Characterisation of Endothelial Function in Conduit and Resistance Arteries in Chronic Renal Failure

Thesis presented for the Degree of PhD

in the Faculty of Medicine of the University of London

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For William
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Publications Arising from this Work


Associated Publications


Contributions Made to this Thesis

Dr Jenny Cross performed the majority of the Flow mediated dilatation studies with the exception of those in which quality control was being assessed or where simultaneous preparation of biochemical studies was required (vitamin C studies). In these cases studies were performed by Mrs Ann Donald. Dr Jenny Cross performed all plethysmography studies. Dr Jenny Cross performed all HPLC analysis for plasma arginine and its analogues. Dr Tony Briddon performed analysis of homocysteine levels. Dr Jenny Cross collected and prepared blood samples for analysis of Oxidant stress which were performed by Dr Sarah Nuttal.
Abstract

Cardiovascular disease remains the leading cause of death in patients with renal failure. In common with other risk factor groups associated with accelerated atherogenesis such as diabetes mellitus and hyperlipidaemia, atherosclerosis is preceded by vascular dysfunction in renal failure. This thesis investigates the mechanisms of vascular dysfunction in renal failure, with particular reference to reduced bioactivity of the nitric oxide (NO) pathway. I have used in vivo techniques to study the activity of the NO pathway in both conduit and resistance arteries. To assess the effect of circulating uraemic toxins on endothelial function, flow-mediated vasodilatation (FMD) of the brachial artery was measured before and after dialysis. I demonstrated that haemodialysis resulted in rapid clearance of uraemic toxins from plasma and transiently increased FMD; in contrast, automated peritoneal dialysis treatment did not reduce levels of uraemic toxins or improve FMD. These data suggest that acute reduction of circulating inhibitors of endothelial function is associated with improved endothelial function in conduit vessels. To explore this further, the role of competitive inhibition of NO synthase by L-arginine analogues was investigated by administering the natural substrate, L-arginine. Despite normalising the ratio of plasma arginine/NO synthase inhibitors, there was no improvement in either conduit or resistance artery endothelial function. These findings imply that in renal failure, impaired endothelium-dependent vasodilatation is not solely due to competitive inhibition of NOS.

I examined whether reactive oxygen species may contribute to impaired endothelial function in uraemia. The acute administration of the anti-oxidant, vitamin C, reduced biochemical markers of oxidant stress and improved endothelial function of resistance, but not conduit arteries.
In conclusion, the mechanisms responsible for endothelial dysfunction in uraemia remain obscure. I have shown, however, that endothelial function can be corrected by both acute haemodialysis and by an acute increase in plasma anti-oxidant capacity. The observed beneficial effect of haemodialysis on vascular function may explain reports of improved cardiovascular outcome in patients who routinely undergo long hour or daily haemodialysis treatments. It remains to be seen whether other interventions, such as statin therapy, which are known to improve endothelial function can confer similar outcome benefit in this high risk patient group.
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Chapter 1

Introduction
1.1 Cardiovascular Disease In Renal Failure

Cardiovascular disease is the major cause of morbidity and mortality in patients with renal failure. It accounts for 50-60% of deaths (1) and 33% of first hospital admissions in haemodialysis patients (2). To put this in perspective, the impact of cardiovascular disease in dialysis patients should be compared with other causes of death in the general population. For example, the 5-year survival of men over the age of 64 commencing dialysis is lower than those with colonic or prostatic cancer; similarly, the 5-year survival rates for women commencing dialysis are lower than for those with breast or colonic cancer (25). This dramatic difference in survival is illustrated in Figure 1.1, which compares survival of dialysis patients with the general population. Though less well-documented, cardiovascular morbidity and mortality in patients with chronic renal failure not yet requiring dialysis (pre-dialysis), also exceeds that experienced by the general population (3). Individuals with renal failure tend to be excluded from large scale epidemiological studies because they exhibit multiple risk factors, thus making the relative contribution of each one difficult to determine. Similarly, this patient group has been deliberately excluded from most important cardiovascular intervention trials, including those from which they would have been most likely to benefit, such as angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, and lipid lowering agents. Ironically, it is in just this population that preventative strategies for cardiovascular disease may have the greatest benefit.
Over the last three decades, reduction in cardiovascular morbidity and mortality in the general population has resulted from improved understanding of vascular biology and specific pharmacological interventions in targeted risk groups. Given the increased cardiovascular risk of the chronic renal failure population, the yields from appropriate intervention are likely to be more dramatic.

1.1.1 Epidemiology of cardiovascular disease in patients with impaired renal function

In a 10-year follow-up study of 147 pre-dialysis patients, Jungers et al. reported a 3-fold increase in the incidence of myocardial infarction compared with the general population (4). The prospective British Regional Heart Study followed 7690 men, and found that the graded relationship between the incidence of cardiovascular events (stroke and
myocardial infarction) and plasma creatinine started at a level as low as 116 μmol/l. This relationship persisted after adjustment for conventional cardiovascular risk factors, and was also independent of blood pressure (5). A similar progressive relationship between renal function and cardiovascular risk was demonstrated in a prospective analysis of mortality related to coronary heart disease in 417 hyperlipidaemic individuals with established coronary vascular disease. Each 9 μmol/l increment in the baseline serum creatinine increased the relative risk of death by 36% and the relative risk of subsequent death due to coronary heart disease by 47% (6). Of 6228 participants screened in the Framingham study, 8% had renal impairment, and 20% of these individuals had cardiovascular disease. During 15 years of follow up, the presence of baseline renal impairment was associated with a 31% increase in the relative risk of death. However, this study provided no clear evidence of an independent relationship between baseline renal impairment and subsequent incident cardiovascular events in either men or women, because there was no association between renal impairment and cardiovascular deaths after correcting for the presence of traditional risk factors (7).

In summary, cardiovascular disease is more common in patients with some degree of renal impairment; several studies support an intriguing independent graded relationship between the degree of renal impairment and cardiovascular risk.

1.1.2 Epidemiology of cardiovascular disease in patients on dialysis

Despite the effectiveness of dialysis in prolonging life in patients with dialysis-dependent renal failure, annual mortality remains high and usually results from cardiovascular disease. This observation has been consistently reported in maintenance haemodialysis patients since Lindner et al. made the initial observation in 1974 (8). The increased prevalence of cardiovascular disease applies to all modalities of renal
replacement therapy (haemodialysis, peritoneal dialysis and renal transplantation) and outcomes have been most comprehensively recorded by two large renal registries, the United States Renal Data System (USRDS) and the equivalent European Registry of patients (EDTA). The USRDS data set is more complete because of the relationship with Medicare, which provides central funding for renal replacement therapy in the USA. USRDS data indicate that overall cardiovascular mortality in dialysis-dependent renal failure is approximately 9% per annum, 30 times that of the general population. Among patients receiving either haemodialysis or peritoneal dialysis, the prevalence of coronary artery disease is approximately 40% (2). Although the increased prevalence of cardiovascular disease in dialysis patients is clear, these data do not reveal whether the increased risk is secondary to clustering of classical cardiovascular risk factors, or is an independent effect of uraemia and its treatment.

In Europe, dialysis patients aged less than 40 years experience a 150-fold increase in the incidence of cardiovascular events and a 10-fold increased incidence of cerebrovascular events compared to age-matched controls (9). There are few prospective data, but Brown et al. (1996) reported a 10-fold increase in the standardised mortality ratio for cardiovascular disease in a group of 305 patients entering a dialysis programme in the north of England, compared to non-uraemic controls. The outlook was much worse for diabetics, who experienced a rate 44 times that of the general population (10). The same pattern exists in an exaggerated form in the elderly dialysis population. The North Thames Dialysis Study examined clinical outcomes in 221 individuals aged over 70 years who started dialysis. The overall 1-year mortality rate was 29% and progressively increased with age and co-morbid conditions. The most powerful independent predictor of survival in this elderly cohort of patients was the presence of peripheral vascular disease, which conferred a relative risk of death of 2.8 (11).
1.1.3 Pathology of cardiovascular disease in renal failure

Although the most frequent underlying cause of cardiovascular morbidity and mortality is atherosclerosis, characterised by the presence of atheromatous plaques in conduit arteries, there is a spectrum of vascular pathology. These include diseases involving small vessels (arteriolosclerosis), left ventricular hypertrophy and valvular heart disease, all of which are more common in individuals with renal disease.

1.1.3.1 Conduit artery disease (coronary, cerebral, and peripheral vascular disease)

Atherosclerosis is a systemic disease, which affects many vascular beds including the coronary circulation. Post mortem studies indicate an increased prevalence of carotid, coronary atheroma and aortic aneurysm in dialysis patients compared with controls (12). Savage et al. reported that 70% of dialysis dependent patients studied had occult carotid atheromatous plaques compared with 20% of non-uraemic controls (13).

1.1.3.1.1. Coronary artery disease

The prevalence of coronary artery disease (based on history and ECG findings) reported by the national registries at the time of commencing RRT varies between 41% in the United States of America, 36% in Australia and New Zealand and 28% in Canada (14-16). These statistics probably underestimate the true prevalence of coronary artery pathology because they rely on symptomatic disease. The prevalence of coronary artery disease in published coronary angiography series, most of which studied prospective renal transplant recipients, often diabetics, ranges from 25-60% (17;18). The prevalence
of coronary artery disease in the dialysis-dependent population is uncertain because much of the published data is derived from younger individuals selected for cardiac catheterisation based on either symptoms or risk factors for coronary artery disease. Koch et al. reported angiographic findings in 105 consecutive diabetic patients commencing dialysis; coronary artery disease was present in 47% of subjects, of whom 36% had haemodynamically significant stenosis exceeding 50%, (comprising single vessel disease in 16%; two vessel disease in 4%; and triple vessel disease in 14%). Importantly, clinical symptoms were present in only a quarter of these patients with documented coronary lesions, making symptoms a poor guide to the presence of angiographically significant disease.

Although less common, acute myocardial infarction affects 10% of dialysis patients with 53% of these events occurring within 2 years of commencing dialysis. Mortality is greatly increased in dialysis patients who have 33-47% in-hospital mortality, with 59% of all patients dying within 12 months and 73% within 24 months (19). These depressing statistics are worse even than those seen in type II diabetics with coronary artery disease.

1.1.3.1.2 Cerebrovascular disease

The incidence of cerebrovascular accidents in patients with dialysis-dependent renal failure between 1996-1998 was 13 events per 1000 patient years of exposure (25) compared with 3 per thousand (NIH public health statistics Feb 6 1998) in the general population. In a study of 1000 American dialysis patients, the prevalence of stroke disease derived from patients' history was 19% (2). Cerebral haemorrhage is more common than cerebral infarction in the dialysis population with an incidence of 8.7 and 3.7 per 1000 patient years respectively in one study of over 1000 dialysis patients (20).
This probably reflects the prevalence of patients with adult polycystic kidney disease on dialysis programmes and the incidence of hypertension, rather than intra-dialytic anticoagulation. Nevertheless, cerebral infarction remains more common than in the general population presumably due to the high incidence of occult carotid intimal lesions, atrial arrhythmias and cardiac valvular disease (13).

1.1.3.1.3 Peripheral vascular disease

Calcified plaque is common in both the carotid and femoral arteries of patients with dialysis-dependent renal failure in comparison with control subjects (13). In the HEMO study looking at a series of 1000 dialysis subjects, peripheral vascular disease (PVD) was present in 23% (2). The importance of this finding is emphasised by data which show that PVD is a very strong predictor of outcome in subjects commencing dialysis (11).

1.1.3.1.4 Atheromatous plaque composition in uraemia

Not only is the distribution of atheromatous plaque wider in patients with renal failure, but also there is evidence to suggest that plaque morphology is different. The most marked differences observed do not relate to the extent of luminal obstruction, but to plaque composition and the presence of medial calcification (13). This distinction is supported by a post mortem case control study of 54 dialysis patients where it was reported that coronary plaques in patients with end-stage renal failure were characterised by increased media thickness and marked calcification (21). Further support for a fundamental difference in plaque structure in renal failure is provided by the absence of a correlation between the degree of coronary artery stenosis and the frequency of angina or symptomatic ischaemic heart disease. This is in striking contrast
to non-uraemic patients in whom symptoms are usually associated with coronary artery stenosis exceeding 50% of luminal diameter (22). Variation in the proportion of the components that make up an atherosclerotic plaque (such as connective tissue, extracellular matrix, cellular components) result in a spectrum of lesions from the large stable plaques with a thick fibrous cap and a low lipid content to “vulnerable”, highly lipid laden and rupture-prone lesions with thin fibrous caps. Although acute coronary syndromes often result from the rupture of a modestly stenotic but vulnerable plaque, the stability of coronary plaques in uraemic patients remains to be determined. Different plaque composition in renal failure may also contribute to the excessively high re-occlusion rate after PTCA (70% at 1 year in uraemic patients compared with 40% in diabetics and 20% in controls) (23). Plaques of uraemic patients were significantly larger (qualitative assessment of plaque area after a graded classification by Stary et al. (24)) than their non-uraemic counterparts (21).

1.1.3.2 Small artery disease

Ischaemic heart disease in the general population is usually the result of critical coronary artery stenosis. In haemodialysis patients however, ischaemic symptoms are caused by non-atherosclerotic disease in approximately 25% of patients (22). There are a number of reasons why individuals with renal failure experience coronary ischaemia in the absence of occlusive epicardial coronary artery disease (see table 1.1). Cardiac ischaemia results when cardiomyocyte oxygen demand outstrips supply and this may be caused by small vessel disease, secondary to hypertension, diabetes and calcium phosphate deposition. In addition reduced arterial compliance is thought to contribute to reduced myocardial oxygen supply by reduced diastolic perfusion.
Table 1.1 Factors contributing to coronary ischaemia in renal failure in the absence of occlusive coronary disease.

<table>
<thead>
<tr>
<th>Increased Myocardial Oxygen Demand</th>
<th>Reduced Myocardial Oxygen Supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Increased myocardial muscle mass</td>
<td>1. Reduced capillary density</td>
</tr>
<tr>
<td>2. Systolic &amp; diastolic wall stress</td>
<td>2. Increased capillary-cardiomyocyte diffusion distance</td>
</tr>
<tr>
<td>3. Abnormal diastolic relaxation</td>
<td>4. Small vessel disease</td>
</tr>
<tr>
<td>5. Abnormal vasomotor tone</td>
<td>6. Anaemia</td>
</tr>
<tr>
<td>7. Reduced conduit artery compliance (Increased pulse wave velocity)</td>
<td></td>
</tr>
</tbody>
</table>

1.1.3.3 Left ventricular morphology and cardiac failure

Among patients receiving either haemodialysis or peritoneal dialysis, the prevalence of left ventricular hypertrophy (LVH) is approximately 75% (25). The prevalence of LVH increases with progressive renal decline. In a study of subjects with chronic renal impairment using 2D echocardiography, 27% of subjects with a creatinine clearance greater than 50 ml/min had LVH, 31% of those with a clearance above 25 ml/min, and 45% of patients with severe renal impairment (creatinine clearance) below 25 ml/min had LVH (15). LV mass is increased in up to 80% of patients commencing dialysis (26). As LVH progresses, diastolic dysfunction develops with reduced ventricular filling, reduced cardiac output and elevated left ventricular end diastolic pressure which leads to reduced diastolic coronary perfusion.

In common with the general population, LVH predicts the development of congestive cardiac failure and death in dialysis patients. Of 432 patients who survived more than 6 months of haemodialysis, left ventricular mass index independently predicted mortality.
Furthermore, outcome was predicted by the pattern of LVH with left ventricular dilatation, concentric LVH and systolic dysfunction being associated with progressively shorter survival (27). In a study of peritoneal dialysis patients, severe LVH was found in a third of patients and was associated with a significantly greater cardiovascular morbidity and mortality than for subjects with mild LVH (28).

1.1.3.4 Valvular heart disease

The prevalence of valvular heart disease in renal failure is high in comparison with the general population. In a study of unselected dialysis patients, 64% of subjects had structural changes of the cardiac valves on echocardiography involving the mitral annulus in 40% and aortic cusp in 55% of patients (29). Functionally significant aortic stenosis was present in 13% of these individuals. The various aetiologies of valvular disease (the most common is Monckeberg’s degenerative disease) can only be distinguished in the early stages in subjects with renal failure because the later calcific changes are ubiquitous. The pathophysiological mechanisms that promote and accelerate valve calcification and stenosis in renal failure are complex and poorly understood, but appear to involve abnormalities of calcium and phosphate metabolism, hyperparathyroidism, increased stress on valves secondary to hypertension and increased cardiac output (anaemia and arteriovenous fistula).

1.1.3.5 Cardiac arrhythmias

The incidence of cardiac arrhythmias is high in patients with renal failure and includes atrial and ventricular tachycardias, in addition to brady-arrhythmias resulting from abnormalities of the conduction pathway. These probably contribute to the increased
incidence of sudden death amongst dialysis patients. The relative risk of developing arrhythmias and heart block increases with the severity of renal impairment (30).

1.1.4 Conclusion

In summary, patients with renal failure have a massively increased risk of cardiovascular disease, much of it related to the premature development of atherosclerosis. The specific factors that may contribute to accelerated atherogenesis in renal failure will be discussed in the following section.

1.2 Determinants Of Atherosclerosis In Chronic Renal Failure

Even after stratification for age, sex, and the presence of diabetes, the mortality rate in the dialysis population is 10-20 times higher than that of the general population (31). Identification of risk factors that contribute to the development of vascular disease is crucial for the development of strategies for prevention. It is likely that the traditional cardiovascular risk factors derived from the general population are also applicable to subjects with renal failure. One explanation for aggressive, accelerated atheroma formation in renal failure may be that the conventional risk factors cluster in this group. In addition, uraemia may itself confer a unique pro-atherogenic environment that accelerates the development of atherosclerosis.
1.2.1 Conventional cardiovascular risk factors

1.2.1.1 Diabetes mellitus

With a population prevalence of 1-2%, diabetes mellitus is the commonest cause of dialysis-dependent renal failure accounting for between 40-60% of new cases in both Europe and the USA. Between 1996 and 1998, the proportion of cases of ESRF in the USA which were due to diabetes increased by 9.9% (25). This dramatic rise in diabetes in the incident dialysis population reflects the increasing prevalence of obesity leading to carbohydrate intolerance and insulin resistance, as well as important demographic trends. In the USA between 1994 and 1998 there was a 4% increase in Black Americans, an 11% increase in Native Americans and an 8.5% increase in Hispanics compared with a 5% increase in Caucasians treated on the ESRF programme. Each of these groups has a greater prevalence of diabetes than their Caucasian counterparts (25). For example, 60% of Hispanics are diabetic on starting dialysis compared with 42% of non-Hispanics.

Diabetes is an independent risk factor for cardiovascular disease and diabetics with renal failure have a particularly high incidence of peripheral vascular, coronary artery, and cerebrovascular disease. In a group of 1000 dialysis patients, diabetes was associated with a 65% increased relative risk of coronary heart disease and a 3.5 fold increase in the prevalence of peripheral vascular disease (2).

1.2.1.2 Hypertension and volume overload in chronic renal failure

Hypertension complicates most causes of progressive renal impairment with the exception of tubulo-interstitial disease. The complex pathogenesis includes salt and water retention and increased vascular resistance. The determinants of increased
peripheral vascular tone include increased activity of constrictor systems (elevated sympathetic activity, increased levels of circulating catecholamines, increased activity of the renin-angiotensin system, increased local production of endothelin and circulating oubain-like factors), and reduced activity of dilator systems (endothelium derived vasodilator substances including nitric oxide, endothelium derived hyperpolarizing factor and prostacyclin (see section 1.3.1).

