endothelial dysfunction can be measured by invasive cardiac catheterisation; coronary artery diameter can be measured angiographically whilst coronary blood flow can be measured using flow Doppler (Hasdai et al., 1997). In this manner, endothelial dependent vasodilatation can be measured following infusion of acetylcholine and compared to endothelial independent vasodilatation measured following adenosine infusion. A study of 157 patients undergoing coronary angiography found increased cardiovascular event rate in those with the most severe endothelial dysfunction (Suwaidi et al., 2000). Clearly however, the procedure is time consuming, costly, and invasive. Forearm venous occlusion plethysmography entails measuring change in forearm volume after intra-arterial infusion of

vasoactive substances (Mather et al., 2001), but again, the technique is invasive and time consuming.

Microvascular function can also be evaluated non-invasively using either peripheral artery tonometry or laser Doppler flowmetry. In the former, digital blood volume is measured using bilateral finger probes at baseline and during reactive hyperaemia, stimulated unilaterally using a sphygmomanometer inflated to supra-systolic pressure (Rubinshtein et al., 2010). The reactive hyperaemia peripheral artery tonometry (RH-PAT) index is the ratio of digital pulse volume during reactive hyperaemia to a baseline measurement taken on the unstimulated limb, and is a marker of endothelial function which correlates with its measurement using invasive coronary techniques. It has been suggested however that the measured response is not entirely endothelium dependent however, and may be affected by other factors including the sympathetic nervous system. Additionally, the test has not been well validated in CKD in part because the use of both upper limbs may not be possible in this population due to the confounding effects of dialysis vascular access. Laser Doppler flowmetry uses a low powered laser to emit light which penetrates skin a short distance whereby it undergoes a Doppler shift when it is scattered by red blood cells within the microvasculature. The light will therefore comprise both shifted and unshifted blood, the distribution of which is dependent upon the number and velocity of red blood cells and therefore relates to microvascular flow (Swiontkowski, 1991). The drawback of this methodology is that microvascular function is measured only in a small portion of skin, which may not be representative of microvascular function elsewhere.

1.9.2.5 Circulating biomarkers of endothelial dysfunction

As well as directly measuring endothelial function in the ways described above, circulating markers of endothelial damage can also be measured. Examples of these molecules and cells which can be measured in blood as signals of endothelial cell damage are shown in table 1.1.

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Biomarker	
Markers of EC damage and repair	Endothelial progenitor cells
	Circulating Endothelial cells
	Endothelial microparticles
Adhesion molecules	E-selectin
	ICAM-1
	VCAM-1
Markers of EC activation	von Willebrand factor
	Tissue plasminogen activator
	Plasminogen activation inhibitor-1

Table 1.1 List of circulating markers of endothelial dysfunction.

ICAM = Intercellular adhesion molecule, VCAM = Vascular cell adhesion molecule, EC = Endothelial cell.

1.9.3 Evaluation of arterial function

1.9.3.1 Measures of arterial remodelling

Currently, assessment of arterial pressure in routine clinical practice is confined to measurement of peak systolic and end diastolic pressure by sphygmomanometry at the brachial pulse. These measurements provide only a limited summary of the arterial pulse wave however, and furthermore may not be an accurate representation of the pressure within central arteries. Given the complexity of the arterial wave form, a more comprehensive analysis may allow for a better understanding of the cardiovascular sequelae of disturbances in arterial function.

As described in section 1.6, the arterial system has two closely related functions. Firstly, there is a conduit function, by which blood is delivered from the heart to peripheral tissues which is achieved largely due to the composition of peripheral arteries. Secondly, there is a cushioning or 'Windkessel' function, whereby the oscillatory flow produced by left ventricular contraction is converted to smooth flow delivered to peripheral vessels. This is largely achieved by the viscoelastic nature of the large, proximal arteries, whereby a fraction of the energy produced is systole is converted into potential energy within the elastic vessel walls, which recoil during diastole resulting in forward flow of blood reserved within the vessels. This has the effect of dampening peak systolic pressure and increasing end diastolic pressure, protecting tissue capillary systems from extremes of pressure.

The effectiveness of this second function is dependent on the elasticity and structure of the vasculature. This elasticity can be described by a number of intimately related physical parameters (Oliver and Webb, 2003). For example, compliance describes the change in volume which occurs given a certain change in pressure, whilst elasticity, or stiffness, is the inversion of this; meaning the change in pressure which occurs at a given change in volume. Distensibility is the degree of stretch which occurs at a given pressure, whilst the elastic modulus is the opposite; the amount of pressure required to stretch the vessel to double the resting diameter. Elasticity is non-linear, as at low pressure, tension is borne within elastin fibres in the vessel wall, whilst at higher distending pressures stiffer collagen fibres and smooth muscle tend to bear this energy. Knowledge of the distending pressure, meaning the mean arterial blood pressure, is therefore required for interpretation of these stiffness parameters.

Clinically, arterial stiffness is evaluated by measuring the velocity of the pressure wave through the arterial system, by analysis of the pressure wave, or by measurement of the diameter to pressure curve at a certain point of the vasculature. A number of systems are commercially available which facilitate non-invasive measurement of these parameters.

1.9.3.2 Pulse wave velocity

The pulse wave velocity (PWV) is the speed at which the arterial pulse wave is transmitted through the vasculature. In order to measure this, the pulse wave is analysed at two different places along the arterial tree, and the time delay between a particular moment of the pulse wave occurring at each of the two locations is measured, usually by timing against the R wave on an ECG. The carotid and femoral pulses are typically utilised, such that the technique measures the PWV along the aortic and aorto-iliac routes. The distance between the two points is measured as an estimate of the distance the pulse wave travels, and PWV is derived by the distance divided by the time. The Moens-Korteweg equation describes PWV as proportional to the square root of the elastic modulus such that a higher velocity equates to a higher degree of stiffness (Gosling and Budge, 2003). Given that this technique is in effect measuring aortic PWV, this provides information regarding the ability of the central arteries to distend with changes in pressure, such as those which occur during the cardiac cycle.

PWV is raised with ageing, and in association with a number of cardiovascular risk factors including hypertension, obesity, diabetes, and dyslipidaemia (Gottsater et al., 2015). In patients with hypertension, a 5 m/s rise in PWV is associated with a more than doubled likelihood of mortality, after adjustment of other risk factors. PWV is also higher in CKD, has also been shown to rise in proportion to the degree of renal impairment. Furthermore, the CRIC study showed that PWV was independently associated with a variety of vascular outcomes in CKD including heart failure, cognitive impairment, and progressive renal impairment. In a study of 241 patients with end stage renal disease receiving maintenance haemodialysis, PWV was a significant independent predictor of outcome, with patients with the highest third of PWV at 5.9 times risk of a cardiovascular event and 5.4 times risk of death.

1.9.3.3 Pulse wave analysis

In addition to PWV, analysis of the central arterial waveform also allows assessment of large arterial function and stiffness. The antegrade pressure wave generated during left ventricular

systole encounters many points of bifurcation as it travels along the arterial tree away from the heart. Wave reflections are generated at these points of impedance mismatch, where flow exceeds capacity, and are propagated in a retrograde direction back towards the heart. The shape of the arterial waveform therefore results from the summation of forward and backward flowing waves. Given that the effect exerted by the reflections will vary depending on their timing relative to the antegrade pressure wave, this summation will differ at different sites along the vasculature, and the arterial waveform accordingly varies at different sites from central to peripheral arteries. At sites of bifurcation in peripheral arteries where the reflections are generated, reflected waves are in phase with the antegrade pressure wave, resulting in augmentation of systolic pressure. Wave reflections reach central arteries during diastole, augmenting diastolic pressure with the physiologically desirable result of improving coronary blood flow, without increasing systolic pressure. This, together with the Windkessel effect, reduces pulse pressure whilst optimising afterload (Nichols et al., 2008).

Arterial stiffness influences the nature of the waveform by a number of mechanisms. Firstly, the amplitude of the pressure waveform is increased when the left ventricle ejects into stiff central arteries. Secondly, higher PWV results in earlier return of reflected waves to the central arteries, augmenting systolic rather than diastolic pressure. Additionally, the amplitude, as well as the timing of reflected waves, is also affected by the elasticity of small arteries. The degree to which peak systolic pressure is augmented by reflected waves is termed the augmentation index (Aix) and is used as a marker of arterial stiffness. In addition to the timing and amplitude of reflections, left ventricular ejection time will also affect the overlap of forward and backward waves, such that when ejection time is short, such as in tachycardia, reflected waves are more likely to return during diastole. Aix is often adjusted to a heart rate of 75 bpm for this reason (Safar et al., 2003).

Conventionally, applanation tonometry is used to non-invasively evaluate the arterial waveform at a readily accessible peripheral site, typically the radial artery, and a transfer function is used to derive the central arterial waveform from this. Whilst PWA has good reproducibility in both healthy and unhealthy populations, the use of a generalised transfer function may result in systematic error. One study compared invasively measured central waveforms and those derived from radial PWA, and found that individualised transfer functions were only marginally superior to the use of a generalised transfer function, although Aix tended to be underestimated by the use of radial PWA (Segers et al., 2000). Other studies have found that adjustment of the transfer function by parameters such as

gender and the presence of vascular disease provides more accurate analysis of central pulse pressures (Hope et al., 2002).

1.9.4 Renal blood flow

1.9.4.1 Measuring perfusion

Renal perfusion is a crucial component of normal renal function, being one of the main determinants of glomerular filtration rate and tissue oxygenation. Blood flow to the kidney arrives via the renal artery, which divides in turn into the segmental arteries, the interlobar arteries, and the arcuate arteries, before forming the afferent arterioles which supply individual nephrons. A portion of blood, termed the filtration fraction, is filtered through the glomerular membrane into the Bowman's capsule, whilst the remainder enters the peritubular capillary system. Discrimination can be made between renal blood flow, which refers to the velocity of blood travelling through the vasculature measured in millilitres per minute, and perfusion, which refers to the quantity of blood delivered to the capillaries at the tissue level, measured in millilitres per minute per unit mass of kidney tissue.

Measurement of renal haemodynamics may provide important biomarkers in health and disease, but this has been hindered in both research and clinical practice by inherent drawbacks of existing methodologies. Clearance techniques have conventionally been used to measure effective renal blood flow, with para aminohippuric acid (PAH) clearance being the gold standard. However, this process is labour intensive, time consuming, invasive and inappropriate for use in clinical practice or in large clinical trials. Furthermore, availability of PAH in the UK is limited due to debate as to whether it meets the legislative requirements regarding its transmissible spongiform encephalopathy status. Dynamic perfusion studies performed using CT or MR imaging both require administration of an exogenous contrast compound. In the case of CT, iodinated contrast agents are nephrotoxic such that they tend to be avoided in CKD, and the modality also carries a radiation burden. Dynamic contrast enhanced (DCE) MR allows measurement of renal perfusion using kinetics of a non-diffusible paramagnetic tracer containing gadolinium, however these agents, while generally safe, are inappropriate for use in renal impairment due to concerns regarding an association with nephrogenic systemic fibrosis (Collidge et al., 2007). Nuclear scintigraphy also requires exposure to ionising radiation rendering it inappropriate for repeated use or in research.

Arterial spin labelling magnetic resonance imaging (ASL MRI) is an emerging technique which utilises magnetically labelled water protons in blood as an endogenous contrast agent,

and as such represents a non-invasive method of measuring renal perfusion without exposure to ionising radiation or exogenous contrast agents.

1.9.4.2 Arterial spin labelling magnetic resonance imaging

During an ASL MRI acquisition, water protons in blood within the arterial in flow of the tissue undergoing interrogation are magnetically labelled by an inversion or saturation pulse and an image is acquired after a defined time delay which is equal to the arterial transit time. The labelled water exchanges with water in tissue to alter magnetisation, and thereafter the labelling decays over a time related to the local T1 longitudinal relaxation time. A control image is also acquired without the labelling, and subtraction of the control from the labelled image produces a signal difference which is related to blood flow (figure 1.3).
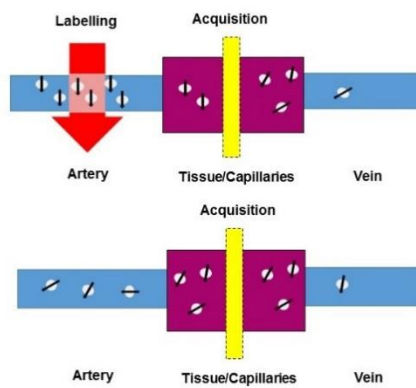
Figure 1.3 Pictorial representation of arterial spin labelling MRI

A quantitative measure of perfusion can be extrapolated from this using modelling extended from the Bloch equations (Roberts et al., 1995). The standard ASL kinetic model (figure 1.4) disregards arterial transit time and assumes that there is complete macromolecular saturation which occurs over a negligible time period. Other parameters must be known including λ, the tissue-blood partition coefficient (0.8 mL/g in kidney), the T1 longitudinal relaxation time, and M0, the equilibrium magnetisation, in addition to ΔM, which is signal difference between the labelled and control images, at inversion time TI. Perfusion, f, is then determined by the equation (Martirosian et al., 2004):

$$f = \frac{\lambda}{2TI} \frac{\Delta M(TI)}{M_0} \, exp\frac{TI}{T1}$$

Figure 1.4 The ASL standard kinetic model.

By measurement of the ASL signal, in addition to T1 and M0, renal perfusion can be quantified. TI = Inversion time, λ = Tissue-blood partition coefficient, M0 = Equilibrium magnetisation, ΔM = ASL signal, T1 = T1 longitudinal relaxation time

In reference to the earlier discussion of the differences between renal perfusion and blood flow, the ASL signal is produced by alteration of tissue magnetisation by labelled water protons in blood, and is therefore a true measure of perfusion: delivery of blood at the tissue level.

The ASL signal has inherently low signal to noise ratio (SNR), such that a number of image acquisitions are performed to produce several subtractions, the average of which is used to measure perfusion. This has the drawback that a number of labelled and control images must be registered together such that motion artefact can confound the acquisition of the eventual perfusion map. A number of techniques have been used to minimise this problem including respiratory gating, breath holding, and automated post processing strategies. On the other hand, an advantage of ASL is that the half-life of the labelling is measured in milliseconds, so the technique can be repeated infinitely even over short time periods; in comparison, gadolinium contrast used in DCE MRI has a half-life of hours and accurate perfusion measurements cannot be made until the tracer is cleared.

1.9.4.3 Validation of arterial spin labelling MRI

ASL MRI has been validated in animal models and phantoms against a number of techniques. In a study of 11 swine, ASL MRI was compared to perfusion measured using a microsphere technique (Artz et al., 2011b). Fluorescent microsphere probes were infused into the left ventricle, and measured in renal cortical tissue at different time points and following a number of interventions designed to modify kidney perfusion. ASL MRI showed strong correlation with the microsphere technique ($r = 0.81$, $p < 0.0001$), albeit underestimating perfusion measurements at lower values. This agreement persisted during manipulation of renal blood flow by fluid bolus, cooling, and administration of acetylcholine or isofluorane. Similarly, ASL MRI was validated in explanted porcine kidneys undergoing haemoperfusion with whole blood at a number of flow rates (Warmuth et al., 2007b). There

was good agreement between the measurements made by ASL MRI and by the reference values, with the values differing by a maximum of 13%.

1.9.4.4 Arterial spin labelling MRI in humans

Similarly, in healthy volunteers and in certain disease cohorts ASL MRI has been validated against other methods of measuring renal blood flow. For example, Ritt et al determined renal perfusion using ASL MRI and compared these measurements with renal plasma flow measured using PAH clearance, in 24 patients with metabolic syndrome, before and after 2 weeks of therapy with telmisartan (Ritt et al., 2010). The baseline measurements of perfusion made by the two methods showed moderate correlation (r = 0.575), as did the measured change in perfusion following ARB treatment (r = 0.548, p = 0.015). Similarly, Cutajar et al compared renal blood flow measured using ASL MRI with DCE MRI, and found reasonable agreement between the two techniques (Cutajar et al., 2014).

Additionally, the use of ASL MRI has been studied in certain disease cohorts. In a study of 12 patients with renal artery stenosis and 6 healthy volunteers, ASL MRI was able to discriminate severe stenosis from moderate, mild, or no renal artery stenosis (Fenchel et al., 2006). In a small study of patients with early CKD stages 2 -3, with a mean inulin clearance of 69 mL/min, renal blood flow was reduced (Rossi et al., 2012), and similarly, in a transplant population, reduced renal blood flow was observed in patients suffering from acute transplant dysfunction manifest as a deteriorating eGFR (Lanzman et al., 2010). Studies of ASL MRI in human participants are summarised in table 1.2.

Nevertheless, there has been little investigation into the use of ASL MRI in patients with CKD stages 3 – 5. Furthermore the application of ASL MRI into wider use, either in routine clinical practice or as a surrogate marker of renal function in trials of novel therapeutics, has been limited, hindered by lack of standardisation in acquisition technique, image sequence, and post processing strategies.

Author	Journal	Year	ASL	Field strength	Population & number	Perfusion (mL/min/100g)		
						Whole kidney	Cortex	Medulla
(Breidthardt et al., 2015)	*Eur. Radiol.*	2015	FAIR	1.5T	HF eGFR < 60 mL/min/100g (n=10)		146 ± 50	
					HF eGFR > 60 mL/min/100g (n=10)		171 ± 31	
					Age matched HV (n=10)		274 ± 65	
					HV < 40 years (n=10)		278 ± 59	
(Heusch et al., 2014)	*J. Magn. Reson. Imaging*	2013	FAIR True FISP	1.5T & 3T	Kidney transplant			
					eGFR > 30	282.7 ± 60.8		
					eGFR < 30	178.2 ±63.3		
(Rossi et al., 2012)	*Invest. Radiol.*	2012	FAIR True FISP	3T	HV (n=8)	301 ± 51	329 ± 52	
					CKD (mean eGFR 69 ± 12 mL/min by inulin clearance) (n=9)	244 ± 77	263 ± 81	
(Artz et al., 2011a)	*Magn. Reson. Imaging.*	2011	FAIR b-SSFP	1.5T	HV (n=5)		427 ± 20	85 ± 33
					CKD (n=5)		225 ± 85	60 ± 23
					Kidney transplant with eGFR > 60 mL/min/1.73m^2 (n=5)		314 ± 41	37 ± 21
					Kidney transplant with eGFR < 60 mL/min/1.73m^2 (n=10)		235 ± 91	36 ± 14
(Lanzman et al., 2010)	*Eur. Radiol.*	2010	FAIR True FISP	1.5T	Kidney transplant with stable function (n=6)		304.8 ± 34.4	
					Recent kidney transplant (n=7)		296.5 ± 44.1	
					Acute transplant dysfunction (n=7)		181.9 ± 53.4	

(Fenchel et al., 2006)	*Radiology*	2006	FAIR True FISP	1.5T	Patients with RAS (n=12)	Asymmetry of perfusion values Significant differences between perfusion in kidney with high grade compared to no or low grade RAS		
					HTN but no RAS (n=6)	243 ± 59	323 ± 79	113 ± 22
(Michaely et al., 2004)	*Invest. Radiol.*	2004	FAIR HASTE	1.5T	CKD (renovascular or other aetiology) (n=46)	Not quantified but reduced ASL signal on semi- quantitative analysis		
(Ott et al., 2013)	*CJASN*	2013	FAIR True FISP	1.5T	HTN, before and after renal denervation (n=19)	256.8 (IQR 241 – 278)		
(Schneider et al., 2012)	*CJASN*	2012	FAIR True FISP	1.5T	HTN, before and after 4 weeks of oral aliskiren therapy (n=34)	272 ±25		
(Ritt et al., 2010)	*NDT*	2010	FAIR True FISP	1.5T	Males with metabolic syndrome before and after 2 weeks of oral telmisartan therapy (n=24)	253 ±20		
(Cutajar et al., 2014)	*Eur. Radiol.*	2014	Multi TI FAIR 3D GRASE	1.5 T	HV (n=16)		263± 41	
(Gillis et al., 2014a)	*BMC Nephrol.*	2014	FAIR True FISP	3 T	HV (n=12)	229 ± 41	327 ± 63	
(Park et al., 2013)	*Magn. Reson. Imaging.*	2013	Pseudocontinuo us ASL	3T	HV (n=1)		320	

(Wang et al., 2012)	*Acad. Radiol.*	2012	FAIR SSFSE	3T	HV, before and after intravenous furosemide (n=11)		366.6 ± 41.2	118.59
(Cutajar et al., 2012)	*MAGMA*	2012	Multi TI FAIR 3D GRASE	1.5T	HV (n=20)	147 ± 30.8	178 ± 40.7	
(Gardener and Francis, 2010)	*Magn. Reson. Imaging.*	2010	FAIR True FISP SE-EPI with & without BS	1.5T	HV (n=9)\nFAIR TRUE FISP\nSE-EPI with BS\nSE EPI without BS		367 ± 50\n284 ± 75\n334 ± 65	103 ± 27\n139 ± 55\n122 ± 48
(Kiefer et al., 2009)	*Acad. Radiol.*	2009	FAIR TrueFISP	3T	HV (n=11)		245 ± 11	109 ± 5
(Karger et al., 2000)	*Magn. Reson. Imaging.*	2000	FAIR UFLARE	1.5T	HV (n=10)	213 ± 55		

Table 1.2. Human studies in arterial spin labelling MRI.

Perfusion measurements are displayed as mean ± standard deviation unless otherwise stated. Studies are grouped by nature of cohort. TI = inversion time, FAIR = flow sensitive inversion recovery, GRASE = gradient and spin echo, FISP = fast imaging with steady state precession, SSFSE = single shot fast spin echo, SSFP = single shot free precession, spin-echo echo planar imaging, BS = background suppression, HASTE = half Fourier acquisition single shot turbo spin echo, UFLARE = ultra-fast low angle rare, T = Tesla, HF = heart failure, HV = healthy volunteer, HTN = hypertensive, CKD = chronic kidney disease, RAS = renal artery stenosis, eGFR = estimated glomerular filtration rate.

1.10 Hypothesis and aims

1.10.1 Oxidative stress in chronic kidney disease

In summary then, CKD is a state characterised by oxidative stress, with endothelial and vascular dysfunction ensuing as a result, together with disturbance in renal haemodynamics that may play a role in the pathophysiology of the presence and progression of kidney disease. Antioxidant administration may ameliorate the oxidative stress which results from CKD and as a result improve endothelial and vascular function, and enhance renal haemodynamics.

1.10.2 Aims

The aims of this thesis are as follows:

1. Characterise a population of patients with CKD in comparison with a control population matched for age, blood pressure, and other cardiovascular risk factors, in terms of baseline markers of oxidative stress and vascular function.

2. Determine the effect of administration of ascorbic acid on the oxidative status and vascular function in CKD, in comparison to its effects on individuals with normal renal function.

3. Establish a robust and accurate method for evaluating renal blood flow non-invasively.

4. Characterise a population of patients with CKD in comparison with a control population in terms of renal perfusion and other MRI parameters.

5. Determine the effect of administration of ascorbic acid on renal perfusion in CKD, in comparison to its effects on individuals with normal renal function.

Chapter 2

Methods

2.1 Study design

2.1.1 Oxidative stress in Chronic Kidney Disease

2.1.1.1 Participant recruitment

Patients with chronic kidney disease were recruited from general nephrology and peritoneal dialysis (PD) clinics associated with the Glasgow Renal and Transplant Unit in the New Victoria and Stobhill Hospitals. A control group consisting of subjects with hypertension and normal renal function was recruited from the tertiary referral professorial hypertension clinic at the Western Infirmary Glasgow. Participants were excluded if they had diabetes, renovascular disease, or had active liver disease or infection. Participants were also excluded where there was a history of vitamin C supplementation, renal stone disease, or systemic oxalosis, given the oxalate loading associated with ascorbic acid (Baxmann et al., 2003). All patients gave written informed consent and the study was approved by the West of Scotland Research Ethics Committee. The study was carried out in concordance with the Declaration of Helsinki and is registered with a clinical trials registry (ISRCTN 31272864) (Levey et al., 2009).

2.1.1.2 Study protocol

Participants attended at the same time of day after an overnight fast. A control infusion of 100mL 0.9% normal saline was administered over 10 minutes, and after a further 10 minutes, phlebotomy was performed. After this assessments of arterial stiffness and endothelial function were carried out as described below. An intravenous infusion of 2000 mg ascorbic acid in 100mL 0.9% normal saline was then given over 10 minutes, and after 10 minutes phlebotomy was repeated, and the same assessments of arterial stiffness and endothelial function carried out. Phlebotomy was carried out for the third time 1 hour after administration of ascorbic acid.

2.1.1.3 Biochemical measurements

Baseline measurements were made of urea, creatinine, cholesterol, lipid subfractions, glucose, CRP, albumin, calcium and phosphate, and urinary protein quantification. The estimated glomerular filtration rate (eGFR) was calculated from serum creatinine using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula.

2.1.1.4 Measures of oxidant stress

Blood samples were taken via an intravenous cannula contralateral to the infusion arm after the control infusion of saline, immediately after ascorbic acid administration, and 1 hour after ascorbic acid administration. A panel of markers of oxidative stress were measured, including total antioxidant potential (TAP), serum ascorbic acid concentration, ADMA, F2-isoprostanes, and the ratio of glutathione (GSH) to its oxidised equivalent glutathione disulphide (GSSG). Electron paramagnetic resonance (EPR) spectrometry was also used to measure the rate of O_2^- production. The protocol for sample collection and processing is described in appendix 4.

2.1.1.5 Vascular function tests

Flow mediated dilatation and SphygmoCor® pulse wave analysis was performed as described in sections 2.2 and 2.3 respectively.

2.1.1.6 Statistics

Results are displayed as mean ± standard deviation, unless otherwise stated. Between group data were compared using a two tailed Student's t test or Mann Whitney U test where data were non-parametrically distributed. For related data, a paired samples Student's t test, or Wilcoxon's test was used. P values < 0.05 were considered significant. The relationship between the change in ADMA following ascorbic acid and eGFR was examined via stepwise multiple linear regression analysis, with inclusion of variables at p values less than 0.05. A one-way analysis of variance (ANOVA) was performed in a subgroup analysis, to compare the change in vascular function and oxidative stress parameters between the HTN, CKD and PD groups. All statistical testing was performed using SPSS Version 22.0 (IBM, Armonk, NY, USA).

2.1.2 Renal Arterial Spin Labelling Magnetic Resonance Imaging in Healthy Volunteers

2.1.2.1 Study design and patient recruitment

Healthy volunteers were recruited via local advertisement. Subjects attended on three occasions; firstly for clinical assessment and sampling of blood and urine, followed by ASL MRI performed during the second and third visits. Participants were fasted for 6 hours prior to imaging. All visits were completed within 4 – 28 days. All subjects gave written informed

consent and the study was approved by the College of Medicine, Veterinary and Life Sciences University of Glasgow Ethics Committee.

Baseline serum biochemistry and haematology measurements and urinary protein and creatinine quantification were obtained at initial visit. Estimated glomerular filtration rate (eGFR) was calculated using CKD–EPI formula (Levey et al., 2009). The full methodology behind the ASL MRI sequence employed is described in section 2.4.

2.1.2.2 Statistics

Results are expressed as mean ± standard deviation. Comparison of renal perfusion between right and left kidney, and ASL MRI 1 and 2, were made using paired Student's t tests with $p < 0.05$ deemed to demonstrate significant differences between methods. Pearson correlation coefficients were used to determine correlation between MRI measurements, and between MRI measurements and serum and urine parameters. Bland Altman plots were made of the mean perfusion values against the difference between the values, with the 95% limits of agreement calculated as the mean difference plus or minus 1.96 times the standard deviation of the difference. Repeatability was also assessed using intra-class correlation (ICC), which measures the contribution of between subject variances to total variance. ICC lies between zero and one, with values closer to one indicating a stronger agreement between measurements. A two way random effect model was used with a 95% confidence interval. The within subject coefficient of variance (CV_{ws}) is also expressed, which represents the ratio of the standard deviation of the differences between visits to the mean of all the perfusion measurements. Values closest to zero suggest good agreement between measurements. SPSS Statistics Version 19 was used for data analysis (IBM, Armonk, New York, USA).