Between 70% and 80% of patients commencing dialysis are hypertensive and require medication, although blood pressure may be normalised by prolonged dialysis to optimise volume control (32). Blood pressure is an important determinant of survival on haemodialysis; the life expectancy of dialysis patients with mean arterial pressure (MAP) < 99 mmHg exceeds that of patients with MAP > 99 mmHg at 5, 10 and 15 years after commencing dialysis (33). Abnormalities in blood pressure control appear early in renal disease; in a study of patients with biopsy-proven IgA glomerulonephritis, but normal renal function, mean 24 hour, daytime and nocturnal blood pressure recordings were higher than in control patients despite equivalent renal function and casual clinic blood pressure measurement of below 140/90 mmHg (34). The prevalence of nocturnal dip decreases as renal failure develops and an inverse correlation between the percentage decline in nocturnal systolic and diastolic blood pressure and plasma creatinine has been shown in patients with CRF (35). Loss of nocturnal dip is recognised as an adverse outcome indicator in renal failure and this, together with higher mean blood pressures, may contribute to the observed increases in left ventricular mass (LVM) and reduced ventricular diastolic compliance, which appear early in the course of renal disease (36).
1.2.1.3 Physical exercise

Several studies in the general population have demonstrated a link between activity level and mortality (37;38). Patients on dialysis have extremely limited physical fitness and exercise capacity. Poor physical functioning has been linked to both low quality of life and high mortality in this population. The pathogenesis of this debility is multifactorial and includes the anaemia of chronic renal failure, cardiac dysfunction, depression, neuropathy, myopathy and the resulting decreased muscle oxygen consumption (39). Uraemic myopathy is thought to result from elevated levels of calcium, acidosis, low carnitine levels and secondary hyperparathyroidism, in addition to an element of disuse atrophy (40). It is reported that exercise beneficially affects aerobic capacity, and quality of life (41). The contribution of anaemia and the role of limited physical training is supported by studies of the effects of correcting both these factors using erythropoietin therapy and exercise training which resulted in increased exercise capacity, mobility and well being (42).

1.2.1.4 Age

In a retrospective cohort study of the general population, Capewell et al. analysed deaths after ischaemic cardiac events and found that age was the most powerful predictor of outcome, and out-of-hospital cardiac deaths were 3 times more common in those over 85 years old compared with those below 55 years. Developed nations are facing demographic shifts in which the proportion of the population over the age of 65 will double in the next few decades. By the year 2050 the number of Americans age 90 years old and above will have increased 10 fold (43;44). The life expectancy for this age group has increased dramatically in the past 4 decades, such that one quarter of individuals aged 65 years old in 2001 can now expect to survive until they are 90 years
old. This is reflected in the demographics of the dialysis population. The percentage of new patients starting dialysis in England and Wales over the age of 65 years has increased from 11% to 39% in the past decade (45). This equates to an anticipated doubling in the UK dialysis population in the coming decade. It seems likely therefore that we can anticipate an increase in the cardiovascular disease burden in the ageing dialysis population.

1.2.1.5 Smoking

Cigarette smoking has long been known to have adverse effects on health causing ischaemic heart disease, stroke, chronic obstructive lung disease and cancers of the respiratory and upper digestive tract, pancreas, kidney and urinary tract. Despite this, USRDS data indicates that 21% of subjects receiving renal replacement therapy continue to smoke and a similar percentage have been smokers in the past (46). This reflects both socio-economic status of patients and the problems of living with chronic disease. The adverse cardiovascular effects of smoking include increase in mean arterial pressure and heart rate mediated by catecholamines and beta-adrenergic mechanisms. Conversely, there is also evidence that tobacco smoking has important adverse effects on renal outcome in primary hypertension, diabetic nephropathy, primary glomerular diseases, systemic diseases involving the kidney and after renal transplantation (47;48).

1.2.2 Cardiovascular risk factors of particular relevance to chronic renal failure

Many of the conventional cardiovascular risk factors are over represented in subjects with renal failure. In addition however, renal failure is associated with a number of
additional abnormalities that are thought to contribute to the burden of cardiovascular
disease that these individuals experience.

1.2.2.1 Anaemia

Anaemia is associated with tachycardia and increased LV volume and is an independent
predictor of LV mass index (LVMI). In a longitudinal study, LVH and its progression
were independently associated with decreased haemoglobin level (49). Consistent with
this pathogenic role, the partial reversal of anaemia with erythropoietin reduces LV
mass (50) and is associated with improved survival (51). Normalization of haemoglobin
concentration may not, however, be beneficial, with reduced survival reported in a
group of dialysis patients with congestive cardiac failure and ischaemic heart disease
who underwent aggressive treatment with iron and erythropoieten therapy (52).
However, this disappointing outcome may have been due to associated reduction in
haemodialysis efficiency due to rheological consequences of an elevated haematocrit or
the excessive administration of parenteral iron leading to increased oxidative stress (53).

1.2.2.2 Disturbance of calcium and phosphate metabolism

In addition to their familiar actions on bone, kidneys and gut, parathyroid hormone
(PTH) and vitamin D have been shown to influence cardiac and vascular smooth muscle
cell growth in human subjects with normal renal function (54). In renal failure,
secondary hyperparathyroidism and reduced active vitamin D levels are common, with
evidence to suggest that these abnormalities may contribute to the development of
hypertension, abnormal left ventricular morphology, vascular dysfunction and
calcification (55;56). Abnormalities of calcium and phosphate metabolism correlate
with cardiovascular morbidity and mortality. Hyperphosphatemia, which may in part
relate to under-dialysis, predicts reduced survival due to excess cardiovascular mortality in haemodialysis patients (57). Individuals in the highest quintile for plasma phosphate had a 52% greater risk of death from coronary artery disease, 39% greater risk of stroke, 34% greater risk of death from other cardiac causes and a 26% increased risk of sudden death compared to those in the lowest quintile. A growing body of evidence suggests that abnormal regulation of calcium, phosphate, calcium phosphate product, PTH and PTH-like substances promote vascular calcification (58).

1.2.2.2.1 Vascular calcification

Vascular calcification correlates with and predicts mortality from cardiovascular events in the general population (59). The clinical significance of these observations relates to the biophysical alterations that they induce in the vascular tree. Calcification around atherosclerotic lesions increases plaque fragility by making the vessel wall more vulnerable to shear stress and precipitating plaque rupture and thrombosis. This may explain the correlation between coronary calcification and mortality observed in the general population, and it seems likely to be important in individuals with renal impairment. The haemodynamic consequences of more rigid calcified vessels with reduced compliance are increased systolic blood pressure, a fall in diastolic pressure (widened pulse pressure), increased pulse wave velocity, and increased left ventricular after load. These result in LVH and reduced coronary diastolic perfusion (60).

Both ultrasound imaging and pathological series have shown that atherosclerotic plaques are more highly calcified in uraemic patients (13;61;62). The mechanism of accelerated cardiovascular calcification in uraemia remains uncertain. Duration of dialysis, elevated calcium phosphate product and raised PTH levels all predict both vascular and valvular calcification. There is increased local production of a bone matrix
like protein (osteopontin) by osteoblastic cells present in the arterial wall. These osteoblasts may promote vascular repair in the presence of high wall shear stress, but in the presence of high concentrations of parathormone and parathyroid related peptide (PTHrP) they initiate ectopic calcification (63). Expression of PTHrP is widespread in the vasculature and is upregulated by increased wall shear stress associated with hypertension, fluid overload, and upregulation of vasoconstrictor systems, as seen in renal failure.

1.2.2.2 Left ventricular hypertrophy

In a study of patients with this condition, left ventricular diastolic function was found to be abnormal and to correlate closely with PTH levels suggesting that the hormone itself may affect cardiovascular function. PTH and PTHrP have been demonstrated to increase myocardial contractility and are trophic for myocardial, vascular smooth muscle, and interstitial cells. Clinical and experimental data suggest that PTH, calcium, and phosphate participate in the pathogenesis of LVH, systolic dysfunction, diastolic failure and vascular dysfunction in renal failure (54). The hypothesis that PTH plays a causal role in LVH is supported by the observation that many of these effects on cardiac morphology improve after parathyroidectomy (64). Similarly, parathyroidectomy improved endothelium-dependent vasodilatation in patients with severe secondary hyperparathyroidism (65).

PTH/PTHrP have been shown to increase acutely the force and frequency of contraction in isolated beating rat cardiac myocytes. This is mediated by increased intracellular calcium concentrations effected by the classical PTH/PTHrP coupled receptors, which are widely distributed throughout both cardiac and vascular smooth muscle cells (55). Support for the hypothesis that these biochemical observations result in the
morphological changes observed in vivo come from the observation that they can be reproduced experimentally by a calcium ionophore and blocked by verapamil (56). In addition to these direct effects, there is evidence that parathyroid hormone itself may have an inhibitory effect on the transcription of endothelial nitric oxide synthase (e-NOS) which generates the important atheroprotective vasodilator nitric oxide (NO) (see 1.3.1.1 Nitric oxide). Vaziri et al. demonstrated, that the down-regulation of NO synthase observed in the rat 5/6 nephrectomy model could be reversed by parathyroidectomy. This finding suggests that PTH is directly involved in the dysregulation of NO production in renal failure (66). Loss of peripheral vasodilator function results in increased peripheral resistance and cardiac work, which also stimulates LVH.

1.2.2.3 Dyslipidaemia

1.2.2.3.1 Normal lipid metabolism

Lipids are insoluble in the aqueous phase, and are transported in plasma in complexes called lipoproteins containing amphiphilic substances, which permit the transport of a lipid core (see figure 1.2). These lipoprotein particles are spherical with a polar monolayer of lipoproteins and specific apoproteins on the outside. The liver and intestines produce apoproteins, and their function is to stabilise and facilitate recognition and enzymatic degradation of lipoprotein moieties. Very low-density lipoproteins (VLDL) are the major transport mechanism for plasma triglycerides destined for fat storage or oxidation to free fatty acids and glycerol by the endothelial bound lipoprotein lipase (LPL). Circulating cholesterol is mainly transported in the form of low-density lipoproteins (LDL) formed as a by-product of VLDL metabolism via the intermediate form, intermediate density lipoprotein (IDL).
Cholesterol is also transported from the periphery to the liver by high-density lipoprotein (HDL) within which free cholesterol is converted to the esterified form by lecithin-cholesterol-acyl-transferase (LCAT). High concentrations of triglyceride rich lipid sub-fractions, and low levels of cardioprotective HDL occur in both diabetes mellitus and chronic renal failure and are associated with an increased risk of cardiovascular disease in the general population (67).
1.2.2.3.2 The dyslipidaemia of renal failure

Dyslipidaemia in renal failure is characterised by an accumulation of incompletely metabolised triglyceride-rich VLDL and IDL remnants. This results from reduced tissue lipase activity, which leads to hypertriglyceridemia and reduced concentrations of cardioprotective HDL (for review, see (68)). Early in the course of chronic renal failure, triglyceride levels rise without change in total cholesterol levels, although HDL concentrations are reduced. These abnormalities progress with renal impairment (69). Very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL) all have moderately elevated levels of triglyceride. Before and after the initiation of dialysis, cholesterol concentrations (a negative acute phase reactant) remain similar to those seen in the general population and may even be lower. This observation probably reflects chronic malnutrition, which is closely associated with the acute phase inflammatory response which in turn relates to mortality in renal failure (see section 1.2.2.7). Relative normal or low cholesterol concentrations conceals a highly abnormal lipid sub-fraction profile with an accumulation of pro-atherogenic small dense LDL particles, which are more susceptible to oxidation and have a higher cholesterol content (70). Rather than increased production, the abnormal triglyceride/cholesterol ratio is due to reduced activity of the enzymes, which control lipoprotein degradation. Lipoprotein lipase and hepatic triglyceride lipase activity are the two enzymes responsible for binding to circulating VLDL and subsequently degrading it to LDL via IDL. Lipoprotein lipase (LPL) and hepatic triglyceride lipase are bound to capillary endothelium in the systemic and hepatic circulation respectively. Inhibition of LPL activity by cytokines and parathyroid hormone impedes conversion of VLDL to LDL, and may result in remnant accumulation and hypertriglyceridemia. LPL hydrolyses circulating triglyceride into free fatty acid only while it is bound to the luminal surface of endothelial cells via the high affinity attachments to heparan sulfate
proteoglycans forming binding lipolysis sites. Heparin competes with heparan sulphate binding sites for attachment to LPL leaving the heparin/LPL complex free, but inactive, in the circulation. Detachment from endothelial cells and inactivation of this enzyme system by heparin has two potentially opposing effects. On the one hand it prolongs the life of circulating lipoprotein moieties and may contribute to the accumulation of triglyceride-rich VLDL, LDL and IDL in the circulation of haemodialysis patients routinely anticoagulated during treatment. On the other hand, it prevents these complexes from binding to the endothelial cell surface and thereby reduces the delivery of lipoproteins to underlying macrophages which are thought to contribute to foam cell formation and atherogenesis.

1.2.2.3.3 Oxidised lipids

Oxidative stress has been implicated in the atherogenesis of renal failure (see 1.4.3) and this may be due to the oxidation of VLDL and LDL. Oxidized low-density lipoprotein (Ox-LDL) has recently received much attention because of its potential cytotoxic and pro-atherogenic properties. LDL particles can penetrate the arterial intima easily and are prone to further oxidation possibly because they contain less antioxidant protection. Experimental studies in animals and humans provide strong evidence that atherosclerotic lesions contain oxidatively modified LDL (71). Patients on dialysis have increased concentrations of the oxidation products of LDL and VLDL and elevated titers of autoantibodies against oxidised lipid fractions (72). Ox-LDL inhibits endothelium dependent vasodilatation and may be implicated early in the genesis of atheroma (73). Oxidised LDL has also been demonstrated to induce apoptosis of human endothelial cells (74). They are taken up by macrophage scavengers and contribute to
accelerated foam cell generation, a histological feature which precedes the development of atheroma.

1.2.2.3.4 Lipoprotein (a)

Lipoprotein (a) [Lp(a)], a large apoprotein associated with LDL particles, has emerged as an important predictor of cardiovascular morbidity in the general population (75). Levels of Lp(a), particularly the high molecular weight isoforms, are known to be especially pro-atherogenic and are elevated in early renal impairment, in patients with proteinuria and in patients with dialysis dependent renal failure, particularly those receiving peritoneal dialysis (76). In contrast to the hypertriglyceridemia of renal failure, this probably results from over-production rather than failure of clearance. Lp(a) accumulates in the vascular wall at sites of atheroma formation and is prone to oxidation (77).

1.2.2.4 Homocysteine

Based on recent retrospective and prospective studies, it is now widely accepted that increased total plasma homocysteine is a significant risk factor for cardiovascular disease. Impaired enzyme function resulting from genetic mutation of the cystathione β-synthase or deficiency of the essential B vitamins (B₆ and B₁₂) and folic acid can lead to hyperhomocysteinemia. Although there is uncertainty whether increased homocysteine is causal or merely a proxy for cardiovascular disease, several lines of evidence suggest that it may play a role in atherothrombotic disease. Plasma homocysteine levels are elevated in renal failure, correlate closely with plasma creatinine and GFR, and are inversely related to whole-blood folate concentrations (78). Hyperhomocysteinaemia has been identified as an independent risk factor for cardiovascular disease in dialysis-dependent subjects (79). Folic acid is effective in lowering homocysteine levels in
various groups including renal failure, but it remains to be seen whether this will reduce cardiovascular morbidity and mortality.

1.2.2.4.1 Normal homocysteine metabolism

Interest in the role that this sulphur-containing amino acid plays in cardiovascular disease stemmed from the observation made in 1969 by Dr. Kilmer McCully(46). He noted that patients suffering from the rare genetic disorder of hyperhomocystinuria, which most commonly results from a deficiency of the enzyme cystathione β-synthase (see figure 1.3), experience accelerated atherogenesis with severe widespread vascular disease. Homocysteine levels in these individuals are markedly elevated (> 50 μmol/l, normal range 5-15 μmol/l).

![Diagram of Normal homocysteine metabolism](image)

**Figure 1.3 Normal homocysteine metabolism**
Homocysteine is an intermediary amino acid in the methionine to cysteine metabolic pathway (see figure 1.3). Methionine is metabolised to homocysteine, which is then irreversibly catabolized to cysteine, in the vitamin B₆ (pyridoxal phosphate) dependent trans-sulfuration pathway, through the actions of β-cystathione synthase and cystathione lyase. A large proportion of homocysteine is, however, re-methylated to methionine through the cobalamin (vitamin B₁₂) and methyl-tetrahydrofolate dependent pathway, catalysed by the enzyme methionine synthase. Any reduction in the activity of either pathway, through defects or deficiencies in the critical enzymes or cofactors, results in the accumulation of homocysteine in plasma. The average plasma level of homocysteine is approximately 10 µmol/l with a 95th centile of 15 µmol/l in the general population. The concentration rises with age, and it tends to be greater in men than women. In the general population, mild to moderate elevation of homocysteine (15 to 30 µmol/l) is common and may result from inherited enzyme variation or relative deficiency of the cofactors essential for normal metabolism of homocysteine.

1.2.2.4.2 Homocysteine and cardiovascular disease: epidemiology

There is clear evidence from both retrospective and prospective studies of cardiovascular disease groups that hyperhomocysteinaemia is associated with atherosclerosis. A meta-analysis based on 27 studies including 4000 patients suggested that homocysteine is an independent, graded risk factor for coronary, cerebral and peripheral vascular disease (80). The Canadian Nutritional Survey looked at 5000 individuals with 15 years follow up and concluded that low serum folate levels correlated with the risk of fatal cardiovascular events (81). In a study of angiographically-proven coronary vascular disease, although homocysteine levels did not predict extent of vascular disease, there was a strong, graded relationship between
plasma homocysteine levels and subsequent all-cause mortality in 587 individuals (82). These retrospective studies could also be consistent with the conclusion that cardiovascular disease is associated with increased plasma homocysteine levels, rather than the consequence. However, longitudinal data may support a pathogenic role for homocysteine. Stampfer et al., in a case control study, demonstrated that homocysteine levels were higher at baseline in individuals who went on to develop vascular events and the relative risk of a cardiovascular event was 3 times greater in the highest quartile compared with the lowest (83). In a similar study, Perry et al. demonstrated a 2.8 fold increase in risk of stroke in individuals with a homocysteine level in the upper quartile (84).

1.2.2.4.3 Metabolism of homocysteine in renal failure

Plasma homocysteine concentrations are elevated in renal failure, correlate closely with plasma creatinine and GFR, and are inversely related to whole blood folate concentrations (78). In healthy individuals, however, urinary excretion of homocysteine is low (approximately 0.25 μmol/hr). Since 30% of plasma homocysteine is not protein-bound and is freely filtered by the glomerulus, this suggests that 99% of filtered homocysteine is subject to tubular reabsorption and enzymatic metabolism by tubular epithelial cells. In chronic renal failure, not only is urinary clearance reduced, but also more importantly tubular metabolism is reduced and consequently plasma levels rise. Plasma concentrations of the water-soluble co-factors of homocysteine metabolism (folate, vitamin B₁₂ and vitamin B₆) correlate inversely with plasma homocysteine in renal failure and are themselves reduced by dialysis. Hyperhomocysteinaemia is associated with vascular disease in pre-dialysis renal failure (85) and dialysis-dependent renal failure (79), though not renal transplant recipients (86)
1.2.2.4.4 Mechanism of action of homocysteine in cardiovascular disease

The mechanism of homocysteine-induced vasculopathy is not fully understood. Homocysteine is a thiol, which is metabolised by auto-oxidation with the production of reactive oxygen species (hydrogen peroxide and superoxide anion radicals). Free radicals derived from homocysteine metabolism might oxidise lipid moieties (particularly lipoprotein (a)) and cause endothelial damage. In support of this hypothesis, two groups have demonstrated that experimental hyperhomocysteinaemia results in acute endothelial dysfunction which can be abolished by pre-treatment with an antioxidant (87;88). In addition, homocysteine increases thrombin activation of protein C and activates procoagulant co-factors such as factor V to promote thrombosis (89;90). Finally, homocysteine induces the expression and secretion of chemokines including monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) in vascular endothelial cells (91). Increased production of these chemokines by endothelial cells attracts monocytes and neutrophils to sites of vascular injury, where they may take up residence in the intimal space and could contribute to atheroma formation.