2.1.3 Renal Arterial Spin Labelling Magnetic Resonance Imaging in Chronic Kidney Disease

2.1.3.1 Patient recruitment

Patients with CKD were recruited from the general nephrology clinic at the Glasgow Renal and Transplant Unit, whilst healthy volunteers (HV) were recruited via local advertisement. All subjects gave written informed consent and the study was approved by the West of Scotland Regional Ethics Committee. The study is registered with a clinical trials database (ISRCTN 12301736) and was carried out in compliance with the Declaration of Helsinki.

2.1.3.2 Baseline biochemical measurements

Baseline serum and urine samples for biochemistry and haematology analysis were obtained. eGFR was calculated from the measured serum creatinine using the CKD-EPI formula. Historical biochemical measurements were taken from the electronic patient record (SERPR, VitalPulse, UK). Proteinuria was measured using a spot protein to creatinine ratio (PCR) from a random urine sample.

2.1.3.3 Study protocol in healthy volunteers

Participants attended for clinical and biochemical assessment, and underwent ASL MRI according to the protocol described in section 2.4.

2.1.3.4 Study protocol in chronic kidney disease

After clinical and biochemical assessment, participants were cannulated in the left antecubital fossa. 100 mL of normal saline was administered and the first ASL MRI was performed. The participant remained in situ within the scanner, whilst 2000 mg of ascorbic acid in 100 mL of normal saline was administered intravenously. After 20 minutes, a second ASL MRI scan was performed.

2.1.3.5 Statistics

Results are expressed as mean ± standard deviation. T1 time and perfusion were measured in cortical and whole kidney ROIs, and ASL measurements are expressed by unit of mass (100 g) which is typical in the standard kinetic model. Whole kidney perfusion was factored with renal volume to give total kidney perfusion. Between group differences in T1 time, CMD, and perfusion were evaluated using Student's t tests. Changes in perfusion before and after administration of ascorbic acid were assessed with a paired Student's t test. Evaluation of correlation between MRI measurements and serum and urine biochemistry parameters was performed using Pearson's correlation coefficient. Throughout, p values < 0.05 were deemed significant. Data were analysed using IBM SPSS Statistics version 22.0 (IBM, Armonk, New York, USA).

2.2 Flow mediated dilatation

2.2.1 Brachial artery ultrasound

Subjects were supine with the upper limb held at 90° abduction at the level of the heart and held in situ within a semi-cylindrical frame, with the ultrasound transducer stabilised using

a pneumatic probe holder (figure 2.1). An inflatable cuff was positioned on the forearm, at least 10 cm distal to the recording site. Longitudinal recordings of the brachial artery were taken using 7MHz ultrasound transducer and a Siemens Accuson Sequoia ultrasound system (Siemens AG, Erlangen, Germany). Recordings were ECG gated with image capture occurring simultaneous with the R wave. A baseline recording was made for 2 minutes prior to cuff inflation. The cuff was inflated to 240 mmHg for 5 minutes. Following cuff release, a 5 minute recording was made. Endothelium independent vasodilatation was then measured using glyceryl trinitrate (GTN). Firstly, a second baseline measurement was made for 2 minutes, before 25 micrograms of sublingual GTN was administered and a further 5 minute recording made. Brachial artery ultrasound was performed by the same operator on each occasion.

This protocol was performed in adherence to the 2002 guidelines (Corretti et al., 2002) and recent update (Thijssen et al., 2011) regarding measurement of FMD, with the intention of measuring a purely endothelium dependent NO response. A brachial artery approach was used as endothelial function at this site has previously been shown to have close correlation with the coronary vasculature. ECG gated recordings were made in order to minimise the variability occurring during the cardiac cycle, and image capture at the end of diastole reduces the effect of vasculature compliance on arterial diameter. Peak brachial arterial diameter typically occurs within 180 seconds in healthy volunteers, but a 5 minute recording was made in order to capture peak FMD in the CKD cohort, which occurs later in patients with endothelial dysfunction (Black et al., 2008). As previously discussed, placement of the cuff distal to the recording site is more specific for a NO specific response. Response to GTN was measured in order to evaluate endothelium independent dilatation.
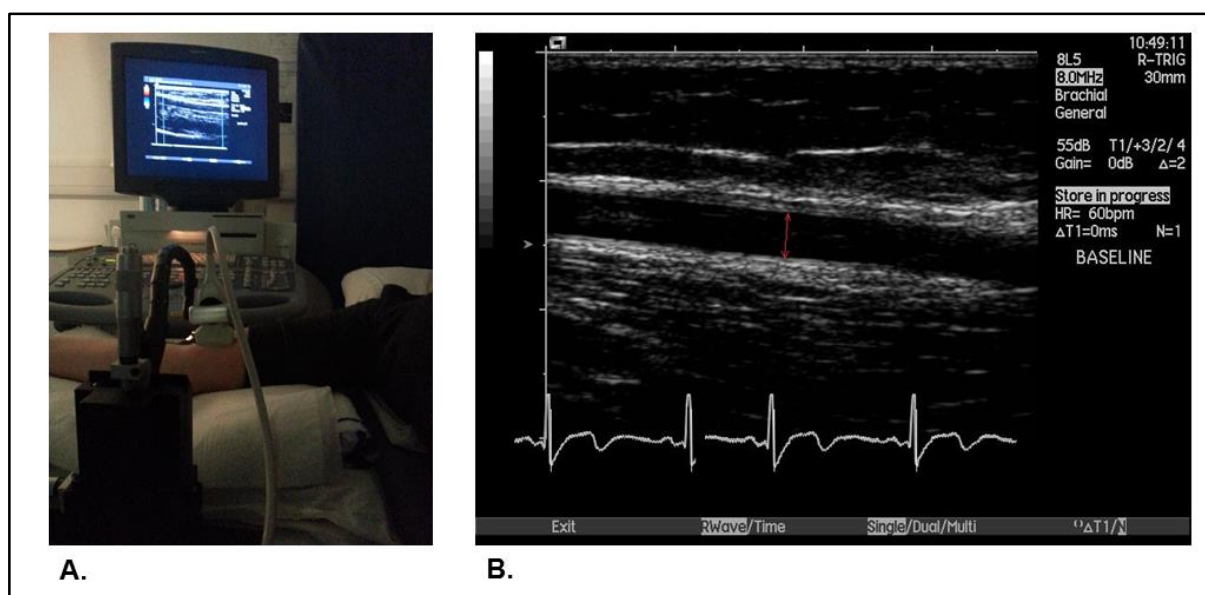
Figure 2.1 Flow mediated dilatation measured using brachial artery ultrasound.
A pneumatic probe holder and cylindrical limb rest is used to position the participant (A). Image analysis was then performed off line (B) using dedicated software utilising edge detection and tracking functionality.

2.2.2 Image analysis

Computer assisted analysis was carried out off line using dedicated edge detection and wall tracking software (Brachial Analyzer 5, Medical Imaging Applications LLC, Iowa, USA)(figure 2.1). The use of automation in FMD analysis has been shown to significantly improve accuracy (Woodman et al., 2001) and reduce intra-observer and inter-institution variability (Sonka et al., 2002). Baseline diameter is the mean diameter of the brachial artery measured over the 2 minute recording. The time point of the peak dilatation occurring following either cuff release or GTN was found, and peak diameter was taken as the mean of the measurements immediately before and after this. Degree of dilatation has been shown to be significantly and negatively correlated with baseline diameter; with larger vessels exhibiting proportionally less dilatation than smaller vessels. As such, FMD is expressed both as a proportion to baseline diameter as is conventional, as well as an absolute measurement, which has been proposed as a solution to this confounder (Atkinson et al., 2013).

2.3 Arterial stiffness

Arterial remodelling was evaluated using the SphygmoCor® Vx system (AtCor Medical, Sydney, Australia) of applanation tonometry, which allows measurement of Aix and PWV, as well as measurement of central blood pressures via a Fourier transformation. The theoretical basis for this has already been discussed in section 1.9.3 and subject preparation is discussed in section 2.1.1.

Patients were semi-recumbent for 10 minutes prior to measurement, and ambient temperature was controlled at $21 - 23$ °C. Three lead electrocardiogram (ECG) monitoring was performed throughout. Pulse wave analysis is confounded by arrhythmia such as atrial fibrillation and significant aortic valve disease, and the presence of this was assessed during clinical evaluation and ECG monitoring. All measurements were taken by the same trained investigator.

2.3.1 Augmentation index

Augmentation index was measured at the radial artery with the wrist in dorsiflexion. A 15 second recording was obtained when a satisfactory waveform tracing was obtained. Waveforms were assessed for quality and consistency as shown in figure 2.2. The analysis software provides an operator index, which is a quality control rating comprised of a number of individual accuracy indices (average pulse height > 100 units, pulse height variation < 5%, diastolic variation < 5%, shape variation < 5%). Measurements were deemed satisfactory if the operator index was greater than 85%.

As described in section 1.9.3, peripheral waveforms are converted to a central waveform by a standardised Fourier transformation, and Aix is corrected to a heart rate of 75 bpm to derive the adjusted Aix. Three measurements of Aix@75bpm were made and an average was taken.
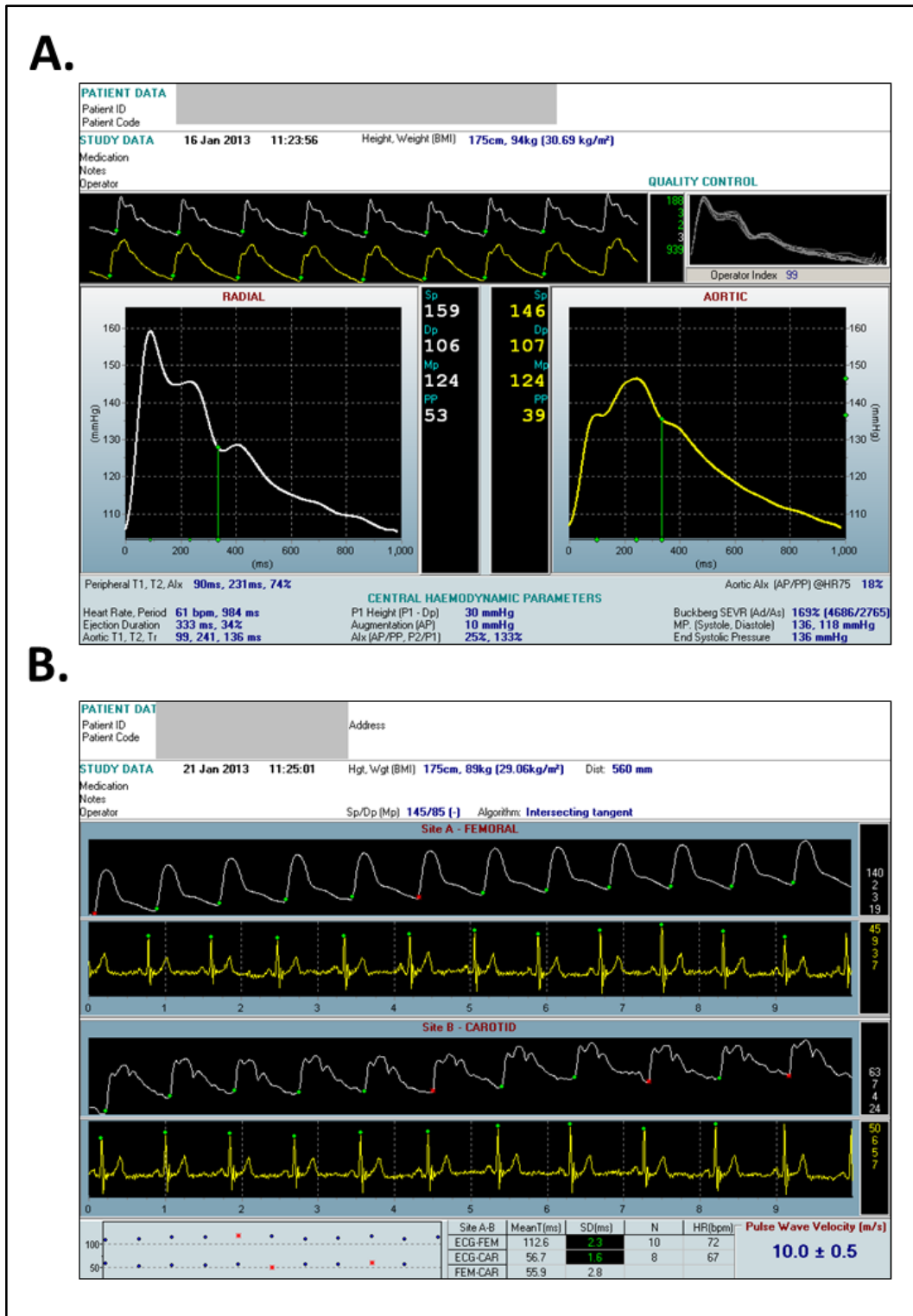
Figure 2.2 The SphygmoCor® system.

Pulse wave analysis (A) and measurement of pulse wave velocity (B).

2.3.2 Central blood pressure

Central blood pressure is derived from the central pulse wave which is constructed by transformation of the measured radial pulse wave. Peak systolic pressure, lowest diastolic pressure, and mean arterial pressure were measured.

2.3.3 Pulse wave velocity

Firstly the distances between the left carotid pulse and sternal notch, and between the sternal notch and the right femoral pulse via the umbilicus, were measured in millimetres. The SphygmoCor® system uses a subtraction method to calculate length, which represents the distance in the vasculature between the two pulses. Pulse wave analysis was then performed at the left carotid and right femoral pulses. The time between the R wave of the ECG and the upstroke of the arterial waveform was measured at each pulse. The difference between the proximal pulse upstroke and the distal pulse upstroke was calculated as the transit time. Velocity was then calculated as the distance travelled in metres divided by the transit time measured in seconds. PWV therefore represents a direct measurement of aortic pulse wave velocity.

A 15 second recording was made, and SphygmoCor® calculates a PWV for each cardiac cycle within the recording and provides the mean and standard deviation. Measurements were deemed acceptable if the standard deviation was less than 10% of mean PWV. Three PWV recordings were made, and an average was taken (figure 2.2).

2.4 Arterial spin labelling magnetic resonance imaging

2.4.1 ASL MRI method development

ASL MRI was performed using a Siemens work-in-progress sequence. Prior to the studies described here, there was little experience in the use of the method in the institute, necessitating an extensive period of method development prior to the conduct of clinical studies. This entailed the evaluation of different sequence parameters, breathing strategies, and imaging planes by scanning in-house volunteers in order to optimise image acquisition and data capture, whilst minimising scan time and participant inconvenience. Ultimately the sequence described below was found to obtain the clearest anatomical images and allow measurement of ASL signal.

2.4.2 Acquisition protocol

The principles behind ASL MRI were described in section 1.9.4. Briefly, an inversion pulse is used to alter the spin of protons in blood proximal to a tissue of interest. An image is taken of the tissue before and after application of the inversion pulse and the subtraction of the two equates to a contrast image. Quantification of perfusion from this ASL contrast signal is also dependent on the T1 relaxation time of tissue, the tissue-blood partition coefficient, and the equilibrium magnetisation, and perfusion can therefore be measured using the ASL standard kinetic model. T1 longitudinal relaxation time is an inherent property of tissues, representing the duration of time taken for 63% of the longitudinal magnetisation vector to return after a 90° radiofrequency pulse. An individual voxel is comprised of both cellular and extracellular compartments such that its signal intensity is a composite of the T1 time of both these structures. T1 has the tendency to be longer in fluids and shorter in fat, such that tissue which is more fluid in nature is darker on MRI, and vice versa.

MRI was performed on a Siemens Magnetom Verio 3.0 Tesla scanner (Siemens AG, Erlangen, Germany), using a 6-channel phased array body coil. A half Fourier acquisition single shot turbo spin echo (HASTE) localiser sequence was used to identify the location of the kidneys and the major vessels. ASL was performed using a flow-sensitive alternating inversion recovery (FAIR) perfusion preparation with true fast imaging with steady state free precession (True-FISP) sequence. Sagittal double oblique images were taken of both kidneys, with a single slice obtained at the mid-point of each axis, moved posteriorly to avoid major vessels. Three images with alternating selective and non-selective inversions were obtained in a single acquisition during a 12 second breath hold, and this was repeated five times. In addition, an image with no ASL preparation was acquired to measure equilibrium magnetisation. Fair True FISP parameters were: inversion time 900ms, repetition time 3.65ms, echo time 1.83ms, flip angle 60°, field of view 380mm by 380mm, in plane resolution 256 x 256 and slice thickness 10mm. T1 maps were obtained during a separate breath hold using a modified Look-Locker inversion recovery (MOLLI) sequence.

2.4.3 Image analysis

Renal anatomy was evaluated on the localiser images using a commercially available multi-modality post processing workstation (Siemens Syngo, Siemens AG, Erlangen, Germany). Kidney length was measured on the coronal images and volume was measured using a voxel count method by tracing contours on each slice of a 22 slice transverse image. A conventional post processing workstation was also used to measure T1 time at the cortex,

medulla, and whole kidney, and corticomedullary differentiation (CMD) was calculated as the cortex to medulla T1 time. Image analysis was then performed off line in house software MATLAB (MATLAB 8.4 R2014b, MathWorks, Natick, Massachusetts, USA). The analysis software registered the ASL images, subtracted non-selective from selective images, and averaged the subtractions. The averaged ASL subtraction image was fitted to the M0 and T1 data using the standard ASL kinetic model (Martirosian et al., 2004) to produce a perfusion map, onto which regions of interest (ROI) were drawn to measure cortical and whole kidney perfusion. ASL perfusion measurements, expressed by unit of mass (100 g), were factored with renal volume to give kidney perfusion.

2.5 Biomarkers of oxidative stress

2.5.1 F2-isoprostanes

Measurement of F2-isoprostanes was carried out by Dr Andrew Duncan and his team at the Biochemistry Department, Glasgow Royal Infirmary. A competitive immunoassay with colourimetric quantification (Direct 8-iso-Prostaglandin F2α Enzyme Immunoassay Kit, Assay Designs, Enzo Life Science, New York, USA) was used to measure 8-iso-prostaglandin F2α (8-iso-PGF2α), also termed F2-isoprostane, as a marker of lipid peroxidation. Blood samples were collected as described in section 2.1.1, and illustrated in appendix 4.

Frozen serum samples were thawed at room temperature and mixed 4:1 with 10 mol/L sodium hydroxide, then heated at 45 $^{\circ}$C for 2 hours in order to hydrolyse the sample. 500 µL of hydrolysed sample was then added to 100 µL of 12.1 mol/L hydrogen chloride and then 50 µL of this to 50 µL of a neutralising reagent. 50 µL each of a blue solution of alkaline phosphatase conjugated with 8-iso-PGF2α, and a yellow solution of a rabbit polyclonal antibody to 8-iso-PGF2α was added. The plate was incubated at room temperature on a shaker for 2 hours at 500 rpm. The contents of the wells were emptied and washed three times with wash solution. 200 µL of p-nitrophenyl phosphate solution was added and the plate incubated for 45 minutes at room temperature before stop solution was applied. The optical density of the plate was then read at 405 nm. 8-iso-PGF2α was quantified by comparison to a standard curve derived from known concentrations.

2.5.2 Glutathione to glutathione disulphide ratio

Measurement of GSH/GSSG ratio was performed by Mr Jim McCulloch and his team at the BHF Glasgow Cardiovascular Research Centre. The ratio of GSH to its oxidised equivalent, GSSG, was taken as a measure of GSH availability, and this was measured using a colorimetric assay (Bioxytech GSH/GSSG-412, OxisResearch, Percipio Biosciences, Burlingame, USA). The assay relies on the reaction of GSH with Ellman's reagent, or 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), which produces a spectrophotometric product at 412 nm. To measure GSSG, the assay uses the thiol scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP) to scavenge existing GSH within the sample, and then generates GSH from the remaining GSSG using glutathione peroxidase, as is shown in figure 2.2.
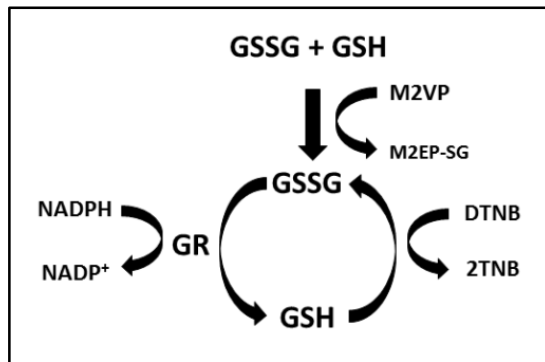
Figure 2.3 Measurement of GSH:GSSG.

The measurement of oxidised glutathione (GSSG) is difficult due to its low concentration and the tendency for reduced glutathione (GSH) coexistent within the sample to become oxidised during the reaction. The assay used here measures GSH by reaction with Ellman's reagent. To measure GSSG, firstly any GSH in the sample is scavenged using 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP). GSSG is then reduced by glutathione reductase (GR) with NADPH as a cofactor of the reaction to form GSH. During reaction with Ellman's reagent (DTNB), GSH is oxidised to GSSG whilst producing a spectrophotometrically detectable product.

Samples were obtained according to section 2.1.1. To prepare the GSSG samples, 10 µL of M2VP was added to a microcentrifuge tube, and 100 µL of whole blood was added to the bottom of the centrifuge tube. Samples were mixed and then frozen at –70°C. The sample was thawed at room temperature, 290 µL 5% metaphosphoric acid was added, and the sample vortexed for 15 – 20 seconds, and centrifuged at 1000 g for 10 minutes. 50 µL of the extract was added to 700 µL GSSG buffer and the diluted extract placed on ice. A control sample was made by adding 50 µL metaphosphoric acid to 700 µL GSSG buffer.

To prepare GSH samples, 50 µL of whole blood was added to the bottom of a microcentrifuge tube and was frozen at –70°C. The sample was thawed, and 350 µL cold 5% metaphosphoric acid added to the tube. The sample was vortexed for 15 seconds and centrifuged at 1000 g for 10 minutes. 50 µL metaphosphoric acid extract was then added to 3 mL assay buffer (NaPO$_4$ with EDTA).

The assay was then performed by adding 200 µL of standards, controls, or samples to the cuvettes. 200 µL of DTNB, and then 200 µL of glutathione peroxidase was added to each cuvette. The samples were mixed and incubated at room temperature for 5 minutes. 200 µL of NADPH was added and the change in absorbance at 412 nm was recorded for 3 min.

To quantify the concentrations of both GSH and GSSG, firstly the reaction rate was calculated as the slope of the regression line of the change in absorption per unit of time. Calibration curves were constructed using GSH and GSSG standards of known concentrations, and the concentration of each analyte was determined using these references. The GSH to GSSH ratio was then calculated.

2.5.3 Electron paramagnetic resonance

EPR was performed by Mr Jim McCulloch and his team at the BHF Glasgow Cardiovascular Research Centre. Samples used to measure ROS using EPR were obtained in the manner described in section 2.1.1 and in appendix 4. Plasma which was obtained for EPR was immediately placed on ice and analysed using a Bruker e-scan EPR system.

Firstly, Krebs-HEPES buffer was prepared, filtered, and adjusted to pH 7.4. A 10 mM solution of deferoxamine was produced, as was a 10 mM solution of diethyldithiocarbamic acid (DETC), and a 1 mM solution of the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH). 100 µL of deferoxamine and 20 µL of DETC was added to the 40 mL of buffer, and then 900µL of the buffer and chelator solution was combined with 100 µL of CPH. The CPH solution was then added 1:1 to the sample and 50 µL of this was placed into a glass EPR capillary tube. ROS within the sample react with CPH to produce stable CPH radicals, and the spectra produced when subject to EPR were recorded for 5 minutes. The EPR signal is proportional to the quantity of ROS in the biological sample, and the rate of this production during the EPR measurement was taken as a measure of oxidative stress

2.5.4 Total antioxidant potential

Quantification of total antioxidant potential (TAP) was performed by Mr Jim McCulloch and his team at the BHF Glasgow Cardiovascular Research Centre. TAP was measured using a qualitative colorimetric assay (Bioxytech AOP-450 Quantitative Assay for Total Antioxidant Potential, OxisResearch, Percipio Biosciences, Burlingame, USA). The assay is based upon the reduction of Cu^{2+} to Cu^+ by the combined activity of all antioxidants within the sample. A chromogenic reagent selectively forms a stable complex with reduced copper, with a maximum absorption at 450 nm. A known concentration of Trolox (a vitamin E analogue) was used to create a calibration curve, such that TAP is expressed quantitatively as millimoles of Trolox equivalents.

Samples were obtained and processed as described in section 2.1.1 and appendix 4, and then thawed at room temperature. Different concentrations of Trolox were made by dilution with deionised water. Samples and Trolox standards were diluted 1:40 in dilution buffer, and 200 µL of mixture was placed into each well. The plate was read at 450 nm for a reference measurement. 50 µL of copper solution was then added to each well, which was incubated at 3 minutes at room temperature. Stop solution was then added and the plate was read for a second time at 450 nm. Net absorption of a sample is derived by subtracting the second measured absorption from the first measured absorption. A calibration curve was produced plotting the known concentration of Trolox along the X axis and the net absorption on the Y axis. Quantification of TAP was then calculated by referencing the absorption of a given sample to the calibration curve.

2.5.5 Ascorbic acid

High performance liquid chromatography was used to measure ascorbic acid, performed by Dr Andrew Duncan at the Biochemistry Department, Glasgow Royal Infirmary. Biological samples were obtained as described in section 2.1.1 and shown in appendix 4. Samples destined for ascorbic acid quantification underwent deproteinisation using 6% metaphosphoric acid prior to freezing. After thawing an aliquot of the sample was injected on to a C18 reverse phase chromatographic column and the ascorbic acid concentration assayed using an electrochemical detector.

Chromatographic measurements were made using a Waters HPLC pump and autosampler (Waters Corporation, U.S.A), an ESA Coulochem 5100A Electrochemical Detector, and ESA Analytical Cell Model 501 (Dionex, U.S.A). A 25 cm Apex ODS HPLC column with

5µm particle size, and 1cm Apex ODS guard column with 5µ particle size was used (Grace Discovery Sciences, U.S.A). The mobile phase comprised chloroacetic acid 7.1 g, sodium hydroxide 2.3 g, sodium EDTA 0.43 g, L-octane sulphonic acid100 mg, dissolved in 500 mL distilled water, and filtered using a 0.2 µm filter. The mobile phase was then pumped at 1.5 mL per minute. Samples, quality control material and standards were allowed to thaw thoroughly at room temperature and were mixed thoroughly before use. Samples were vortexed then centrifuged at 3000 rpm for 10 minutes.

200 µL of sample supernatant was then combined with 800 µL of a solution of dithiothreitol and metaphosphoric acid (750 mg dithiothreitol in 500 mL of 3% metaphosphoric acid) in an Eppendorf tube. Tubes were vortexed for 5 seconds then centrifuged in the Eppendorf microcentrifuge at 10,000 rpm for 5 minutes. The sample was then decanted into glass autosampler vials, and placed on the carousel. The ascorbic acid peak was identified by comparison with that of the ascorbic acid standard, and quantification was carried out using an external standard.

2.5.6 Asymmetric dimethylarginine

ADMA was measured by using HPLC methodology (Blackwell et al., 2009) by Dr Andrew Duncan at the Biochemistry Department, Glasgow Royal Infirmary, using cation exchange solid phase extraction (SPE), utilising a solvent delivery system and fluorimeter (Waters Corporation, U.S.A). Samples were obtained as described in section 2.1.1 and appendix 4.

The analyte was extracted from plasma using Isolute PRS cation exchange SPE column (Biotage, Sweden) with a Vac Elut extraction system (Agilent Technologies, U.S.A). The columns were activated with 2mL methanol, then 2mL 50 mmol/L borate buffer. 200 µL of sample was then mixed with 720 borate buffer and 80 of the internal standard monoethylarginine, and loaded onto the SPE column. After washing with borate, water, and methanol, the analyte was eluted with a solution of methanol and ammonia. The eluent was then evaporated at 80 ^{0}C. HPLC was performed, with a mobile phase comprising 50 mmol/L acetate buffer with 9% acetonitrile, filtered via a 0.45 µ filter and pumped at 1.5 mL/min. ADMA was then measured fluorometrically at an excitement of 340 nm and emission of 455 nm. Quantification was performed by comparison to a standard curve.