1.2.2.5 Oxidant stress

Any imbalance between the production of free radicals and anti-oxidant defences leads to an excess of oxidative free radicals and tissue damage. This may result from a deficiency of anti-oxidants (such as glutathione, ascorbate or alpha-tocopherol), a reduction in antioxidant enzyme activity together with an increase in the formation of reactive oxygen species (ROS). ROS have been implicated in the pathogenesis of atherosclerosis in humans.
Observational data in humans have suggested that antioxidant vitamin intake is associated with reduced cardiovascular disease. Animal studies are largely consistent with the concept that dietary supplementation with antioxidant vitamins reduces the progression of atherosclerosis. However, recent prospective, controlled clinical trials of vitamin E, including the Cardiovascular Disease, Hypertension and Hyperlipidemia, Adult-Onset Diabetes, Obesity, and Stroke (CHAOS) study (92), the Heart Outcomes Prevention Evaluation (HOPE) trial (93), Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial (94), the Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE) trial (95), and the Heart Protection Study (HPS) (96) present a confused picture. The CHAOS study was a secondary prevention trial of 1035 patients demonstrating that vitamin E supplementation reduced the rate of re-infarction. Similarly, the GISSI trial of 11,304 patients after myocardial infarction demonstrated a reduced death rate and re-infarction rate in the polyunsaturated fatty acid limb but no change in the vitamin E arm of the study. The HOPE study examined subjects with cardiovascular risk factors treated with vitamin E and demonstrated no effect on cardiovascular outcomes and the antioxidant limb of the Heart Protection Study failed to show any improvement in the 5 year mortality or cardiovascular outcome in the antioxidant group. It has been suggested that the reason for these discrepancies relate, in part, to the failure to target subjects at high risk of oxidant stress, who are most likely to gain benefit, such as diabetics, smokers and patients with renal failure (92;97). In patients with renal failure, there is biochemical evidence of increased oxidative stress in those receiving haemodialysis, peritoneal dialysis and transplantation (98). Sources of increased oxidative free radicals in renal failure include recurrent leucocyte activation and superoxide generation during haemodialysis; free radical liberation during the metabolism of homocysteine; renal anaemia, is itself associated with increased markers...
of oxidative stress (99); and intravenous iron and recombinant human erythropoietin therapy are associated with enhanced oxidative stress. In addition, many of the antioxidant defence systems are deficient in renal disease. The activities of the enzymes superoxide dismutase, catalase and glutathione peroxidase are reduced (100) and the redox state of the main intracellular antioxidant glutathione is disturbed in renal failure in favour of elevated concentrations of the oxidised form glutathione disulphide (GSSG) (101). In addition, plasma levels of water and lipid soluble antioxidant vitamins are reduced as a result of low dietary intake and increased plasma clearance during both haemo- and peritoneal dialysis (102). Thus, imbalance between antioxidant production and defence in chronic renal failure may result in an accumulation of free radical species with the capacity for direct vascular damage.

1.2.2.6 Advanced glycation end products

Advanced glycation end products (AGEs) are a heterogeneous group of molecules that accumulate in plasma and tissues with advancing age, diabetes and renal failure. AGEs are formed by irreversible non-enzymatic glycation and oxidation reactions (Browning reactions) involving carbohydrates and lipids (103). These substances accelerate atherosclerosis through cross-linking of proteins, modification of matrix components, platelet aggregation, defective vascular relaxation, and abnormal lipoprotein metabolism (for a recent review, see (104)). AGE-modification of apolipoprotein B results in interference with its normal attachment to the LDL receptor, thereby delaying LDL clearance and prolonging its circulation (105). There is emerging evidence that AGEs may have a role in the pathogenesis of vascular complications associated with renal failure. AGE excretion is inversely related to creatinine clearance and both haemodialysis and peritoneal dialysis are relatively ineffective at clearing these large molecular weight substances. Toxicity of circulating AGE-modified peptides is
mediated by a specific endothelial membrane receptor protein (RAGE), which is induced by uraemia. RAGE activation promotes neutrophil chemotaxis, angiogenesis, oxidative stress, cell proliferation and apoptosis (106) and probably contributes to atherogenesis in uraemia.

1.2.2.7 Atherosclerosis: a chronic inflammatory condition

C-reactive protein (CRP), an acute-phase protein, is a predictor of cardiovascular mortality in the general population and in patients with chronic renal failure. The process of atheroma formation from the initial development of foam-laden macrophages through to fatty streaks and culminating in the advanced lesions of atherosclerotic plaque is tightly linked with an inflammatory response. There is increasing evidence that atherosclerosis is a chronic inflammatory disorder with activation of the acute phase response, cytokines, clotting pathways, complement, leucocytes, endothelial cells and platelets. Infections provide an inflammatory stimulus and several epidemiological studies have shown a relationship between infection and risk of subsequent cardiovascular events. For example, high CRP levels (the prototypical acute phase protein produced in response to infection) are related to future cardiovascular event rate in both stable and unstable angina (107). Data from the prospective Physicians' Health Study show that healthy men at low risk of cardiovascular disease, but with C reactive protein levels in the upper quartile, have a threefold increased risk of acute cardiac events and twofold increased risk of stroke compared with those with a CRP in the lower quartile, CRP was demonstrated to be a better predictor of cardiovascular risk than cholesterol (108). In a population based cross-sectional study of 303 men, there was a strong association between CRP and a number of other inflammatory indices and the presence of cardiovascular disease (109). The mechanisms that underlie these
observations are incompletely understood, and it is not clear whether the link between inflammatory markers and the atherosclerotic process is causal or secondary. Evidence that HMG-co reductase inhibitors (statins) and aspirin reduce the acute inflammatory response suggests that inflammation may contribute to atherogenesis and plaque instability (108;110)

1.2.2.7.1 Inflammation and outcome in chronic renal failure

Several observations in the last decade have demonstrated that inflammatory markers including CRP are elevated in one third of dialysis dependent patients without any obvious evidence of ongoing infection or inflammatory source (111). Haemodialysis generates an acute transient cytokine response, beginning at 60 minutes and becoming maximal after 240 minutes. This reflects bio-incompatibility of membranes and contact activation of immune cells within the extra-corporeal circuit, impurities and endotoxin in the dialysate water, and indwelling dialysis catheters or vascular grafts in some patients. The extent of activation is dependent on the dialyser material used and is considered an index of biocompatibility of the treatment. Cytokines, such as interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and IL-6, may induce an inflammatory state and are believed to play a significant role in dialysis-related morbidity; for a review see (112). Increases in CRP are not detectable until 24 hours after haemodialysis (113;114). In a recent study of dialysis patients without overt infection, CRP levels were elevated in almost 50% of haemodialysis patients and 30% of those receiving peritoneal dialysis (115). Zimmerman et al. (116) confirmed elevation of both CRP and serum amyloid A protein (SAA) in haemodialysis patients and went on to demonstrate a correlation between these markers and other atherogenic risk factors such as elevated LP(a), fibrinogen and low levels of HDL-cholesterol.
Importantly, they also showed CRP to be an independent risk factor for all cause and cardiovascular mortality in this group.

Recent studies in pre-dialysis patients have demonstrated elevated CRP (117;118), TNF-α and IL-1 (119). These data provide evidence that renal failure is an inflammatory condition even in the absence of renal replacement therapy.

The aggressive atherosclerosis observed in advanced CRF may therefore be caused by a synergism of different mechanisms, which includes dyslipidaemia, inflammation, and oxidative stress. Each factor interacts with the others and this interaction may potentiate the effect of individual risk factors. The next section describes the role that the vascular endothelium may play in the pathogenesis of atherosclerosis in general, and more specifically in chronic renal failure.

1.3 The Vascular Endothelium

In the past two decades, it has become clear that the vascular endothelium, the single cell layer lining all blood vessels, plays a central role in the control of vascular physiology. The endothelium separates the vessel wall from circulating components of blood and it plays a pivotal role in the defence against atherogenesis.

1.3.1 Physiology of the endothelium

The endothelium regulates vascular tone, cell adhesiveness, coagulation, inflammation, permeability, and vascular smooth muscle cell proliferation (for a review see (120)). It responds to a number of stimuli including neurotransmitters released from autonomic
and sensory nerves (acetylcholine, norepinephrine, ATP substance P), circulating
hormones (catecholamines, vasopressin angiotensin II, insulin) and locally active
autocoids produced both by endothelial and smooth muscle cells (bradykinin, adenine
nucleotides and nucleosides, angiotensin and endothelin). In addition, the endothelium
responds to physical stimuli, in particular changes in shear stress, which elicits flow-
mediated vasodilatation (FMD). In response to these various stimuli, endothelial cells
produces a variety of vasoactive substances including nitric oxide (NO), arachidonic
acid derivatives, angiotensin II, endothelins, and endothelium derived hyperpolarising
factor (EDHF) (121). The specific contributions made by each of these mediators to the
endothelium-dependent regulation of vascular tone are both species and tissue
dependent (122).

1.3.1.1 Nitric oxide

Nitric oxide (NO) is synthesised from the terminal guanidino nitrogen atom(s) of the
amino acid L-arginine in a reaction catalysed by three isoforms of the enzyme nitric
oxide synthase (NOS): endothelial, inducible and neuronal NOS (see figure 1.4). In the
vasculature, under physiological conditions, the principal source of NO is the
constitutively expressed endothelial isoform (123). The enzyme is located within
caveolae in the plasma membrane and bound to caveolin. Increased intracellular
calcium promotes calmodulin formation, which displaces the caveolin to activate the
enzyme. Stimuli that increase intracellular calcium levels include receptor-mediated
agonists, such as acetylcholine, bradykinin, substance P and the physical stimulus of
endothelial cell shear stress. NO is a highly diffusible signalling molecule that freely
crosses cell membranes to combine directly with target proteins and exert its biological
effect both within the lumen and on the vessel wall. It diffuses to adjacent smooth
muscle where it activates guanylate cyclase and increases production of cyclic 3',5' guanosine monophosphate (cGMP). cGMP in turn reduces intra-cellular calcium within smooth muscle cells, and results in smooth muscle relaxation. In humans *in vivo* the basal release of NO by endothelial cells contributes to blood pressure control (124).

In addition to its effect on vascular dilatation, NO exhibits many of the anti-atherogenic characteristics of the endothelium. *In vitro*, the proliferation of vascular smooth muscle cells and lymphocytes in culture is inhibited by the addition of exogenous NO donors as well as endogenous NO (125-127). Platelet adhesion and aggregation as well as monocyte adhesion and chemotaxis are reduced by NO (128). These observations suggest that NO plays an important role in suppressing some of the key processes involved in atheroma formation and infer that any reduction in the production or bioactivity of NO will promote atherogenesis.

\[
\text{Acetylcholine} \quad \text{Bradykinnin} \quad \text{Substance P} \quad \text{Shear stress}
\]

\[
\text{O}_2 \quad \text{H}_2 \quad \text{OONO}^{-}
\]

\[
\text{L-arginine} \quad \text{NOS} \quad \text{BH} \quad \text{BH}_2 \quad \text{NADPH} \quad \text{NADP}^{+}
\]

\[
\text{C-GMP} \quad \text{Cyclic guanosine monophosphate} \\
\text{NO} \quad \text{Nitric oxide} \\
\text{BH}_{(x)} \quad \text{Biopterin} \\
\text{NOS} \quad \text{Nitric oxide synthase}
\]

Figure 1.4 A schematic representation of the nitric oxide pathway
1.3.1.2 Arachidonic acid metabolites

Endothelial cyclooxygenase-1 (COX-1) synthesises prostanoids from arachidonic acid, including the vasodilators prostacyclin (PGI$_2$) and prostaglandin E$_2$ (PGE$_2$). PGI$_2$ exerts its effect via a receptor mediated cyclic-AMP dependent mechanism. Inhibitors of cyclo-oxygenase reduce the production of this family of prostanoids and exacerbate hypertension in rats and humans (129;130).

![Figure 1.5 A schematic representation of arachidonic acid metabolism](image-url)
In man, recent publications have implicated a role for dilator prostanoids in the physiological regulation of human vascular tone, and the involvement of constrictor prostanoids in diseases such as hypertension (131;132) and atherosclerosis (133). Vasoconstrictor prostanoids such as prostaglandin H$_2$ and thromboxane A$_2$ act on endoperoxide and thromboxane receptors expressed on smooth muscle cells to induce vasoconstriction. In addition to this vasoconstrictor action, thromboxane has platelet-aggregating effects, activates adhesion molecule expression on monocytes and is a mitogen for smooth muscle cells. In animal models of atherosclerosis, thromboxane A$_2$ levels are increased and administration of selective inhibitors retard the development of atherosclerosis (134).

1.3.1.3 Endothelium derived hyperpolarising factor

The existence of another vasodilator factor is suggested by the observation that endothelium-dependent hyperpolarisation of vascular smooth muscle is resistant to the combined effect of nitric oxide synthase and cyclo-oxygenase inhibition in both animals and humans (135). A component of the vasodilator response has therefore been ascribed to endothelium-derived hyperpolarizing factor (EDHF), the identity of which remains controversial. EDHF seems to act by opening K+ channels in vascular smooth muscle. The release of EDHF from the endothelium can be mediated by activation of pertussis toxin-sensitive (alpha 2-adrenoceptor activation, serotonin, aggregating platelets, leukotrienes) and insensitive (adenosine diphosphate, bradykinin) G proteins; for a review, see (136). In both animals and humans, the importance of EDHF as a vasodilator increases as vessel calibre decreases (137), which suggests that it may play an important role in the regulation of local blood flow in the resistance vasculature. The
relevance of EDHF to the control of human vascular tone has been difficult to assess, however, in the absence of a selective inhibitor.

1.3.1.4 Endothelins

The endothelins are a family of proteins consisting of four closely related peptides (ET-1, ET-2, ET-3, ET-4), which are converted by ET-converting enzymes from “big endothelin”. They are produced by endothelial, smooth muscle, neuronal, renal, and some inflammatory cells. The most important of these isopeptides in the cardiovascular system is ET-1. Its major vascular effects are potent vasoconstriction, cellular proliferation, but it also influences myocardial contractility, and renal sodium excretion through activation of specific ET<sub>A</sub> receptors. ET-1 contributes to maintenance of basal vascular tone and blood pressure in humans (137). ET-1 (via ET<sub>B</sub> receptors) also tonically stimulates the production of vasodilator agents such as NO, PGI<sub>2</sub> and EDHF, which offset its vasoconstrictor effect. Thus, infusion of a selective ET<sub>A</sub> receptor antagonist results in vasodilation, whereas infusion of an ET<sub>B</sub> receptor antagonist results in vasoconstriction (138). ET-1 has a number of other pro-atherogenic actions acting as a chemo-attractant for monocytes and macrophages and a mitogen for smooth muscle cells (139;140).

Endothelins are thought to play an important pathogenic role in hypertension, congestive cardiac failure, and atherosclerosis. Patients with hypertension exhibit an exaggerated vasodilator response to ET-receptor blockade (132), suggesting that hypertension may, in part, be driven by elevated levels or greater sensitivity to endogenous ET. ET-1 levels are also elevated in congestive cardiac failure and correlate with clinical state (141) presumably due to their effects on systemic and pulmonary vascular resistance, myocardial ischaemia and renal sodium retention. Consistent with
this, selective ET\textsubscript{A} receptor antagonists have been studied in patients with heart failure where they reduce peripheral vascular resistance and increase cardiac output (142). Endothelins may contribute to the process of plaque generation by promotion of vasoconstriction, smooth muscle proliferation, neutrophil adhesion and platelet aggregation. Selective blockade of ET\textsubscript{1} receptors reduces development of atherosclerosis in animal models, and in humans, tissue ET-1 levels correlate with the severity of coronary artery disease and increase as the clinical presentation becomes unstable (143;144). These data provide indirect evidence of a role for endothelin in atherosclerosis.

Normally the production of vasodilator and vasoconstrictor substances is balanced to regulate the resistance of the vascular bed and maintain appropriate tissue perfusion. In health, the balance of production favours vasodilators. In most risk factor groups for vascular disease, this balance is disturbed in favour of vasoconstriction. This may reflect reduced production of vasodilators, increased production of vasoconstrictors, or an alteration in the sensitivity of the target organ. Given the anti-atherogenic actions of many vasodilators, a switch from the normally predominant release of relaxing factors to contracting factors is likely to play an important role in the development of atherosclerosis.

In the next section, the evidence for endothelial dysfunction in the pathogenesis of atherosclerosis will be reviewed.

1.3.2 Endothelial dysfunction and atherosclerosis

Given that endothelial function plays a central role in vascular homeostasis, it follows that endothelial dysfunction may contribute to disease states characterized by
vasoconstriction, vasospasm, thrombosis, and smooth muscle vascular proliferation such as atherosclerosis. Endothelial function is abnormal in animal models of both cardiovascular risk factors and of established atherosclerosis (73;145;146). Moreover, pharmacologically-induced endothelial dysfunction (using inhibitors of NO synthesis) induces atherosclerosis in experimental animals. (147;148)

Endothelial dysfunction was first demonstrated in patients by Ludmer et al. in 1986, when they showed that intra-coronary infusion of acetylcholine (ACh) caused coronary vasoconstriction in patients with ischaemic heart disease (149). This response represents ACh-induced smooth muscle contraction unopposed by ACh-induced endothelium-dependent vasodilatation in the diseased coronary artery. Since then, endothelial dysfunction has been described in many risk factor groups (including advanced age, smoking, diabetes mellitus, family history of vascular disease, hypertension and hypercholesterolaemia), in the absence of anatomically obvious disease (150-153). This data suggests that impaired endothelial function may anticipate structural disease.

1.3.2.1 Hypertension

In human hypertension, endothelial dysfunction has been documented in peripheral conduit arteries (154), coronary arteries (155) and forearm resistance arteries (156); (157). Whilst several small studies have demonstrated reduced responsiveness of the forearm to muscarinic agonists (158;159), the largest single study in hypertensive patients failed to demonstrate a reduced response to acetylcholine (160). NO synthesis has been measured in one study of hypertensives using \(^{15}\)N-arginine, and indicates that NO synthesis is reduced (161). Other studies have implicated increased constrictor prostanoids in the mechanism of endothelial dysfunction in hypertension (131), and reduced EDHF-activity (162). Whether endothelial dysfunction is a consequence rather
than a cause of hypertension remains unclear; several studies have reported that endothelial function is improved by anti-hypertensive therapy (163-167), whereas others have found no change (168-171).

1.3.2.2 Hyperlipidaemia

Hypercholesterolaemic patients have impaired conduit and resistance artery dilator responses (153;172). L-arginine administration, the substrate for NOS, partially restores endothelium-dependent dilatation in both the conduit (brachial and endocardial) and resistance vasculature of hypercholesterolaemics (173;174). This finding is consistent with relative substrate deficiency which results in reduced NO bioactivity due to either reduced NO production or accelerated NO metabolism. Other studies have reported that endothelial function is also restored by supplemental tetrahydrobiopterin (175) which is consistent with a relative deficiency of this co-factor for NO synthesis (176).

1.3.2.3 Diabetes mellitus

Clinical studies looking at endothelial dysfunction in patients with type I and type II diabetes are conflicting. Some authors have demonstrated impaired endothelial function in conduit (brachial and epicardial) and resistance vasculature (177-179) but others have failed to confirm these findings (180-183). These negative studies excluded microalbuminuric subjects and dilator dysfunction was consistently observed when this subgroup was included. The largest single study of Type I diabetes demonstrated impaired response to endothelium-dependent and independent dilators, which is consistent with impaired smooth muscle function (184).
In type II diabetes (in common with renal failure) studies are often confounded by the presence of hypertension and dyslipidaemia both of which may independently affect endothelial function so the specific contribution of diabetes is difficult to evaluate. This subject is extensively reviewed in reference (146).

1.3.2.4 Oxidative stress

Oxidative stress might cause endothelial dysfunction through reduction in the activity of endothelium-derived mediators (including NO, Fig 1.4). Consistent with this mechanism, anti-oxidant therapy improves endothelial dilator function in patients at risk of atherosclerosis. Acute interventional studies in a wide variety of cardiovascular risk groups indicate that anti-oxidant vitamins (including vitamin C) improve endothelial function in both conduit and resistance arteries. Investigators have demonstrated improved conduit artery endothelial function after the administration of antioxidants in subjects with cardiac failure (185;186), established coronary artery disease (187;188), hypertension (189-191) and hyperhomocysteinaemia (87;192). Similarly, there is an increased dilator response in forearm resistance vascular beds after administration of antioxidants in subjects with hypertension (191;193;194), hypercholesterolaemia (195) and diabetes (196).

Despite these findings, the results of long-term clinical trials with anti-oxidants in these groups have produced inconsistent evidence of benefit (see section 1.2.2.5)(92). In renal failure, antioxidant treatment (including administration of vitamin C) reduced mortality (95), and improved endothelial function in renal transplant recipients (197).

1.3.2.5 Endothelial dysfunction predicts cardiovascular outcome

In a study of 157 patients with mild coronary vascular disease (proven angiographically) followed for a mean of 28 months, coronary artery endothelial function was a good
predictor of the risk of a major cardiac event (defined as death, myocardial infarction, coronary artery bypass surgery, or angioplasty) (198). Similarly, in a study with median follow up of 92 months, coronary endothelial function predicted cardiovascular event rate, independent of other risk factors and the degree of coronary atherosclerosis (199). One study of endothelial function in the forearm of hypertensive individuals demonstrated a similar relationship (200). These studies support the intriguing idea that the health of the endothelium is a better predictor of the risk of developing an acute coronary syndrome than the burden of atherosclerosis, and suggests the possibility that transition to an acute syndrome is not dependent upon the anatomy, but upon the overall function of the endothelium.