Chapter 3

Oxidative Stress in Chronic Kidney Disease

3.1 Introduction

The risk of CVD is significantly elevated in patients with chronic kidney disease (CKD) with cardiovascular risk increasing in parallel with the decline in renal function (Culleton et al., 1999). Indeed most patients with CKD will die of a cardiovascular event before they progress to the need for renal replacement therapy. This cardiovascular risk can be explained, in part, by shared risk factors and the age of the CKD population, but does not account fully for the degree of observed CVD. Indeed, such shared risk factors can confound the study of CVD in CKD, but nevertheless, the prevalence of CVD remains higher in this group even when adjusted for age and co-morbidities (Go et al., 2004).

The presence of non-conventional risk factors specific to CKD has been suggested as a mechanism contributing to CVD, such as hyperphosphataemia, anaemia, chronic inflammation and structural heart disease specific to CKD, so called 'uraemic cardiomyopathy'. Oxidative stress is another cardiovascular risk factor characterised by an excess of reactive oxygen species (ROS) and other oxidants, leading to a derangement in redox signalling which activates pathways which lead to deleterious changes to vascular biology (Jones, 2008).

In vivo, this is demonstrated by evidence of endothelial dysfunction and by an increase in oxidative modification of macromolecules. For example, advanced oxidative protein products (AOPPS), 8-hydroxydeoxyguanosine, and F2-isoprostanes are all elevated in CKD, and renal transplantation results in a significant reduction in these biomarkers of oxidative stress (Simmons et al., 2005, Yilmaz et al., 2005). Furthermore, circulating levels of the nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) are increased in renal impairment which also leads to increased ROS production via uncoupling of endothelial NOS (Fujiwara et al., 2011, Valli et al., 2007, Oberg et al., 2004).

Previous studies have investigated the use of exogenous antioxidants either as therapy, or as manipulators of redox homeostasis as a means to explore the mechanisms behind oxidative stress in CKD. For example, antioxidant supplementation has been shown to improve markers of oxidative stress and blood pressure in animal models of CKD and hypertension (Tian et al., 2006, Shing et al., 2014) . In humans, however, the data is conflicting, with some studies showing certain benefits of antioxidant therapy in patients with CKD (Boaz et al., 2000, Williams et al., 2001), and other studies finding no such effect (Cross et al., 2003, Ramos et al., 2011).

The effect of antioxidant administration in renal impairment on vascular function was therefore investigated in order to explore the mechanisms underlying oxidative stress and endothelial dysfunction in CKD. A cross over study of intravenous ascorbic acid and normal saline was carried out in a population of patients with CKD stages 3 – 5 (Kidney Disease Improving Global Outcomes, 2012), in comparison to a matched population of patients with hypertension (HTN).

3.2 Methods

The methodology behind the Renox study was described in detail in section 2.1.1 and shown in figure 3.1. Three groups of participants were recruited: patients with CKD were recruited from general nephrology clinics, patients receiving peritoneal dialysis were recruited from the PD clinic, and a control group of hypertensives with normal renal function were recruited from a tertiary referral blood pressure clinic.

To summarise the study protocol, after clinical evaluation had taken place, 100 mL of 0.9% normal saline was administered intravenously. After 10 minutes, blood samples were taken in order to measure baseline biochemistry, and biomarkers of oxidative stress. Arterial stiffness was then assessed using the SphygmoCor® system, as described in section 2.4, and evaluation of endothelial function was undertaken by measurement of flow mediated dilatation using brachial artery ultrasound, as described in section 2.2. After a rest period, 2000 mg of ascorbic acid was administered intravenously in 100 mL 0.9% normal saline. After ten minutes, blood samples were taken for a second occasion for measurement of oxidative stress biomarkers and the vascular function studies were repeated. Phlebotomy was performed for a third occasion 60 minutes after administration of ascorbic acid.

Biomarkers of oxidative stress which were measured are shown below, in table 3.1. The pathophysiological basis for these markers was discussed in section 1.9, and the methodology behind their measurement described in detail in section 2.5.
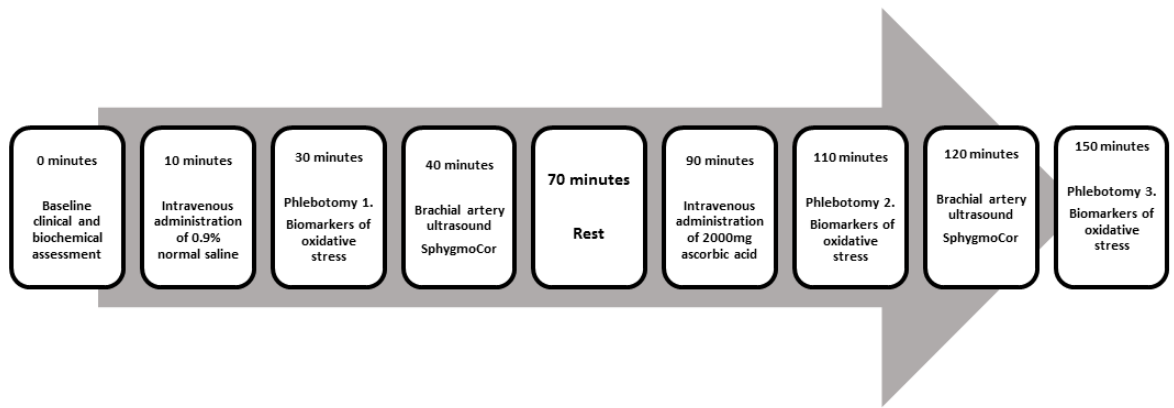
Figure 3.1 Renox study protocol

Biomarker	Rationale	Methodology
GSH/GSSG	Reduced glutathione is an important buffer, and ratio of glutathione to its oxidised equivalent is a marker of oxidative stress	Colourimetry
F2-isoprostanes	Indicator of lipid oxidative modification	Immunoassay with colourimetric quantification
O_2^-	ROS are a physiologically important radical species	Electron paramagnetic resonance
Ascorbic acid	Water based antioxidant vitamin	High performance liquid chromatography
Total antioxidant potential	Measure of non-enzymatic reducing potential in serum	Colourimetry
ADMA	Circulating inhibitor of NO production which is elevated in CKD	High performance liquid chromatography

Table 3.1 Biomarkers of oxidative stress measured in the Renox study

3.3 Results

3.3.1 Clinical characteristics

30 subjects with CKD (22 with stages 3- 5 CKD and 8 patients receiving PD) and 20 subjects with HTN were recruited. eGFR was 22.4 ± 12.6 mL/min/1.73m^2 in the CKD group compared to 94.4 ± 11.7 mL/min/1.73m^2 in the HTN group ($p < 0.001$). The two groups were well matched for age, body mass index (BMI), smoking status and medication history, but blood pressure was higher in the HTN group. Baseline parameters are fully described in table 3.2, and medication history is shown in table 3.3.

3.3.2 Biomarkers of oxidative stress

Baseline ascorbic acid was significantly lower in patients with CKD than in HTN (22.45 ± 27.49 vs. 43.96 ± 14.13 µmol/L, $p = 0.023$), whilst ADMA (0.61 ± 0.14 vs. 0.48 ± 0.07 µmol/L, $p < 0.001$) and TAP (0.66 ± 0.15 µM Trolox vs. 0.58 ± 0.10 µM Trolox, $p = 0.027$) were significantly higher. There were no significant differences in F2-isoprostanes (1073.98 ± 1048.38 pg/mL vs. 898.96 ± 643.02 pg/mL, $p = 0.61$), GSH/GSSG (110.71 ± 266.17 vs. 140.03 ± 448.80, $p = 0.75$), and O_2^- production (0.50 ± 0.29 arb. units vs. 0.37 ± 0.95 arb. units, $p = 0.08$) between the two groups at baseline (table 3.4).

After ascorbic acid, TAP and O_2^- production both transiently rose and then fell to a level higher than baseline in both groups. In CKD, TAP changed from 0.66 ± 0.15 to 1.24 ± 0.20 ($p < 0.001$) to 1.11 ± 0.22 µM Trolox ($p < 0.001$), whilst O_2^- production altered from 0.50 ± 0.29 to 0.63 ± 0.22 ($p = 0.038$) to 0.55 ± 0.18 arb. units ($p = 0.42$). In HTN, TAP changed from 0.58 ± 0.10 to 1.15 ± 0.22 ($p < 0.001$) to 0.85 ± 0.14 µM Trolox ($p < 0.001$) and O_2^- changed from 0.37 ± 0.10 to 0.50 ± 0.14 ($p = 0.005$) to 0.43 ± 0.10 arb. units ($p = 0.007$).

There was no reduction in F2-isoprostanes observed in the HTN group (899 ± 643 to 729 ± 464 pg/mL, $p = 0.18$) or the CKD group (1074 ± 1048 to 862 ± 389 pg/mL, $p = 0.37$), and neither was there a change in GSH/GSSG observed in either HTN (140.0 ± 448.8 to 111.3 ± 390.4, $p = 0.35$) or CKD (94.6 ± 256.2 to 46.70 ± 83.2, $p = 0.21$) (figure 3.2).

After administration of ascorbic acid there was a reduction in ADMA in the CKD group (0.61 ± 0.14 to 0.58 ± 0.14 µmol/L, $p = 0.039$), but not the HTN group (0.48 ± 0.07 to 0.51 ± 0.07 µmol/L, $p = 0.36$), which reached statistical significance in the second but not the

third phlebotomy time point. The degree of change in ADMA was also significantly different between the two groups (-0.04 ± 0.09 vs 0.02 ± 0.06, p = 0.013).

Table 4 shows the result of multivariate regression analysis on the determinants of the response of ADMA to ascorbic acid with age, eGFR, BMI, blood pressure and gender entered into the model. Age (B = 0.002 (95% CI 0.000, 0.004), p = 0.041) and eGFR (B = 0.001 (95%CI 0.000, 0.002), p = 0.005) were independent determinants, whilst BMI, blood pressure, and gender were not significant predictors of response to ADMA.

Parameter	HTN	CKD	p value
	n = 20	*n = 30*	
Age (years)	56 ± 10	59 ± 14	0.34
Body mass index (kg/m2)	30.9 ± 5.4	28.1 ± 6.7	0.13
Peripheral BP (mmHg)	150/95 ± 17/10	141/82 ± 15/10	0.04*
Mean arterial BP (mmHg)	112 ± 12	101.6 ± 10.2	0.002*
Primary renal diagnosis (n)			
Glomerulonephritis		14	
Renovascular disease		1	
Reflux nephropathy		3	
Polycystic kidney disease		4	
Unknown		4	
Other		4	
CKD stage (n)			
3a		1	
3b		7	
4		14	
5		0	
	n = 20	*n = 22*	
Creatinine (µmol/L)	71 ± 11	359 ± 274	< 0.001*
eGFR (mL/min/1.73m2)	94.4 ± 11.7	22.4 ± 12.6	<0.001*

Table 3.2 Baseline parameters in the Renox study.

Asterisks (*) indicate a significant difference. CKD participants receiving peritoneal dialysis are not included in comparison of creatinine and eGFR. HTN = Hypertension, CKD = Chronic kidney disease, BP = Blood pressure, eGFR = Estimated glomerular filtration rate.

Current medications	HTN n (%)	CKD n (%)
ACE inhibitor	9 (45)	15 (50)
Angiotensin receptor blocker	6 (30)	7 (23)
Beta blocker	3 (15)	10 (33)
Calcium channel blocker	10 (50)	15 (50)
Statin	6 (30)	16 (53)
Allopurinol	2 (10)	10 (33)
Spironolactone	2 (10)	0 (0)
Current smoker	4 (20)	6 (20)

Table 3.3 Medication and smoking history

3.3.3 Arterial function

Table 3.6 demonstrates measurements of vascular function studies before and after ascorbic acid. Baseline central blood pressure was significantly higher in the HTN group (132/85 ± 15/11 vs. 142/97 ± 18/10 mmHg, p = 0.042) but there was no difference in adjAix (25.6 ± 8.0% vs. 24.4 ± 12.3%, p = 0.70). PWV was significantly higher in the CKD group (10.2 ± 2.5 vs. 8.6 ± 1.7m/s, p = 0.022).

Following administration of ascorbic acid central blood pressure fell significantly in both CKD (132/85 ± 15/11 to 120/75 ± 21/11 mmHg, p = 0.005) and HTN (142/97 ± 18/10 to 130/81 ± 25/15 mmHg, p = 0.002). There was no significant difference in the degree of change between CKD and HTN (p = 0.86).

A significant reduction in adjAix was also seen in CKD (25.6 ± 8.0% to 15.8 ± 10.4%, p < 0.001) and HTN (24.4 ± 12.3% to 17.6 ± 11.3%, p < 0.001), and again, the degree of change was not different between groups (p = 0.09). No change in PWV was observed in either group following ascorbic acid in either group (figure 3.3).

3.3.4 Endothelial dysfunction

At baseline, no significant difference was observed in the diameter of the brachial artery between the HTN and CKD groups (4.71 ± 0.81mm vs. 4.40 ± 0.67mm, p = 0.18). Neither was there a between group difference in FMD in either absolute (0.19 ± 0.07mm vs. 0.19 ± 0.12mm, p = 0.27) or proportional (4.13 ± 1.99% vs. 4.70 ± 3.21%, p = 0.51) terms.

In the HTN group, after administration of ascorbic acid, FMD changed from 0.19 ± 0.07 to 0.49 ± 0.25 (p = 0.12) and percentage FMD changed from 4.13 ± 1.99 to 4.95 ± 2.86 (p = 0.31). In the CKD group FMD changed from 0.19 ± 0.12 to 0.35 ± 0.21 mm (p = 0.10) and proportional FMD changed from 4.71 ± 3.21 to 5.96 ± 3.66 (p = 0.35) after ascorbic acid. There was a significant reduction in endothelial independent dilatation after ascorbic acid administration, in both the HTN group (12.30 ± 5.91 vs. 6.68 ± 4.95 %, p < 0.001) and the CKD group (14.66 ± 6.22 vs. 7.73 ± 5.30 %, p <0.001) (figure 3.4).

Parameter	HTN	CKD	p value
Ascorbic acid (µmol/L)	44.0 ± 14.1	23.7 ± 27.7	0.023*
ADMA (µmol/L)	0.48 ± 0.07	0.61 ± 0.14	<0.001*
F2-isoprostanes (pg/mL)	899.0 ± 643.0	1074.0 ± 1048.4	0.61
Total antioxidant potential (µM Trolox)	0.58 ± 0.10	0.66 ± 0.15	0.027*
GSH/GSSG	140.0 ± 448.8	110.7 ± 266.2	0.75
O_2^- production	0.37 ± 0.10	0.50 ± 0.29	0.08

Table 3.4 Comparison of biomarkers of oxidative stress at baseline.

Asterisks (*) indicate a significant difference. HTN = Hypertension, CKD = Chronic kidney disease, ADMA = Asymmetric dimethylarginine, GSH = Glutathione, GSSG = Glutathione disulphide, O_2^- = Superoxide.

	Unstandardized coefficient	Significance	95% confidence interval
	B	p	
R^2 = 0.212			
Constant	-0.170	0.006	-0.288, -0.051
eGFR	0.001	0.005	0.000, 0.002
Age	0.002	0.041	0.000, 0.004

Table 3.5 Determinants of the change in ADMA in response to ascorbic acid.

Evaluated using a multiple stepwise linear regression analysis with a stepwise paradigm which included variables with p values less than 0.05.

Parameter	HTN			CKD		
	Before	After	p value	Before	After	p value
Central blood pressure (mmHg)	142/97 ± 18/10*	130/82 ± 25/15†	0.002	132/85 ± 15/12*	120/75 ± 21/11†	0.005
Adjusted augmentation index (%)	24.4 ± 12.3*	17.6 ± 11.3†	<0.001	25.6 ± 8.0*	15.8 ± 10.5†	<0.001
Pulse wave velocity (m/s)	8.62 ± 1.69*	8.42 ± 1.45	0.28	10.19 ± 2.45*	9.70 ± 3.04	0.11
Brachial artery diameter (mm)	4.71 ± 0.81	5.15 ± 0.84†	< 0.001	4.40 ± 0.67	4.76 ± 0.87†	<0.001
Flow mediated dilatation (mm)	0.19 ± 0.07	0.25 ± 0.13	0.12	0.19 ± 0.12	0.27 ± 0.15	0.10
Percent flow mediated dilatation (%)	4.13 ± 1.99	4.95 ± 2.86	0.31	4.71 ± 3.21	5.96 ± 3.66	0.35
GTN mediated dilatation (mm)	0.55 ± 0.25	0.33 ± 0.24†	<0.001	0.63 ± 0.21	0.35 ± 0.21†	<0.001
Percent GTN mediated dilatation (%)	12.30 ± 5.91	6.68 ± 4.95†	<0.001	14.66 ± 6.22	7.73 ± 5.30†	<0.001

Table 3.6 Vascular function tests before and after administration of ascorbic acid.

The p value refers to a paired Student's t test comparing the parameter before and after ascorbic acid. Asterisks (*) indicate parameters where there was a significant difference in baseline values between the two groups, whilst the dagger (†) indicates a significant difference values measured before and after ascorbic acid. HTN = Hypertension, CKD = Chronic kidney disease, GTN = Glyceryl trinitrate.
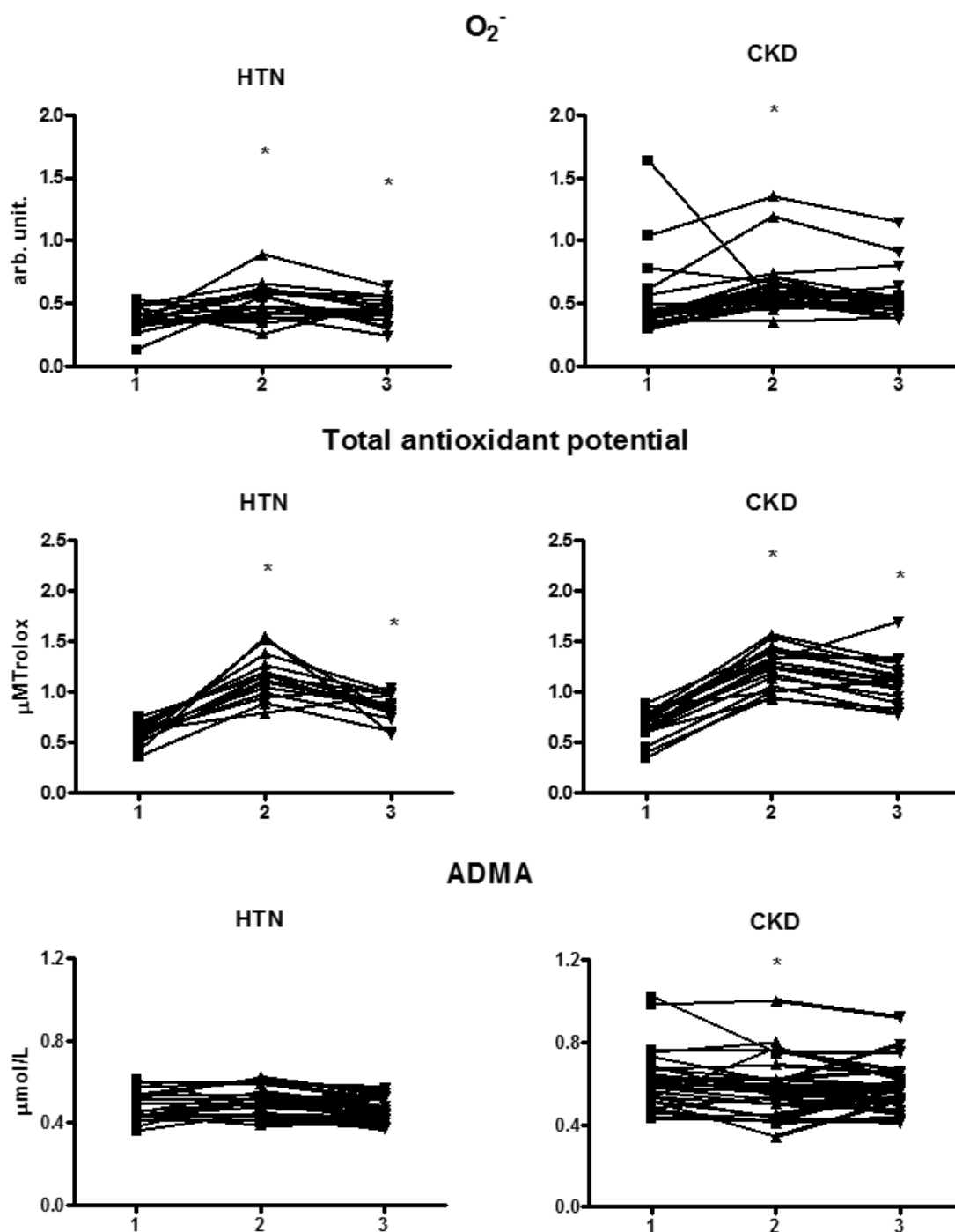
Figure 3.2 Effect of ascorbic acid on biomarkers of oxidative stress.

Ascorbic acid increases O_2^- production (A) and total antioxidant potential (B) in both groups, whilst reducing ADMA in patients with CKD (C). Measurements of biomarkers of oxidative stress made in both groups are shown at baseline (1), after ascorbic acid (2) and at one hour after ascorbic acid (3) (see figure 1). Asterisks (*) indicate a significant difference ($p < 0.05$) in comparison with baseline. HTN = Hypertension, CKD = Chronic kidney disease, O_2^- = Superoxide, ADMA = Asymmetric dimethylarginine.
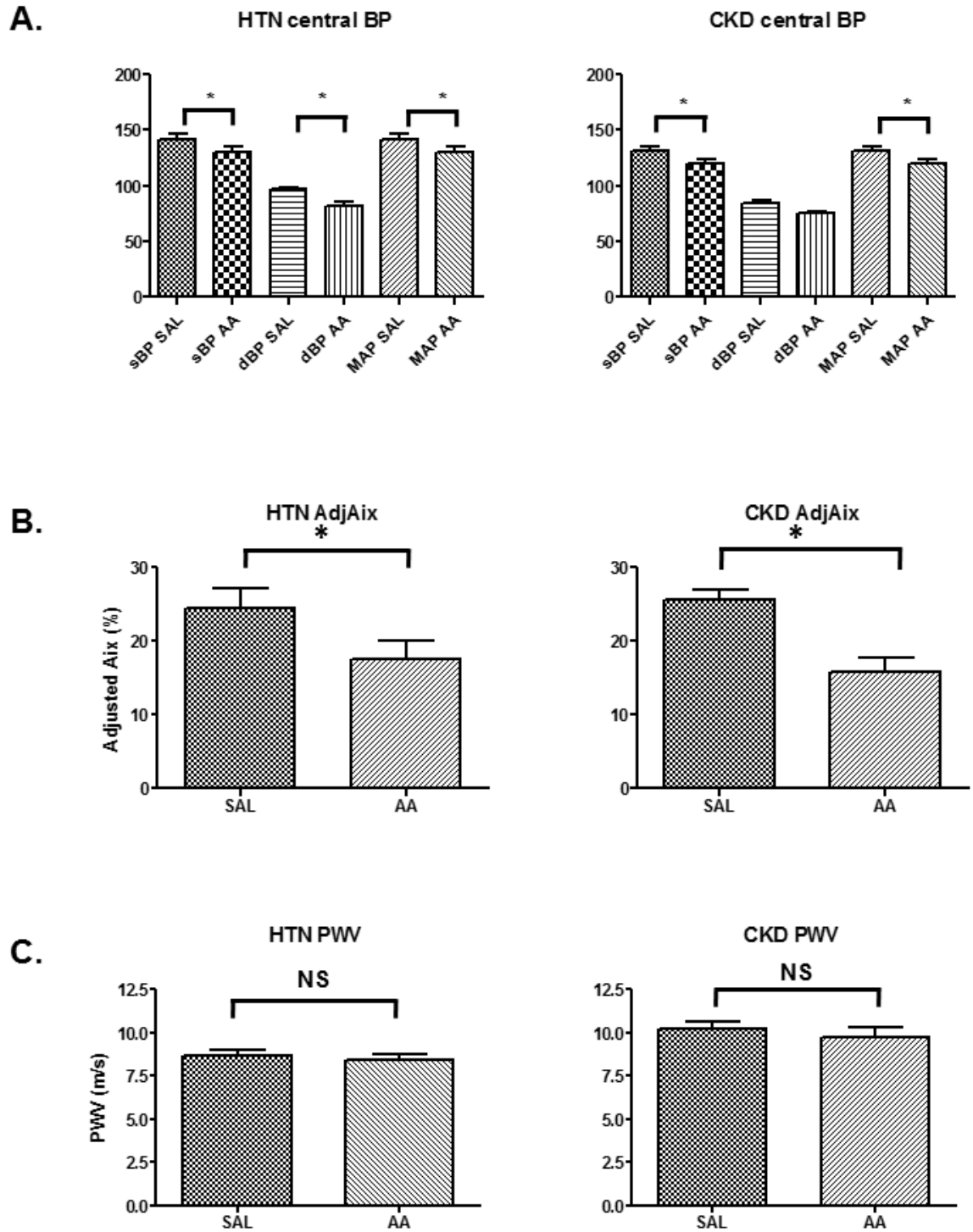
Figure 3.3 Effect of ascorbic acid on large arterial function.

Ascorbic acid reduces central blood pressure (A) and augmentation index (B) in both hypertension and CKD. Changes in central BP (A), adjusted augmentation index (B) and pulse wave velocity (PWV) are shown in both groups. Asterisks (*) indicate a significant difference (p < 0.05) occurring after ascorbic acid. Error bars represent standard error of the mean. HTN = Hypertension, CKD = Chronic kidney disease, SAL = Normal saline, AA = Ascorbic acid, NS = Not significant.
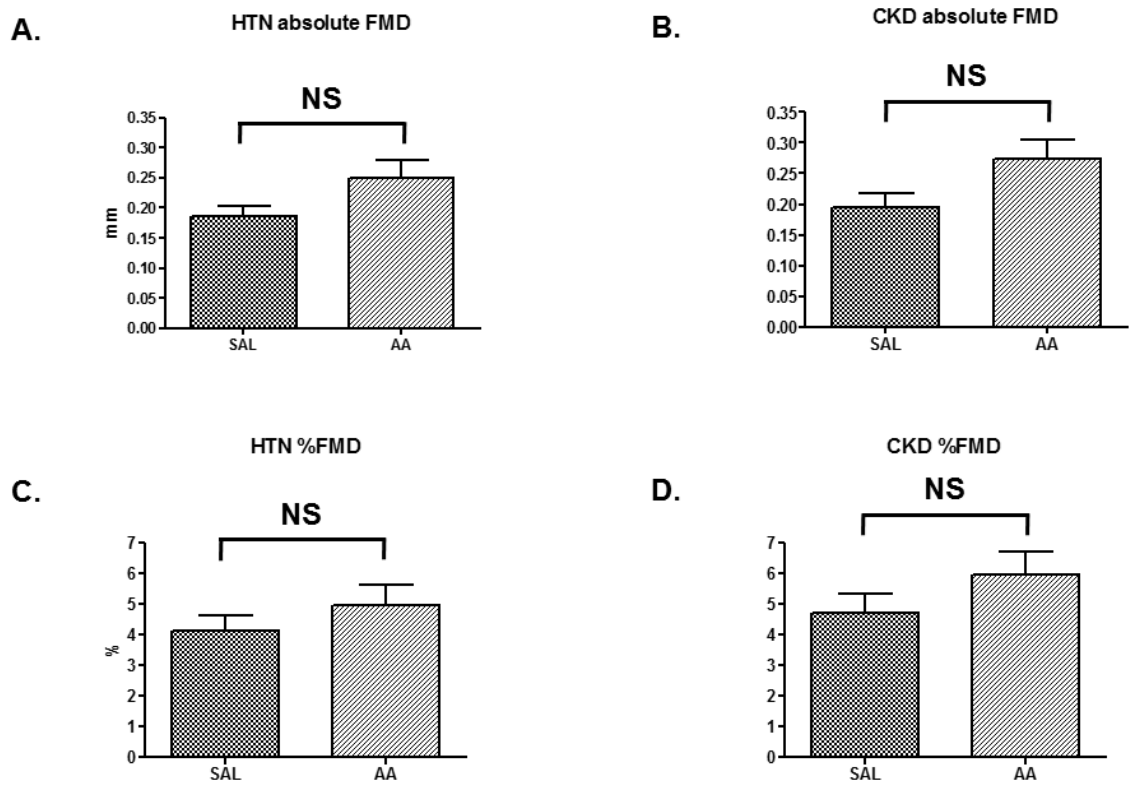
Figure 3.4 Effect of ascorbic acid on flow mediated dilatation.

No significant change was seen in endothelial function after administration of ascorbic acid. Results of brachial artery dilatation are shown, in terms of both absolute (A & B) and relative (C & D) measurements. Error bars represent standard error of the mean. HTN = Hypertension, CKD = Chronic kidney disease, SAL = Normal saline, AA = Ascorbic acid, NS = Not significant.

3.3.5 Subgroup analysis

The results of a subgroup analysis comparing the change in FMD, adjAix, PWV, blood pressure and ADMA which occurred in the HTN, CKD and PD groups are shown in table 3.7. There was no significant difference in the change in FMD ($F(2, 37) = 0.02$; $p = 0.98$) or PWV ($F(2, 42) = 2.58$; $p = 0.09$) between groups. There was significant difference in the change in MAP ($F(2, 44) = 4.26$; $p = 0.02$), adjAix ($F(2, 47) = 4.75$; $p = 0.01$) and ADMA ($F(2,44) = 3.45$; $p = 0.04$). Lower reduction of blood pressure and adjAix and greater reduction of ADMA was observed in the PD group.

	HTN	CKD	PD	p
Flow mediated dilatation (mm)	0.07 ± 0.18	0.08 ± 0.17	0.06 ± 0.29	0.98
Augmentation index (%)	-6.8 ± 5.5	-11.4 ± 6.4	-5.5 ± 3.4	0.01*
Mean arterial blood pressure (mmHg)	-14.1 ± 14.1	-14.5 ± 12.7	-0.1 ± 6.9	0.02*
Pulse wave velocity (m/s)	-0.19 ± 0.74	-0.76 ± 1.40	0.47 ± 1.81	0.09
ADMA (µmol/L)	0.02 ± 0.06	-0.3 ± 0.09	-0.05 ± 0.10	0.04*

Table 3.7 Subgroup analysis

The change in parameters from measurement 1 to measurement 2 is shown and compared across groups. Values representing the mean ± standard deviation of the change in parameter between measurements. In the PD group there is lower reduction of blood pressure and augmentation index, with greater reduction of ADMA. Asterisks (*) demonstrate a significant difference between groups. HTN = Hypertension, CKD = Chronic kidney disease, PD = Peritoneal dialysis, ADMA = Asymmetric dimethylarginine.

3.4 Discussion

CVD is the commonest cause of death in patients with CKD. The prevalence of atherosclerotic coronary artery disease, congestive cardiac failure and arrhythmia are all elevated in patients with CKD in proportion to the severity of renal impairment (United States Renal Data System, 2011a). Whilst part of this can be explained by the presence of shared risk factors, the burden of disease is not fully accounted for by these alone, and the existence of non-conventional risk factors specific to CKD may also contribute to cardiovascular risk (Go et al., 2004).

3.4.1 Oxidative stress in CKD

Oxidative stress describes a state of disturbed redox signalling due to an excess of ROS and their derivatives, and a consequent depletion of cardioprotective signalling molecules such as nitric oxide (NO). Oxidative stress occurs in CKD due to a number of mechanisms, such as stimulation of NADPH oxidase by an activated renin angiotensin system (DuPont et al., 2014) and downregulation of the antioxidant master gene regulator Nrf-2 (Kim and Vaziri, 2010). Clinically, oxidative stress is manifest by endothelial dysfunction and previously, several studies have shown reduction in endothelial function in a step wise manner relative to the stage of CKD (Yilmaz et al., 2011). Novel therapeutics which modify these pathways may reduce the burden of CVD in patients with CKD.

3.4.2 Ascorbic acid in CKD

In this study, it was found that serum ascorbic acid levels were significantly lower in CKD than hypertensive controls. Whilst it is generally accepted that symptoms of scurvy occur with serum ascorbic acid levels below 11.4 µmol/L, the lower limit of the recommended range is around 40 µmol/L (Gey et al., 1993) and blood concentrations below this may be associated with adverse outcome. Our findings are in keeping with earlier studies showing reduced ascorbic acid levels in CKD, both with and without diabetes (Takahashi et al., 1252), and lower still in patients receiving haemodialysis (Deicher and Horl, 2003). Patients with CKD are often advised to adhere to a low potassium diet, which can limit intake of micronutrients such as ascorbic acid. Furthermore, CKD is associated with low grade inflammation which can increase utilisation of ascorbic acid (Langlois et al., 2001), and clearance of ascorbic acid is increased by proteinuria and diuretic use (Hirsch et al., 1998). Interestingly, the reduction in ascorbic acid is offset by an apparent increase in the other non-

enzymatic antioxidant systems present in serum which comprise the total antioxidant capacity, such that TAP was in fact higher in CKD than HTN.

Administration of ascorbic acid not only increased antioxidant capacity in this study, but also increased O_2^- levels in both groups, in keeping with some previous studies showing mixed pro- and antioxidant effects. Ascorbic acid reacts with several transition metal ions facilitating their interaction with hydrogen peroxide in the Fenton reaction to generate hydroxyl radicals. That said, it remains unclear to what extent these reactions are relevant *in vivo*, as most transition metals are sequestered to chelates such as transferrin and caeruloplasmin. Our study has shown in controlled conditions that infusion of ascorbic acid, as well as having antioxidant effects, results in increased production of O_2^-.

3.4.3 Regulation of ADMA in CKD

ADMA is a by-product of the methylation of arginine residues, which acts as a competitive inhibitor of L-arginine to reduce NO production, and also causes decoupling of eNOS leading to ROS production instead of NO (Sydow and Munzel, 2003). ADMA is increased in CKD in proportion to the severity of renal impairment and is an important predictor of cardiovascular outcomes. Oxidative stress also increases ADMA concentration by upregulating the synthetic enzyme protein arginine methyltransferase-1 (PRMT-1) and downregulating the degradation enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Matsuguma et al., 2006). It was found that intravenous ascorbic acid level transiently reduces ADMA in CKD but not HTN, echoing the results of earlier studies which have shown a reduction in ADMA levels in patients with CKD supplemented with vitamin E (Saran et al., 2003). Given that ADMA is a significant predictor of cardiovascular outcome (Zoccali et al., 2001), this may represent an important mechanism by which antioxidants exert a beneficial cardiovascular effect.

3.4.4 Effect of ascorbic acid on central BP

Conversely, ascorbic acid reduced central blood pressure in both HTN and CKD groups by 12/15, and 12/10 mmHg respectively, and this was associated with a reduction in Aix. This was also reflected by an increase in the diameter of the brachial artery at the baseline stage of FMD, suggesting that this might have occurred due to systemic vasodilatation. Juraschek *et al* (Juraschek et al., 2012) previously conducted a meta-analysis of the effect of longitudinal ascorbic acid supplementation on blood pressure, finding that systolic and diastolic blood pressures were reduced by 4.85 and 1.67 mm Hg, respectively. It should be

remembered that Aix is dependent on blood pressure and ventricular contractility as well as velocity of reflected pulse waves (Lemogoum et al., 2004, Laurent et al., 2006), such that adjAix is a less direct method of measuring arterial stiffness than PWV, which is a direct measure of aortic stiffness. As such, it is not surprising that PWV is unchanged where Aix is reduced, and suggests that the acute action of ascorbic acid lies primarily in lowering blood pressure, rather than altering vascular geometry. That baseline brachial artery diameter was increased after ascorbic acid suggests a vasodilatory effect. Interestingly, the reduction in blood pressure was significantly lower in the PD group; it is possible that in this group blood pressure and Aix is more dependent on fluid status than other dynamic factors.

3.4.5 Effect of ascorbic acid on endothelial function

With regards to FMD, it is notable that there was no difference in baseline endothelium dependent vasodilatation between groups. Baseline FMD in the CKD group was similar to other studies in this cohort (Recio-Mayoral et al., 2011, Takahashi et al., 1252, Yilmaz et al., 2006), but in the HTN group, FMD was 4.13%, which is lower than in many other studies in similar populations. Having been recruited from a tertiary referral blood pressure clinic, it might be the case that this represents a population with higher cardiovascular risk and inherently worse endothelial function than other populations with hypertension and normal renal function (Shimbo et al., 2010). Similarly, although the two study groups were well matched for age, BMI, smoking status and medication history, blood pressure was significantly higher in the HTN group, such that higher blood pressure might offset the difference in endothelial function due to renal impairment.

In both study cohorts, there was a numerical increase in FMD following administration of ascorbic acid but this did not reach statistical significance. Previous studies have shown conflicting effects of ascorbic acid on vascular function in CKD populations. Cross *et al* (Cross et al., 2003) found no improvement in FMD after parenteral administration of ascorbic acid, although William*s et al* (Williams et al., 2001) have previously shown in improvement in endothelium dependent vasodilatation after ascorbic acid supplementation, in a cohort of transplant patients with mildly impairment kidney function. Additionally, it was found that impaired endothelium independent vasodilatation after administration of ascorbic acid. This may be another consequence of the pro-oxidant effects of ascorbic acid, or may simply be artefactual and due to nitrate tolerance or vessel fatigue.

3.4.6 Conclusion

In summary, in comparison to matched hypertensive controls, CKD patients have ascorbic acid deficiency but otherwise similar levels of oxidative stress. Parenteral ascorbic acid reduces central blood pressure and augmentation index in subjects with hypertension and CKD, in a manner independent of endothelial function. Further studies are required to assess the effects of chronic ascorbic acid on vascular function in these populations.

Chapter 4

Renal Arterial Spin Labelling Magnetic Resonance

Imaging in Healthy Volunteers

4.1 Introduction

As described in section 1.7, renal perfusion is a crucial component of normal renal function, being one of the main determinants of glomerular filtration rate and tissue oxygenation (Leong et al., 2007, Rosen et al., 1992). Serum creatinine and the derived estimated glomerular filtration rate (eGFR) are the conventional measures of renal function (Levey et al., 2009) used in clinical practice, however these are less sensitive to alterations in renal physiology. Furthermore, alterations in renal perfusion and endothelial function may represent pathophysiological mechanisms involved in the perpetuation of CKD. Existing methods of measuring renal perfusion have inherent drawbacks, as has already been discussed in section 1.9.4, but ASL MRI shows promise as a methods of non-invasively measuring perfusion without the administration of a potentially harmful tracer

Most perfusion MRI imaging in the literature is carried out at field strengths of 1.5 Tesla (Cutajar et al., 2012, Ritt et al., 2010). As magnetic labelling decays over the relaxation time T1, which is longer at higher field strengths, 3.0 Tesla MRI is associated with greater signal to noise ratio (SNR), which should result in enhanced image quality and allow more accurate analysis of renal perfusion. To this end, the reproducibility of ASL at 3.0 Tesla MRI in healthy volunteers with normal renal function was investigated.

4.2 Methods

The methodology of this study has already been discussed in section 2.1.2, but is summarised again in figure 4.1. Participants were recruited by local advertisement. ASL MRI was performed as described in section 2.4, and image analysis was performed offline using bespoke software written with MATLAB. The primary outcome measure was repeatability of ASL MRI parameters.

Figure 4.1 VolRASL study protocol.

Participants were recruited by advertisement. The study comprised three visits, on the first a clinical and biochemical assessment was carried out. On the second and third ASL MRI was performed.

4.3 Results

4.3.1 Participant demographics

12 participants completed the study protocol with a mean age of 44.1 ± 14.6 years. Mean blood pressure was 136/82mmHg and no participants receiving antihypertensive therapy. All subjects had normal renal function with a mean CKD EPI eGFR of 98.3 ± 15.1mL/min/1.73m² (table 4.1) without proteinuria. Images of appropriate quality for analysis were obtained at both visits for all participants (figure 4.2).

4.3.2 Renal morphology

Mean kidney length was 10.6 ± 0.8 cm at ASL MRI 1 and 10.8 ± 0.8cm at ASL MRI 2 (table 4.2) with significant correlation between the two ($r = 0.89$, $p < 0.001$). Kidney volume measured using the ellipsoid formula was 120.5 ± 26.1cm³ at ASL MRI 1 and 126.4 ± 24.9cm³ at ASL MRI 2. Kidney volume measured using the voxel count method was 155.7 ± 29.2cm³ at ASL MRI 1 and 157.7 ± 28.6cm³ at ASL MRI 2. Volume measurements made by the voxel count method were 30% higher than those made by the ellipsoid method, and there was significant correlation between both methods ($r = 0.70$, $p < 0.001$) (figure 4.3).

Parameter	Result
	n = 12
Age (years)	44.1 ± 14.6
Male (number)	5
BMI (kg/cm^2)	26.5 ± 6.6
Systolic BP (mmHg)	136 ± 23
Diastolic BP (mmHg)	82 ± 9
Urea (mmol/L)	5.0 ± 1.1
Creatinine (µmol/L)	72.3 ± 10.6
CKD EPI eGFR (mL/min/1.73m^2)	98.3 ± 15.1
Total Cholesterol (mmol/L)	4.8 ± 0.7
HDL cholesterol (mmol/L)	1.8 ± 1.2
Glucose (mmol/L)	5.2 ± 1.0
Haemoglobin (g/L)	144 ± 12
Protein to creatinine ratio (mg/µmol)	0.58 ± 2.02
Albumin to creatinine ratio (mg/µmol)	0.17 ± 0.32
Adjusted serum calcium (mmol/L)	2.47 ± 0.05
Serum phosphate (mmol/L)	0.96 ± 0.35
Calcium phosphate product (mmol/L)	2.38 ± 0.89

Table 4.1 Baseline clinical and biochemical assessment in the VolRASL study.
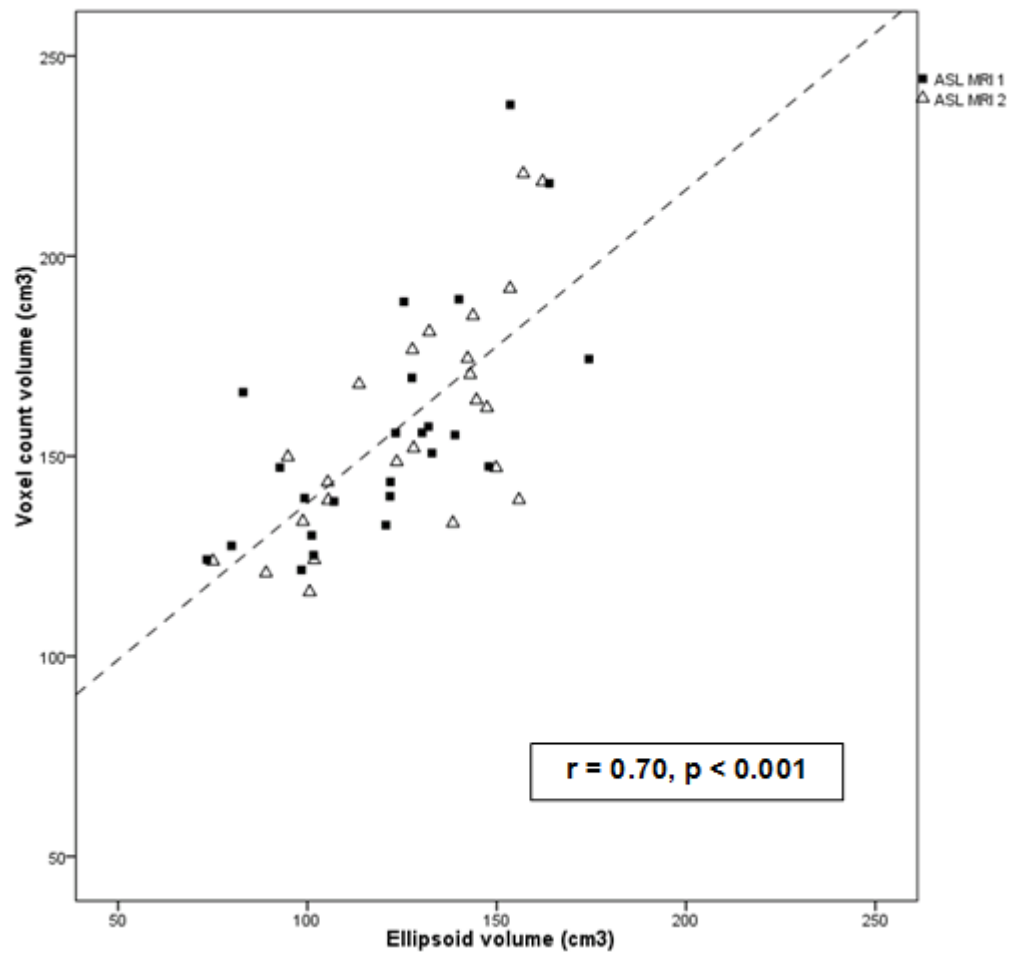
Figure 4.2 Renal anatomy correlation plot.

Correlation between ellipsoid formula and voxel count methods of measuring renal volume. r is the Pearson correlation coefficient.
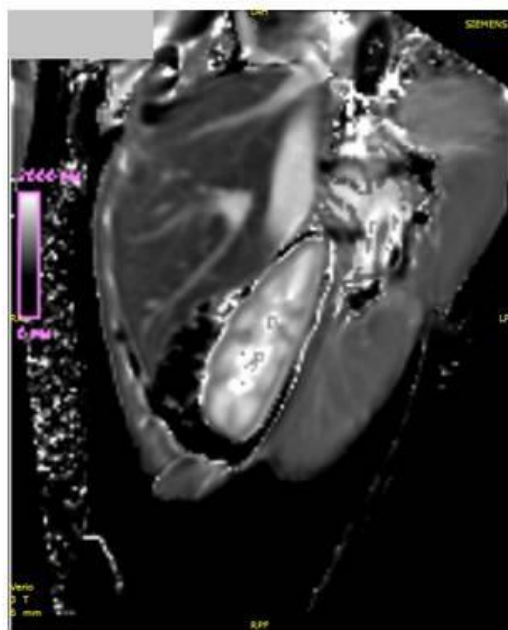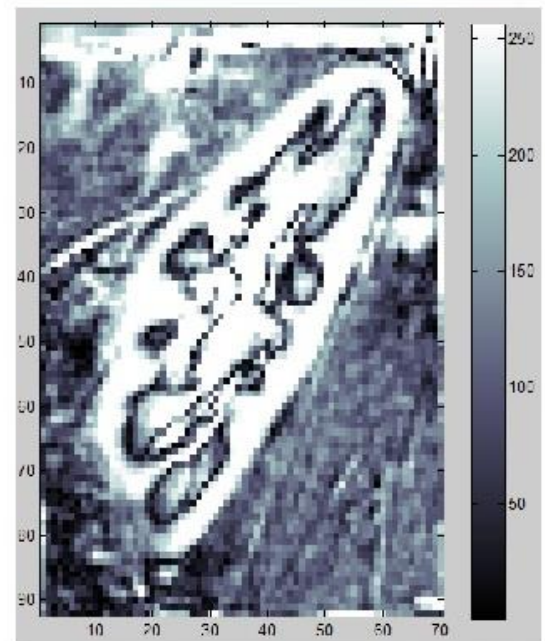
Figure 4.3 Renal ASL MRI in a healthy volunteer.

Representative renal arterial spin labelling MRI images in a healthy volunteer with normal renal function showing **(A)** ASL subtraction image, **(B)** M0 image demonstrating magnetisation equilibrium, **(C)** T1 map and **(D)** composite image of 5 ASL MRI contrast images registered with post processing.

4.3.3 Comparison of right and left kidneys

Measurements of right and left kidneys were compared to exclude any confounding effect by differences in adjacent tissue types. No significant difference was observed in the T1 relaxation time of the cortex (p = 0.74), nor whole kidney (p = 0.56). Neither was there a difference in perfusion of the cortex (p = 0.93), or whole kidney (p = 0.28). There was a significant correlation between the perfusion measured in the right and left kidneys of each participant between visits 1 and 2, both in the cortex (r = 0.79; p < 0.001), and the whole kidney (r = 0.80; p < 0.001) (figure 4.4).

4.3.4 T1 relaxation time

No difference in T1 longitudinal relaxation time was observed between visits either in the whole kidney ROI (1491 ± 61 vs 1499 ± 52 ms; p = 0.52), cortical ROI (1376 ± 104 vs 1406 ± 96 ms; p = 0.07), or medullary ROI (1651 ± 86 vs 1639 ± 80 ms; p = 0.38).

4.3.5 Perfusion

Similarly, no difference in perfusion measured with ASL MRI was observed between visits. Whole kidney perfusion was 228 mL/min/100g at ASL MRI 1 and 230 mL/min/100g at ASL MRI 2 (p = 0.66), with significant correlation between the two MRIs (r = 0.75, p < 0.001). Cortical perfusion was 321 mL/min/100g then 334 mL/min/100g (p = 0.18), with significant correlation between the two (r = 0.74, p < 0.001). Absolute kidney perfusion was 367 ± 66 mL/min at ASL MRI 1 and 379 ± 86 mL/min at ASL MRI 2 (p = 0.33), whilst total perfusion was 734 ± 117 mL/min at ASL MRI 1 and 757 ± 156 mL/min at ASL MRI 2 (p = 0.42) (figure 4.5).

4.3.6 Indices of repeatability

Bland Altman plots were constructed of the cortical and whole kidney perfusion measurements made at ASL MRIs 1 and 2 (figure 4.6). These showed good agreement between measurements, with a random distribution of means plotted against differences observed. The intra-class correlation for cortical perfusion was 0.85 (95% confidence interval 0.65 – 0.94), whilst the CV_{ws} was 9.2%. The intra-class correlation for whole kidney perfusion was 0.86 (0.68 – 0.94), whilst the CV_{ws} was 7.1%.

	MRI 1	MRI 2	p value
Kidney length (cm)	10.6 ± 0.8	10.8 ± 0.8	0.022*
Kidney volume Ellipsoid formula (cm³)	120.5 ± 26.1	126.4 ± 24.9	0.019*
Kidney volume voxel count (cm³)	155.7 ± 29.2	157.7 ± 28.6	0.39
Whole kidney T1 (ms)	1491 ± 61	1499 ± 52	0.52
Cortical T1 (ms)	1376 ± 104	1406 ± 96	0.07
Medullary T1 (ms)	1651 ± 86	1639 ± 80	0.38
Whole kidney perfusion (mL/min/100g)	228 ± 40	230 ± 41	0.66
Cortical perfusion (mL/min/100g)	321 ± 63	334 ± 63	0.18
Absolute perfusion (mL/min)	367 ± 66	379 ± 86	0.33

Table 4.2 MRI measurements of T1 longitudinal relaxation time and ASL perfusion.

p value refers to a Student's t test comparison between the two visits. Asterisks (*) indicate parameters where there was a significant difference.
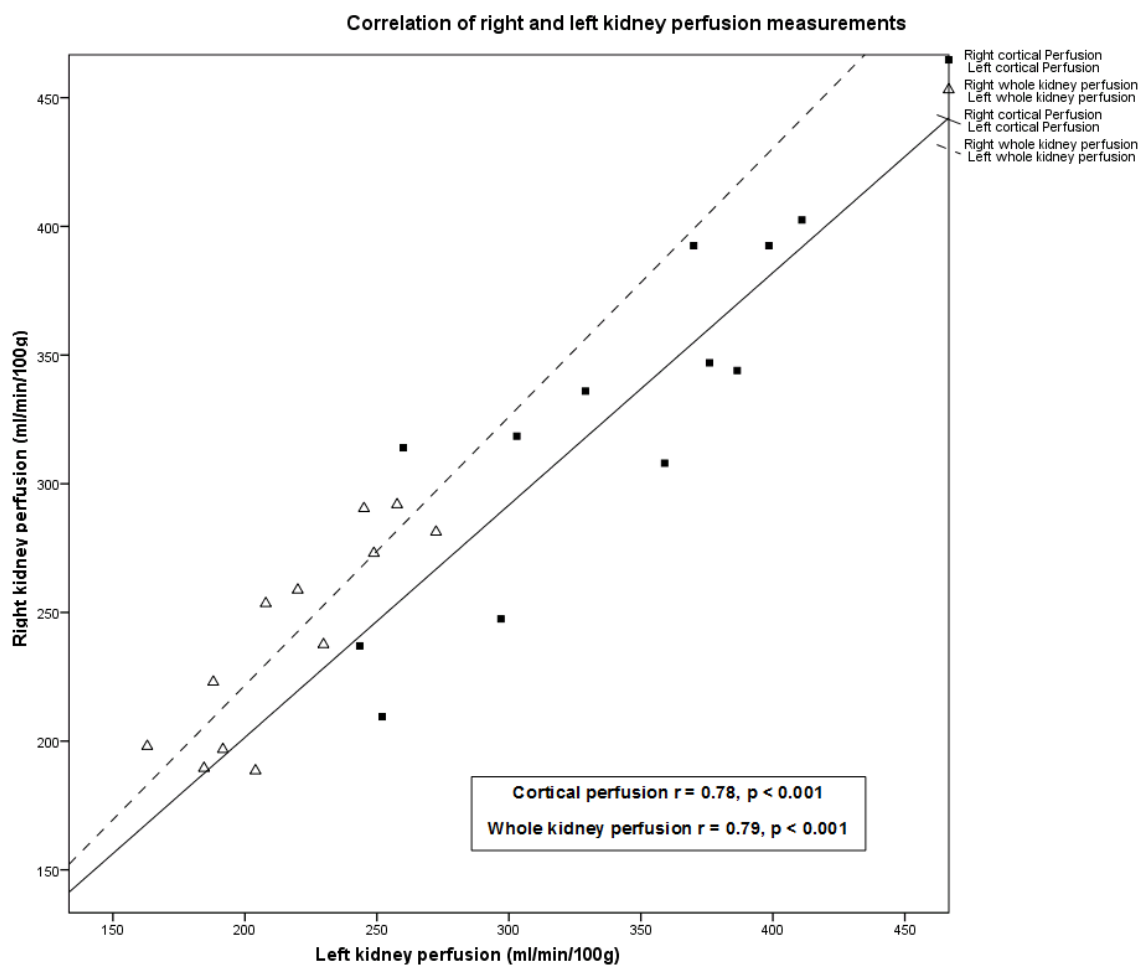
Figure 4.4 Correlation of right and left kidney perfusion measured by ASL MRI.
r is the Pearson correlation coefficient.
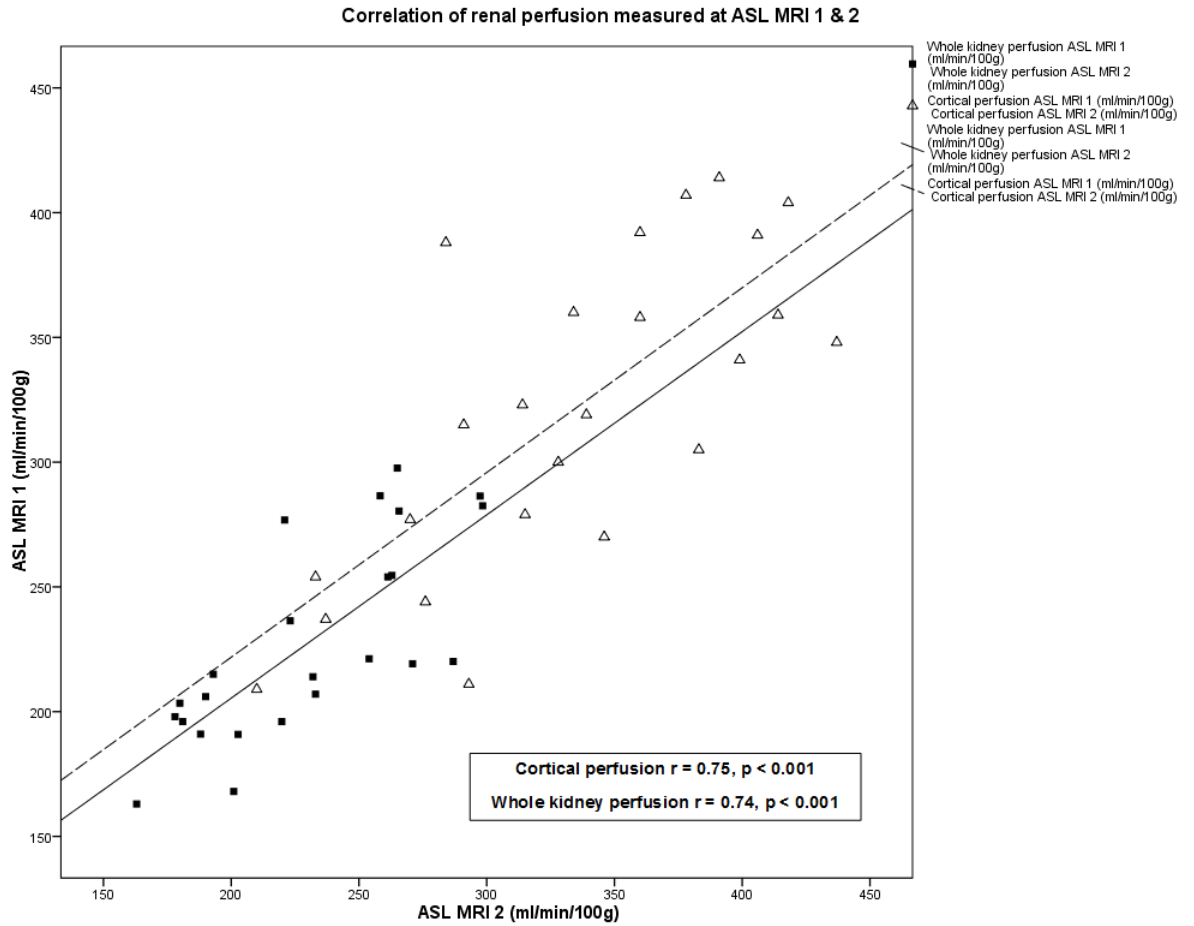
Figure 4.5 Perfusion correlation plot