In summary, there is evidence in humans that endothelial dysfunction is associated with the major cardiovascular risk factors for atherosclerosis. The mechanism of endothelial dysfunction remains poorly understood, with evidence for reduced NO activity in many of these groups. In the next section, the evidence for endothelial dysfunction in patients with renal failure will be discussed.

1.3.3 Endothelial function in renal failure

In common with many other risk factors groups for atherosclerosis, renal failure is associated with endothelial dysfunction both in animal models and in humans in both conduit and resistance vasculature. The mechanisms that underlie these observations remain unclear.
1.3.3.1 Animal studies of endothelial function in renal failure

In the rat model of acute ischaemic renal failure, Kakoki et al. found reduced acetylcholine-induced vasodilatation compared with sham operated animals (201). Similarly, Ruschitzka et al. demonstrated that endothelium-dependent, but not-independent, vasodilatation of aorta and renal artery was impaired 24 hours after renal injury (202).

In experimental chronic renal failure, Falloon et al. demonstrated endothelial dysfunction in the mesenteric vessels using flow myography (145). Using a similar experimental model, Thurasingham et al. were, however, unable to demonstrate any abnormality in agonist-induced endothelium-dependent dilatation in spontaneously hypertensive and non-hypertensive rats with renal failure compared with controls (203). Rats with renal mass reduction (caused by ligation of one renal artery) show reduced urinary excretion of NO metabolites and a reduction in NOS expression in the remnant kidney, which implies reduced systemic and renal NO production. In animals subjected to surgical resection rather than arterial ligation, which results in only mild hypertension rather than the severe hypertension seen in the ligation model, there was a profound reduction in both renal and systemic NO production (66). However, neither model developed significant uraemia. These findings contrast with the finding of increased histochemical staining for e-NOS in aortic walls in a similar experimental model (204). Thus the experimental data from animal models of renal failure provides inconsistent evidence of endothelial dysfunction.
1.3.3.2 Biochemical assessment of endothelial function in human renal failure

Several studies of chronic renal failure in vivo suggest a chronic state of endothelial activation and endothelial injury. Plasma levels of substances secreted by healthy endothelial cells, including endothelium-derived vWF, tPA, urokinase-type plasminogen activator, and soluble thrombomodulin, were elevated in subjects with renal failure when compared with age- and sex-matched controls (205). In common with the physiological measures, abnormalities of biochemical markers are present long before the onset of renal replacement therapy (206). These results were confirmed in renal failure patients by Haaber et al. who observed that in addition to elevated vWF, the presence of another cardiovascular risk factor (smoking) had a multiplicative effect on plasma levels of circulating markers of endothelial injury (207).

There is controversy about the effect of renal failure on the systemic production of NO metabolites. Some reports suggest that production is reduced (208-211), while others report that it is increased (84;212-220). Many of the studies that reported increased NO production in renal failure used platelet and mononuclear cells that had been stimulated by contact with bio-incompatible membranes often during dialysis sessions with no restriction on dietary nitrate intake. Schmidt et al. controlled for these variables and demonstrated a reduction in whole body nitrite/nitrate excretion over 24 hours. These data are in agreement with isotopic plasma enrichment studies of $^{15}$N arginine conversion to $^{15}$N citrulline, which reflects the composite result of the activities of the NO isoforms. Thus although NO production does appear to be reduced in renal failure, this does not exclude the possibility that normal or even excessive amounts are produced under the influence of stimuli such as inflammation, sepsis and bioincompatible treatment modalities (for a review see (221)). Overall, these results suggest that in humans, whole body NO production is reduced in uraemia.
1.3.3.3 Assessment of resistance artery endothelial function in humans with chronic renal failure

Pannier et al., however, examined post ischaemic flow as a measure of vascular function using unilateral venous occlusion plethysmography in 60 subjects with end stage renal failure. These authors found that subjects with renal failure had an impaired dilator response to ischaemia, compared with matched controls. (222). Morris et al. demonstrated impaired vasodilatation to the endothelium-dependent dilator carbachol, but preserved dilatation to the endothelium-independent NO donor, sodium nitroprusside in a study of patients with pre-dialysis renal failure (223). Passeur et al. demonstrated that even in the absence of hypertension, endothelium-dependent dilatation to acetylcholine was reduced in subjects with dialysis-dependent renal failure (224). Finally, Annuk et al. examined 56 patients with a mean creatinine clearance of 30 mls/min using bilateral venous occlusion plethysmography and found that endothelium-dependent dilatation was impaired in renal failure and that the magnitude of this impairment was related to the degree of renal failure (225). Similarly, in skin microvasculature, the dilator response to acetylcholine is impaired in patients with renal failure (226).

1.3.3.4 Assessment of conduit artery endothelial function in humans with chronic renal failure

Much of the work assessing the bioactivity of the nitric oxide pathway in renal failure is based on the dilator response of conduit vessels to a high flow stimulus using external high-resolution ultrasound scanning techniques (flow mediated dilatation, FMD). Kari et al. examined the influence of uraemia alone on conduit artery endothelial function, deliberately selecting subjects who were not complicated by confounding factors such
as hypertension, concurrent vasoactive medication and dyslipidaemia, and compared the arterial dilator response to matched controls. They found that endothelium-dependent dilatation was impaired in subjects with renal failure (227). Similarly, in an adult population with less severe renal impairment, creatinine > 130 μmol/l, Thambyrajah et al. were able to demonstrate impaired flow-mediated dilatation. This effect was independent of conventional cardiovascular risk factors and an association was observed between FMD and plasma levels of von Willebrand factor at this relatively early stage of renal failure (228). Impaired endothelium-dependent dilatation has also been demonstrated in patients receiving haemodialysis, peritoneal dialysis and transplant recipients (229-233). Of interest, haemodialysis patients have more profoundly impaired brachial artery FMD when compared with subjects receiving peritoneal dialysis (3.5 ±1.2 and 3.7±1.1 (234;230) versus 5.7±1% (231)).

Therefore, in conduit and resistance arteries, subjects with varying degrees of renal failure, receiving different forms of renal replacement therapy demonstrate impaired endothelium-dependent dilatation. The degree of endothelial impairment varies with different modalities of renal replacement therapy.

1.4 Mechanisms of Reduced Nitric Oxide Bioavailability in Renal Failure

Impaired endothelium-dependent dilatation (which is in part NO-mediated) together with the evidence of reduced NOx production, suggests that the NO pathway is impaired in chronic renal failure. Substrate availability may limit NO production either, because plasma levels are reduced or local concentrations at the site of NOS activity are reduced. Competitive inhibition of NOS by circulating arginine analogues, which
accumulate in chronic renal failure, may reduce NO production. A number of co-factors (including tetrahydrobiopterin (BH4)) are necessary to catalyse the production of NO and any deficiency may reduce NO production (see figure 1.4). NO may be produced in normal quantities but prematurely inactivated in the presence of reactive oxygen species known to be present in high concentration in subjects with renal failure. Alterations in the level of expression of the e-NOS gene product may result in changes in NO bioavailability (235). It is also possible that, in addition to a reduction in the bioactivity of NO, effector responsiveness may be attenuated. Passauer et al. demonstrated that the vasodilator response to GTN was impaired in subjects with dialysis dependent renal failure (224). Though the number of subjects examined in this study were small, given the observed changes in vascular morphology (chronic vessel dilatation, medial calcium deposition) (236) and reduction in distensibility (237), the capacity to vasodilate may be impaired either because of the physical insensitivity of the smooth muscle to NO or because of the loss of vascular compliance in renal failure.

1.4.1 Circulating inhibitors of NO synthesis

Analogues of L-arginine that are chemically modified at the terminal guanidino nitrogen group (see figure 1.6), such as N⁰-monomethyl-L-arginine (L-NMMA), compete with L-arginine as substrate for NOS to inhibit NO production. These analogues can be used to probe the physiological and pathophysiological role of this chemical pathway in vitro and in vivo. Several methylated L-arginine analogues, including L-NMMA, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) exist endogenously. These are formed by N-methyltransferases, a family of enzymes that methylate L-arginine residues of proteins which are subsequently released following proteolytic cleavage.
ADMA levels are elevated, in renal failure and in a number of other diseases, which include hypertension, hypercholesterolaemia, heart failure (238;239). Evidence is accumulating that ADMA contributes to endothelial dysfunction in these conditions. Although the mechanism responsible for ADMA accumulation in renal failure is understood, it is less clear for these other diseases.

1.4.1.1 Accumulation of methylarginine analogues in renal failure

ADMA is a small water-soluble molecule that diffuses freely and is normally filtered and excreted by the healthy kidney. In humans, the kidney is an important route of excretion and this explains the substantially elevated plasma concentrations found in renal failure. The plasma concentration of both ADMA and SDMA in healthy controls is around 0.4 μM, which compares with a 3 fold increase in ADMA and a 10 fold increase in SDMA in dialysis-dependent patients (240).
In addition to urinary excretion, plasma methylarginine analogue concentrations depend on enzymatic degradation by a pair of enzymes dimethylaminohydrolase type I and II (DDAH I, II). The activity of this enzyme system may become more relevant in conditions where the substrate concentration is elevated such as renal failure. Recent evidence suggests that elevated ADMA levels can result from down regulation of DDAH which leads to reduced enzymatic degradation of L-arginine adducts (241;242). Because ADMA is small, water soluble and freely filtered, it is removed from plasma along with other uraemic toxins during the process of dialysis (240;243). Dialysis therefore represents a mechanism by which levels can be acutely manipulated in vivo.

1.4.1.2 Evidence that methyl arginine analogues cause vascular dysfunction

Several in vitro and in vivo studies support the hypothesis that methylarginine analogues play a pathophysiological role in hypertension and deranged vascular responsiveness in renal failure. Data from experimental studies suggest that ADMA concentrations in the same range as those found in renal failure (1 to 5 μmol/l), significantly inhibit vascular NO formation by NOS in cultured endothelial cells, cultured macrophages and in isolated human blood vessels (243;244). Similarly, plasma from subjects with renal failure inhibits NOS activity in cultured human endothelial cells in vitro (245). Further evidence of an important role for these substances in determining vascular tone is suggested by the observation that plasma taken after a haemodialysis session no longer displayed the inhibitory effect on NOS activity of plasma taken before dialysis (246). Similarly, increased dialytic clearance of methylarginine analogues using high flux dialysis techniques is associated with improved inter-dialytic blood pressure control, which may be due to, increased clearance of L-arginine analogues (247). In a cross sectional study, Valconen et al have shown a relationship between plasma ADMA levels
and cardiovascular events (248). Similarly in renal failure Zoccali et al demonstrated that in renal failure, plasma ADMA levels were a strong independent predictor of both cardiovascular outcome and all cause mortality(249). The same group performed a prospective study examining the relationship between plasma ADMA, C-reactive protein, and carotid intima-media thickness (IMT) in 90 dialysis patients. They demonstrated that plasma ADMA levels are related to carotid IMT and that ADMA and CRP interact and together predict the progression of carotid intimal lesions (250). Finally, defective venodilatation in patients with renal failure can be corrected by both haemodialysis which clears the inhibitors) and by L-arginine supplementation (251), which overcomes competitive inhibition.

1.4.1.3 Aim of this work

In chapter 3, the possible role of ADMA as a modulator of endothelial dysfunction in humans will be investigated by assessing the effect of dialysis treatment on ADMA concentrations and endothelial function.

1.4.2 L-arginine availability

In healthy subjects, the concentration of L-arginine is not rate limiting for NOS activity. The half-saturating ($K_m$) concentrations of each of the three NOS isoforms is in the region of 1-10 μmol and physiological plasma arginine concentrations are greatly in excess of this (0.1-0.8 mmol). e-NOS therefore should be fully saturated at physiological concentrations and the delivery of ‘excess’ L-arginine should theoretically not further increase NO production. Consistent with this, L-arginine supplementation in healthy volunteers has no effect on vascular tone (252). In some diseases, however, L-arginine supplementation in patients with normal plasma L-arginine concentrations has
been shown to significantly improve endothelium-dependent dilatation. This effect has been reported in hypercholesterolaemic subjects in conduit (253), forearm resistance (174) and the coronary arterial bed (173). L-arginine has also been demonstrated to improve endothelial function in smokers (254). These studies illustrate that despite the apparently saturating concentrations of arginine for NOS, 2-4 fold increases in plasma arginine concentration will increase NO production. The mechanism for this paradox is unknown but the chemical similarity between L-arginine and its methyl analogues (see figure 1.6) has prompted interest in possible competitive inhibition of NOS by L-arginine adducts. L-arginine analogues may occupy the active site to displace or exclude the physiological substrate and thereby reduce NO production and diminish endothelium-dependent vasodilation. In renal failure, absolute plasma arginine levels in chronic renal failure are normal (4-10 mmol/l) (220;255). The ratio of L-arginine to its endogenous competitive inhibitors is, however, reduced in renal failure, and this mechanism may contribute to reduced NO production.

Extracellular concentrations of L-arginine and its analogues may not reflect intracellular concentrations. The transport of L-arginine through the plasma membrane is mediated by Y+ cationic amino acid transporters (CAT), a family of transport proteins. Based on immunofluorescence staining of endothelial cells, CAT-1, the transporter responsible for arginine influx into vascular endothelial cells, co-localises with e-NOS and caveolin in specialised micro-compartments called caveolae. This suggests that CAT-1 might preferentially supply substrate directly to e-NOS in this sequestered compartment containing L-arginine and ADMA at concentrations potentially independent of both the plasma concentration and the overall intracellular concentration (256). Therefore it is possible that the activity of e-NOS in endothelial cells will be dictated by the relative concentration of arginine and arginine analogues achieved in the caveolae of the cell where NOS, DDAH and the Y+ CAT proteins exist together. Little is known about the
intracellular or microcompartmental concentrations of these substances so this hypothesis remains untested.

1.4.2.1 Aim of this work

In chapter 4, the effects of acute elevation of plasma L-arginine concentrations on endothelial function will be assessed in patients with chronic renal failure.

1.4.3 Oxidative stress and endothelial dysfunction in renal failure

NO bioactivity is determined by factors that influence not only NO production but also its breakdown (257). Reactive oxygen species (ROS), such as superoxide ($O_2^-$) hydroxyl (OH), hydrogen peroxide ($H_2O_2$), and indeed NO itself are continually formed in health in vivo by activated macrophages, endothelial cells and smooth muscle cells. Under physiological conditions in healthy subjects, there is a balance between the production of reactive oxygen species and the activity of the antioxidant enzyme systems (superoxide dismutase, catalase and glutathione peroxidase). In pathological conditions, there is an imbalance between the formation and consumption of free radicals which may contribute to the premature inactivation of NO and result in damage to cellular constituents, characteristic of increased oxidative stress. Both NO and superoxide are free radicals and they can react together to produce another free radical, peroxynitrite (ONO$\mathrm{O}^-$). Enzyme kinetics determine the biochemical destiny of NO in pathophysiological situations. The rate of oxidation of $O_2^-$ by superoxide dismutase is three times slower than the rate of its oxidation to peroxynitrite (ONOO$^-$). Thus within the vasculature, in the presence of excess $O_2^-$ there may be a preferential use of NO to form ONOO$^-$. Whether NO has its biological effect (for example, vasodilatation) before
being inactivated or not depends upon the relative concentrations of free radicals and NO in specific compartments within the cell. Consistent with this hypothesis, experimental evidence suggests that arterial segments from hypercholesterolaemic animals produce excess $O_2^-$ before the development of macroscopic atherosclerosis (258). The effect of excessive free radicals production in *vitro* and in *vivo* can be attenuated by supplying free radical scavengers such as ascorbic acid or vitamin E. The interaction between NO and oxygen free radicals may contribute to the observed loss of endothelium-dependent relaxation in hypercholesterolaemia, congestive heart failure, diabetes, hyperhomocysteinaemia, hypertension and smoking which is reversed in the presence of exogenous antioxidants (see Table 5.1).

1.4.3.1 Aim of this work

In chapter 5 the effect of antioxidant therapy on endothelial function will be investigated in patients with chronic renal failure.

1.5 Summary

The endothelium regulates many aspects of vascular function and abnormalities may predispose to the generation of atherosclerosis. It is the source of a wide range of regulatory molecules, which, in health, function in concert to provide a carefully balanced anti-atherogenic environment. Endothelial dysfunction is detectable long before the onset of anatomically identifiable disease and appears to be useful in the prediction of morbidity and mortality in certain cardiovascular risk groups. One of the most intensively studied and important endothelium-derived mediators is NO whose
production is reduced in CRF. This thesis will explore possible mechanisms of impaired endothelial function present in renal failure by probing various aspects of the NO pathway. A clearer understanding of the pathogenesis of endothelial dysfunction in CRF has potential clinical implications. It may provide avenues for therapeutic interventions before the onset of clinically obvious cardiovascular disease in this high-risk patient group.

1.6 Hypotheses to be Tested

In this thesis, I will examine the role of substrate availability, circulating competitive inhibitors of NOS, and the part played by oxidative free radicals in the reduced bioavailability of NO observed in patients with chronic renal failure. The following hypotheses will be tested:

1. Dialysable small molecules cause reversible endothelial dysfunction in patients with renal failure.

2. Competitive inhibition of NOS is an important contributor to defective endothelium-dependent vasodilatation in chronic renal failure.

3. High levels of oxidative stress reduce NO bioactivity in renal failure.
Chapter 2

Methods
2.1 Assessment Of Endothelial Function In Humans In Vivo

Several techniques may be used to assess arterial endothelial function in vivo ranging from the biochemical to the physiological. No single measure describes, however, all aspects of its varied function. Furthermore, endothelial function is not uniform between different vascular beds or at different sites in the same vascular bed. Endothelial function in veins may differ from conduit arteries, resistance arteries may respond differently to larger conduit arteries and the endothelium at arterial branch points exposed to turbulent flow may behave differently from that in areas exposed only to laminar flow. The role of the endothelium in the regulation of vascular tone has become one of the most comprehensively studied areas of vascular biology. A variety of biochemical assays (von Willebrand factor vWF, platelet activating factor inhibitor type 1 (PAI-1), tissue plasminogen activator (tPA) and thrombomodulin) and physiological assays (flow mediated dilatation (FMD), receptor mediated vasodilatation) have been used to assess endothelial function. The correlation between these biochemical and physiological measures of endothelial dysfunction tends to be weak in the clinical setting (116). In part, this reflects the lack of tissue and cell specificity of these biochemical substances.

The limitations in interpreting the significance of soluble endothelial products means that physiological measures of endothelial function remain central to the assessment of the endothelium. Such techniques rely on the measurement of blood vessel tone or diameter in response to endothelium-dependent stimuli that cause vasodilatation or vasoconstriction. In the studies described in this thesis, I have chosen to examine dilatation in the resistance arteries in the muscular bed of the human forearm in response to infused pharmacological agents (venous occlusion plethysmography) and dilatation of conduit arteries in response to a high flow stimulus (flow mediated
dilatation, FMD). These techniques were chosen because they are reproducible, reliable, and relatively non-invasive. In addition, their mechanisms have been well characterised and correspond largely with the activity of the NO pathway (259;260). Moreover, forearm conduit artery endothelial function has been shown to correlate well with endothelial function in the coronary vasculature (261). Throughout these studies, I have performed parallel assessment of intrinsic smooth muscle responsiveness. The smooth muscle response is the final common pathway both for the dilator signal derived from the endothelium and for exogenous endothelium independent vasodilators.

2.1.1 Flow Mediated Dilatation (FMD)

Studies performed in the 1980s indicated that large arteries (conduit vessels) responded to alterations in blood flow by increasing vessel diameter (262), and that this phenomenon was endothelium-dependent (263). Early techniques were aimed at the assessment of coronary artery endothelial function, and were necessarily invasive and difficult to repeat over time. In 1989, a non-invasive ultrasound technique was developed to assess endothelial function in peripheral conduit vessels (262), which has subsequently been shown to correlate well with invasive testing of endothelial function in the coronary circulation (261) and more importantly, with cardiovascular outcome (264). This technique provides a dynamic assessment of conduit artery endothelial function that is both accurate and reproducible (265). FMD of the brachial artery has been used to study endothelial dilator function in a variety of cardiovascular risk groups and has been used in large clinical studies (151). The dilatation of human conduit arteries in response to increased flow has been shown to be mediated in part by endogenous NO, and can be diminished by the inhibition of NO synthesis (259;260). In these studies, I have used FMD to assess endothelial function of the conduit vessels.
(brachial and radial) in the human forearm in subjects with dialysis-dependent and pre-dialysis renal failure and normal controls.

FMD relies on the observation that blood vessels dilate in response to high blood flow. Increased conduit artery blood flow is generated after a period of distal cuff occlusion, which induces reactive hyperaemia on cuff release. The increase in arterial diameter in response to this high flow stimulus is measured, and the magnitude of this dilatation is taken as a measure of the activity of the NO pathway. High-resolution vascular ultrasound is used to image the vessel in longitudinal section. The operating parameters of the ultrasound machine are adjusted to maximise the differentiation between the arterial lumen and wall. Once a stable clear image is obtained (figure 2.1), the transducer position is fixed over the artery using a stereotactic clamp and micrometer adjustment screws (figure 2.2), which permit adjustment in the coronal and sagittal plane to maintain a constant, focused image of the vessel being studied.

**Figure 2.1** Stable image of a longitudinal section of the brachial artery demonstrating digital callipers on the left.
2.1.1.1 Measurement of arterial diameter

Several methods are available to measure arterial diameter, and these have been developed since the first description of the technique in 1989 (151). In the original description of the technique, the image of the vessel was recorded in M-mode. Arterial diameter was measured using manual on-screen callipers, which were placed at the M-lines of the anterior and posterior walls, at a single point in the artery. This method is extremely labour intensive. In order to reduce analysis time, researchers have identified specific time points of interest (baseline, peak dilatation and recovery of baseline) to analyse rather than analysing each frame of a 9-minute study. To overcome the
limitations of this method, quantitative ultrasound techniques, using B-mode images, have been developed. Ultrasound acquired end-diastolic images of the vessel are subject to 8 bit analogue to digital conversion. Proprietary commercial digital edge detection software (Information Integrity, Boston, USA) is used to calculate the internal diameter of the artery over a 2-3 cm length. Images are acquired at 3-second intervals and the internal diameter of the vessel measured for each image (figure 2.3). In addition, this allows measurement of a longer segment of the artery than previous methods. Secondly, this method allows continuous assessment of vessel diameter throughout an experimental protocol, which results in a more comprehensive description of the changes in vessel diameter that occur over time in response to a flow stimulus.

**Figure 2.3 Region of interest box during automatic diameter measure**

**2.1.1.2 Data analysis and presentation of arterial diameter**

Comparison of the arterial dilator response in a single subject measured on multiple occasions, or between groups of subjects is possible if there are no major differences in basal blood flow, or arterial diameter. Data can be expressed as absolute dilatation, or as percentage change from baseline. It is possible to plot the whole time course of the
dilatation together with return to baseline and then calculate both the peak dilatation and the area under the curve for the whole dilatation profile, Figure 2.4.

![Figure 2.4 Area under the curve (AUC) of the arterial diameter / time profile during flow mediated dilatation in human conduit arteries.]

2.1.1.3 Inducing high blood flow in a conduit artery

In these studies, high blood flow was generated by inducing reactive hyperaemia. A blood pressure cuff, distal to the site of arterial diameter recording (Figure 2.5), is inflated to supra-systolic pressure for five minutes, the optimal occlusion period to achieve maximal dilation (266). At cuff release there is a rapid, transient increase in blood flow which lasts approximately 90 seconds (illustrated in figure 2.6).
Figure 2.5 Distal blood pressure cuff placement to induce reactive hyperaemia. Figure 2.5a demonstrates a brachial artery study and figure 2.5b which includes an intra arterial intervention in which FMD of the radial artery is examined.

Figure 2.6 Blood flow /time profile during reactive hyperaemia

Increased blood velocity results in increased wall shear stress, which stimulates the endothelium to release vasodilators (predominantly nitric oxide). This causes relaxation of the vascular smooth muscle and results in a measurable dilatation of the vessel approximating to 3-8% of the baseline diameter measure. In the studies described in this

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thesis, the occluding cuff was placed *distal* to the segment of artery being studied. When analysing the effect of intrabrachial infusions on FMD the radial artery was studied and hence the occluding cuff was placed at the wrist instead of just below the elbow. Other investigators have placed the cuff *proximal* to the study segment, which results in a greater dilator response, but this dilatation is thought to result from a combination of increased flow and ischaemia of the conduit vessel. The latter is a complex signal which is mediated by the release of local factors in addition to NO (267).

### 2.1.1.4 Measurement of blood flow

Blood flow velocity was measured using pulsed wave Doppler. The Doppler signal is the velocity-time profile for a single cardiac cycle and is displayed as a spectral Doppler curve. The area under the curve of the velocity-time profile is the velocity-time integral (VTI), and approximates to the average distance, measured in metres, travelled by a pulse of blood during one cardiac cycle, typically 0.01-0.05 m.

![Figure 2.7 Calculating the velocity time integral](image)

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VTI = Velocity (m/s) x time (seconds)

\[ \text{VTI} = \left[ \frac{\text{Distance (m)}}{\text{time (seconds)}} \right] \times \text{time (seconds)} \]

\[ \text{VTI} = \text{Distance (m)} \]

Volume flow is that volume of blood that passes a point in a specified period. Assuming that we can approximate a section of artery to a cylinder, we can calculate the blood flow volume as follows:

Volume / pulse = artery cross-sectional area x average distance travelled by pulse

\[ \text{Volume / pulse} = \pi r(t)^2 \int v(t) \, dt, \]

where \( r(t) = \text{the measured instantaneous vessel radius}, \)

and \( v(t) = \text{the instantaneous blood velocity}. \)

The Doppler signal is measured at an angle of approximately 70° to the axis of the blood vessel, giving \( \int v(t) \, dt = \cos 70° \times \text{measured VTI}, \) and thus:

Volume / pulse = \( \cos 70° \cdot \pi r(t)^2 \cdot \text{VTI} \)

Volume per minute is calculated by multiplying this value by heart rate (HR).

Volume / min = HR \( \cos 70° \cdot \pi r(t)^2 \cdot \text{VTI} \).

VTI is proportional to volume flow since heart rate, the Doppler angle of incidence and arterial diameter remain essentially constant during the period of study. Measurement of the radius for the majority of the period of interest (up to 90s after cuff release) shows
that $\Delta r$ is much smaller than baseline radius. Figure 2.8 below shows both VTI and the arterial dilator response (equal to $\Delta r/r$) as a function of time.

![Diagram showing VTI and Dilatation as a function of time](image)

*Figure 2.8 VTI & Dilatation as a function of time*

The most significant error in the assumption that the volume flow per minute is proportional to VTI is due to variation in the radius. To calculate this error, we expand the square of the radius at time $t$, $r(t)^2$, as follows:

\[
r(t) = r(t=0) + \Delta r \\
\]

\[
r^2(t) = (r(t=0) + \Delta r)^2 \\
\]

\[
r^2(t) = r^2 (t=0) + 2r(t=0)\Delta r + \Delta r^2 \\
= r^2 (t=0)[1+2\Delta r/ r(t=0) + 2\Delta r^2/ r^2 (t=0)]
\]

Now as $\Delta r << r(t=0)$, we can ignore the last term, and the error in volume is:

\[
Volume/min = HR \cos 70^\circ \pi r(t=0)^2. VTI [1 + 2\Delta r/ r(t=0)]
\]
So fractional error is typically \((2\Delta r / r(t=0))\), and thus varies as dilatation. From figure 2.8, the maximum error occurs at \(t=60s\) and is typically \(\sim 6\%\). For the majority of the period of interest, however, this is negligible.

2.1.1.5 Data analysis and presentation of arterial flow

Blood flow is the stimulus to endothelium-dependent arterial dilatation and can be expressed in several ways: absolute volume flow, ratio of peak to baseline absolute volume flow, percentage change in volume flow between baseline and peak, or as area under the curve (AUC) for the whole flow profile as it returns to baseline values over a period of 90 seconds. These parameters can similarly be expressed for the VTI. In these studies, VTI is used as a measure of volume flow and expressed as both peak VTI and AUC of VTI for the 90-second flow envelope.

2.1.1.6 Assessing intrinsic smooth muscle function; endothelium independent dilatation

To assess intrinsic smooth muscle reactivity (endothelium independent dilatation), glyceryl-trinitrate (GTN) is given sublingually and arterial dilatation is recorded. Another NO donor that may be used is sodium nitroprusside (SNP). Both GTN and SNP supply NO directly to vascular smooth muscle and cause relaxation, which is independent of the endothelial cell layer. Early in the development of this technique conventional anti-anginal doses of GTN (400 µg) were given. This had two major disadvantages, firstly administration was commonly associated with headache, which though short lived and self-limiting, is undesirable. Secondly, the dilator response of the conduit vessel to large doses of GTN was far in excess of that following reactive hyperaemia. This meant that it was possible to miss subtle differences in intrinsic
smooth muscle function because of the excess of NO applied to the system. Leeson et al. examined the relationship between sub-lingual isosorbide-dinitrate (another NO donor) dose and dilator response in healthy volunteers and established that no dilatation occurred at doses of 10-20 µg, but there was a graded response to doses up to 100 µg and no further increase at 200 µg and above (266). During the current series of experiments, I have attempted to match the magnitude of the dilator response achieved in response to the generated flow stimulus with that which results from the nitric oxide donor GTN. In order to identify an appropriate dose of GTN in subjects with renal failure I performed a preliminary series of experiments constructing dose response curves to sublingual GTN in both healthy volunteers and subjects with dialysis-dependent renal failure. Cumulative dilatation dose response curves of the brachial artery to GTN (12.5, 25, 50, 100, 400 µg) were constructed in 12 healthy volunteers recruited from hospital staff; and 12 individuals with end stage renal failure (5 receiving peritoneal dialysis (PD) and 7 receiving haemodialysis (HD)). The two groups were matched for age (controls 35.9 ± 3.6 years vs renal failure 37.8±4.1 years; p=0.2), and sex (controls M:F 5:7 vs renal failure 5:7) though not for mean arterial blood pressure (controls 95.4 ±3.6 mmHg vs renal failure 111.5±3.4 mmHg; p=0.04). The dilator response was analysed statistically by calculating the area under the GTN dose/dilatation response curve for each subject and this summary measure was analysed using an unpaired Student’s t test (see figure 2.9).
Figure 2.9: Cumulative dose response curves of the dilator response to sublingual GTN in healthy volunteers and subjects with chronic renal failure.

The graphs of dilator response in healthy volunteers versus subjects with end stage renal failure superimpose (healthy volunteers 5084.7± 432.2 %s vs renal failure 4740.9±405.9 %s; P=0.6). In order to match the expected dilator response to flow in the groups to be studied in this thesis, a dose of 25 µg of GTN was selected for the assessment of smooth muscle function. This corresponds to the expected dilator response to flow of 3-6 % recorded in the literature for this clinical group.

2.1.1.7 Experimental technique

Longitudinal, ECG-gated end-diastolic images were acquired every 3 seconds, using customized software, and arterial diameter over a 1-2 cm segment was determined for each image, using an automatic edge-detection algorithm (as described in section 2.1.1.1). Pulsed wave Doppler was used to measure blood flow velocity expressed as the velocity time integral (VTI) for a single cardiac cycle. Brachial or radial artery
diameter and VTI were measured for 1 minute (baseline), during 5 minutes of reduced
blood flow (induced by inflation of a pneumatic cuff to 300 mmHg placed at the
forearm/wrist, distal to the segment of artery being analysed), and for 5 minutes during
reactive-hyperaemia after release of the wrist cuff. The dilator response of the radial
artery to administration of sublingual GTN (25 μg) was used to assess endothelium-
independent dilatation. Blood flow was expressed as VTI, or area under the VTI–time
curve. Dilatation was expressed as peak percentage dilatation and area under the
dilatation-time curve.

2.1.1.8 Accuracy and reproducibility of the technique of flow mediated dilatation

In any measuring technique, there are a number of potential sources of error. Ideally, a
measure should be accurate and reproducible over both time and between observers.
The problem of measurement inaccuracy in flow mediated dilatation was addressed by
Sorenson et al. (265) using high resolution ultrasound to record the absolute dimensions
of ‘phantom’ arteries of known diameter (mock blood vessels with the same ultrasound
characteristics as the original embedded in a block of latex). These investigators
recorded the observer’s ability to distinguish between small differences in phantom
vessel diameters. Different observers were able to discern the absolute diameter
correctly to within 0.04 mm and were able to distinguish between pairs of phantom
arteries with diameter differences 0.1 mm in 61% of cases using this technique (265).
These data suggest that the technique is accurate. It is also important to establish
reproducibility of this technique over time (intra-observer variation) and between
observers (inter-observer variation) since, in the studies described in this thesis,
individuals have been examined on multiple occasions. In order to reliably detect a
treatment effect, we need to be confident that we are able to distinguish between
changes in the measure that relate to background measurement variability (noise) and changes in the signal that relate to the intervention.

Sorenson et al. have assessed inter-observer variability using the technique of FMD in a study in which four independent observers recorded arterial diameter (265). They reported that no measurement was more than 0.1 mm from the measure made by the other 3 observers and the coefficient of variation was 1.8% (265). In the first study presented in this thesis, two independent observers assessed a random selection of traces from 10 patient studies. The results for two observers are shown in figure 2.10.

![Figure 2.10 Inter-observer variability in the measurement of FMD](image)

In unpublished work carried out in the vascular physiology unit by Mrs Ann Donald, the variation of FMD over time has been investigated using this technique. 50 healthy volunteers were examined repeatedly by the same observer at three-month intervals and analysis of variance of repeated measures showed no significant difference between the measures over a period of 6 months (see figure 2.11)
Any measure that requires observer judgement in the processing of data before generating a result is prone to bias. In this thesis, observer bias is minimised by blinding the operator to the intervention and the use of wholly automated computerised analysis packages.

2.1.2 Venous occlusion plethysmography

Forearm vasodilator responses to brachial artery infusion of endothelium dependent dilators (acetylcholine and bradykinin) at sub-systemic doses have been used to demonstrate endothelial dysfunction in conditions associated with atherosclerosis. In this thesis, this technique was used to assess endothelial function of resistance vessels in the human forearm in vivo in subjects with renal failure (see figure 2.12).

Forearm blood flow depends upon resistance vessel tone in the forearm muscular bed, as microvascular smooth muscle relaxes so resistance falls and forearm blood flow increases. The technique of venous occlusion plethysmography is based on the observation that vasodilation of the forearm microvasculature is associated with a proportional increase in blood flow into the forearm. An upper arm occluding cuff is
inflated to 40 mmHg (see fig 2.12), which is sufficient to occlude venous return, but insufficient to impair arterial filling. Arterial blood continues to enter, and forearm volume expands. The rate of arterial blood flow into the forearm is proportional to the initial linear rate of rise in forearm volume. We used electrically calibrated mercury-in-silastic strain gauges attached to the forearm to derive forearm blood flow from changes in forearm circumference / volume induced by intermittent venous occlusion. The forearm gauges act as variable electrical resistors and stretching increases the electrical resistance of the strain gauge.

![Figure 2.12 Experimental set-up for venous occlusion plethysmography](image)

Using computer software (Macleab version 3.5) it is possible to define the change in resistance associated with a standard one percent change in forearm volume, after this calibration, the rate of change in forearm volume (and hence blood flow) can be calculated by analysing the rate of rise (gradient) of the recorded slope.
Pharmacological agents can be administered directly into the arterial bed by cannulation of and infusion into the brachial artery. Delivering drugs in this way has the advantage of allowing observation of the physiological effect of much higher local concentrations of the agent without inducing systemic side effects. The effects of dilator (acetylcholine, bradykinin, glyceryl trinitrate (GTN) and sodium nitroprusside (SNP), arginine) or constrictor agents (noradrenaline) on skeletal muscle blood flow can then be directly examined.

2.1.2.1 Experimental technique

Mercury-in-silastic strain gauges were used to measure forearm blood flow in both arms simultaneously (268). Blood pressure cuffs were applied to both upper arms and wrists. Upper arm congestion cuffs were set to 40 mmHg and inflated for 10 seconds in every 15-second cycle. Wrist occlusion cuffs were set to 200 mmHg and remained inflated for 11 minutes during construction of individual dose response curves. Cuff inflation was achieved using a Hokansen E 20 rapid cuff inflator. Strain gauges were placed at the point of maximum forearm circumference and taped to ensure placement stability. Measurement of forearm circumference was achieved by connecting the strain gauges to a plethysmograph. The transduced signal was digitised by a Maclab, 400 Analogue to digital converter and displayed on a monitor in real time (MacLab, 4E, AD Instruments, UK).

An unmounted 27-G needle (Cooper's Needle Works, UK) was attached to a 16G epidural catheter and sealed with sterile commercial glue. The needle was inserted into the non-dominant brachial artery under sterile conditions using 2 ml of 1% lignocaine subcutaneously. After insertion of the needle into the brachial artery and a rest period of 15 minutes to establish basal flow conditions, measurements of blood flow were made for 5 minutes in every 10 minutes to establish resting control values of forearm blood flow.
The strain gauges were calibrated at the start of each experiment. Forearm blood flow was expressed as ml (flow)/100 ml (forearm volume)/minute. Forearm blood flows were measured in response to infusion of the endothelium-dependent dilator (acetylcholine: Ach 25, 50, 100 nmol/min) or endothelium-independent dilator agents (glyceryl trinitrate: GTN 4, 8, 16 nmol/min: sodium nitroprusside: SNP 4, 8, 16 nmol/min). The maximum dilator response was recorded when steady state was reached (3 minutes of infusion at each dose for Ach and 5 min of infusion at each dose for GTN and SNP). These dose ranges were selected on the basis of published data in this field such that the dose response curves fall on the linear portion of the sigmoidal dose response curve for the individual drugs (269;270). Blood flow was calculated as the mean of the last 4 inflations of the occluding cuffs at each dose using MacLab chart software (MacLab version 3.5).

2.1.2.2 Accuracy and reproducibility of the technique of venous occlusion plethysmography

Even when experimental conditions are carefully controlled during venous occlusion plethysmography, forearm blood flow can vary in both arms from moment to moment in response to changes in mental arousal, sympathetic nervous activity and ambient temperature in addition to those responses, which result from intra-arterial test substance infusion. These random fluctuations in flow can have significant and misleading effects on the measured responses to intervention if simple unilateral flow changes are reported. Many of these difficulties can be overcome by measuring forearm blood flow bilaterally, using the contralateral arm as a concurrent control, and recording the background variation in forearm blood flow independent of infused agents under study. Results can be then be expressed as percentage change in forearm blood flow.
ratio \((\text{infused/control} \times 100\%)\). The assumption is that any background changes in
blood flow detected in the contralateral arm reflect background changes to which the
study arm is also exposed, independent of the infused agent under test. This bilateral
technique was first described by Greenfield (271). In support of this method of data
presentation, Petrie et al. reported that forearm blood flow ratios are more reproducible
than unilateral forearm blood flow measures, (coefficient of variation of 19% for ratio
analysis and 39% for unilateral analysis). Implicit in this is the assumption that flow in
the infused arm will be influenced by background conditions to the same degree as flow
in the control arm. This is generally true if the vasodilator/constrictor results in
relatively small changes in blood flow. Large changes in blood flow in the infused arm
are, however, unlikely to be affected to the same extent by changes in basal flow in the
control arm. In contrast to Petrie et al., Chowienczyk and colleagues found that within
subject variation was greater when expressed as a ratio compared with presentation as
unilateral absolute flow (coefficient of variation of approximately 50% for ratio analysis
and 25% for unilateral analysis) (272). So in situations where there are large changes in
infused arm blood flow, expressing data in ratio form may actually produce less reliable
results because of the disproportionate arithmetic impact that correcting for contralateral
changes in baseline flow create.

In this thesis, both methods of analysing plethysmography data were used. However, the
conclusions drawn were similar with either method of analysis and I have therefore
presented the data using absolute flows rather than ratios of flows.

The accuracy of venous occlusion plethysmography is the extent to which the technique
produces results that compare with other methods of recording blood flow. Pallares et
al. (273) demonstrated a close correlation between blood flow measured simultaneously
using Doppler ultrasound and occlusion plethysmography, correlation coefficient of
0.57 (273).
2.1.2.3 Data analysis and presentation

Forearm blood flow was expressed as ml/100ml forearm volume/min. When an intervention is assessed, comparisons of blood flow response to each dose of the agonist can be made using repeated measures analysis of variance (ANOVA) or alternatively the data can be converted to a summary measure of the whole dilator response (area under the dose response curve) and comparisons made between summary measures using a conventional paired or unpaired students t test as appropriate. Both have been employed to analyse results, however, the technique used had little impact on the final result and for consistency ANOVA has been reported. Studies have a 90% power to detect intraindividual differences of 1% in FMD, based on the standard deviation observed in studies of repeated baseline measures within individuals.