Correlation between whole and cortical kidney perfusion measurements made at MRI 1 and 2. r is the Pearson correlation coefficient.
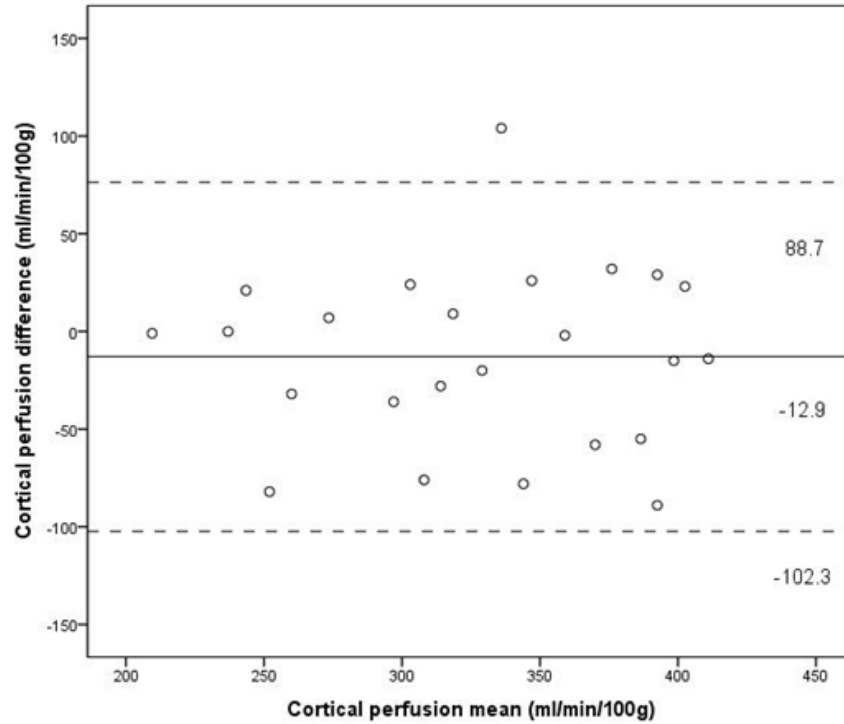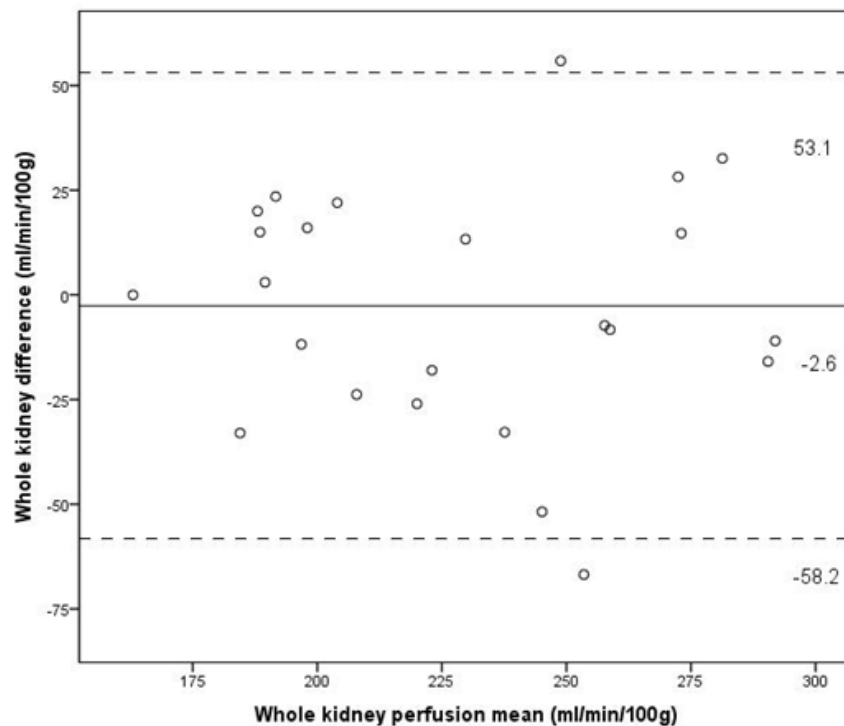
Figure 4.6 Reproducibility of ASL MRI.

Bland Altman plots of cortical (A) and whole kidney (B) perfusion measurements made at MRI 1 and 2. Solid line and adjacent number indicates mean difference, whilst dashed line and number indicates limits of agreement.

4.4 Discussion

This study demonstrates the reproducibility of perfusion measurements made using FAIR True FISP ASL on a 3 T MRI in healthy volunteers, with no significant differences found between the first and second measurements made of both T1 relaxation time and renal perfusion.

4.4.1 T1 and ASL MRI data in the literature

T1 values were somewhat higher than reported in the literature, at 1491 ms for the whole kidney, 1375 ms at the cortex, and 1651 ms at the medulla. This is in comparison to reported values of 1142 ms at the cortex and 1545 ms at the medulla reported in one previous study which used an inversion recovery method with different inversion times, in contrast to the multiple look locker sequence used here. MOLLI determination of T1 has advantages such as faster scan time and smaller limits of agreement between repeated measures than traditional T1 mapping techniques, and has been validated at least in cardiac tissue.

Published data using ASL to measure renal perfusion in subjects with normal renal function show a wide range of values from 197 mL/min/100g to 329 mL/min/100g. This variation may represent physiological or population differences but factors pertaining to the ASL method are likely to also contribute. A number of ASL protocols exist differing in labelling and image acquisition strategy. In addition to the numerous ASL sequences in use, different acquisition strategies have been employed in order to minimise the error caused by respiratory motion. Consistent with the approach here, other studies have employed breath-holding techniques, which whilst minimising respiratory motion, can prove difficult for participants to comply with. In this study all of the healthy volunteers were able to comply with a 25 second breath hold, however this strategy may not be appropriate for patients with kidney or co-morbid diseases. Other strategies which have been employed include prolonged acquisition during light free breathing, respiratory triggering, navigator echo and parallel imaging methods. Post-acquisition image realignment has been shown to improve visual quality and background noise suppression can be combined with parallel imaging to allow whole kidney data to be collected during free breathing. The same study demonstrated that background suppression resulted in under estimated perfusion measurements, whilst motion correction led to improved estimation of perfusion. Further research is required to improve the application of these techniques in order to give accurate quantification of renal perfusion.

4.4.2 Reproducibility of ASL MRI

Good reproducibility was demonstrated in the perfusion measurements made at 3.0 T MRI, with within subject coefficient of variation calculated at 9.2 % for cortical perfusion, and 7.1 % for whole kidney perfusion. This is similar to measures of reproducibility found in other studies at 1.5 T (Cutajar et al., 2012). Therefore there was no demonstrable difference in the reproducibility of ASL measurements made at 1.5 T or 3.0 T in healthy volunteers. Signal to noise ratio is greater at higher field strengths, and whilst no advantage in terms of reproducibility was observed in this study, this may be of more benefit in the imaging of patients with chronic kidney disease where image quality may be compromised by patient factors such as obesity or breath holding ability, or tissue factors such as kidney fibrosis.

Perfusion maps generated via post processing result in a heterogeneous appearance of the renal medulla, probably due to the presence of larger vessels and the renal pelvis. This resulted in a degree of variability in the measurement of perfusion in this region, hence the measurement of whole kidney perfusion in preference to medullary perfusion. Improved post processing techniques may allow for differential quantification of cortical and medullary perfusion and measurement of these values may reveal differences in relative perfusion in patients with chronic kidney disease. Ideally automated detection of the differentiation between cortex and medulla using a digital threshold for signal intensity would generate more reliable, less operator dependent and hence more reproducible data.

This study provides further evidence for the reproducibility of FAIR ASL and confirms this at higher field strength than in previous work. To ensure normality of renal function and minimal variation in scan conditions in this cohort, subjects underwent biochemical screening of blood and urine and physical assessment to confirm normal kidney function prior to imaging. Furthermore, participants attended at a fixed time of day after a stated period of fasting to ensure constant study conditions. It is possible that differences in the ASL sequence, MRI systems or subject factors used in different studies may account for the variation in perfusion measurements.

Future work is required to refine renal perfusion measurements using ASL. Quantitative analysis of ASL images is possible using a model derived from the extended Bloch equation. A limitation of this is that the model ignores transit time and exchange effects of water molecules in blood. Some of the labelling is lost during transit from the location of tagging to the location of capillary exchange in tissue, to a degree which is relative to the transit

time, such that perfusion values might be confounded by this. These limitations will have to be borne in mind during the use of ASL MRI in patients with chronic kidney disease given there may be inter individual differences in transit time between CKD and healthy populations, and intra individual differences which accrue over time, as CKD progresses. Transit time could be measured as part of the ASL imaging protocol and perfusion values adjusted for this; further research is required to determine if this would be the appropriate approach.

4.4.3 Conclusions

In summary, development of renal ASL MRI represents a technique, which may be applicable, both for diagnostic purposes and for monitoring response to therapeutic interventions. This study shows that ASL MRI is reproducible in healthy volunteers with normal kidney function at 3.0 Tesla. Further research is required to investigate its utility across a spectrum of renal disease.

Chapter 5

Renal Arterial Spin Labelling Magnetic Resonance Imaging in Chronic Kidney Disease

5.1 Introduction

As was discussed in section 1.7, renal perfusion is an important physiological parameter in health and disease. In normal physiology, renal blood flow is an important determinant of oxygen supply and glomerular filtration rate (Evans et al., 2013). In chronic kidney disease (CKD), renal microvascular dysfunction is one of a number of pathological mechanisms involved in the progression of disease, irrespective of the initiating insult. Endothelial dysfunction is typically association with oxidative stress, and there is accumulating evidence implicating these processes in the progression of CKD (Baylis, 2012). Furthermore, previous studies have shown an improvement in renal plasma flow following administration of antioxidants such as ascorbic acid (Delles et al., 2004).

In chapter 4, satisfactory reproducibility of ASL measurements in volunteers with normal renal function was demonstrated, with a coefficient of variance of 9.2% and 7.1% for cortical perfusion and whole kidney perfusion respectively. The utility of ASL MRI as a marker for disease severity and progression in CKD, and as a measure of response to therapy, is yet to be determined however. This study was therefore designed to investigate the use of ASL MRI for the assessment of patients with CKD and their response to administration of ascorbic acid.

5.2 Methods

The methodology of this study has already been described in section 2.1.3 and is summarised by figure 5.1. The CKD group was formed of patients attending the local general nephrology clinic, and are a separate cohort from those who participated in the Renox study. Healthy volunteers were recruited from the live donor transplant clinic. ASL MRI was performed according to the protocol described in section 2.4 and image analysis was performed offline using bespoke software written with MATLAB.
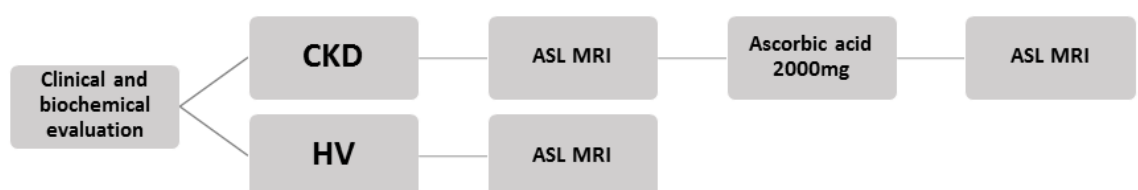
Figure 5.1 KidRASL study protocol

5.3 Results

5.3.1 Baseline data

24 HV and 17 patients with CKD were recruited; the demographic data for each group is displayed in table 5.1. The CKD group was significantly older (47 ± 14 vs 56 ± 10 years, p = 0.040), and had higher blood pressure ($132/83 \pm 15/8$ vs $151/90 \pm 26/14$ mmHg, p = 0.006/0.047). CKD-EPI eGFR was 39.8 ± 25.2 mL/min/1.73m² in the CKD group and 99.6 ± 14.0 mL/min/1.73m² in the HV group (p < 0.001).

5.3.2 Renal anatomy

Renal anatomical data is shown in table 5.2. Kidney length was significantly shorter in the CKD group compared with the HV group (10.5 ± 0.8 vs 9.7 ± 0.9 cm, p = 0.005) however renal volume was no different between the two (167.1 ± 35.0 vs 160.1 ± 53.4 cm^3, p = 0.62). The CKD group had significantly higher T1 longitudinal relaxation time both measured in the cortex (1366 ± 122 vs 1529 ± 77 ms, p < 0.001) and the whole kidney (1472 ± 91 vs 1550 ± 81 ms, p = 0.007) ROI (figure 5.2). Furthermore, CMD was significantly higher in CKD than in HV (0.84 ± 0.07 vs 0.94 ± 0.07, p < 0.001).

Parameter	Healthy volunteers	Chronic kidney disease	p value
	n = 24	*n = 17*	
Age (years)	47 ± 14	56 ± 10	0.040*
Body mass index (kg/m^2)	26.5 ± 5.3	29.3 ± 3.4	0.06
Blood pressure (mmHg)	132/83 ± 15/8	151/90 ± 26/14	0.006/0.047*
Mean arterial blood pressure (mmHg)	99 ± 9	110 ± 17	0.011*
CKD-EPI eGFR (mL/min/1.73m²)	99.6 ± 14.0	39.9 ± 25.2	< 0.001*
Serum creatinine (μmol/L)	68 ± 10	184 ± 69	<0.001*
Serum creatinine 1 year previously (μmol/L)	66 ± 11	165 ± 57	< 0.001*
Primary renal diagnosis (n)			
Diabetes		*2*	
Glomerulonephritis		*8*	
Renovascular disease		*4*	
Other		*2*	
Unknown		*1*	
CKD stage (n)			
1		*2*	
2		*1*	
3		*4*	
4		*10*	
5		*0*	

Table 5.1 Baseline clinical and biochemical data in the KidRASL study.

Asterisks (*) indicate significant a difference between the two groups.

Parameter	Healthy volunteers	Chronic kidney disease	p value
Kidney length (cm)	10.5 ± 0.8	9.7 ± 0.9	0.005*
Kidney volume (cm^3)	167.1 ± 35.0	160.1 ± 53.4	0.62
Cortical T1 time (ms)	1366 ± 122	1529 ± 77	<0.001*
Whole kidney T1 time (ms)	1472 ± 91	1550 ± 81	0.007*
Corticomedullary differentiation	0.84 ± 0.07	0.94 ± 0.07	<0.001*
Mean cortical perfusion (mL/min/100g)	279 ± 69	136 ± 37	<0.001*
Mean whole kidney perfusion (mL/min/100g)	221 ± 38	146 ± 24	<0.001*
Mean kidney perfusion (mL/min)	366 ± 79	223 ± 75	<0.001*
Total renal perfusion (mL/min)	731 ± 159	446 ± 150	<0.001*

Table 5.2 Baseline T1 longitudinal relaxation time and perfusion
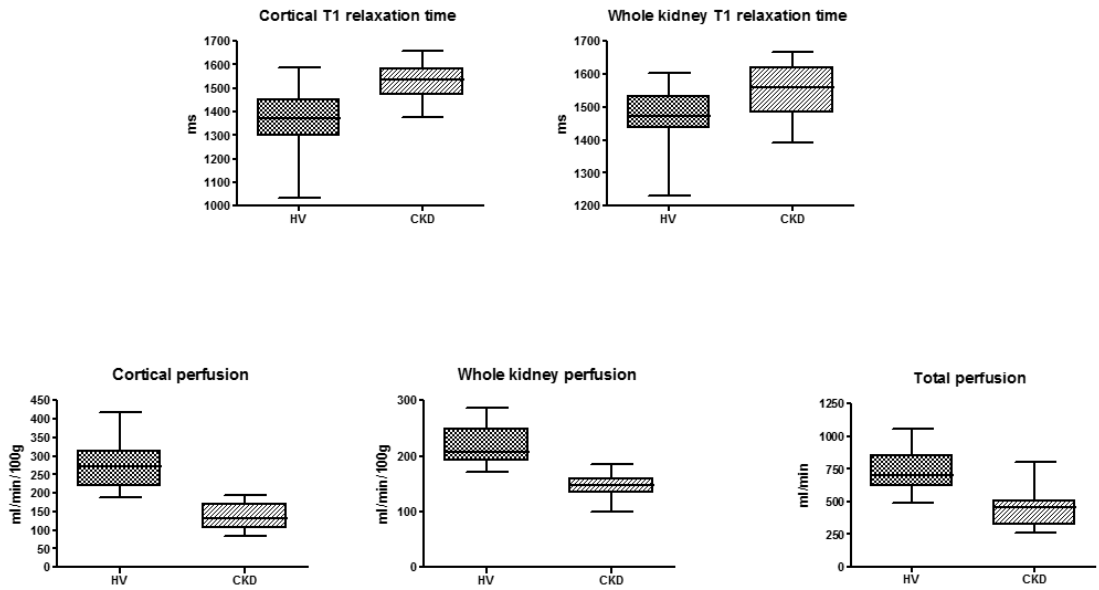
Figure 5.2 T1 and renal perfusion in CKD and HV

Box and whisker plots showing T1 longitudinal relaxation times and ASL MRI perfusion measurements for CKD and HV groups.

5.3.3 Intra-observer variability

Intra-observer variation of cortical perfusion measurements was 7.3% with intra-class correlation (ICC) of 0.98, whilst variation of whole kidney perfusion measurements was found to be 4.4% with ICC of 0.96.

5.3.4 Acquisition strategy

Whole kidney perfusion was higher when measured from a single acquisition compared to using five acquisitions (241 ± 40 vs 227 ± 41 mL/min/100g, $p < 0.01$) but the two techniques were significantly correlated ($r = 0.86$, $p < 0.001$). ICC was 0.82 (95% CI $0.53 - 0.93$; $p < 0.001$) and mean variation was 7.8%. Cortical perfusion measured from a single acquisition was not significantly different from that measured from 5 acquisitions (292 ± 58 vs 296 ± 75 mL/min/100g), and these measurements were significantly correlated ($r = 0.84$, $p < 0.001$). ICC was 0.82 (95% CI $0.63 - 0.92$; $p < 0.001$) and mean variation was 10.7%. Image quality, however, was subjectively poorer when perfusion maps were derived from a single acquisition (figure 5.3).

5.3.5 Renal perfusion

Renal perfusion was significantly lower in the CKD group (table 5.2 and figure 5.4). In the CKD cohort, mean cortical perfusion was 136 ± 37 mL/min/100g in comparison to 279 ± 69 mL/min/100g in the HV cohort ($p < 0.001$). Similarly, whole kidney perfusion was reduced in the CKD group, at 146 ± 24 mL/min/100g compared to 221 ± 38 mL/min/100g ($p < 0.001$). Furthermore, total renal perfusion was 446 ± 150 mL/min in CKD compared to 731 ± 158 mL/min in HV ($p < 0.001$). Typical perfusion maps from both groups are shown in figure 5.4.

5.3.6 Correlation of clinical, biochemical and MRI parameters

Both cortical and whole kidney perfusion were found to have a negative association with age (respectively, $r = -0.48$, $p = 0.002$; $r = -0.51$, $p = 0.001$). Whilst there was no association between blood pressure and cortical perfusion, a negative correlation was observed between whole kidney perfusion and mean arterial blood pressure ($r = -0.33$, $p = 0.049$).

Correlation was seen between CKD EPI eGFR and both whole kidney T1 longitudinal relaxation time ($r = -0.40$, $p = 0.011$) and cortical T1 time ($r = -0.58$, $p < 0.001$). Furthermore, significant correlation was seen between CKD-EPI eGFR and both cortical perfusion ($r =$

0.73, p < 0.001) and whole kidney perfusion (r = 0.69, p < 0.001). There was also significant correlation between total renal perfusion and eGFR (r = 0.69, p < 0.001). PCR was negatively correlated with both cortical (r = -0.60, p = 0.002) and whole kidney perfusion (r = -0.43, p = 0.037). The percentage rise in serum creatinine over the preceding 12 months prior to the study was also negatively correlated with total perfusion (r = -0.52, p = 0.028) (figure 5.5).

5.3.7 Change in perfusion following ascorbic acid

Perfusion measurements made before and after administration of ascorbic acid are shown in table 5.3. No statistically significant differences were observed (table 5.3). Cortical perfusion was 137.2 ± 38 pre and 128.4 ± 40.0 mL/min/100g post ascorbic acid (p = 0.38), whilst whole kidney perfusion was 146.6 ± 17.8 mL/min/100g pre and 156.0 ± 22.5 mL/min/100g post (p = 0.31). Total kidney perfusion was 471.0 ± 156 mL/min before and 515.2 mL/min after ascorbic acid administration (p = 0.32).
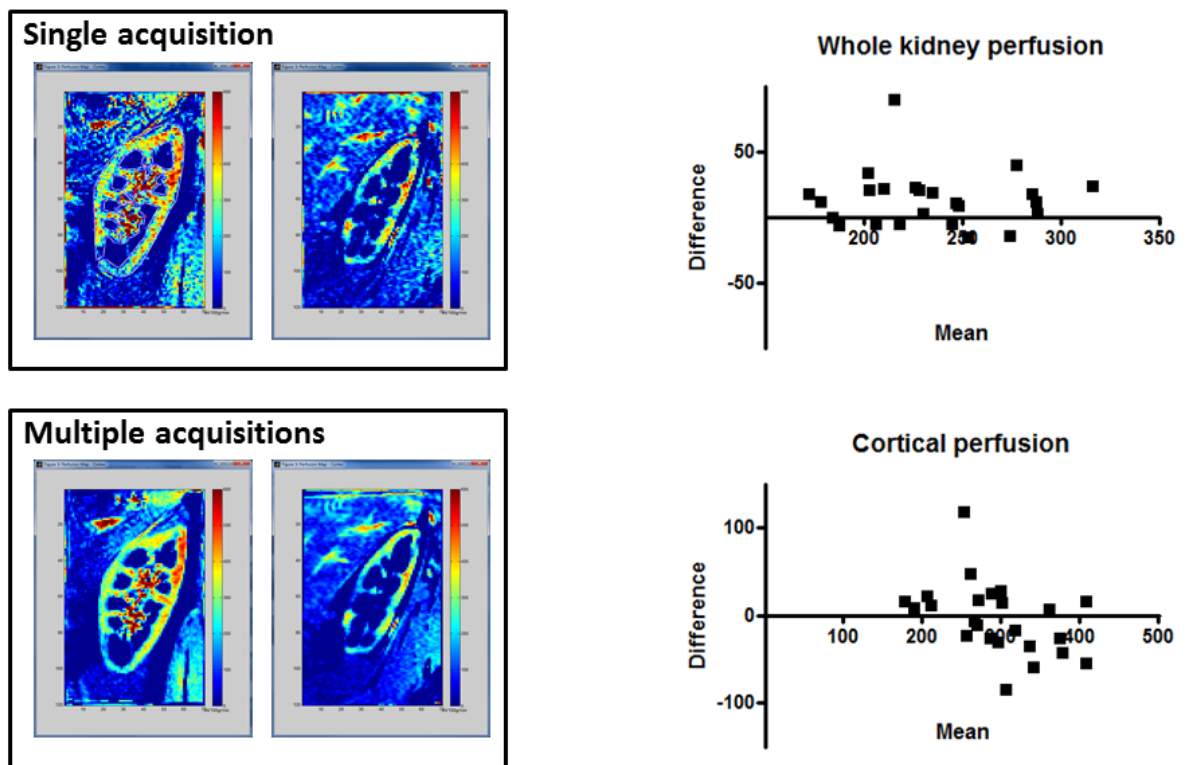
Figure 5.3 Single versus multiple acquisitions

Comparison images and Bland Altman plot of using a single acquisition versus using multiple acquisitions.

	Before ascorbic acid	After ascorbic acid	p value
Cortical perfusion (mL/min/100g)	137.2 ± 38.2	128.4 ± 39.7	0.38
Whole kidney perfusion (mL/min/100g)	146.6 ± 17.8	156.0 ± 22.5	0.31
Mean kidney perfusion (mL/min)	235.5 ± 78.0	257.6 ± 108	0.32
Total kidney perfusion (mL/min)	471.0 ± 156.1	515.2 ± 216.0	0.32

Table 5.3 Perfusion measurements before and after ascorbic acid
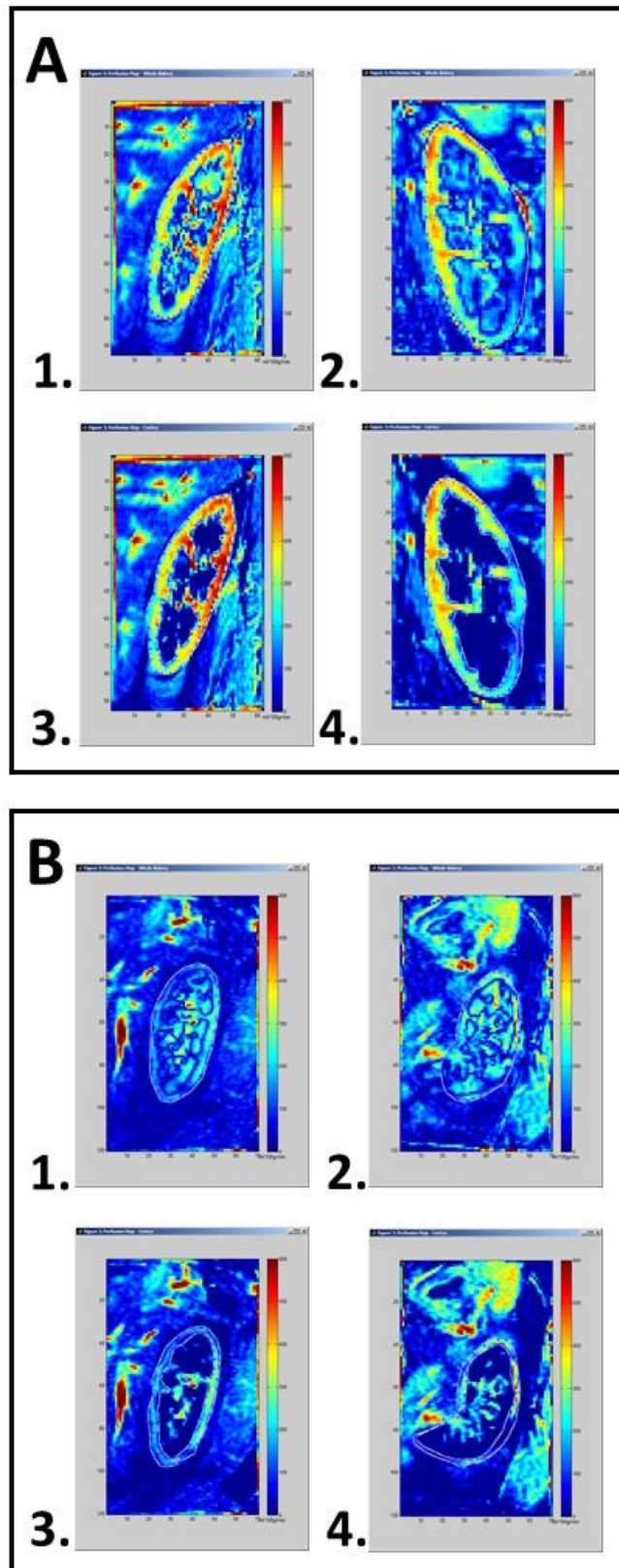
Figure 5.4 Perfusion maps in CKD and HV

ASL MRI perfusion maps from patients with healthy volunteer (A), in comparison to a participant with CKD (B). Demonstrates right and left whole kidney perfusion (1 & 2), and right and left cortical perfusion (3&4).
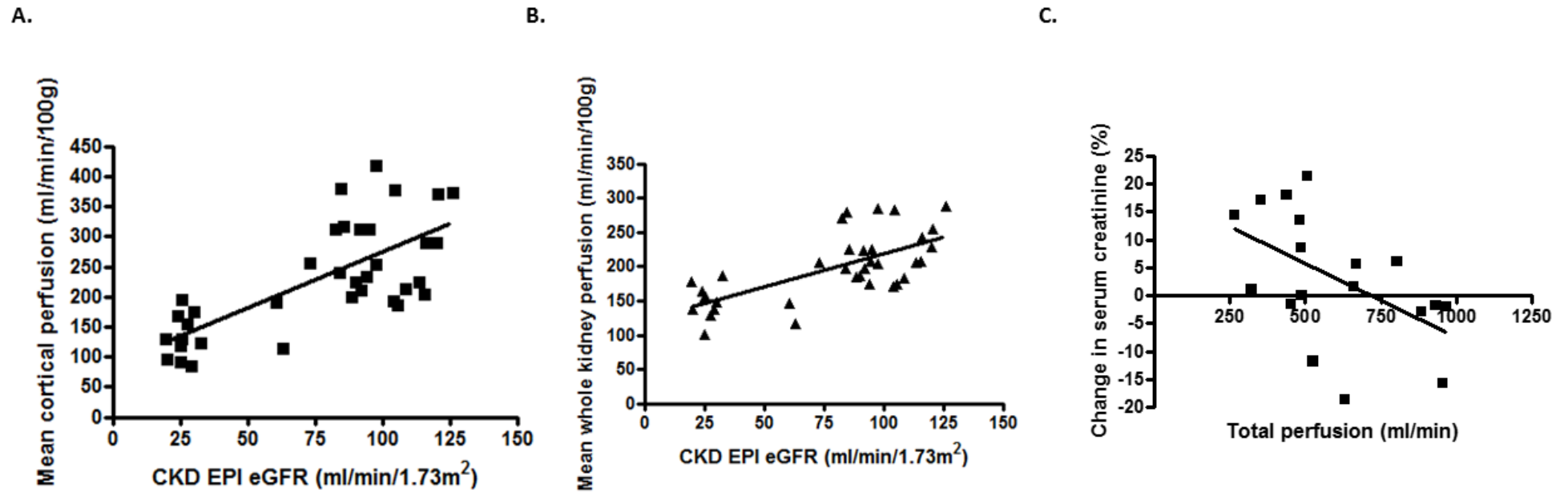
Figure 5.5 Perfusion vs clinical parameters

Correlation plots showing associations between (A) cortical perfusion and eGFR, (B) whole kidney perfusion and eGFR, and (C) change in serum creatinine over the preceding year and total perfusion.

5.4 Discussion

CKD has a tendency to worsen despite treatment of blood pressure and any other reversible or aetiological factors, and there is evidence that common pathological mechanisms are responsible for this irrespective of the original renal insult. Renal damage has been shown to correlate primarily with tubulointerstitial injury (Schainuck et al., 1970), characterised by a vicious cycle of microvascular dysfunction leading to tubular atrophy and fibrosis (Bohle et al., 1990). *In vivo* biomarkers to assess renal progression are lacking and emerging techniques such as ASL MRI may provide much needed insight into renal perfusion and thus extent of renal damage.

5.4.1 Renal perfusion in CKD

Cortical perfusion is lower in CKD, at 136 mL/min/100g compared to from 279 mL/min/100g in HV with correlation between perfusion and degree of renal impairment quantified by CKD-EPI eGFR. Whole kidney perfusion is similarly reduced, from 221 mL/min/100g to 146 mL/min/100g. This is in keeping with previous measurements of renal perfusion in health and disease, and the finding of reduced native kidney perfusion in CKD has also previously been demonstrated (Artz et al., 2011a, Rossi et al., 2012). Whilst these perfusion values are lower than found in other studies, this CKD cohort represents the largest to undergo ASL MRI and included patients with more advanced renal impairment than previously studied. These findings demonstrate strong correlation of renal function to perfusion across a broad range of CKD-EPI eGFR, ranging from 20 to 126 mL/min/1.73m^2. Furthermore, an association was found between perfusion and decline in renal function, such that patients with lower total perfusion were more likely to have undergone progression of their disease over the preceding year. ASL MRI may therefore be used to identify patients who will benefit from interventions used to optimise renal haemodynamics, such as inhibitors of the renin angiotensin system, in order to improve outcomes. Earlier pilot studies have shown the utility of ASL MRI in monitoring the response to such interventions (Ritt et al., 2010).

Previous human studies using ASL MRI are summarised in table 1.2, which demonstrates the range of perfusion values previously found using this technique, in both health and disease. As discussed in chapter 4, this could be ascribed to differences in ASL sequence, imaging strategy, and post processing as well as true differences in study population. For example, different strategies have been employed to circumvent the problem of renal

respiratory motion, including breath-holding, respiratory gating, or post processing registration. Gardener & Francis (Gardener and Francis, 2010) found no difference in perfusion measurements made with either breath-holding or free breathing, but found reduced perfusion when background suppression was used to improve image quality, showing that some variations in imaging approach cause differences in perfusion measurements. Typically, a number of acquisitions are taken given the low signal to noise inherent to ASL, however this increases scan time and leads to patient tiring. No difference in perfusion measurements with increasing acquisitions was found here, but greatly improved image quality was observed, such that repeated image acquisition is to be recommended where tolerated. This ASL protocol resulted in a scan time of 15 minutes, and breath-holding time of 12 seconds, which was tolerated by all participants.

ASL has been validated in animal models using microsphere techniques and using explanted organs undergoing haemoperfusion. In normal renal function, strong correlation between ASL and both DCE MRI perfusion (Cutajar et al., 2014), and PAH clearance (Ritt et al., 2010) has been shown. Validation of ASL against a gold standard perfusion technique has never been undertaken in a CKD population. Given that quantitative measurement of perfusion using the standard ASL kinetic model is dependent on T1 time, it is possible that structural changes in CKD are at least partly responsible for the functional changes suggested by ASL MRI. In keeping with previous studies (Huang et al., 2011), T1 time was significantly higher in CKD, and showed strong correlation with CKD-EPI eGFR. Lee et al (Lee et al., 2007) previously showed that cortical T1, but not medullary T1 time showed strong correlation with single kidney GFR measured by renography. These differences may be accounted for by changes in extracellular composition, fibrosis, or changes in the microvasculature. Further investigation into how structural changes affect measurement of functional MRI parameters is needed, to confirm that ASL provides true assessment of perfusion in CKD. That said, T1 time itself has been shown to have biological relevance in cardiac imaging, with prolongation observed in association with the diffuse fibrosis of hypertrophic cardiomyopathy (Puntmann et al., 2013). Further research is required in larger patient cohorts to determine the utility of T1 time as a biomarker in CKD.

5.4.2 Effect of antioxidant administration on renal perfusion

It was hypothesized that antioxidant administration would ameliorate oxidative stress, reduce endothelial dysfunction and improve renal perfusion, however no increase in renal blood flow was seen following administration of ascorbic acid in patients with CKD. In

contrast to these findings, an earlier study by Delles et al (Delles et al., 2004) showed that L-arginine increased renal plasma flow measured by PAH clearance in patients with type 2 diabetes and healthy volunteers, whilst co infusion of ascorbic acid further increased perfusion only in patients with diabetes. This contradiction could be explained by methodological differences between PAH clearance and ASL, or by the fact that this study administered ascorbic acid as a monotherapy. Alternatively, the renal perfusion abnormalities seen in this cohort with advanced CKD may be fixed and less responsive to changes in oxidative stress than in the population of patients with early diabetic nephropathy. Regardless, this study demonstrates the feasibility of the use of ASL for measuring the acute response to an intervention highlighting the utility of this technique for assessing response of renal physiology to novel therapeutics. The half-life of the ASL tracer is measured in seconds, relating to the T1 time of blood, such that the technique is well suited for the rapid repeated acquisition such as that performed in this study.

5.4.3 Study limitations

This study has a number of limitations. This CKD cohort has a variety of renal pathologies and whilst common pathological mechanisms underpin all chronic kidney disorders it is possible that perfusion abnormalities may predominate in certain aetiologies of CKD over others. Despite being one of the largest ASL studies in CKD, even larger studies are required to confirm these findings and exclude the possibility of group effects confounding some of the associations with the biochemical parameters which were measured. Furthermore, the standard ASL kinetic model was used here which is primarily validated in healthy volunteers and assumes constant arterial transit time and blood tissue exchange. Differences in these factors may artefactually alter perfusion measurements in CKD, and as previously discussed further research is necessary to validate the use of ASL in the CKD population. Lastly, this study was carried out using 3.0 T MRI, which is in general less available in clinical use and further work will be required to translate these findings to 1.5 T platform, as it is more commonly used in clinical practice.

5.4.4 Conclusions

In conclusion, this study demonstrates significant differences in renal perfusion measured with ASL MRI in a group of patients with advanced CKD, and shown correlation to renal parameters such as eGFR. Correlation between progression of CKD over the preceding year and perfusion measurement was also shown. In the future, non-invasive imaging such as ASL MRI may be used as a biomarker to identity patients at high risk of progressive renal

impairment who may benefit from aggressive therapy, or as a surrogate marker in clinical trials of novel therapeutics.

Chapter 6

Discussion

6.1 Principle findings

6.1.1 Oxidative stress in chronic kidney disease

CKD is associated with excess cardiovascular morbidity and mortality which is not entirely explained by conventional cardiovascular risk factors. As discussed in chapter 1, CKD causes a state of oxidative stress due to increased ROS production and depletion of antioxidant capacity. In this state of oxidative stress, there is disruption of redox signalling and depletion of signalling molecules such as NO, leading to vascular and endothelial dysfunction which have an important role in the early stages of atherosclerosis and other forms of CVD. Interventional studies using antioxidants to modulate oxidative stress to improve endothelial function and reduce cardiovascular risk have had conflicting results. The aim of the oxidative stress in CKD study was therefore to determine the effect of parenteral ascorbic acid on vascular biology in a cohort of patients with CKD stages 3 – 5.

A crossover comparison of intravenous saline and ascorbic acid was carried out in patients with stages 3 – 5 CKD and a group of matched hypertensive controls. Biomarkers of oxidative stress were measured at baseline, and at 10 and 60 minutes following ascorbic acid. Arterial function was evaluated using the SphygmoCor® system, and brachial artery FMD was measured after each intervention.

Participants with CKD had lower ascorbic acid, higher TAP and ADMA, together with higher adjAix and PWV. There was no difference in baseline FMD between the groups. Intravenous ascorbic acid increased TAP and O_2^-, and reduced central BP and adjAix in both groups, and lowered ADMA in the CKD group only. No effect on FMD was observed. As discussed, there is conflicting evidence regarding the effect of ascorbic acid on vascular function; this study highlighted the mixed pro- and antioxidant effects of ascorbic acid, and whilst there was an effect on vascular biology in terms of a central blood pressure and ADMA, there was no change in endothelial function.

The strengths of this study include the recruitment of a well characterised cohort of patients without a history of vascular disease. The effects of ascorbic acid administration were investigated using gold standard techniques performed by a single operator. Despite there being no effect on endothelial function, this study lends credence to the idea of ascorbic acid as a vasoactive substance.

6.1.2 Renal arterial spin labelling MRI in healthy volunteers

As discussed in section 1.7, regulation of renal perfusion is also in part sensitive to redox regulation. Established methods of measuring renal perfusion non-invasively have inherent drawbacks, including the need for administration of a contrast agent which may be nephrotoxic or harmful in renal impairment. ASL MRI is an emerging technique which allows quantitative measurement of kidney perfusion without administration of a harmful contrast agent. In earlier studies, ASL MRI showed good agreement with perfusion measured using a microsphere technique in a porcine animal model (Artz et al., 2011b), and had good accuracy in measuring perfusion in explanted organs undergoing haemoperfusion (Warmuth et al., 2007a). In humans, ASL MRI has good agreement with other *in vivo* measures of kidney perfusion such as DCE MRI (Cutajar et al., 2014) and PAH clearance (Ritt et al., 2010). At the onset of this work, local experience with ASL MRI was limited and few studies had examined the repeatability of ASL MRI at 3.0T in healthy volunteers. The VolRASL study was designed to establish local experience with the technique and investigate the reproducibility of the technique in healthy volunteers at a field strength of 3.0T.

Healthy volunteers attended on three occasions: firstly for biochemical and clinical assessment, and on the second and third occasions underwent ASL MRI. Renal anatomy was evaluated on sagittal HASTE images, T1 longitudinal relaxation time was measured using a MOLLI sequence and FAIR True-FISP ASL MRI was carried out to measure perfusion.

T1 time was 1376 ms in the cortex, 1651 ms in the medulla, and 1491 ms for whole kidney ROI. Whole kidney perfusion was found to be 228 mL/min/100g, cortical perfusion was 321 mL/min/100g and absolute perfusion was 367 mL/min per kidney. In terms of reproducibility, the CV_{ws} was 9.2 % for cortical perfusion and 7.1 % for whole kidney perfusion, and good agreement was demonstrated on Bland Altman analysis.

A broad range of perfusion values have been found in previous studies employing ASL MRI and our findings lie within this range. The T1 longitudinal relaxation times are higher than in earlier literature, although this is to be expected at higher magnetisation, and there is limited data regarding renal T1 values at 3.0 T. Good reproducibility was shown, with acceptable values of coefficient of variation, and good agreement on Bland Altman analysis.

6.1.3 Renal arterial spin labelling MRI in chronic kidney disease

Subsequently, the redox regulation of kidney perfusion was investigated in the KidRASL study, by measuring the response to parenteral ascorbic acid using renal ASL MRI. Earlier work has showed that renal function is regulated in a redox sensitive manner. For example, infusion of the NOS inhibitor L-NAME reduces medullary blood flow and filtration fraction in rats with normal renal function (Zatz and de Nucci, 1991), whilst chronic supplementation results in proteinuria and renal impairment (Baylis et al., 1992). Conversely, supplementation with the SOD mimetic tempol ameliorates renal impairment and tubular atrophy in 5/6 nephrectomised mice (Ding et al., 2015). There are limited human studies into the redox regulation of renal perfusion, and so the KidRASL study was designed to investigate the effect of ascorbic acid on renal perfusion, measured using ASL MRI.

No significant difference in kidney perfusion was shown following administration of ascorbic acid, either measured in the cortex or in the whole of the kidney. This is in contrast to an earlier study by Delles et al, in which parenteral administration of ascorbic caused increased perfusion in patients with type 2 diabetes co-infused with L-arginine (Delles et al., 2004). In this earlier study, participants had much earlier renal disease in comparison to the population of patients with CKD stages 3 – 5 recruited here. The discrepancy in the two studies could therefore be accounted for by the need for the simultaneous administration of NOS substrate to increase renal perfusion, or by the fact that irreversible changes to renal function have developed in this later stage of CKD.

The strengths of this study include the fact that this is one of the largest studies employing ASL MRI to investigate patients with CKD, especially such a well characterised cohort of patients and at a field strength of 3.0 T. Ascorbic acid was administered in a controlled environment and perfusion was measured using a technique that was well validated, at least in healthy volunteers. Although no change in renal perfusion was found following ascorbic acid, it was found that ASL MRI demonstrated significant differences between those with normal renal function and participants with CKD stages 3 – 5, with increased cortical and whole kidney T1, and reduced cortical and whole kidney perfusion. Interestingly, absolute perfusion showed a weak but significant correlation with progression of kidney disease over the preceding year.

6.2 Limitations and criticisms

6.2.1 Antioxidant intervention

Intravenous parenteral ascorbic acid was used as a modulator of oxidative stress and a number of criticisms of the choice and administration of the agent could be made. For example, ascorbic acid has not only antioxidant actions but acts as an essential cofactor to several other enzymes including dopamine β-monooxygenase, peptidyl glycine α-monooxygenase, 4-hydroxyphenylpyruvate and prolyl hydroxylase, and as shown here, also has pro-oxidant effects in addition to its antioxidant activity. As such it could be argued that ascorbic acid is not the "cleanest" of antioxidant agents, however this criticism could also be made of other antioxidant agents available for use in humans.

For example, N-acetylcysteine administered parenterally is associated with drug reaction in over 10% of patients, with anaphylactoid reactions occurring in 8% to 18% of patients (Schmidt and Dalhoff, 2001). In addition to their antioxidant effect, tocopherols also have enzyme regulatory activity, for example in relation to protein kinase C. Whilst the route of administration of ascorbic acid could be criticised for being non-physiological, the intravenous route avoids the problems of inter- individual or group differences in absorption and compliance which are associated with oral supplementation. The intravenous route therefore allows for study of the effects of ascorbic in a controlled and standardised manner.

6.2.2 Participants and recruitment

In the Renox study, patients with CKD were recruited from general nephrology clinics, whilst controls were recruited from a tertiary referral blood pressure clinic, in order to match for blood pressure and other non-renal aspects of cardiovascular risk. Whilst participants were well matched for age and BMI, the CKD group had significantly lower blood pressure. In contrast to earlier studies, there was no difference in FMD between groups, and it was postulated that the excess endothelial dysfunction associated with CKD might have been offset by the lower blood pressure in the cohort. Alternatively, it is possible that uraemia itself is a smaller contributor to oxidative stress in comparison to other cardiovascular risk factors. Whilst many studies have shown an association between CKD and FMD, other studies have failed to show this relationship. Unfortunately the Renox study failed to comprehensively answer this question due to the discrepancy in matching. Whilst avoidance of participants with pre-existing vascular disease, diabetes, or in the case of the control

group, microalbuminuria, should be considered a strength of the study, these exclusion criteria contributed to difficulties in recruitment.

Similarly, in the KidRASL study the CKD group had significantly higher age and blood pressure than the control group. Given that there was an association between perfusion and both age and systolic blood pressure, this discrepancy in matching could have confounded the differences in ASL measurements, however, on stepwise linear regression analysis eGFR remained a significant determinant of renal perfusion after adjustment for age. Nevertheless the conclusions regarding differences in MRI findings could be stronger with better matching between CKD and control groups.

6.2.3 Biomarkers of oxidative stress

As discussed in chapter 1, there are numerous molecules previously posited as ideal markers of oxidative stress, including ROS and RNS themselves, as well as the downstream end products of oxidative damage to lipids, proteins and other macromolecules. There remains a lack of consensus however regarding the most informative and appropriate biomarkers which should be measured during clinical studies into oxidative stress. A major criticism of any biomarker is that measurement of its circulating concentration in serum or blood, may not reflect its concentration in the relevant tissues being studied, for example, in endothelial cells or vascular smooth muscle. The situation is complicated by the fact that there may not be a direct relationship between burden of oxidative stress, and resultant endothelial dysfunction and cardiovascular outcome.

In the Renox study, CKD was associated with lower ascorbic acid, and higher TAP and ADMA, whilst there was no difference in F2-isoprostanes, GSH/GSSG ratio, or O_2^-. This contradicts earlier studies where an increase in markers of oxidative stress were observed. This disagreement could be related to true differences in populations, methodological differences, or related to lack of power. Certainly, with regards to the latter, the study was powered to detect a difference in FMD rather than the secondary aims of oxidative stress biomarkers, and all biomarkers were numerically higher in CKD. With regards to methodological issues, all biomarkers were tested in whole blood or serum samples, which in some cases may not be appropriate. The vast majority of glutathione, for example, is found intracellularly such that measurement of erythrocyte or polymorphonuclear leukocyte glutathione may be more appropriate (Jacobson and Moldeus, 1994).

6.2.4 Magnetic resonance imaging

Significant differences in T1 longitudinal relaxation time and renal perfusion measured by ASL MRI were measured in patients with CKD compared to controls. A number of criticisms could be made of the imaging strategy employed here, and there are inherent limitations to the ASL MRI technique which should also be acknowledged.

The signal to noise ratio (SNR) describes how much true physiological signal is measured compared to background noise (Dietrich et al., 2007). Increasing the number of acquisitions increases SNR, as the contribution of random noise to the MRI signal has the tendency to cancel itself out with repeated imaging, such that SNR is proportional to the square root of the number of acquisitions. ASL MRI has an inherently low signal to noise ratio, and most centres aim to lessen this by acquiring a number of images and producing an average. This demands meticulous alignment of images in order to generate an accurate average value of each voxel, which necessitates the application of one or more of a number of imaging strategies or post processing techniques. In this study, acquisitions were taken during exhaled breath holding in order to reduce artefact derived from respiratory motion and post processing image registration was performed using in house developed software. Other studies have used respiratory gating techniques which use an MR prepulse to identify the position of an anatomical landmark such as the diaphragm, in order to synchronise image acquisition. Others have trained participants in free light breathing in order to reduce the movement which occurs during respiration, acquired dozens of images during an extended scan time, and subsequently applied post processing image registration. A number of techniques were tested prior to commencement of the VolRASL study, and it was found that the breath holding strategy provided the clearest pictures and most accurate image registration. Respiratory gating was found to prolong scan time without improving image quality, and respiratory training was felt to be impractical in a diverse patient population. Future work should seek to formally measure SNR produced by available strategies in order to allow standardisation of ASL MRI protocols and allow fair comparisons of measurements made in different centres.

Additionally, in the standard kinetic model used to facilitate quantification of perfusion from the ASL signal (Martirosian et al., 2004), a number of assumptions are made which may be unfounded especially in a CKD cohort. The principle of ASL MRI is that water is labelled with an inversion pulse at an intravascular location proximal to the organ of interest, with the labelling then subsequently travelling to the imaging region to exchange with tissue

blood, after a period of time in transit. This transit time is the aggregate of arterial transit time, meaning the interval from the point of labelling to the tissue microvasculature, and the tissue transit time which is the duration of tissue capillary exchange. Image acquisition is therefore designed to occur after a fixed time delay following labelling. The standard ASL kinetic model assumes a fixed arterial transit time which may not be correct either between individuals or in CKD. As a result, image acquisition may be mistimed in relation to labelling, leading to an inaccurate measure of perfusion. Furthermore, perfusion quantified from the ASL signal is inversely proportional to the T1 longitudinal relaxation time of tissue. Given that T1 is longer in patients with CKD, as shown here and in previous studies (Huang et al., 2011), it is possible that variation in perfusion measurements in kidney disease may relate to differences in T1 than to ASL signal. Further research is needed to clarify how much ASL perfusion reflects true changes in microvascular perfusion as opposed to reduction in arterial transit time of labelled blood or increased T1 time due to structural changes to the interstitium.

6.3 Future directions

6.3.1 Oxidative stress in chronic kidney disease

As has been discussed in chapter 1, ROS and other radicals are not simply harmful by-products, but signalling molecules involved in a number of normal cellular processes. Exogenous antioxidants such as ascorbic acid have effects outwith their antioxidant properties, and in certain cases have pro-oxidant effects. As such, it is unsurprising that studies investigating the administration of antioxidants have had conflicting results and this is reflected in the conflicting results of interventional trials of antioxidant therapy.

Given these conflicting results and negative outcome of systematic review of antioxidant interventions, future studies into the manipulation of redox homeostasis may be more fruitful by investigating modulators of endogenous antioxidant pathways. Whilst the recent studies using bardoxolone methyl raised concerns regarding cardiovascular safety, specific effects relating to activation of endothelin receptors, and electrolyte abnormalities have been identified which are potentially modifiable. Novel therapeutics which agonise Nrf-2 without these adverse effects are also under investigation.

6.3.2 Renal imaging in kidney disease

Significant differences in kidney perfusion were measured in patients with CKD using ASL MRI, and interestingly, there was a weak but significant association with progression of renal impairment. At present, evaluation of kidney disease relies on measurement of serum creatinine in order to derive the eGFR, and quantification of proteinuria, both of which provided prognostic information regarding progression to ESRD and likelihood of complications such as CVD. Both are imperfect measures, being insensitive to small changes in renal function as well as being confounded by factors such as muscle mass and diet. Novel biomarkers which allow more accurate quantification of renal function and prognosis are greatly needed in order to guide more aggressive therapy, and to act as outcome measures in trials of novel therapeutics aimed at ameliorating progression of CKD.

MRI has many desirable qualities as an imaging technique, allowing avoidance of ionising radiation and potentially nephrotoxic contrast agents, which renders it ideal for repeated, longitudinal use in chronic disease, and in clinical studies where avoidance of harm to participants is crucial. ASL is one of a number of emerging MRI techniques which may have utility in CKD. Many of these techniques, discussed below, also allow conversion of "pictures to numbers" by modelling techniques validated in phantom and animal studies which allow quantification of anatomical or physiological properties relevant to the underlying disease process.

Blood oxygen level dependent (BOLD) MRI measures tissue oxygenation using the principle that deoxyhaemoglobin has paramagnetic properties whilst oxyhaemoglobin does not. The paramagnetic effects of deoxyhaemoglobin cause local tissue magnetic field inhomogeneity, which results in faster transverse dephasing of photons, represented by a shorter $T2^*$ relaxation time and a lower signal intensity on $T2^*$ weighted images (Logothetis and Pfeuffer, 2004). The BOLD MRI measurement is often expressed as the relaxation rate, $R2^*$, which is the inverse of T2* such that high signal equates to a low concentration of oxyhaemoglobin. The utility of BOLD measurements in renal imaging is still under investigation. Chrysochou et al investigated the use of BOLD to identify patients who would respond favourably to stenting of an atherosclerotic renal artery stenosis (Chrysochou et al., 2012). Patients whose eGFR rose following revascularisation had a higher ratio of R2* to single kidney GFR at baseline, such that the authors postulated that R2*/GFR represented living tissue undergoing cellular respiration and converting oxygenated to deoxygenated haemoglobin. Tissue with a lower ratio of R2* to single kidney GFR represented kidneys

which had undergone permanent physiological changes which would not benefit from revascularisation. In a later study of 342 patients undergoing BOLD MRI however, there was no association between R2* and eGFR (Michaely et al., 2012). A criticism of both these studies is the use of a single measurement of R2*, given that BOLD measurements are a relative, rather than absolute indicator of tissue function, such that change in BOLD signal following a challenge with a stimulus which alters tissue oxygenation, such as a loop diuretic or intravenous fluid, provides a much more useful measure.

Another emerging MRI strategy which shows promise in the characterisation of renal disease is diffusion weighted imaging (DWI). DWI maps the Brownian motion of water molecules of each voxel within a given tissue, such that the signal represents the average rate of diffusion within that site (Bammer, 2003). The DWI signal therefore relates to a number of complex factors such as the composition of both intra- and extracellular fluid, and the diffusion which occurs both within, and between the two compartments. Furthermore, diffusion in certain tissues is anisotropic, meaning that the direction of flow is not equal in all directions due to the presence of certain tracts within the tissue microstructure. The direction of diffusion within a voxel can be represented by a mathematical matrix expressing the forces occurring in each direction, and diffusion tensor imaging (DTI) is the technique by which this information is represented pictographically in a 2- or 3- dimensional image. Anisotropy is higher in some tissues in comparison to others (for example in white, rather than grey matter), and may be altered by certain disease states, allowing discrimination of anatomical compartments and identification of certain pathologies. For example, in neuroimaging DWI is used clinically to diagnose stroke, as a measureable change in diffusion develops early in the disease process, visible as a reduction in the DWI signal prior to any abnormality becoming visible using other imaging modalities. DTI has been used to identify white matter tracts within the cerebral cortex, allowing investigation of connectivity between different regions of the brain in neurological research. Patients with CKD have significantly lower fractional anisotropy (FA) regardless of eGFR, and there is a correlation between eGFR and FA and apparent diffusion coefficient (ADC). There is also a negative association between scores of glomerular and tubulointerstitial injury on biopsy with FA in patients with CKD (Li et al., 2014).