2.2 Measurement of L-arginine and its methyl analogues

Arginine, ADMA and SDMA were measured by reverse phase high-pressure liquid chromatography (HPLC) after a method described by Vallance et al. (274). Figure 2.13 shows a schematic of the equipment used. HPLC allows the resolution, identification, and quantification of chemical compounds in plasma samples.

The sample for study is first chemically purified, dissolved in solvent and then perfused at a constant flow rate through an HPLC column (figure 2.13). Molecules with different physicochemical properties pass through the column at different speeds and are eventually eluted from the end of the column at which point they can be detected and quantified.
Arginine and its analogues were detected and quantified by absorption of UV light, at an optimal wavelength of 200 nm. UV detectors have a sensitivity to approximately $10^{-8}$ or $10^{-9}$ gm/ml. Quantifying the compounds of interest is achieved by comparing the area under the chromatographic curve with peaks that result from a known quantity of synthetic standard.

![HPLC setup diagram](image)

*Figure 2.13 Experimental set up for the detection and quantification of L-arginine adducts using HPLC, after Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 27.*

2.2.1 Methylarginine Purification Procedure

In order to purify human plasma before analysis, venous blood was collected into lithium heparin containers, numerically coded and centrifuged at 3000 RPM for 15 minutes at 4°C to separate plasma. Samples were frozen at -40°C until batch analysis, defrosted and centrifuged once more at 3000 RPM for 5 minutes. One ml of plasma was loaded onto a 2 ml ‘Bondelut SCX column’ (Anachem Ltd, Luton, Beds UK) previously washed and primed with double distilled filtered water and 100% HPLC Analar grade.
methanol (BDH, UK). Dimethylarginines were serially eluted in 25% ammonia/methanol followed by 50% ammonia/methanol. The eluate was collected and evaporated at 90°C on a hotplate under gaseous nitrogen. The dried extract was re-suspended in 2 ml of double distilled water and applied to a washed and primed ‘Bondelut CBA column’. Dimethylarginines were again eluted using a 10% ammonia/methanol solution, this final eluate collected, dried at 90°C on a hotplate under nitrogen and finally re-suspended in 100 µl of double distilled water. To estimate the extraction efficiency of this procedure, each 1 ml sample of plasma was spiked with a known quantity of LNMMA (1 µg) before purification.

2.2.2 Applying samples to the HPLC column

Extracted samples were applied to a C18 analytical high performance liquid chromatography column (BDH cat no765452) using a solvent consisting of 1.71 ml of 0.025 M orthophosphoric acid (HiPerSolv BDH), 1.88 g hexanesulphonic acid (Romil laboratories, Loughbourough), 10 ml acetonitrile (v/v) super purity, far UV (Romil Laboratories, Loughbourough). 40 µl of the final 100 µl extracted sample is injected automatically onto the column. Detector absorbance was set at 200 nm. At the beginning and end of each series of sample analysis, we applied 1 µg standards dissolved in double distilled water of arginine, LNMMA, ADMA and SDMA to the column. This allowed us to confirm the position of peaks associated with each of these substances in the plasma sample traces, which can vary from day to day depending upon the ambient temperature and the precise quantity of acetonitrile in the running buffer. Typically, arginine is detected at 10 minutes, L-NMMA at 17 minutes, ADMA at 30 minutes and finally SDMA at 32 minutes. Figure 2.13 shows a typical trace demonstrating peaks for each substance obtained using this technique.
Figure 2.13 Chromatograph of L-arginine and related compounds following injection onto a chromatography column.
2.2.3 Quantifying plasma arginine and methyl arginines

Quantification of methylated arginine compounds by HPLC involves injecting a known concentration of standard solution onto the HPLC column for detection. The chromatograph of these known concentrations will give a peak whose area correlates with the concentration of the sample. The plasma concentrations of methylarginines were calculated by recording the area under the chromatographic curve for each substance. The area under the standard peak generated by applying 1 μg of standard to the column was measured and the quantity of methyl arginines were calculated by simple proportion. Since the automatic sampler applies exactly 40 μl of the extracted sample to the column, we can calculate the concentration of the methylated arginine in the extracted sample and the concentration in the original 1 ml plasma sample. This however assumes 100% extraction efficiency. We calculated individual sample extraction efficiencies by comparing the 1 μg LNMMA standard curve to the LNMMA peak in our spiked samples. In these studies the extraction efficiency was approximately 30%, which is, similar to that reported previously (240).

2.2.4 Assessment of plasma homocysteine concentrations

Clinically the measurement of total homocysteine (tHcy) (free plus bound), is more useful diagnostically than measurement of free homocysteine, cysteine and their mixed disulphide which can be regarded as artefacts of sample oxidation during imperfect sample handling (275). In this thesis, total plasma homocysteine concentrations were estimated by Dr Tony Briddon, Chief Biochemist at the Hospital for Neurological Diseases, London, using the technique of ion-exchange chromatography (IEC) with ninhydrin post-column derivatisation. Analyses were carried out on a biochrom 20
amino acid analyser (Pharmacia, Biotech, Camb. UK) and a high-resolution lithium column. Plasma samples were de-proteinised using equal volumes of 10% sulphosalicylic acid using 200 μmol/l norleucine as an internal standard. After centrifugation 70 μl of supernatant was applied to the column. Sample concentrations were calculated from the area under the peak on the chromatogram calculated with reference to standards as previously discussed.

2.3 The Assessment Of Oxidant Stress

2.3.1 Direct assessment of oxidative free radicals

Direct measurement of oxidative stress in vivo is difficult, because free radicals are highly reactive, have a short half-life, and are present in very low concentrations. The only direct way to detect radicals is by using electron spin resonance spectroscopy, which relies on immediate collection and analysis of fresh plasma samples in an oxygen-free environment. The interaction between free radical species and specific chemical agents with the emission of detectable light is a common assay technique with which to detect and quantify free radicals, however it can be non-specific. More recently, there have been modifications to these assays that allow measurement of individual radical species. Among the specific assays for O$_2^-$ the reduction of nitroblue-tetrazolium or cytochrome-C are among the most widely used and other specific agents exist for the detection of H$_2$O$_2$ and OH$^-$. 
2.3.2 Measuring the products of oxidant stress

Measuring the secondary products of oxidative stress is an indirect way of assessing the impact of prevalent levels of free radicals upon tissue components (for example, lipids, proteins and DNA). The detection of increased levels of oxidation products in tissue and biological fluids is an indicator of the relationship between free radical production and the development of pathology. Many techniques now exist which measure the products of oxidation in human tissues and plasma; these techniques include tests for oxidized lipids, volatile hydrocarbons in breath, and oxidized DNA bases. The most important assays of this kind quantify lipid peroxides and assay for thiobarbituric acid-reactive substances (TBARS), DNA strand breaks, and interstrand crosslinks.

2.3.3 Assessment of antioxidant capacity

An estimate of the tissue impact of ambient levels of free radicals can be gleaned from assessing the residual capacity that exists in tissue and plasma to resist oxidant injury. There are several specific antioxidant systems in vivo including vitamins C & E and a number of antioxidant enzyme pathways. The total antioxidant capacity (TAC) in plasma, however, represents a more reliable estimate of plasma antioxidant capability than the measurement of each of these individual antioxidant systems.

No single measurement of antioxidant status is sufficient to describe prevalent oxidant stress, but a "battery" of measurements including a measure of individual antioxidant levels, total antioxidant capacity (TAC), lipid and protein oxidation products are necessary to adequately assess oxidative stress in biological systems.

In this thesis, a number of measures of oxidant status were made in collaboration with Dr Sarah Nuttall (Biochemistry Laboratories, Queen Elizabeth Hospital, University of
Birmingham UK). Serum total antioxidant capacity was determined by an enhanced chemiluminescence method developed in these laboratories (276). This method depends on the continuous production of a free-radical-mediated luminescent light signal. When a sample containing antioxidants is added, the light signal is temporarily stopped by its free radical-scavenging action. The time taken for the light signal to return is then directly proportional to the amount of antioxidant in the sample.

Concentrations of the antioxidant vitamins C in plasma are determined by reverse-phase high performance liquid chromatography (HPLC) using UV-detection at wavelengths of 254 and 292 nm respectively (277,278).

Total plasma glutathione was determined by enzyme rate assay using the colorimetric dye 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) (279). This method relies on the recycling of the oxidized and reduced forms of glutathione by the enzyme glutathione reductase, which is determined by spectrophotometry at a wavelength of 412 nm.

Lipid hydroperoxides were analysed spectrophotometrically by the ferrous oxidation of the colorimetric dye xylenol orange at a wavelength of 560 nm in conjunction with the specific hydroperoxide reductant triphenylphosphine (280).

All studies performed in this thesis were reviewed by University College Local Research Ethics Committee and The Institute of Child Health and Great Ormond Street Hospital Research Ethics Committee.
Chapter 3

The Effect of Dialysis on Endothelial Function

in Humans
3.1 Introduction

Patients with renal failure have increased cardiovascular risk that relates in large part to the development of atherosclerosis and its complications. Early onset of endothelial dysfunction, with the loss of physiological anti-thrombotic and anti-atherosclerotic properties, may contribute to cardiovascular risk in these patients. Endothelial dysfunction with reduced NO bioactivity occur in patients on haemodialysis (HD) (230), peritoneal dialysis (231) and in those with chronic renal failure prior to the introduction of renal replacement therapy (227). It is therefore possible that endothelial dysfunction, and reduced NO bioactivity, could be the consequence of accumulation of inhibitors of endothelial function. Water-soluble, dialysable substances (uraemic toxins) have been implicated in the pathogenesis of clinical uraemia for over 80 years, and removal by dialysis is thought to account for many of the beneficial effects of renal replacement therapy. Of the many different molecules that have been proposed as possible uraemic toxins (281), some have the potential to inhibit endothelial function, including \( \text{N}^\text{G} \text{N}^\text{G} \) dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA; an inhibitor of endothelial NO production), and homocysteine (tHcy; which directly damages the endothelium and may inactivate nitric oxide). If the plasma concentrations and effects of these, and other as yet unidentified endothelial toxins, could be rapidly lowered by dialysis then improved endothelial function would be expected. To test this hypothesis, we compared the acute effect of a single episode of haemodialysis with an episode of automated peritoneal dialysis on endothelial function. A single treatment of haemodialysis results in larger, more rapid reductions in dialysable compounds than automated peritoneal dialysis. Our hypothesis predicts that endothelial function would fluctuate with the clearance of endothelial toxins, with the greatest changes being observed during haemodialysis.
3.2 Methods

3.2.1 Subjects

Two groups of adults with end stage renal failure, 16 (8 male) receiving HD and 14 (7 male) receiving APD were studied. Outpatients receiving renal replacement therapy for at least 3 months were recruited. Exclusion criteria included diabetes, age > 70 years, smoking and a fasting cholesterol exceeding 6 mmol/l (recognised independent risk factors for impaired endothelial function). Between 60 and 70% of subjects in both groups were hypertensive with a mean arterial pressure >100 mmHg despite concurrent treatment with an average of two anti-hypertensive agents (table 3.1). Subjects taking nitrates discontinued therapy 48 hours prior to the study but other regular medications were continued. HD patients received dialysis for 4 hours 3 times per week using a cellulosynthetic membrane (Hemophane), a dialysate calcium of 1.5 mmol/l, and freshly prepared bicarbonate buffer.

Table 3.1 Subjects' antihypertensive medication profile.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>HD</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>α adrenergic blocker</td>
<td>7 (44)</td>
<td>5 (36)</td>
</tr>
<tr>
<td>β adrenergic blocker</td>
<td>10 (63)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>6 (38)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>ACE I</td>
<td>9 (56)</td>
<td>5 (36)</td>
</tr>
<tr>
<td>Minoxidil</td>
<td>1 (6)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Frusemide</td>
<td>1 (6)</td>
<td>3 (21)</td>
</tr>
</tbody>
</table>

Mean antihypertensives per subject 2 2

ACE I (Angiotensin converting enzyme inhibitor)
Data are expressed as absolute number (percentage of whole group). There were no significant differences between the HD (hemodialysis), APD (automated peritoneal dialysis) groups in medications (P=0.9; Fischer’s exact test).

Dialysate quality was within Renal Association guidelines (U.K.) (<0.03 endotoxin units/ml and < 8 viable bacterial units/ml). Dialysate temperature was maintained at 37 °C throughout. A mean of 2.2±0.3 kg was removed during HD (table 3.2), 1 kg of fluid was ultrafiltered in the first hour of haemodialysis and the remainder was removed evenly over the dialysis session to achieve the subjects prescribed dry weight. Patients treated with overnight APD received a mean of 12±3 l exchanges (range 10-15 l) using a combination of 1.36, 2.27 and 3.86 % lactate-based PD fluid (Homechoice, Baxter UK).

3.2.2 Assessment of endothelial function

Endothelial function was determined by recording the dilator response of the brachial artery to increased blood flow generated during reactive hyperaemia of the downstream forearm as described previously (see 2.1.1). In each case, endothelium independent dilatation was assessed by recording the dilator response to 25 μg of sublingual GTN as previously described (see section 2.1.1.6).

3.2.2.1 Effect of haemodialysis on brachial artery reactivity

Endothelium dependent and independent dilatation was assessed in haemodialysis patients immediately before and after dialysis (n=16), and then sequentially at 2 hours (n=11), 5 hours (n=7), and 24 hours (n=6) hours after completion of the treatment. In
both groups there was a significant drop-out rate at later time points, reflecting the inconvenience to these patients of repeatedly measuring endothelial function. Time-control studies were performed in 5 HD subjects on a non-dialysis day at the pre- and post-dialysis time points.

3.2.2.2 Effect of APD on brachial artery reactivity

Endothelium dependent and independent dilatation was assessed in automated peritoneal dialysis patients immediately before and after dialysis and then sequentially at 2 hours (n=14), 5 hours (n=13) and 24 hours (n=9) after completion of a dialysis session.

3.2.2.3 Effect of volume expansion on brachial artery reactivity

To determine whether intravascular volume changes might directly alter endothelial function, the effect of volume expansion on brachial artery reactivity was assessed in 7 healthy volunteers, drawn from hospital staff, who were normotensive, non-smokers with a mean fasting plasma cholesterol of 3.9 ±0.2 mg/dl. Subjects were volume loaded with 1 litre of 0.9% saline (w/v) infused intravenously over 30 minutes. Diameter changes and FMD of the brachial artery were assessed immediately before and after saline infusion.
3.2.3 Biochemical measurements

Blood samples were taken before and after dialysis for analysis of haemoglobin, haematocrit, total cholesterol, creatinine, potassium, glucose and C reactive-protein (CRP). L-arginine and asymmetric dimethyl-L-arginine (ADMA) were measured by reverse phase high pressure liquid chromatography (HPLC) as previously described (274) (see section 2.2). Total plasma homocysteine (tHcy) was measured by ion-exchange chromatography using a Biochrom 20 amino acid analyser, (Pharmacia Biotech, Cambridge, UK) and a high-resolution lithium column with a standard physiological separation programme as previously described (see section 2.2.5(282)).

3.2.4 Calculations and statistics

Blood flow and FMD were expressed as described in section 2.1.1.2. Data was analysed using two tailed Students t test or by analysis of variance of repeated measures as appropriate. Significance was assumed at P<0.05 (Graph Pad Prism statistical package). The study had a 90% power to detect intraindividual differences of 1% in FMD, based on the standard deviation observed in studies of repeated baseline measures within individuals.

3.3 Results

The HD and APD groups were well matched for cardiovascular risk factors (table3.2). No studies were excluded based on technical inadequacy (inter-observer variability of FMD <1%) (see figure 2.10).
Table 3.2 Patient baseline clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>HD (n=16)</th>
<th>APD (n=14)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43 ± 3.5</td>
<td>44 ± 2.7</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>8/8</td>
<td>7/7</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Months on dialysis</td>
<td>59±14</td>
<td>49±22</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Cholesterol mg/dl</td>
<td>4.2±0.2</td>
<td>4.2±0.3</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Mean arterial pressure mm/hg</td>
<td>108±4.6</td>
<td>102±5.6</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Heart rate (BPM)</td>
<td>76±3</td>
<td>74±4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>10.0±0.3</td>
<td>10.7±0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Weight loss during dialysis (Kg)</td>
<td>2.2±0.3</td>
<td>1.8±0.7</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

HD (haemodialysis), APD (automated peritoneal dialysis)

3.3.1 The effects of dialysis (haemodialysis and automated peritoneal dialysis) on brachial artery reactivity

Baseline arterial diameter, mean arterial pressure and heart rate were similar in HD and APD subjects (table 3.2 & 3.3). Arterial diameter during baseline recording was identical before and after both HD and APD (table 3.3). HD did not change mean arterial blood pressure (113.9±3.5 mmHg versus 108.6±3.7 mmHg; P=0.1) or diastolic blood pressure (80.7±2.9mmHg versus 79.8±2.5 mmHg; P=0.7) but reduced systolic blood pressure (169.6±4.9mmHg versus 158.2±5.0 mmHg after HD; P=0.02). In the APD group mean arterial pressure (102.7±2.8 mmHg versus 101.6±3.4 mmHg; P=0.5) and systolic pressure (136.2±3.7 mmHg versus 134.1±4.0mmHg; P=0.2) remained constant after dialysis. Diastolic pressure, however, fell (80.0±2.9 mmHg versus
77.0±2.8 mmHg; P=0.03). The flow stimuli generated during reactive hyperaemia were similar in both groups before and after treatment (AUC 15.9±2.1 ms versus 15.3±2.3 for HD; P=0.5; 17.1±2.1ms versus 18.8±2.7 for APD; P=0.5).

Table 3.3 The effect of dialysis on measured parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HD Pre</th>
<th>HD Post</th>
<th>P</th>
<th>APD Pre</th>
<th>APD Post</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>9.7±0.4</td>
<td>9.2±0.5</td>
<td>0.1</td>
<td>11.2±1</td>
<td>11.2±1</td>
<td>0.1</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.30±0.01</td>
<td>0.31±0.01</td>
<td>0.1</td>
<td>0.35±0.02</td>
<td>0.35±0.02</td>
<td>0.3</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.2±0.2</td>
<td>3.75±0.3</td>
<td>0.5</td>
<td>4.2±0.4</td>
<td>3.9±0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>26.4±1.2</td>
<td>9.1±0.6</td>
<td>0.001</td>
<td>21.1±2.4</td>
<td>19.7±2.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>842±46</td>
<td>356±29</td>
<td>&lt;0.0001</td>
<td>977±59</td>
<td>956±59</td>
<td>0.2</td>
</tr>
<tr>
<td>ADMA (μmol/l)</td>
<td>0.68±0.1</td>
<td>0.43±0.06</td>
<td>&lt;0.001</td>
<td>0.49±0.1</td>
<td>0.49±0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>Arginine (μmol/l)</td>
<td>35.6±4.7</td>
<td>32.8±4.5</td>
<td>0.6</td>
<td>45.1±12</td>
<td>51.3±12</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.0±0.3</td>
<td>4.9±0.3</td>
<td>0.7</td>
<td>4.7±0.2</td>
<td>4.5±0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>tHcy (μmol/l)</td>
<td>38.3±5.9</td>
<td>23.4±3.0</td>
<td>&lt;0.001</td>
<td>52.4±15</td>
<td>45±13</td>
<td>0.6</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>12.0±4.2</td>
<td>12.1±4.6</td>
<td>0.5</td>
<td>11.5±4.2</td>
<td>9.8±4.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Baseline arterial diameter</td>
<td>4.3±0.2</td>
<td>4.2±0.2</td>
<td>0.2</td>
<td>4.4±0.2</td>
<td>4.6±0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are mean ±SEM. Abbreviations: HD (Haemodialysis), APD (automated peritoneal dialysis), tHcy (total plasma homocysteine).

FMD was increased by HD (4.0±1.0% versus 5.8±1.2%; P<0.002; fig 3.1a), but remained unchanged following APD (5.7±1.1% versus 5.4±0.8%; P>0.5; fig 3.1b).
Similarly the area under the dilatation time curve increased after HD (466±84 %s versus 657±116 %s; P<0.05; fig 3.1c), but remained unchanged after APD (638±107 %s versus 633±111 %s; P>0.9; fig 3.1d). There were no significant changes in endothelium-independent dilatation in response to GTN after HD (5.9±1.0% versus 6.4±1.1%; P>0.5; fig 3.1a) or APD (6.3%±1.2% versus 7.3±1.2%; P>0.5; fig 3.1b).

3.1a)

3.1b)

Key
HD  Haemodialysis
APD  Automated peritoneal dialysis
NS  Not significant
FMD  Flow mediated dilatation
GTN  Glyceryl-trinitrate
Figure 3.1 (a-d) The effect of dialysis on flow-mediated dilatation (FMD). (For clarity, in Figures 3.1c) and 3.1d) standard error bars have been omitted; data represents the whole population). Haemodialysis (HD) increased FMD but did not alter dilatation to glyceryl trinitrate (GTN; fig 3.1a). Automated peritoneal dialysis (APD) had no effect on FMD or dilatation to GTN (fig 3.1b). The profile of FMD for the 4-minute period after cuff release is shown for HD and APD (fig 3.1 c&d). Analysis of area under the dilatation time curves showed improvement after HD, (n=16) but not APD, (n=14).

The improvement in FMD after completion of HD persisted for at least 5 hours but returned to baseline by 24 hours (fig 3.2a). Pre treatment FMD was lower in the HD compared with the APD group (4.0±1.0% versus 5.7±1.1% respectively), but this did
not reach statistical significance (P=0.2). In 5 patients assessed on a non-dialysis day, there were no time-dependent changes in FMD in the absence of dialysis (4.1±0.7% versus 3.8±1.4% 4 hours later; P>0.5) or GTN dilatation (5.7±1.0% versus 5.5±0.8% 4 hours later; P>0.5).

![Graph](image)

**Figure 3.2 The effect of dialysis on the 24-hour time course of FMD.**

3.3.2 The effect of changes in plasma volume on brachial artery reactivity

In 7 healthy volunteers, volume expansion had no effect on baseline arterial diameter (3.5±0.3mm versus 3.5±0.3mm; P>0.2), FMD (5.3%±0.9% versus 6.3%±1.4%; P>0.2), or flow profiles (14.3±2.3 ms versus 14.6±2.46 ms; P=0.5).
3.3.3 The effect of dialysis on plasma biochemistry

HD significantly reduced the plasma concentration of ADMA and tHcy (table 3.3). Plasma L-arginine concentration was unaltered by HD (P>0.5), but the ADMA/L-arginine ratio fell significantly after HD (P<0.03). APD did not significantly reduce the plasma concentrations of ADMA, L-arginine or tHcy (table 3.3), although levels of methylarginines were lower and tHcy higher than those seen in the HD group. HD resulted in a greater immediate reduction in plasma creatinine and urea compared with APD but neither treatment affected the plasma concentration of haemoglobin, C reactive protein, glucose, or total cholesterol (table 3.3).

3.4 Discussion

The purpose of this study was to test the hypothesis that clearance of circulating inhibitors of endothelial function by dialysis improves arterial endothelial function. A single haemodialysis treatment caused rapid clearance of uraemic markers and endothelial toxins, and transiently increased FMD. In comparison, a single APD treatment caused only small changes in these substances and did not acutely alter FMD. Neither treatment altered smooth muscle responsiveness to GTN. These data suggest that acute reduction of the concentration of circulating inhibitors of endothelial function associated with uraemia improves endothelial function in the arterial vasculature.

Dialysis relieves the symptoms of uraemia by removing low to middle molecular weight molecules and the therapeutic effect of renal replacement therapy indicates that these substances contribute to the pathophysiology of chronic renal failure. A variety of candidate toxins have been proposed including products of amino acid and protein
metabolism (281). These include the guanidino compounds ADMA, methylguanidine, and \( \text{N}^6\)-monomethyl-L-arginine (LNMMA) that are excreted by the kidney (see section 1.4.1.1), and homocysteine (see section 1.2.2.4.3), a product of methionine metabolism that requires renal clearance. ADMA, methylguanidine and LNMMA are inhibitors of NO synthesis and have been shown to reversibly block endothelium-dependent relaxation \textit{in vitro} and \textit{in vivo} (156;244). Homocysteine has been implicated in the generation of oxidative free radicals that might impair endothelial function through inactivation of NO (87). Another candidate endothelial toxin is oxalate. In common with arginine analogues and homocysteine, it is known to accumulate in renal failure, is small, water soluble and cleared on haemodialysis and is toxic to the endothelium (283). The accumulation in uraemia of endothelial toxins could contribute to the impaired NO-mediated dilatation reported in patients with renal failure (274).

In the present study, we used FMD of human conduit arteries to assess NO-mediated endothelial function. Dilatation of human conduit vessels is dependent on NO synthesis in the brachial, and coronary vascular beds, because dilatation in response to flow is reduced by inhibitors of NO synthesis (259). FMD of the brachial artery is approximately 6-8% in healthy volunteers (265) and has been consistently reported between 3-4% in subjects with impaired in renal function see section 1.3.3.4).

In the present study, there was a 45% increase in FMD of the brachial artery after treatment with HD, along with a reduction in plasma concentration of ADMA, homocysteine, creatinine and urea. In contrast, a single treatment with APD had no effect on uraemic markers or FMD.

There are other possible explanations for these observations. Haemodialysis was associated with a modest, though statistically insignificant increase in haematocrit (table 3). It is difficult to predict the effect of increased haematocrit on FMD, which could
augment FMD by increasing blood viscosity and luminal shear stress, or reduce FMD through, increased NO scavenging by haemoglobin. Perhaps because of these opposing effects, blood transfusion to increase haemoglobin does not alter FMD in humans (284) and it appears unlikely that the observed small changes in haematocrit contributed to the increase in FMD after HD. Both dialysis treatments caused similar weight loss, suggesting that changes in blood volume did not explain the increase in FMD observed after HD. This conclusion is further supported by the observation that rapid volume expansion does not alter FMD in healthy volunteers. Elevation of core body temperature during HD (due to peripheral vasoconstriction and reduced heat loss) has been implicated in increased NO production and dialysis associated hypotension (285). This is unlikely to explain the increase in FMD observed because there were no changes in basal forearm blood flow or basal arterial diameter after HD. Although mean arterial blood pressure did not change in the present study, there was a reduction in systolic pressure following HD that could alter FMD. However the relationship between endothelial function and short term changes in blood pressure is unclear (170;286) and systolic blood pressure changes did not correlate with change in FMD. The profile of antihypertensive medication was similar between the two groups (table 3.1), and unlikely to explain the differential effects of dialysis on endothelial function. Changes in plasma calcium levels after haemodialysis in which the dialysate solution contains 1.5mmol/l calcium, may have contributed to the increased vascular dilatation observed. However a reduction in plasma levels of calcium (when dialysate calcium is low relative to plasma) would result in a reduction in calmodulin production and consequently reduced NOS activity. This would be expected to be associated with a reduction in dilatation and not the increase that I observed. If we postulate that the flux of calcium in to cells reduces in the presence of low dialysate calcium levels it may be that this may mimmick the effector response to NO. However if this were the case
you would expect this to affect endothelium dependent and independent responses equally which we did not observe. Other factors known to affect endothelial function such as plasma glucose, CRP and cholesterol were unaffected by either mode of dialysis.

These findings contrast with a previous report, demonstrating that HD is detrimental to FMD of the brachial artery, an effect ascribed to oxidative stress associated with dialysis (287). This discrepancy may be explained by methodological differences, including the use of less biocompatible and potentially more pro-oxidant cellulose membranes in that study. Whilst further work is necessary to resolve these discrepancies, our results are consistent with the demonstration that HD improves endothelium-dependent dilatation in hand veins (251).

The data do not suggest that APD is a less effective form of dialysis. Indeed the APD group had slightly greater baseline FMD than the HD group, although this did not reach statistical significance, probably because of the relatively small numbers of patients studied. This difference was not explained by baseline differences between the two groups in known determinants of endothelial function. It is possible that the higher pre-treatment FMD in the APD group indicates that the APD dialysis schedule maintains endothelial function without re-bound between treatments, at a level that HD only achieved for the few hours immediately after a dialysis session. This conclusion is supported by previous studies where FMD of the brachial artery has been reported to be 5.7±1% in patients on peritoneal dialysis (231) and between 3.5±1.2 (234) and 3.7±1.1 (230) in haemodialysis patients respectively. Whether this effect of APD is mediated by a differential effect on the clearance of larger molecular weight toxins remains to be seen (288). What is clear from this study is that HD is associated with greater swings in dialysable endothelial toxins and endothelial function than APD.
3.5 Conclusions

In conclusion, there is increasing evidence that the presence of endothelial dysfunction reflects increased cardiovascular risk (199). This data shows that endothelial function can be improved by dialysis. These findings have implications for the concept of dialysis adequacy, particularly when therapy is directed towards reduction of cardiovascular morbidity and mortality in this high-risk group. In the next chapter, the specific contribution of ADMA to endothelial dysfunction in renal failure will be addressed.
Chapter 4
The Effect of Acute Administration of L-Arginine
on Arterial Endothelial Function
in Chronic Renal Failure
4.1 Introduction

Endothelial dysfunction which results in the reduced bioavailability of NO has been implicated in the pathogenesis of uraemic vasculopathy (289). In experimental animals, reduced NO bioactivity accelerates atherogenesis (147), and strategies to increase NO are anti-atherogenic (290). As previously described, reduced bioactivity of the NO pathway has been demonstrated in patients with renal impairment (227;230;231). The mechanism remains unclear, but may include reduced activity of NO synthase (NOS; secondary to reduced substrate or co-factor availability), or inhibition of NOS by endogenous inhibitors.

ADMA is a potent naturally occurring NOS inhibitor (244) and plasma concentrations are raised four-fold in patients with renal failure (240). The specific impact of ADMA on endothelial function remains, however, to be determined (291). In the preceding chapter, the effect of acute alterations in the concentration of uraemic toxins, including ADMA, on endothelial function was examined. If L-arginine adducts act as competitive inhibitors of NOS and contribute to the endothelial dysfunction observed in renal failure, then this effect ought to be overcome by the administration of sufficient substrate to normalise the analogue / substrate ratio. In this study, I have tested the hypothesis that in renal failure, NO-dependent dilatation might be improved by acute supplementation with L-arginine to overcome competitive inhibition of NOS by ADMA and other uraemic toxins.

4.2 Methods

4.2.1 Subjects

Eighteen subjects receiving haemodialysis and 8 subjects with severe chronic renal
failure prior to the initiation of renal replacement therapy were studied. Patients were excluded if aged >70 years of age, or if there was a history of smoking, diabetes or a fasting cholesterol >6 mmol/l (recognized independent risk factors for impaired endothelial function (150;151;153;177). As part of their regular medication, patients were being treated with an average of 2 anti-hypertensive agents (22% α blockers, 56% β blockers, 72% calcium channel blockers, 56% angiotensin converting enzyme inhibitors, 6% on potassium channel openers and 22% on loop diuretics). Regular medications were continued, though subjects were asked to omit nitrates for the 48-hour period prior to study.

4.2.2 Assessment of conduit artery endothelial function

Endothelial function was determined by recording the dilator response of the brachial or radial artery to increased blood flow generated during reactive hyperaemia of the downstream forearm (brachial artery studies) or hand (radial artery studies) as previously described (see section 2.1.1.1). The brachial or radial arteries of the non-fistula or non-dominant arm were examined using the ultrasound technique. Analysis was performed by an experienced vascular technician blinded to the subject and order and repeated by a second investigator in a random selection of one third of scans (studies where dilatation differed by more than 1% were deemed technically inadequate). Using pulsed wave Doppler, blood flow was recorded continuously throughout the study and is expressed as the velocity time integral (VTI; area under the blood velocity/time curve for a complete cardiac cycle, see section 2.1.1.4). Baseline recordings of arterial diameter were made for one minute before inflation of a blood pressure cuff placed distal to the site of arterial imaging. Recording continued for 5 minutes during cuff inflation to 300 mmHg and for 4 minutes after deflation (see section
2.1.1.1). Endothelium-independent dilatation of the brachial artery was assessed by measuring the dilator response to a submaximal dose of the nitric oxide donor, glyceryl trinitrate (GTN, 25μg sublingually) (see section 2.1.1.6)

4.2.3 Assessment of forearm blood flow (resistance vessel function)

Mercury-in-silastic strain-gauge plethysmography was used to measure forearm blood flow (ml/100 ml of forearm/minute) in both arms as described previously (see section 2.1.2 (156)). For each study, the brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper’s Needle Works) inserted under local anesthesia (2 ml of 1% lignocaine). Drug or saline (sodium chloride 0.9% wt/vol) were infused continuously at 0.5 ml/min. During recording periods, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mm Hg (292).

Protocol 4.1

Six normotensive, normocholesterolaemic healthy volunteers (3 male) were studied to determine whether 10 g of L-arginine (Martindale Pharmaceuticals, Essex, England) infused systemically was sufficient to overcome NOS inhibition. After measurement of basal radial artery FMD the NOS inhibitor N\textsuperscript{G}monomethyl-L-arginine (LNMMA), was infused via the upstream brachial artery at a dose (4 μmol/min) known from previous studies to induce maximal reduction of radial FMD (260). After infusion of LNMMA for 10 minutes, radial artery FMD was recorded. Systemic L-arginine was then administered intravenously over 30 minutes in the contralateral arm, after completing the infusion FMD was assessed again.
Protocol 4.2

This protocol was designed to determine the effect of systemic L-arginine on FMD of the brachial artery in patients with dialysis dependent renal failure. Eighteen subjects (7 male) on renal replacement therapy for at least 3 months were recruited and studied immediately prior to a haemodialysis treatment. Brachial artery FMD and blood flow were measured at baseline and 10 minutes after completion of an intravenous infusion of L-arginine (10 g in 100 ml of 0.9% saline over 30 minutes). Because L-arginine has been reported to elevate plasma potassium, subjects were excluded if serum potassium was greater than 5.6 mmol/l at the time of study. Mean arterial blood pressure was recorded using an automated oscillometric system (Critikon, Dinamap vital signs monitor 1846 8X), and measurements obtained before and after each scan and at 5-minute intervals throughout the L-arginine infusion using the non-study arm or the calf (in the presence of a fistula).

Protocol 4.3

This protocol was used to determine the effects of high local concentrations of L-arginine on conduit and resistance artery endothelial function in patients with chronic renal failure, not yet receiving renal replacement therapy. Eight subjects (5 male) approaching dialysis dependent renal failure (calculated GFR < 20 ml/min estimated from plasma creatinine using the Cockcroft-Gault formula) were recruited. Figure 4.1 summarizes the experimental protocol. The brachial artery of the non-dominant arm was cannulated and radial FMD was measured during saline infusion. Then forearm blood flow response was measured during intrabrachial infusion of the endothelium-dependent vasodilator acetylcholine (ACh; 25, 50, 100 nmol/min; each dose given for 3 minutes). L-arginine was infused via the brachial artery (50 μmol/ min) to achieve a predicted
local concentration of approximately 2-4 mM (252). Radial FMD and the dilator response to ACh was determined in the presence of local L-arginine.

Figure 4.1 Schematic representation of the experimental setup for protocol 4.3 to examine the effect of a local intrarterial infusion of L-arginine on endothelial function of conduit and resistance arteries in patients with predialysis renal failure.

4.2.4 Biochemical measurements

Blood samples were taken before each study for analysis of total fasting cholesterol, creatinine, potassium and glucose. L-arginine and ADMA were measured by reverse phase high-pressure liquid chromatography (HPLC) before and 20 minutes after completion of the intravenous L-arginine infusion (protocol 4.2) as previously described (see section 2.20 (274)).

4.2.5 Calculations and statistics

For conduit vessel responses, the flow stimulus generated by reactive hyperaemia was measured 5 seconds after cuff release and subsequently at 15-second intervals for 90
seconds. Dilatation was expressed as the percentage change from baseline diameter of the brachial or radial artery (mean ±SEM) and analyzed by comparing both the maximal dilatation (mean of three consecutive observations) and also the area under the curve of dilatation against time (AUC; units %/(t)) as previously described (see section 2.1.1.2). FMD and GTN dilatation were compared before and after L-arginine infusion. Using venous occlusion plethysmography for resistance vessel studies, the dilator response to each dose of ACh was derived from the absolute blood flow in the infused arm, as described previously (156). Data were compared using two tailed students t test or by analysis of variance as appropriate. A P value <0.05 was considered significant (Graph Pad Prism statistical package).

4.3 Results

The study population characteristics are outlined in table 4.1. In each group baseline arterial diameter, blood pressure, and heart rate were similar before, during and after drug infusions (data not shown). No studies were excluded because of technical inadequacy.
Table 4.1 Subject characteristics

<table>
<thead>
<tr>
<th>Mean (range)</th>
<th>Hemodialysis</th>
<th>Pre Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 2</td>
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<td>N=8</td>
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<tr>
<td>Age (years)</td>
<td>42±4</td>
<td>39±7</td>
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<td>MAP (mmHg)</td>
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<tr>
<td>Pulse (BPM)</td>
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<td>76±8</td>
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<td>Baseline arterial diameter (mm)</td>
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</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.4±0.2</td>
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</tr>
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<td>Creatinine clearance mls/min</td>
<td>ESRF</td>
<td>14±4</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
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<td>5.6±0.5</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>5.0±0.1</td>
<td>4.6±0.1</td>
</tr>
</tbody>
</table>

4.3.1 The effect of systemic L-arginine on plasma biochemistry

Intravenous infusion of 10 g of L-arginine increased the plasma concentration four-fold (102.6±14.8 µmol/l before and 405.4±34.2 µmol/l after; n=18; P=0.0001) but did not affect ADMA (0.89±0.12 µmol/l before and 0.99±0.05 µmol/l after; n=18; P=0.4) see figure 4.2.
Figure 4.2 Demonstrates a four-fold increase in plasma L-arginine concentrations after systemic infusion. Figure 4b) shows the effect of this infusion on the ratio of arginine to ADMA.

4.3.2 The effect of systemic L-arginine on radial artery FMD in the presence of NOS inhibition in healthy volunteers (protocol 4.1).

The flow stimulus was unaffected by LNMMA or L-arginine infusion (figure 4.3a). FMD of the radial artery was reduced by LNMMA (6.8±1.6% before and 3.4±0.9% after LNMMA; n=6; P<0.01). L-arginine (10 g intravenously) fully restored FMD in the presence of LNMMA (6.2±1.3%; n=6; P>0.05; fig 4.3b).
Figure 4.3 Competitive inhibition of NOS by L-arginine analogues is reversible in healthy volunteers.

Neither L-arginine nor LNMMA had any effect on arterial blood flow (fig 4.3a). FMD was reduced by LNMMA (P<0.01 by ANOVA) and restored by intravenous infusion of L-arginine (P>0.05 by ANOVA, fig 4.3b)
4.3.3 The effect of systemic L-arginine on brachial artery FMD in patients with dialysis-dependent renal failure (protocol 4.2)

The flow stimulus generated during reactive hyperaemia before and after systemic L-arginine was similar (peak VTI (m) before 0.27±0.03 and 0.26±0.03 after; n=18; P=0.5; and the flow envelope over 90 seconds were similar; n=18; ANOVA P=0.1; fig 4.4). FMD did not increase after L-arginine infusion (4.1±1.1% before and 3.0±1.2% after; n=18; P=0.07; fig 4.5a). Similarly, the area under the dilatation time curve before and after intravenous L-arginine infusion remained unchanged (513.2±89.8 %s before and 478.1±109.6 %s after; n=18; P=0.6; fig 4.5b). Endothelium-independent dilatation in response to GTN was not affected by L-arginine infusion (5.3±1.0% before and 4.5±0.8% after; P= 0.2; fig 4.5a).

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**Figure 4.4** Demonstrates the flow stimulus before and after systemic infusion of L-arginine.
Figure 4.5 The effect of systemic L-arginine on endothelium dependent and independent arterial dilator function. (For clarity standard error bars have been omitted; data represents the whole population.)
4.3.3 The effect of local L-arginine on radial artery FMD in pre-dialysis renal failure (protocol 3)

The flow stimulus generated during reactive hyperaemia was similar before and after L-arginine infusion (peak VTI 0.20±0.02m before and 0.21±0.03m after; n=8; P>0.5; and the flow envelopes over 90 seconds were similar; n=8; ANOVA; P>0.5). Radial FMD remained unchanged after intra-arterial L-arginine infusion (6.5±1.2% before and 6.3±0.8% after; n=8; P>0.5; fig 4.6).