In CKD, there is reduction in corticomedullary differentiation (CMD) with progressive renal impairment, primarily due to an increase in cortical T1 (Lee et al., 2007). The histopathological determinates of the variation in T1 in CKD are yet to be determined, but

presumably relate in some way to changes in the extracellular volume or composition which reflect and increased fluid content in the region of interest. There are significant differences in T1 time in CKD compared to in health, and changes in T1 may be another parameter of use in determining prognosis.

In a small cohort of patients with CKD and healthy volunteers, absolute perfusion measured using ASL MRI predicted progression of kidney dysfunction over the preceding year. Future work should firstly investigate the histopathological determinates of renal fibrosis which predict poor outcome and determine the MRI correlates of this, and which MRI techniques are most useful in the investigation of this. It is possible that complementary techniques will strengthen the diagnostic power of renal imaging, for example combining measures of perfusion such as ASL and BOLD with measures of kidney structure such as DTI and T1.

6.4 Conclusions

In patients with hypertension and CKD, parenteral administration of ascorbic acid reduces central BP and improves large arterial function, whilst in CKD only, reduces serum ADMA. ASL MRI was established as a robust method of measuring renal perfusion in a group of healthy volunteers. There are significant differences in ASL MRI measured renal perfusion, and in T1 longitudinal relaxation time, in CKD as compared to healthy volunteers, and there with correlation between perfusion and progression of CKD over the preceding year.

Publications and presentations

Publications containing work undertaken for this thesis

Non contrast renal magnetic resonance imaging to assess perfusion and corticomedullary differentiation in health and chronic kidney disease

Keith A Gillis, Christie McComb, Rajan K Patel, Kathryn K Stevens, Markus P Schneider, Aleksandra Radjenovic, Scott TW Morris, Giles H Roditi, Christian Delles, Patrick B Mark

Nephron 2016, *publication pending*

Inter-study reproducibility of arterial spin labelling magnetic resonance imaging for measurement of renal perfusion in healthy volunteers at 3 Tesla.

Keith A Gillis, Christie McComb, John E Foster, Alison HM Taylor, Rajan K Patel, Scott TW Morris, Alan G Jardine, Markus P Schneider, Giles H Roditi, Christian Delles and Patrick B Mark

BMC Nephrology 2014, 15:23

Presentations to learned societies, of work undertaken for this thesis

Comprehensive assessment of kidney anatomy and perfusion with arterial spin labelling magnetic resonance imaging.

Keith Gillis, Christie McComb, Giles H. Roditi, Aleksandra Radjenovic, Scott Morris, Christian Delles, Patrick Mark.

Poster presentation, European Renal Association, London, May 2015.

Effect of Ascorbic Acid on Endothelial Dysfunction and Oxidative Stress in Chronic Kidney Disease.

Keith Gillis, Kathryn K. Stevens, Scott Morris, Christian Delles and Patrick B. Mark.

Poster presentation, American Society of Nephrology Kidney Week, Philadelphia, November 2014.

Renal arterial spin labelling magnetic resonance imaging in health and chronic kidney disease.

Keith Gillis, Christie McComb, Alison Taylor, Rajan Patel, Giles Roditi, Scott Morris, Aleksandra Radjenovic, Christian Delles, Patrick Mark.

Oral presentation, Scottish Renal Association Conference, Edinburgh, September 2014.

Effects of Intravenous Ascorbic acid on Vascular Function and Oxidative Stress in Chronic Kidney Disease.

Keith Gillis, Kathryn K. Stevens, Markus P. Schneider, Scott Morris, Christian Delles, Alan G. Jardine and Patrick B Mark.

Oral presentation, Scottish Renal Association Conference, Glasgow, November 2013.

Reproducibility of Renal Perfusion Measurements Using Arterial Spin Labelling MRI: A Healthy Volunteer Study.

Keith Gillis, Christie McComb, Scott Morris, Christian Delles, Patrick B Mark.

Poster presentation, American Society of Nephrology Kidney Week, Atlanta, November 2013.

Effects of Intravenous Ascorbic acid on Vascular Function and Oxidative Stress in Chronic Kidney Disease.

Keith Gillis, Kathryn K. Stevens, Markus P. Schneider, Scott Morris, Christian Delles, Alan G. Jardine, Patrick B. Mark.

Poster presentation, American Society of Nephrology Kidney Week, Atlanta, November 2013.

Appendix 1

Information about the Research:

Oxidative stress in patients with chronic kidney disease ("Renox")

Version 5.0 (10/05/2012)

Version for patients with chronic kidney disease

<u>Invitation</u>

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We already know that the blood vessels in patients with chronic kidney disease (CKD) do not function properly, and that this can contribute to a higher blood pressure and to stiffer blood vessels. It has been suggested that increased "oxidative stress" is an important cause for the impaired function of blood vessels in CKD, but this has not been studied in detail. In this context, "oxidative stress" means an increased production of highly reactive oxygen molecules that have the potential to impair the function of blood vessels. In this research project, we are investigating whether infusing the antioxidant vitamin C, which can reduce oxidative stress, improves blood vessel function in CKD patients and in a group of healthy volunteers. In future this knowledge could lead to therapies that improve blood vessel function and reduce blood pressure in CKD patients.

Why have I been chosen to take part?

You have been selected because you have chronic kidney disease (CKD).

Do I have to take part?

No. It is up to you to decide whether or not to take part: participation is completely voluntary. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your current or future medical treatment.

What will be involved if I decide to take part?

Study visit 1 will be during one of your regular visits to the renal clinic. We will check whether you are eligible for this study, give you information about the study and take some blood and urine samples. Visit 2 will be at the Clinical Research Facility for the actual test of blood vessel function, including the intravenous infusion of vitamin C.
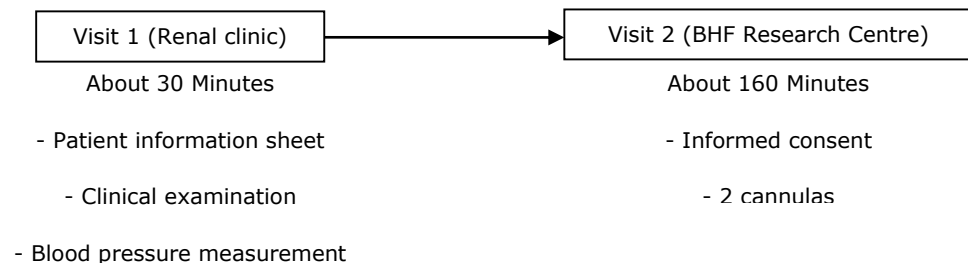
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┌─────────────────────────┐            ┌──────────────────────────────┐
│  Visit 1 (Renal clinic) │ ─────────► │ Visit 2 (BHF Research Centre)│
└─────────────────────────┘            └──────────────────────────────┘
      About 30 Minutes                        About 160 Minutes

  - Patient information sheet                 - Informed consent

    - Clinical examination                      - 2 cannulas

- Blood pressure measurement
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On each occasion, we ask you to come fasted (from midnight for a morning appointment and after breakfast for an afternoon appointment) and not to take any caffeine for 12 hours beforehand.

Visit 1

- After you have read the project information sheet, we will answer any questions that you might have about this study. *This will take about 15 minutes.*

- We will perform a brief clinical examination, measure height and weight and ask you some simple questions about your health and the medication you are taking.

- We will measure your blood pressure using a machine (similar to the one used at the renal clinic and at the GP practice). *Clinical examination and blood pressure measurement should take no longer than 15 minutes.*

- We will also look at the results of the blood and urine taken at your renal clinic appointments (no additional samples are required for visit 1).

Visit 2

- We will put two cannulas in the veins of your arms, one for infusions and one for drawing bloods.

- We will first infuse normal saline over duration of 10 minutes. We will wait 10 minutes and then take a blood sample (about 15 mL, about two teaspoonfuls). The blood will be stored in our lab to be analysed at a later date, to look at markers of oxidative stress.

- We will then examine the blood vessels in your wrist. This examination involves assessment of your pulse with a pencil-like probe. It is carried out at the artery in your wrist and enables us to look at the pulse waveform, which gives us useful information about the blood vessels. *This will take about 15 minutes.*

- We will then measure changes in the diameter of your main artery of your arm with an ultrasound probe. We will apply a jelly-like substance to the skin and the probe will be gently pressed against your upper arm to visualise the main artery. A blood pressure cuff will be placed around your forearm and inflated for 5 minutes. We can then measure the changes in the diameter of the upper arm artery after deflation of the blood pressure cuff. The final part of the test involves a spray of a medication called GTN spray under the tongue. GTN is a very short acting medication which dilates the blood vessel. *Overall, this will take about 20 minutes.*

- We will then infuse vitamin C at a dose of 2 grams over 10 minutes. Subsequently, we will take another blood sample (about 15 mL, about two teaspoonfuls), and repeat the examinations of the blood vessels in your wrist and arm, as described above. We will then take a third blood sample (about 15 mL, about two teaspoonfuls).

- The total time for these tests will be about *160 minutes* (60 minutes for the infusions and 70 minutes for the above described tests of blood vessel function, 30 minutes to take consent, assess basic parameters, and move you through the study process).

Flowchart of Visit 2:

Basic parameters	Saline infusion	Examination of blood vessels in wrist and arm	Vitamin C infusion	Examination of blood vessels in wrist and arm	Exit from BHF
20 Minutes	30 Minutes	35 Minutes	30 Minutes	35 Minutes	10 Minutes

Blood sample   Blood sample   Blood sample

We would also like to look at the renal computer system and in some cases your medical case notes to copy some of the information about any other medical conditions which you may have. Additionally, we will record on the renal computer system that you are taking part in the study to make the other renal doctors looking after you aware.

What are the risks of taking part in this research?

We will take blood from the vein in your arm which in rare cases results in a small bruise. The amount of blood taken for this research does not place you at any risk. The other tests are 'non-invasive' – the probes are attached to the skin and no needles are involved. Assessment of the pulse waveform has no specific side effects or risks. Inflation of the blood pressure cuff during the examination of your main arm artery may lead to some discomfort and numbness in the fingers. This will disappear when the cuff is deflated. A small bruise on your forearm may result from the cuff but will disappear within a couple of days. Some studies have suggested that administration of vitamin C may cause kidney stones. To minimize any such risk, you should not take part in this study if you previously had a kidney stone or if you are already taking daily vitamin C supplements. GTN spray is very short acting. In some people it can cause a headache which is short lived and will disappear within a few minutes.

What are the benefits of taking part?

There is no direct benefit to you in taking part in this study. However, the information we get from this study may help us in the future to have a better understanding of the role of oxidative stress in the function of blood vessels of patients with chronic kidney disease.

What happens if we discover a previously undiagnosed condition?

We may discover previously undiagnosed clinical abnormalities/conditions in yourself, e.g. during the blood tests. We would notify you and your GP of this. Although potentially this could lead to earlier diagnosis and treatment of a condition, this may also lead to further investigations which can turn out to have been unnecessary. Further, the discovery of a new condition can have implications for insurance matters (e.g. you may be denied life insurance or a mortgage because of this newly diagnosed condition).

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation against NHS Greater Glasgow and Clyde, but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the clinical research facility will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research. Further tests on stored samples will again require review and approval by the Ethics Committee.

What will happen to the results of the research study?

The results of the research study will be stored on a computer database and are likely to be published in medical journals. Reports or publications resulting from the study will not contain any personal details. The research doctor will provide a copy of the results on request.

Will I get any reimbursement for my expenses?

After you completed the study, we will pay you a lump sum of £15.- to reimburse you for your expenses (parking, public transport).

Contact for Further Information

Should you have any further questions please feel free to call Dr Patrick Mark at the BHF Glasgow Cardiovascular Research Centre by telephone (0141-330-8218).

If you wish to discuss the study with an independent contact person who is not involved in the study, please contact Mr Marc Clancy by telephone (0141-211-1750).

Information about the Research:

Oxidative stress in patients with chronic kidney disease ("Renox")

Version 5.0 (10/05/2012)

Version for healthy volunteers

<u>**Invitation**</u>

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We already know that the blood vessels in patients with chronic kidney disease (CKD) do not function properly, and that this can contribute to a higher blood pressure and to stiffer blood vessels. It has been suggested that increased "oxidative stress" is an important cause for the impaired function of blood vessels in CKD, but this has not been studied in detail. In this context, "oxidative stress" means an increased production of highly reactive oxygen molecules that have the potential to impair the function of blood vessels. In this research project, we are investigating whether infusing the antioxidant vitamin C, which can reduce oxidative stress, improves blood vessel function in CKD patients and in a group of healthy volunteers. In future this knowledge could lead to therapies that improve blood vessel function and reduce blood pressure in CKD patients.

Why have I been chosen to take part?

You have been selected because you belong to the control group of healthy volunteers for this study.

Do I have to take part?

No. It is up to you to decide whether or not to take part: participation is completely voluntary. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your current or future medical treatment.

What will be involved if I decide to take part?

We would like to invite you to come to our Clinical Research Facility twice. On the first occasion we will check whether you are eligible for this study, ask you to sign the consent form, and take some blood and urine samples. Visit 2 to the Clinical Research facility will be for the actual test of blood vessel function, including the intravenous administration of vitamin C.
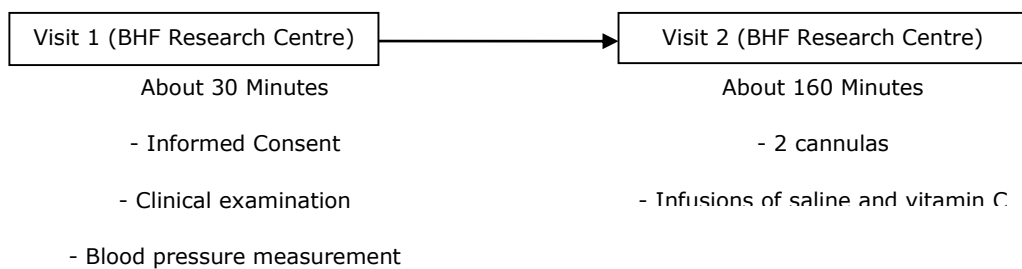
Visit 1 (BHF Research Centre)	→	Visit 2 (BHF Research Centre)
About 30 Minutes		About 160 Minutes
- Informed Consent		- 2 cannulas
- Clinical examination		- Infusions of saline and vitamin C
- Blood pressure measurement		

On each occasion, we ask you to come fasted (from midnight for a morning appointment and after breakfast for an afternoon appointment) and not to take any caffeine for 12 hours beforehand.

Visit 1

- After you have read the project information sheet, we will answer any questions that you might have about this study. *This will take about 15 minutes.*

- We will perform a brief clinical examination, measure height and weight and ask you some simple questions about your health and the medication you are taking.

- We will measure your blood pressure using a machine (similar to the one used at the GP practice). *Clinical examination and blood pressure measurement should take no longer than 15 minutes.*

- We will also take some blood and urine samples.

Visit 2

- We will put two cannulas in the veins of your arms, one for infusions and one for drawing bloods.

- We will first infuse normal saline over duration of 10 minutes. We will wait 10 minutes and then take a blood sample (about 15 mL, about two teaspoonfuls). The blood will be stored in our lab to be analysed at a later date, to look at markers of oxidative stress.

- We will then examine the blood vessels in your wrist. This examination involves assessment of your pulse with a pencil-like probe. It is carried out at the artery in your wrist and enables us to look at the pulse waveform, which gives us useful information about the blood vessels. *This will take about 15 minutes.*

- We will then measure changes in the diameter of your main artery of your arm with an ultrasound probe. We will apply a jelly-like substance to the skin and the probe will be gently pressed against your upper arm to visualise the main artery. A blood pressure cuff will be placed around your forearm and inflated for 5 minutes. We can then measure the changes in the diameter of the upper arm artery after deflation of the blood pressure cuff. The final part of the test involves a spray of a medication called GTN spray under the tongue. GTN is a very short acting medication which dilates the blood vessel. *Overall, this will take about 20 minutes.*

- We will then infuse vitamin C at a dose of 2 grams over 10 minutes. Subsequently, we will take another blood sample (about 15 mL, about two teaspoonfuls), and repeat the examinations of the blood vessels in your wrist and arm, as described above. We will then take a third blood sample (about 15 mL, about two teaspoonfuls).

- The total time for these tests will be about *160 minutes* (60 minutes for the infusions and 70 minutes for the above described tests of blood vessel function, 30 minutes to take consent, assess basic parameters, and move you through the study process).

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Basic parameters	Saline infusion	Examination of blood vessels in wrist and arm	Vitamin C infusion	Examination of blood vessels in wrist and arm	Exit from BHF
20 Minutes	30 Minutes	35 Minutes	30 Minutes	35 Minutes	10 Minutes
		Blood sample		Blood sample	Blood sample

In some cases we may look at your medical case notes to copy some of the information about any other medical conditions which you may have. We can inform your GP that you are taking part in this study.

What are the risks of taking part in this research?

We will take blood from the vein in your arm which in rare cases results in a small bruise. The amount of blood taken for this research does not place you at any risk. The other tests are 'non-invasive' – the probes are attached to the skin and no needles are involved. Assessment of the pulse waveform has no specific side effects or risks. Inflation of the blood pressure cuff during the examination of your main arm artery may lead to some discomfort and numbness in the fingers. This will disappear when the cuff is deflated. A small bruise on your forearm may result from the cuff but will disappear within a couple of days. Some studies have suggested that administration of vitamin C may cause kidney stones. To minimize any such risk, you should not take part in this study if you previously had a kidney stone or if you are already taking daily vitamin C supplements. GTN spray is very short acting. In some people it can cause a headache which is short lived and will disappear within a few minutes.

What are the benefits of taking part?

There is no direct benefit to you in taking part in this study. However, the information we get from this study may help us in the future to have a better understanding of the role of oxidative stress in the function of blood vessels of patients with chronic kidney disease.

What happens if we discover a previously undiagnosed condition?

We may discover previously undiagnosed clinical abnormalities/conditions in yourself, e.g. during the blood tests. We would notify you and your GP of this. Although potentially this could lead to earlier diagnosis and treatment of a condition, this may also lead to further investigations which can turn out to have been unnecessary. Further, the discovery of a new condition can have implications for insurance matters (e.g. you may be denied life insurance or a mortgage because of this newly diagnosed condition).

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation against NHS Greater Glasgow and Clyde, but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the clinical research facility will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this

means you will not be entitled to any future financial reimbursement related to this study and related research. Further tests on stored samples will again require review and approval by the Ethics Committee.

What will happen to the results of the research study?

The results of the research study will be stored on a computer database and are likely to be published in medical journals. Reports or publications resulting from the study will not contain any personal details. The research doctor will provide a copy of the results on request.

Will I get any reimbursement for my expenses?

After you completed the study, we will pay you a lump sum of £15.- to reimburse you for your expenses (parking, public transport).

Contact for Further Information

Should you have any further questions please feel free to call Dr Patrick Mark at the BHF Glasgow Cardiovascular Research Centre by telephone (0141-330-8218).

If you wish to discuss the study with an independent contact person who is not involved in the study, please contact Mr Marc Clancy by telephone (0141-211-1750).

NHS

Greater Glasgow
and Clyde

WoSRES
West of Scotland Research Ethics Service

West of Scotland REC 4

Ground Floor, Tennent Building
Western Infirmary
38 Church Street
Glasgow
G11 6NT
www.nhsggc.org.uk

Dr Paddy Mark
Specialist Registrar
BHF Cardiovascular Research Centre
University of Glasgow
Glasgow
G12 8TA

Date	7 June 2012
Direct line	0141-211-1722
Fax	0141-211-1847
e-mail	evelyn.jackson@ggc.scot.nhs.uk

Dear Dr Mark

Study title:	Oxidative stress in patients with chronic kidney disease
REC reference:	11/WS/0045
Protocol number:	1.0
Amendment number:	AM03
Amendment date:	15 May 2012

The above amendment was reviewed by a Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation. The amendments are as follows:

1. A third blood sample is to be taken at the end of study visit 2.
2. The duration of visit 2 has been altered after consideration of the techniques involved. It is estimated that a realistic duration of the visit is 160 minutes (was 110 minutes previously).

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Information Sheet: Patients with chronic kidney disease	5.0	10 May 2012
Participant Information Sheet: Healthy volunteers	5.0	10 May 2012
Protocol	5.0	10 May 2012
Notice of Substantial Amendment (non-CTIMPs)	AM03	15 May 2012
Participant Consent Form	5.0	10 May 2012

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/WS/0045:	Please quote this number on all correspondence

Yours sincerely

Evelyn Jackson

for **Dr Clair Evans**
Alternate Vice-Chair

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Maureen Travers, R&D Office, Tennent Building, Western Infirmary*

WoSRES
West of Scotland Research Ethics Service

NHS

Greater Glasgow
and Clyde

West of Scotland REC 2
Ground Floor, Tennent Building
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38 Church Street
Glasgow
G11 6NT
www.nhsggc.org.uk

Dr Paddy Mark
Specialist Registrar
BHF Cardiovascular Research Centre
University of Glasgow
Glasgow
G12 8TA

Date   30 April 2012
Direct line 0141-211-1722
Fax   0141-211-1847
e-mail   evelyn.jackson@ggc.scot.nhs.uk

Dear Dr Mark

Study title:	Oxidative stress in patients with chronic kidney disease
REC reference:	11/WS/0045
Protocol number:	1.0
Amendment number:	Minor Amendment AM02
Amendment date:	30 April 2012

Thank you for your e-mail of 30 April 2012, notifying the Committee of the addition of a letter to participants to remind them of the agreed date and time of their appointment,

The Committee does not consider this to be a "substantial amendment", as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	Version	Date
Reminder letter to participants	1-1-0	-
Notification of a Minor Amendment	AM02	30 April 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

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11/WS/0045:	Please quote this number on all correspondence

Yours sincerely

[signature: Evelyn Jackson]

Ms Evelyn Jackson
Committee Co-ordinator

Copy to: Dr Maureen Travers, R&D Office, Tennent Building, Western Infirmary

WoSRES

West of Scotland Research Ethics Service

NHS

Greater Glasgow and Clyde

Dr Markus Schneider
Clinical Senior Lecturer
University of Glasgow
BHF Glasgow Cardiovascular Research
Centre
126 University Place
University of Glasgow
Glasgow
G12 8TA

Date	16th January 2012
Your Ref	
Our Ref	
Direct line	0141 211 2123
Fax	0141 211 1847
E-mail	Liz.Jamieson@ggc.scot.nhs.uk

Dear Dr Schneider

Study title: **Oxidative stress in patients with chronic kidney disease**
REC reference: **11/WS/0045**
Amendment date: **11 January 2012**

Thank you for submitting the above amendment, i.e. a change of Chief Investigator from yourself to Dr Paddy Mark and a change to the exclusion criteria, which was received on 13 January 2012. I can confirm that this is a valid notice of a substantial amendment and will be reviewed by the Sub-Committee of the REC at its next meeting.

Documents received

The documents to be reviewed are as follows:

Document	Version	Date
Participant Consent Form: Patients	4.0	11 January 2012
Participant Information Sheet: Patients with Chronic Kidney Disease	4.0	11 January 2012
Participant Information Sheet: Healthy Volunteers	3.0	11 January 2012
Protocol	3.0	11 January 2012
Notice of Substantial Amendment (non-CTIMPs)	AM01	11 January 2012
Covering Letter		11 January 2012
Investigator CV - Dr Paddy Mark – **TO FOLLOW**		

Notification of the Committee's decision

The Committee will issue an ethical opinion on the amendment within a maximum of 35 days from the date of receipt.

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University of Glasgow | Institute of Cardiovascular & Medical Sciences

West of Scotland
Research Ethics Committee
Ethics Department
Ground Floor, The Tennents Institute
Western Infirmary
38 Church Street
Glasgow G12 6NT

11/01/2012

RE: "Oxidative stress in patients with chronic kidney disease", REC reference number 11/WS/0045
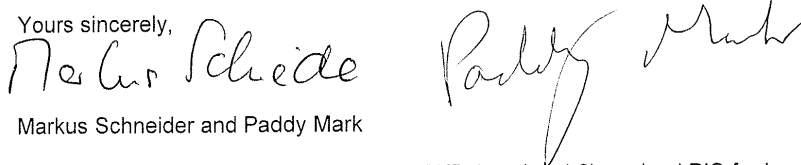
Dear Dr Langridge,

Please find enclosed our application for a substantial amendment to the above study.

1) We apply for a change in the Chief Investigator. Dr. Markus Schneider resigns from the study, and new Chief Investigator is Dr. Paddy Mark. Co-Investigators are Dr. Scott Morris and Dr. Christian Delles. New independent advisor for this study is Mr Marc Clancy, vascular surgeon at the Western Infirmary.

2) Prolonged intake of higher doses of vitamin C has been linked with development of kidney stones in some studies, while others dispute such a risk. Although this issue is contentious, we have mentioned the risk of kidney stones with vitamin C administration as a potential side effect in the PIS. To minimize any such risk we will also exclude subjects with history of kidney stones and those already taking daily vitamin C supplements.

Thank you very much again for reviewing our application. Please direct all future correspondence to Dr. Paddy Mark (Patrick.Mark@glasgow.ac.uk).

Yours sincerely,

Markus Schneider and Paddy Mark

Enclosures: revised PIS for patients with CKD (version 4.0), revised PIS for healthy volunteers (version 3.0), revised consent form (version 4.0), revised protocol (version 3.0)

NHS

Greater Glasgow
and Clyde

West of Scotland REC 2
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
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www.nhsggc.org.uk

Dr Markus Schneider
Clinical Senior Lecturer
University of Glasgow
BHF Glasgow Cardiovascular Research
Centre
126 University Place
Glasgow
G12 8TA

Date	24th November 2011
Your Ref	
Our Ref	
Direct line	0141 211 2123
Fax	0141 211 1847
E-mail	Liz.Jamieson@ggc.scot.nhs.uk

Dear Dr Schneider

Full title of study: **Oxidative stress in patients with chronic kidney disease**
REC reference number: **11/WS/0045**

Thank you for your letter of 10th December 2011. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 15 November 2011. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

Document	Version	Date
Covering Letter		10 November 2011
Participant Consent Form: Patients	3.0	10 November 2011
Participant Information Sheet: Patients	3.0	10 November 2011
Participant Information Sheet: Healthy Volunteers	2.0	10 November 2011

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

11/WS/0045	Please quote this number on all correspondence

Yours sincerely

Mrs Liz Jamieson
Committee Co-ordinator

Copy to: Dr Maureen Travers, NHS Greater Glasgow and Clyde

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West of Scotland
Research Ethics Committee
Ethics Department
Ground Floor, The Tennents Institute
Western Infirmary
38 Church Street
Glasgow G12 6NT

10/11/2011

Dear Dr Langridge,

Thank you very much for the favourable ethical opinion on our project **"Oxidative stress in patients with chronic kidney disease", REC reference number 11/WS/0045**.

We have now addressed the remaining issues:

1) It was requested that we provide an explanation of what "oxidative stress" is in the PIS. This explanation has now been given in the PISs.

2) We have changed line 7 from "We hope to show" to "We are investigating whether".

3) We have replaced the wavy line symbol for "about" with "about".

4) In the consent from, we now refer to the most recent PIS from 10/11/2011.

Thank you very much again for reviewing our application.

Yours sincerely,

Markus Schneider

Markus Schneider MD
Clinical Senior Lecturer

University | Institute of Cardiovascular
of Glasgow | & Medical Sciences

Institute of Cardiovascular and Medical Sciences
BHF Glasgow Cardiovascular Research Centre
College of Medical, Veterinary and Life Sciences
University of Glasgow
126 University Place
Glasgow G12 8TA

Email: Markus.Schneider@glasgow.ac.uk
Tel: +44(0)141-330-5941
Fax: +44(0)141-330-1689

Enclosures: revised PIS for patients with CKD (version 3.0), revised PIS for healthy volunteers
(version 2.0), revised consent form (version 3.0)

Copy to: Dr. Maureen Travers
                  Dr. Scott Morris

Dr Markus Schneider
Clinical Senior Lecturer
University of Glasgow
BHF Glasgow Cardiovascular Research
Centre
126 University Place
Glasgow
G12 8TA

Date	7th November 2011
Your Ref	
Our Ref	
Direct line	0141 211 2123
Fax	0141 211 1847
E-mail	Liz.Jamieson@ggc.scot.nhs.uk

Dear Dr Schneider

Study title: **Oxidative stress in patients with chronic kidney disease**
REC reference: **11/WS/0045**
Protocol number: **1.0**

Thank you for your letter of 18 October 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Other conditions specified by the REC

1) In the Participant Information Sheet (PIS) an explanation of what 'oxidative stress' is should be given
2) In line 7 of the PIS it states "We hope to show' - this should be changed to 'We are investigating whether'.
3) Remove the wavy line symbol for 'about' and replace with 'about'.
4) In the Consent Form bullet point one the wrong date of the PIS has been quoted, i.e. 01/11/2011. This should read 18/10/11.

Please submit revised versions of the documentation as soon as possible.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Advertisement	1.0	26 August 2011
Advertisement	2.0	18 October 2011
Covering Letter		29 August 2011
GP/Consultant Information Sheets	1.0	26 August 2011
GP/Consultant Information Sheets	2.0	18 October 2011
Investigator CV		
Letter of invitation to participant	1.0	26 August 2011
Other: Participation Thank You Letter	1	26 August 2011
Other: CV Co-Investigator - Dr Scott Morris		28 August 2011
Participant Consent Form	1.0	26 August 2011
Participant Consent Form: Patients with Chronic Kidney Disease	2.0	18 October 2011
Participant Information Sheet	1.0	26 August 2011
Participant Information Sheet: Healthy Volunteers	1.0	18 October 2011

Participant Information Sheet: Patients with Chronic Kidney Disease	2.0	18 October 2011
Protocol		26 August 2011
Protocol	2.0	18 October 2011
REC application		29 August 2011
Response to Request for Further Information		18 October 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
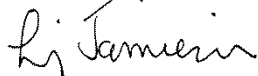
Further information is available at National Research Ethics Service website > After Review

11/WS/0045	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

[signature]

Liz Jamieson
Committee Co-ordinator
On behalf of Dr Sue Dr Sue Langridge, Chair

Enclosures:	List of names and professions of members who were present at the meeting "After ethical review – guidance for researchers"
Copy to:	Dr Maureen Travers, NHS Greater Glasgow and Clyde R&D

Appendix 2

Information about the research

Renal Arterial Spin Labelling MRI in Healthy Volunteers – "Vol-RASL"

Version 2.0 17th November 2012

Version for healthy volunteers

Invitation

You are being invited to kindly donate your time to participate in a research study. Before you take part it is important for you to understand why the research is being carried out and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear or if you would like more information. Take time to decide if you would like to take part and remember that your participation is voluntary.

What is the purpose of the study?

Magnetic Resonance Imaging – or MRI – is a type of scan which allows us to get detailed pictures of internal organs. The advantage of this MRI is that it does not involve exposure to harmful radiation, and as far as we know, there are no long term health risks. It is often useful to examine the blood supply to internal organs and in these cases we have to give the patient a type of dye called 'contrast' into a vein around the time of the scan. Whilst MRI itself is generally harmless, the contrast given can have complications, and certain types of contrast cannot be given to people with kidney disease.

We are developing a type of MRI scan called Arterial Spin Labelling (or ASL) that looks at blood flow to the kidneys without giving potentially harmful contrast and avoids exposure to radiation. This is cutting edge technology which isn't carried out in clinical practice at the moment. We hope that in the future it will allow us to look at the blood flow to the kidneys in people with kidney disease or transplants without exposing them to contrast; this would allow us to improve their treatment.

The purpose of this study is to ensure that the way in which we are carrying out the scan gives us reliable results.

Why have I been selected to take part?

You are a healthy person without kidney problems or any other major health problems.

Do I have to take part?

No – participation is entirely voluntary. Additionally, if you decide to take part but then change your mind you can withdraw at any time.

What will be involved if I decide to take part?

Participation involves three visits to our clinical research facility. On the first visit one of our medical staff will check if you are eligible for the study and after a thorough explanation of what is involved, ask you to sign our consent form. We will take some screening blood tests and urine samples. On the second visit, again to our clinical research facility, you will undergo an MRI scan which will take at the very most, one hour. On the third visit, we will carry out the same MRI scan on two different occasions. The third visit will also take a maximum of one hour.

Visit 1

- You will attend the clinical area of our clinical research facility
- After you have read the Participant Information Sheet we will answer any questions you may have
- A doctor will ask you some simple questions about your past medical history to make sure you are suitable for an MRI scan, and if necessary do a quick clinical examination of your heart and lungs
- We will ask you to sign the consent form
- We will measure your blood pressure and take some samples of blood and urine

Visit 2
- You will attend the MRI department of our clinical research facility
- You will be asked to change into a hospital gown and ensure you don't have any metal objects on your person, storing them in a secure locker in the department
- A trained member of the MRI team will perform an MRI safety questionnaire
- You will then undergo an MRI scan

Visit 3
- You will attend the MRI department of our clinical research facility
- You will be asked to change into a hospital gown and ensure you don't have any metal objects on your person, storing them in a secure locker in the department
- A trained member of the MRI team will perform an MRI safety questionnaire
- You will undergo the same MRI scan twice

What are the risks of taking part in the research?

We will take blood and urine samples to ensure that your kidneys are working normally and that there is no sign of any other health problem of which you are unaware. We will take a small amount of blood from the vein in your arm which in some rare cases can result in minor bruising. The amount of blood taken does not pose any risks. A blood pressure cuff can cause discomfort for the very brief period of time it is inflated, and in extremely rare cases cause a bruise.

Are MRI scans dangerous?

MRI scans are not suitable for people with metal objects in their body such as joint replacements, heart valves, surgical clips or metal shrapnel from previous eye injuries. We will explore this possibility during medical history taking and via the MRI safety questionnaire. The scan takes place in a narrow enclosure, and some people who are extremely claustrophobic will find this disconcerting. Lastly, the scan can be quite noisy however headphones are used to protect you and can also be used to play music for your comfort and relaxation. There are no known health risks associated with MRI so long as those individuals who are unsuitable to undergo the investigation are identified appropriately.

What are the benefits of taking part?

Taking part in this study is of no direct benefit to you. We hope to develop a new type of MRI scan which is currently unavailable which may improve the care of people with kidney problems. If we find that this test gives reliable results in healthy volunteers we will progress to using it in people with kidney disease.

Travel costs can be reimbursed however no other financial incentive will be offered.

What happens if we find a previously undiagnosed condition?

If we discover any previously unknown clinical abnormalities during testing then we will notify you and your GP with your consent. Although this may potentially lead to earlier treatment of a previously unknown condition it may also lead to unnecessary tests and cause anxiety. Furthermore any condition that is found can have implications for insurance and mortgage purposes. The type of MRI scan used does not examine other internal organs aside from the kidneys, and the images that we obtain are not examined by an MRI expert, so that this is not an appropriate scan for finding out about other underlying health problems. This means that if you are experiencing symptoms then this scan will not find out what is causing them. Similarly if you should develop symptoms in the future then you should not be reassured by the fact you have had this scan to rule out the existence of any problem – you should attend your GP as you would have done if you hadn't taken part in this study.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence then you may have grounds for legal action for compensation against the University of Glasgow, but you may have to pay for it. Regardless, if you wish to complain or have any concerns about the way you have been approached or treated during this study then the normal complaints procedure will be available to you.

Will my taking part be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and with any personal details so that all analyses will be carried out anonymously. All information collected about you during the course of the research will be kept strictly confidential.

What will happen to samples that I give?

You will donate blood and urine samples for research purposes. Some examinations will be done straight away whilst others will be done at a later stage when we collect more samples from other participants. We will also store some samples for up to 10 years to perform additional tests if required. The samples are treated as a "gift"; this means you will not be entitled to any future financial imbursement related to this study and related research. Further tests on stored samples will again require review and approval by the ethics committee.

What will happen to the images obtained by MRI?

Images taken by the MRI scan will be kept on a computer database and stored anonymously. The images from the scan are only able to be seen from a few desktop computers in the MRI suite of the Clinical Research Centre, which is secured at all times. We will analyse these images in order to determine the circulation to your kidneys, and compare this to measurements of healthy kidney blood flow found by other studies. We will compare the images from each scan to ensure that they are similar, so that we know the scan is reliable.

Contact for further information

Should you have any further questions please feel free to call Dr Patrick Mark at the BHF Glasgow Cardiovascular Research Centre by telephone on 0141-330-8218

27 July 2016

Dear Dr Keith Gillis

MVLS College Ethics Committee

***Project Title: Vol-RASL: Renal Arterial Spin Labelling in Healthy Volunteers
Project No: 2012098***

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions

- The research should be carried out only on the sites, and/or with the groups defined in the application.

- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.

- If the study does not start within three years of the date of this letter, the project should be resubmitted.

- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Professor William Martin
Professor of Cardiovascular Pharmacology

R507B Level 5
School of Life Sciences
West Medical Building
Glasgow G12 8QQ Tel: 0141 330 4489
E-mail: William.Martin@glasgow.ac.uk

Andrew C Rankin
College Ethics Officer

Appendix 3

Kid-RASL

Renal Arterial Spin Labelling in Chronic Kidney Disease
Information about the research

Version 2.1 25/04/13

Version for CKD

Invitation

You are being invited to kindly donate your time to participate in a research study. Before you take part it is important for you to understand why the research is being carried out and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear or if you would like more information. Take time to decide if you would like to take part and remember that your participation is voluntary.

What is the purpose of the study?

It has been suggested that increased "oxidative stress" is an important cause of the impaired function of blood vessels in chronic kidney disease (CKD), but this has not been studied in detail. In this context, "oxidative stress" means an increased production of highly reactive oxygen molecules that have the potential to impair the function of blood vessels and the flow of blood to the kidneys. In this research project, we are investigating whether infusing the antioxidant vitamin C, which can reduce oxidative stress, improve blood vessel function, and increase blood flow to the kidneys.

Magnetic Resonance Imaging – or MRI – is a type of scan which allows us to get detailed pictures of internal organs. The advantage of this MRI is that it does not involve exposure to harmful radiation, and as far as we know, there are no long term health risks. We have developed a type of MRI scan called Arterial Spin Labelling (or ASL) that looks at blood flow to the kidneys. This is cutting edge technology which isn't carried out in clinical practice at the moment. We hope that in the future it will allow us to look at the blood flow (or "perfusion") to the kidneys in people with kidney disease

or transplants without exposing them to harmful radiation. The purpose of this study is to if the results from the ASL-MRI correlate with markers of kidney function and oxidative stress, and if kidney blood flow is improved by vitamin C.

Why have I been selected to take part?

You have chronic kidney disease and are under follow up at one of the renal clinics.

Do I have to take part?

No – participation is entirely voluntary. Additionally, if you decide to take part but then change your mind you can withdraw at any time.

What will be involved if I decide to take part?

Participation involves one visit to our clinical research facility. After having had time to read this information sheet you will have a chance to ask questions when you meet one of the research team.

Study visit

You will attend the Glasgow Cardiovascular Research Centre next to the Western Infirmary Glasgow having fasted for around 6 hours. After discussing what the study involves, you will be asked to sign a consent form. Your height, weight and blood pressures will be measured and a health questionnaire taken. An infusion of 100ml normal saline will be given and blood samples taken. The ASL-MRI scan will then be carried out. An infusion of vitamin C will then be given and further blood samples taken. A further ASL-MRI scan will then be carried out.

Consent Clinical assessment	Saline infusion	ASL-MRI	Vitamin C infusion	ASL-MRI	Exit from BHF
20 Minutes	20 Minutes	25 Minutes	20 Minutes	25 Minutes	10 Minutes

Blood sample   Blood sample

What are the risks of taking part in the research?

We will take blood and urine samples to ensure that your kidneys are working normally and that there is no sign of any other health problem of which you are unaware. We will take a small amount of blood from the vein in your arm which in some rare cases can result in minor bruising. The amount of blood taken does not pose any risks. A blood pressure cuff can cause discomfort for the very brief period of time it is inflated, and in extremely rare cases cause a bruise.

Are MRI scans dangerous?

MRI scans are not suitable for people with metal objects in their body such as joint replacements, heart valves, surgical clips or metal shrapnel from previous eye injuries. We will explore this possibility during medical history taking and via the MRI safety questionnaire. The scan takes place in a narrow enclosure, and some people who are extremely claustrophobic will find this unpleasant. Lastly, the scan can be quite noisy however headphones are worn for protection and can also be used to play music for your comfort and relaxation. There are no known health risks associated with MRI so long as those individuals who are unsuitable to undergo the investigation are identified appropriately.

Is vitamin C harmful?

Some studies have suggested that administration of vitamin C may cause kidney stones. To minimize any such risk, you should not take part in this study if you previously had a kidney stone or if you are already taking daily vitamin C supplements.

What are the benefits of taking part?

Taking part in this study is of no direct benefit to you. We hope to develop a new type of MRI scan which is currently unavailable which may improve the care of people with kidney problems. Travel costs can be reimbursed however no other financial incentive will be offered.

What happens if we find a previously undiagnosed condition?

If we discover any previously unknown clinical abnormalities during testing then we will notify you and your GP with your consent. Although this may potentially lead to earlier treatment of a previously unknown condition it may also lead to unnecessary tests and cause anxiety. Furthermore any condition that is found can have implications for insurance and mortgage purposes. The type of MRI scan used does not examine other internal organs aside from the kidneys, and the images that we obtain are not examined by an MRI expert, so that this is not an appropriate scan for finding out about other underlying health problems. This means that if you are experiencing symptoms then this scan will not find out what is causing them. Similarly if you should develop symptoms in the future then you should not be reassured by the fact you have had this scan to rule out the existence of any problem – you should attend your GP as you would have done if you hadn't taken part in this study.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation against NHS Greater Glasgow and Clyde, but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and with any personal details so that all analyses will be carried out anonymously. All information collected about you during the course of the research will be kept strictly confidential.

What will happen to samples that I give?

You will donate blood and urine samples for research purposes. Some examinations will be done straight away whilst others will be done at a later stage when we collect more samples from other participants. We will also store some samples for up to 10 years to perform additional tests if required. The samples are treated as a "gift"; this means you will not be entitled to any future financial imbursement related to this study and related research. Further tests on stored samples will again require review and approval by the ethics committee.

What will happen to the images obtained by MRI?

Images taken by the MRI scan will be kept on a computer database and stored anonymously. The images from the scan are only able to be seen from a few desktop computers in the MRI suite of the Clinical Research Centre, which is secured at all times. We will analyse these images in order to determine the circulation to your kidneys, and compare this to measurements of healthy kidney blood flow found by other studies. We will compare the images from each scan to ensure that they are similar, so that we know the scan is reliable.

Contact for further information

Should you have any further questions please feel free to call Dr Patrick Mark at the BHF Glasgow Cardiovascular Research Centre by telephone on 0141-330-8218

Thank you for taking the time to read this Information Sheet

Kid-RASL

Renal Arterial Spin Labelling in Chronic Kidney Disease
Information about the research

Version 2.1 25/04/13

Version for LKD

Invitation

You are being invited to kindly donate your time to participate in a research study. Before you take part it is important for you to understand why the research is being carried out and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear or if you would like more information. Take time to decide if you would like to take part and remember that your participation is voluntary.

What is the purpose of the study?

Magnetic Resonance Imaging – or MRI – is a type of scan which allows us to get detailed pictures of internal organs. The advantage of this MRI is that it does not involve exposure to harmful radiation, and as far as we know, there are no long term health risks. It is often useful to examine the blood supply to internal organs and in these cases we have to give the patient a type of dye called 'contrast' into a vein around the time of the scan. Whilst MRI itself is generally harmless, the contrast given can have complications, and certain types of contrast cannot be given to people with kidney disease.

We have developed a type of MRI scan called Arterial Spin Labelling (or ASL) that looks at blood flow to the kidneys **without** giving potentially harmful contrast and avoids exposure to radiation. This is cutting edge technology which isn't carried out in clinical practice at the moment. We hope that in the future it will allow us to look at the blood flow to the kidneys in people with kidney disease or transplants without exposing them to contrast; this would allow us to improve their treatment.

The purpose of this study is to if the results from the ASL-MRI correlate with markers of kidney function and damage in the blood and urine.

Why have I been selected to take part?

You are healthy and have normal kidney function, and we already have a great deal of information about your kidneys following your kidney donor assessment.

Do I have to take part?

No – participation is entirely voluntary. Additionally, if you decide to take part but then change your mind you can withdraw at any time.

What will be involved if I decide to take part?

Participation involves one visit to our clinical research facility. After having had time to read this information sheet you will have a chance to ask questions when you meet one of the research team.

Study visit

You will attend the Glasgow Cardiovascular Research Centre next to the Western Infirmary Glasgow after having fasted for around 6 hours. After discussing what the study involves, you will be asked to sign a consent form. Your height, weight and blood pressures will be measured and a health questionnaire taken. Samples of blood and urine will be taken. The ASL-MRI scan will then be carried out.

Consent Clinical assessment	ASL-MRI	Exit from BHF
20 Minutes	25 Minutes	10 Minutes

Blood & urine sample

What are the risks of taking part in the research?

We will take blood and urine samples to ensure that your kidneys are working normally and that there is no sign of any other health problem of which you are unaware. We will take a small amount of blood from the vein in your arm which in some rare cases can result in minor bruising. The amount of blood taken does not pose any risks. A blood pressure cuff can cause discomfort for the very brief period of time it is inflated, and in extremely rare cases cause a bruise.

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Thank you for taking the time to read this Information Sheet

WoSRES
West of Scotland Research Ethics Service

AMENDED LETTER – REPLACES
LETTER dated 26 April 2013

West of Scotland REC 5
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow
G11 6NT

Dr Keith Gillis
British Heart Foundation Glasgow
Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA

Date	29 April 2013
Direct line	0141 211 6294
Fax	0141 211 1847
E-mail	Stephanie.keane@ggc.scot.nhs.uk

Dear Dr Gillis

Study title:	**Kid-RASL: Renal Arterial Spin Labelling in Chronic Kidney Disease**
REC reference:	**13/WS/0090**
IRAS project ID:	**120129**

Thank you for your email of 29 April 2013. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 25 April 2013

Documents received

The documents received were as follows:

Document	Version	Date
Participant Consent Form	1-3-0	29 April 2013

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	Version	Date
Advertisement	2-0-0	28 January 2013
Advertisement	2-0-0	28 January 2013
GP/Consultant Information Sheets	1.0	15 March 2013
Investigator CV		16 April 2012

Other: Thank you letter	1-1-0	28 January 2013
Other: Academic Supervisor CV - Dr P Mark		
Other: Academic Supervisor CV - Dr C Delles		
Participant Consent Form	1-3-0	29 April 2013
Participant Information Sheet: LKD	2-1-0	25 April 2013
Participant Information Sheet: RVD	2-1-0	25 April 2013
Participant Information Sheet: CKD	2-1-0	25 April 2013
Protocol	2-1-0	23 January 2013
REC application		03 April 2013

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

13/WS/0090	Please quote this number on all correspondence

Yours sincerely

S. Keane

Stephani Keane
Admin Assistant

Copy to: Dr Maureen Travers, R&D Management Office

NHS
Greater Glasgow
and Clyde

Coordinator: Dr Maureen Travers
Telephone Number: 0141 211 6389
E-Mail: Maureen.travers@ggc.scot.nhs.uk
Website: www.nhsggc.org.uk/r&d

R&D Management Office
Western Infirmary
Tennent Institute
1st Floor 38 Church Street
Glasgow, G11 6NT,

14/05/2013

Dr Keith Gillis
University of Glasgow
BHF Cardiovascular Research Centre
University of Glasgow
Glasgow
G12 8TA

NHS GG&C Board Approval

Dear Dr Keith Gillis

Study Title:	Kid-RASL: renal arterial spin labelling in chronic kidney disease
Principal Investigator:	Dr Keith Gillis
GG&C HB site	Western Infirmary
Sponsor	NHS Greater Glasgow and Clyde
R&D reference:	GN13CA117
REC reference:	13/WS/0090
Protocol no:	2-1-0 (23/01/2013)
(including version and date)	

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Approval** for the above study.

Conditions of Approval

1.   **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004

    a.   During the life span of the study GGHB requires the following information relating to this site

        i.   Notification of any potential serious breaches.

        ii.   Notification of any regulatory inspections.

It is your responsibility to ensure that all staff involved in the study at this site have the appropriate GCP training according to the GGHB GCP policy (www.nhsggc.org.uk/content/default.asp?page=s1411), evidence of such training to be filed in the site file.

2.   **For all studies** the following information is required during their lifespan.

    a.   Recruitment Numbers on a quarterly basis

    b.   Any change of staff named on the original SSI form

NHS
Greater Glasgow
and Clyde

c. Any amendments – Substantial or Non Substantial

d. Notification of Trial/study end including final recruitment figures

e. Final Report & Copies of Publications/Abstracts

Please add this approval to your study file as this letter may be subject to audit and monitoring.

Your personal information will be held on a secure national web-based NHS database.

I wish you every success with this research study

Yours sincerely,

Dr Maureen Travers
Research Co-ordinator

Appendix 4

0.95ml whole blood [on ice] → EPR Team → Rate of superoxide prduction

7ml centrifuge

1ml mixed with 6% metaphosphoric acid for 30s → Freeze at -70oC → Vitamin C
Sample Label : VC

1ml into eppendorf tube → Freeze at -70oC → ADMA
Sample Label : ADMA

1ml into eppendorf tube → Freeze at -70oC → Isoprostanes
Sample Label : IP

0.1ml into eppendorf tube → Freeze at -70oC → Total antioxidant capacity
Sample Label : TAC

**8ml
Green heparin tube**

2.5ml centrifuge

0.5ml into eppendorf tube → Freeze at -70oC → Storage
Sample Label : Plasma storage

0.5ml into eppendorf tube → Freeze at -70oC → Storage
Sample Label : Plasma storage

0.1ml whole blood → Mix with 0.01ml M2VP → Freeze at -70oC → GSSG
Sample label: GSSG

0.05ml whole blood → Freeze at -70oC → GSH
Sample label: GSH

**3ml
Purple EDTA tube**

Storage
Sample Label : Serum storage

3.5ml centrifuge

0.5ml into eppendorf tube → Freeze at -70oC → Storage
Sample Label : Serum storage

0.5ml into eppendorf tube → Freeze at -70oC → Storage
Sample Label : Serum storage

**3.5ml
Yellow SST tube**

Labelling procedure:
• Renox
• Participant ID
• Sample label
• Sample number [1-3]

07/12/2015   Blood & urine sample processing 2-0-0
100512

WIG Routine
Biochem

U&E Bone CRP Bicarb
LFTs
Lipids Glucose Urate
FBC
Urine PCR + Dipstick

X 2
6ml heparin
tube

Centrifuge

6ml plasma

X3 2ml Plasma
Frozen -80oc

X2 5ml
SST tube

Centrifuge

5ml serum

X5 1ml Serum
Frozen -80oc

X1 3.5ml
EDTA tube

X3 1ml Whole Blood
Frozen -80oc

Urine

X2 15ml Urine
Frozen -80oc

Total Tubes:
X 3 Yellow SST tubes
X 2 Green hep tubes
X 2 Purple EDTA tubes
X1 Grey Tube

Total Volume:
35ml venesection

VolRASL Sample Processing 1-1-0 061212

0.95ml whole blood [on ice]

EPR Team

DONT FREEZE!

Rate of superoxide prduction

10ml centrifuge

1ml into eppendorf tube

1ml into eppendorf tube

0.1ml into eppendorf tube

0.5ml into eppendorf tube

0.5ml into eppendorf tube

ADMA
Sample Label : ADMA

Isoprostanes
Sample Label : IP

Total antioxidant capacity
Sample Label : TAC

Storage
Sample Label : Plasma storage

Storage
Sample Label : Plasma storage

0.1ml whole blood

Mix with 0.01ml M2VP

GSSG
Sample label: GSSG

0.05ml whole blood

GSH
Sample label: GSH

3.5ml centrifuge

0.5ml into eppendorf tube

0.5ml into eppendorf tube

Storage
Sample Label : Serum storage

Storage
Sample Label : Serum storage

15ml

Storage
Sample Label : Urine storage

FREEZE

Total Sample Collection:
• 3x 4ml green hep
• 1x 4ml purple EDTA
• 2x 3.5ml yellow SST

Labelling procedure:
• KidRASL
• Participant ID
• Sample label
• Sample number 1-2

07/12/2015

Blood & urine sample processing 1-0-0

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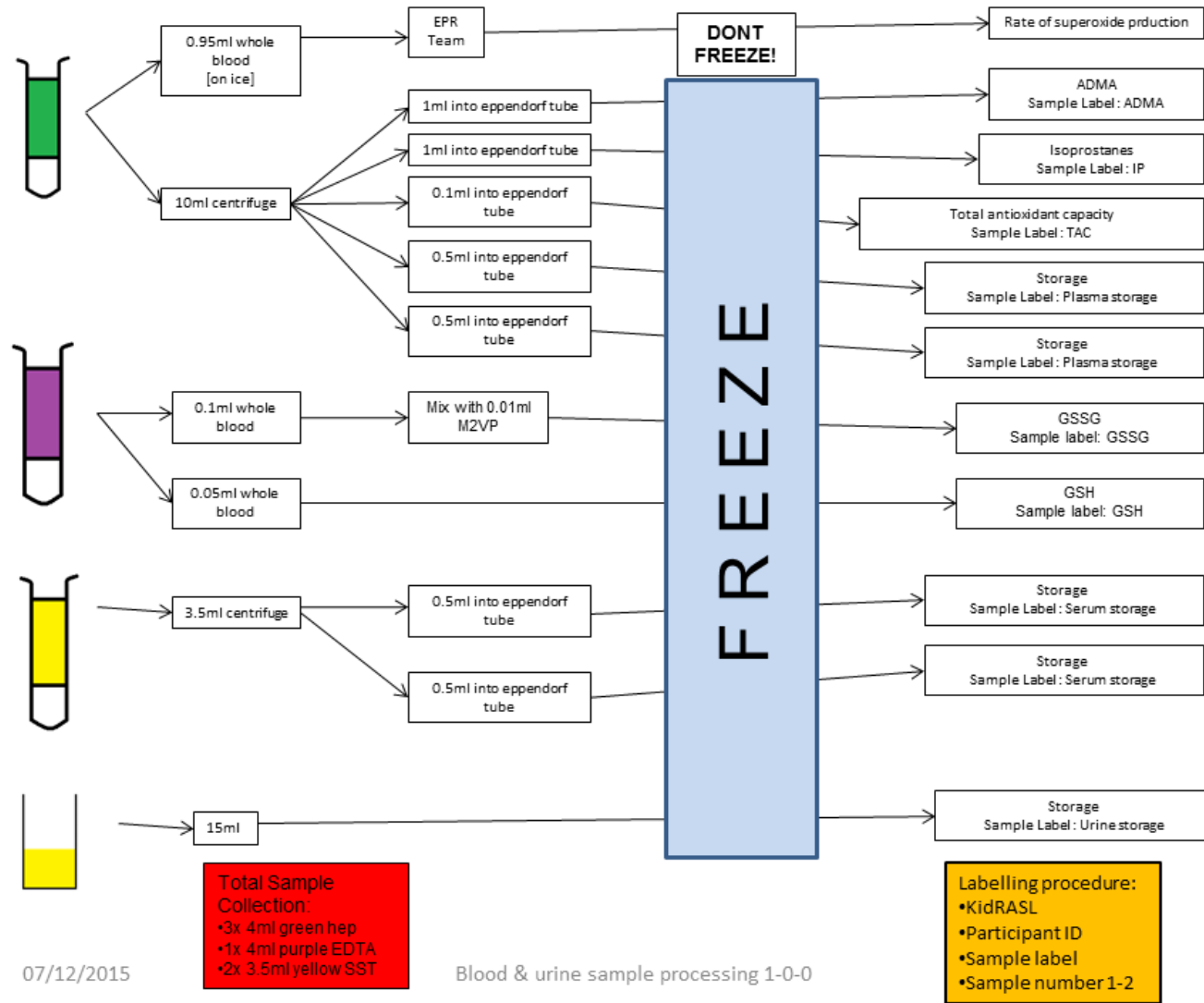ARELLANO-MENDOZA, M. G., VARGAS-ROBLES, H., DEL VALLE-MONDRAGON, L., RIOS, A. & ESCALANTE, B. 2011. Prevention of renal injury and endothelial dysfunction by chronic L-arginine and antioxidant treatment. *Ren Fail,* 33**,** 47-53.

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