Figure 4.6

![Figure 4.6 The effect of intrabrachial infusion of L-arginine on vascular function in subjects with pre-dialysis renal failure. Intrabrachial L-arginine did not improve radial flow-mediated dilatation.](image-url)
4.3.4 The effect of local L-arginine on resistance artery endothelial function in pre-dialysis renal failure

Intrarterial L-arginine had no effect on baseline forearm blood flow measured by venous occlusion plethysmography. Mean baseline blood flow was 5.2±1.3 ml/100ml forearm/minute before L-arginine and 5.5±1.0 ml/100ml forearm/minute after (P=0.1). After co-infusion with L-arginine the dilator response did not increase at any dose of Ach (P=0.4 by ANOVA, fig 4.7), nor was there any improvement in a summary measure of the dose response curve expressed as AUC (33.1 ± 6.4 units before and 31.6 ± 5.3 units after; n=8; P=0.9).

Figure 4.7 Intrabrachial L-arginine did not improve resistance vessel response to the endothelium dependent dilator acetylcholine

4.4 Discussion

Acute administration of L-arginine failed to improve arterial endothelial function in the conduit and resistance vasculature of patients with renal failure. In this series of
experiments, I have demonstrated that the dose of L-arginine used was sufficient to restore a normal arginine/ADMA ratio in subjects with chronic renal failure and to overcome the effect of acute NOS inhibition in healthy volunteers. Despite this, L-arginine failed to improve FMD of the brachial artery in patients on dialysis. Similarly, 10 fold higher concentrations of L-arginine, delivered locally (intra-arterial experiments), had no effect on radial FMD or resistance vessel response to acetylcholine in patients with pre-dialysis renal failure. These observations suggest that competitive inhibition of NOS by circulating toxins alone does not determine endothelium-dependent dilatation in patients with renal failure.

Endothelial dysfunction, assessed by measuring flow-mediated dilatation (FMD) of the brachial artery, has been described in patients with renal failure (227;230;231) as previously discussed (see section 1.3.3.4). FMD of conduit arteries is largely dependent on NO generation (259), and these studies implicate a reduction of NO synthesis or effectiveness in the vasculature of these patients. The generation of NO depends upon a continuing and renewable supply of the substrate, L-arginine. The intracellular concentration of L-arginine is far in excess of that needed to saturate fully eNOS, and additional L-arginine would not be expected to increase NO production. Nonetheless, L-arginine has been reported to augment NO production in healthy humans (293), in patients with hypercholesterolaemia, and in smokers (174;253;254). One explanation for this apparent paradox is that eNOS may be antagonized competitively by high levels of naturally occurring circulating metabolites. Elevated levels of ADMA have been demonstrated in individuals with hypercholesterolemia and other risk factor cohorts for vascular disease (291;294). In patients with renal failure, circulating endogenous L-arginine analogues, including ADMA, are present in the plasma at concentrations 4-8 fold in excess of those seen in subjects with normal renal function. This may account for
reduced NO generation due to competitive inhibition of eNOS resulting in a reduction of FMD.

If competitive inhibition of NOS were an important mechanism in the endothelial dilator dysfunction observed in subjects with renal failure one would anticipate an improvement of endothelium-dependent dilatation after administration of exogenous L-arginine. In the present study, L-arginine was infused systemically and an ADMA/L-arginine ratio comparable to that observed in healthy individuals was achieved (293). In healthy volunteers, this dose of L-arginine overcame the effects of experimental NOS inhibition with LNMMA but in patients with dialysis-dependent renal failure, conduit artery and resistance vessel dilator function did not improve. This implies that endothelium dependent dilatation is not determined in the short term by the provision of exogenous L-arginine in this patient group.

The intravenous L-arginine infusion achieved systemic plasma concentrations in the micromolar range. Higher systemic concentrations of arginine have been reported to induce hyperkalaemia and aggravate acidosis in patients with renal failure. For this reason the effects of millimolar concentrations of L-arginine in conduit and resistance arteries were studied using local intra-brachial infusion. Non-essential brachial artery cannulation in haemodialysis patients is undesirable and we therefore studied patients with severely impaired renal function who would shortly require dialysis. Despite millimolar concentrations of L-arginine, there was no increase in FMD of the radial artery, or dilatation of the resistance vasculature in response to acetylcholine.

The data presented in this chapter suggest that in the short term, NOS inhibitors, which compete with L-arginine, do not determine arterial endothelial dilator function of patients with renal failure. The results of this study are in contrast to those of Hand et al who demonstrated improved venodilator responses after local infusion of L-arginine. Why arteries and veins should behave differently is unclear. It may be an inherent
difference in the dominant dilator mechanisms in each vascular bed. These authors used dorsal hand vein diameter to study endothelial function, a technique prone to high experimental variation (251).

The mechanism of reduced NO-bioactivity in renal failure therefore remains unclear, but could still be secondary to irreversible competitive antagonism, reduced availability of co-factors involved in NO production (e.g. tetrahydrobiopterin (295)), or increased breakdown of NO. In addition, chronic NOS inhibition might have secondary effects (including increased oxidative stress and adhesion molecule expression) resulting in endothelial damage and accelerated atherosclerosis (148). These potential consequences of chronic NOS inhibition might not readily be reversed by acute administration of L-arginine. Therefore, our results do not exclude a beneficial effect on endothelial function of administration of L-arginine earlier in the disease process or in the long term. These possibilities need to be addressed by chronic L-arginine supplementation in patients with renal failure, or the development of strategies to specifically reduce ADMA concentration in vivo (296).

However, linking the results of chapter 3 with the present chapter, I conclude that the effect of dialysis, in causing a short-term improvement in endothelial function, is not explained solely by the removal of ADMA.
Chapter 5

The Effect of Acute Administration of the Antioxidant

Vitamin C on Arterial Endothelial Function
5.1 Introduction

Oxidative stress has been implicated in the pathogenesis of atherosclerosis associated with traditional cardiovascular risk factors (187-190;195;196;297;298). Oxidative stress results from the production of reactive oxygen species (ROS), which include superoxide, hydrogen peroxide and hydroxyl radicals. ROS may contribute to many of the pathogenic mechanisms of atherosclerosis, by causing endothelial damage, smooth muscle proliferation and immune cell activation (257). In patients with atherosclerosis, there is biochemical evidence to suggest that there is increased oxidative stress, which results from an altered balance of endogenous pro-and anti-oxidants. Consistent with a role for ROS in the vasculopathy of renal disease, these biochemical markers are accentuated in uraemia (299). Oxidative stress may cause endothelial dysfunction through inactivation of endothelium-derived vasodilators, (such as nitric oxide, NO) that are known to have atheroprotective properties (see section 1.3.1.1) The effect of antioxidants on endothelium-dependent dilatation has been used to assess the consequences of oxidative stress on vascular function (see Table 5.1) and vitamin C has been found to improve endothelial dilator function in patients at risk of atherosclerosis (194-196;297). Similarly, antioxidant treatment has been shown to improve endothelial function in renal transplant recipients (197) and to reduce overall mortality in patients with renal failure (95).
Table 5.1 Human experimental studies using vitamin C as an antioxidant acutely.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Failure</td>
<td>+</td>
<td>Ellis 2000 (185)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Hornig 1998 (186)</td>
</tr>
<tr>
<td>Coronary disease</td>
<td>+</td>
<td>Gokce 1999 (187)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Levine 1996 (188)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>+</td>
<td>Sherman 2000(193)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Duffy 2001 (189)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Solzbach 1997 (190)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Taddei 1998 (194)</td>
</tr>
<tr>
<td>Hyperhomocysteinaemia</td>
<td>+</td>
<td>Kanani 1999 (191)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Chambers 1999 (87)</td>
</tr>
<tr>
<td>Smokers</td>
<td>+/-</td>
<td>Raitakari 2000 (192)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Heitzer 1996 (300)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>+</td>
<td>Jeserich 1999 (298)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Ting 1997 (195)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>+</td>
<td>Timimi 1998 (196)</td>
</tr>
</tbody>
</table>

+  Increased dilator response
-  Reduced dilator response
+/-  Dilator response unchanged

In the present study, the hypothesis that oxidative stress contributes to the reduced NO bioactivity observed in renal failure was tested. Vitamin C was administered parenterally to patients with renal failure and the short-term effects on NO-dependent endothelial function in conduit and resistance arteries were assessed.
5.2 Methods

5.2.1 Subjects

The study population included 17 subjects (12 male) receiving haemodialysis for a minimum of three months, 33 subjects (19 male) with severe chronic renal failure not yet receiving renal replacement therapy (GFR<20 mls/min, estimated from plasma creatinine using the Cockcroft-Gault formula) and a control group of 8 healthy volunteers recruited from hospital staff. Patients were excluded if they were >70 years of age, were current smokers, or if they had diabetes or a fasting cholesterol >6 mmol/l (recognized independent risk factors for endothelial dysfunction (150;151;153;177). Nitrates were discontinued 48 hours before study and antioxidant vitamin supplements 4 weeks before study. All other regular medications were continued.

5.2.2 Assessment of Resistance Vessel Function

Mercury-in-silastic strain-gauge plethysmography was used to measure forearm blood flow (ml/100 ml of forearm/minute) in both arms as described previously (see section 2.1.2 and (156)). For each study, the brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works) inserted under local anesthesia (2 ml of 1% lignocaine). Drug or saline (sodium chloride 0.9% wt/vol) were infused continuously at 0.5 ml/min.
5.2.3 Assessment of Conduit Artery Endothelial Function

Endothelial function was determined by recording the dilator response of the brachial or radial artery to increased blood flow generated during reactive hyperemia of the downstream forearm (brachial artery studies) or hand (radial artery studies) as previously described (see section 2.1.1). Analysis was performed by an experienced vascular technician blinded to the subject and order and repeated by a second investigator in a random selection of one third of scans (studies where dilatation differed by >1% were deemed technically inadequate). Using pulsed wave Doppler, blood flow was recorded continuously throughout the study and was expressed as the velocity time integral (VTI; area under the blood velocity/time curve for a complete cardiac cycle) as previously described (see section 2.1.1.4). Endothelium-independent dilatation was assessed by measuring the dilator response to a submaximal dose of the nitric oxide donor, glyceryl trinitrate (GTN, 25 µg sublingually) (see section 2.1.1.6).

Protocol 5.1: The effect of intra-arterial vitamin C on resistance artery endothelial function in subjects with pre dialysis renal failure

Sixteen subjects (9 male) approaching end stage renal failure (calculated GFR< 20 ml/min) were recruited. Forearm blood flow responses were measured during intra-brachial infusion of the endothelium-dependent vasodilator acetylcholine (ACh; 25, 50, 100 nmol/min; each dose given for 3 minutes; n=16) followed 15 minutes later by the endothelium-independent dilator glyceryl trinitrate (GTN; 4, 8, 16 nmol/min; n=10; each dose for 5 minutes) or sodium nitroprusside (SNP; 4, 8, 16 nmol/min; n=6; each dose for 5 minutes). After a further 15 minutes (to allow baseline flow to be re-established), vitamin C (25 mg/min) was infused via the brachial artery and the dilator response to ACh and GTN or SNP were determined as above.
Protocol 5.2: The effect of intra-arterial vitamin C on resistance vessel endothelial function in healthy volunteers

The effect of intra-arterial vitamin C on endothelial function in healthy volunteers was determined. Responses to acetylcholine and GTN were measured before and after infusion of vitamin C (as in protocol 1).

 Protocol 5.3: The effect of NOS inhibition on endothelial function in the presence and absence of vitamin C

This protocol was designed to examine the effect of vitamin C on ACh-mediated dilatation in 8 pre-dialysis subjects before and after inhibition of NO synthase with N\(^G\)monomethyl-L-arginine (L-NMMA). Forearm blood flow responses to ACh (25, 50, 100 nmol/min) were determined. After 15 minutes washout, L-NMMA (4 \(\mu\)mol/min) was infused and 10 minutes later the dilator response to ACh was reassessed in the presence of L-NMMA. After a further 15 minutes, vitamin C (25 mg/min) was co-infused with L-NMMA for 10 minutes, after which the response to ACh was measured in the presence of both L-NMMA and vitamin C.

 Protocol 5.4: The effect of vitamin C on conduit artery endothelial function in subjects with predialysis renal failure

In 10 subjects, (6 male) the brachial artery was cannulated to allow infusion of vitamin C into the downstream radial artery. Radial FMD and radial dilatation to GTN were measured during saline infusion, and 15 minutes later in the presence of vitamin C (25 mg/min; pre-infused for 15 minutes).
Protocol 5.5: The effect of systemic vitamin C on conduit artery endothelial function in subjects with end stage renal failure

Because of ethical concerns about arterial cannulation of haemodialysis patients, vitamin C was administered by intravenous infusion and endothelial function assessed by measuring FMD of the brachial artery. FMD was assessed in 17 patients before and after administration of vitamin C in a randomized double blind placebo controlled crossover design. Patients were assigned to receive either vitamin C (3g in 100ml of 0.9% saline over 10 minutes) or placebo (100ml of 0.9% saline over 10 minutes) intravenously and crossed over to the other intervention on the second visit. Each patient was examined immediately before haemodialysis on two occasions 1 week apart. On each occasion, FMD and GTN-induced dilatation (separated by 5 minutes) were measured at baseline and 20 minutes after completion of the intravenous infusion. Blood pressure and pulse rate were recorded before and after each scan using the non-study arm or the calf (in the presence of a fistula) with an automated oscillometric system (Critikon, Dinamap vital signs monitor 1846 8X).

5.2.4 Biochemical measurements:

Baseline measures of total fasting cholesterol, lipid sub-fractions, urea, creatinine, potassium and CRP were made.
5.2.5 Measures of oxidant stress

Blood samples were taken before and during intra-arterial infusion of vitamin C from an ipsilateral antecubital vein (protocol 4) and before and 20 minutes after completion of the intravenous infusion of vitamin C or placebo (protocol 5) for assessment of oxidative stress (see section 2.3.3). EDTA and lithium-heparin samples were placed on ice immediately after sampling. Serum samples were allowed to clot at room temperature for 10 minutes. Samples were centrifuged within 30 minutes of collection at 4500 rpm, 4°C for 15 minutes. Duplicates were collected to assess analysis reproducibility. After individual preparation all samples were stored at -80°C. Assessment of total antioxidant capacity, plasma vitamin C concentration, whole blood and plasma total glutathione concentration and lipid hydroperoxides as previously described (see section 2.3.3).

5.2.6 Calculations and Statistics

Data are presented as mean ± SEM unless otherwise stated. For venous occlusion plethysmography studies (protocols 1-3), mean blood flow for the last 60 seconds of each infused dose was expressed as ml/100 ml forearm volume/min. For conduit vessel responses (protocols 4 and 5), flow stimulus during reactive hyperaemia was measured 5 seconds after cuff release and subsequently at 15-second intervals for 90 seconds. Dilatation was expressed as the percentage change from baseline diameter of the brachial or radial artery and analyzed by comparing both the maximal dilatation (mean of three consecutive observations) and the area under the curve of dilatation against time (AUC; units %t). Data were compared using two tailed students t test or analysis of variance of repeated measures as appropriate where P<0.05 was considered significant.
(Graph Pad Prism). The study had 90% power to detect intra-individual differences of 1% in FMD, based on the standard deviation observed in studies of repeated baseline measures within individuals (see section 2.1.1.8).

5.3 Results

The study population characteristics for the haemodialysis, predialysis and control groups are detailed in Table 5.2. The dialysis-dependent group were receiving an average of 1.6 anti-hypertensive agents (24% α blockers, 35% β blockers, 41% calcium channel blockers, 29% angiotensin converting enzyme inhibitors and 22% potassium channel openers) compared with an average of 1.4 antihypertensive agents in the predialysis group (28% α blockers, 39% β blockers, 37% calcium channel blockers, 24% angiotensin converting enzyme inhibitors and 19% potassium channel openers).

Table 5.2 Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>ESRF</th>
<th>PDRF</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>n(male)</td>
<td>17(9)</td>
<td>33(19)</td>
<td>8(5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37±3</td>
<td>39±4</td>
<td>41±4</td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>ESRF</td>
<td>17±6</td>
<td>110±4</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>171±9</td>
<td>148.8±9.8</td>
<td>137±6</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>84±4</td>
<td>81±5</td>
<td>79±6</td>
</tr>
<tr>
<td>MAP (mmhg)</td>
<td>113±6</td>
<td>103±9</td>
<td>101±6</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.1±0.2</td>
<td>4.0±0.3</td>
<td>4.2±0.3</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7±0.2</td>
<td>1.5±0.2</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2.5±0.1</td>
<td>2.7±0.3</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.2±0.07</td>
<td>1.1±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>CRP</td>
<td>12.1±4.8</td>
<td>8.2±1.5</td>
<td>4.8±1.2</td>
</tr>
<tr>
<td>Haemoglobin (gm/dl)</td>
<td>10.1±0.2</td>
<td>11.4±0.4</td>
<td>12.5±0.3</td>
</tr>
<tr>
<td>Potassium (μmol/l)</td>
<td>5.3±0.3</td>
<td>4.5±0.2</td>
<td>3.7±0.2</td>
</tr>
</tbody>
</table>

PDRF (Pre-dialysis Renal Failure)

ESRF (End Stage Renal Failure)
Blood pressure and pulse rate were unaffected by intra arterial or intravenous infusion of vitamin C (Table 5.3). Blood flow in the non-infused arm (plethysmography studies) did not change during the experiments (data not shown). Intra-arterial vitamin C caused a small increase in baseline forearm blood flow in all groups (protocol 1,2,3); this did not, however, reach statistical significance (Table 5.4). Baseline arterial diameters in both conduit artery studies (protocol 4&5) were unchanged by vitamin C infusion (Table 5.3).

**Table 5.3 The effect of parenteral vitamin C on basal cardiovascular measures**

<table>
<thead>
<tr>
<th></th>
<th>Pre-dialysis (radial artery)</th>
<th>ESRF (brachial artery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protocol 4</td>
<td>Protocol 5</td>
</tr>
<tr>
<td>Before intra-arterial vitamin C</td>
<td>105±2.7</td>
<td>105±2.4</td>
</tr>
<tr>
<td>After intra-arterial vitamin C</td>
<td>105±2.4</td>
<td>115±6</td>
</tr>
<tr>
<td>Before intravenous vitamin C</td>
<td>113±6</td>
<td>116±6</td>
</tr>
<tr>
<td>After intravenous vitamin C</td>
<td>116±6</td>
<td></td>
</tr>
</tbody>
</table>

| Mean arterial pressure (mmHg) | 70±4 | 68±4 | 79±4 | 78±3 |
| Pulse (BPM)                   | 2.6±0.1 | 2.6±0.1 | 4.1±0.2 | 4.1±0.2 |

**Table 5.4 The effect of intra-arterial vitamin C on basal forearm blood flow**

<table>
<thead>
<tr>
<th>Baseline forearm blood flow (ml/100ml forearm/minute)</th>
<th>Pre-dialysis (resistance vessels)</th>
<th>Pre vitamin C</th>
<th>Post vitamin C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1</td>
<td>3.6±1.1</td>
<td>4.2±1.0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Protocol 2</td>
<td>3.9±0.7</td>
<td>4.7±0.9</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Protocol 3 (LNMMA)</td>
<td>1.9±0.3</td>
<td>2.2±0.8</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

5.3.1 Effect of vitamin C on resistance vessel endothelial function

(predialysis subjects and healthy volunteers)

In patients with pre-dialysis renal failure, Vitamin C increased the dilator response to ACh (fig 5.1a; P=0.01; n=16), but did not alter the response to GTN (fig 5.1b; P=0.6;
n=10) or SNP (fig 5.1c; P=0.3; n=6). In contrast, vitamin C had no effect on the dilator response to ACh (fig 5.2a; P=0.7; n=8), or GTN (fig 5.2b; P=0.9; n=8) in healthy volunteers.

Fig 5.1a)

![Graph showing the effect of vitamin C on ACh response](image)

**ANOVA P=0.01**

---

**Baseline** 25 50 100

**ACh (nmol/min)**

---

**Fig 5.1b)**

![Graph showing the effect of vitamin C on GTN response](image)

**ANOVA P=0.6**

---

**Baseline** 4 8 16

**GTN (nmol/min)**

---
Figure 5.1 The effect of intra-arterial infusion of vitamin C on arterial dilator function in subjects with pre dialysis renal failure. Resistance vessel responses to the endothelium-dependent dilator acetylcholine (fig 5.1a), endothelium-independent dilators GTN (fig 5.1b) and SNP (fig 5.1c).

Fig 5.2a)
Figure 5.2 The effect of vitamin C on vascular function in healthy volunteers. Fig 5.2a) demonstrates the effect of intra-arterial vitamin C on resistance vessel responses to the endothelium dependent-dilator acetylcholine. Fig 5.2b) demonstrates the effect of intra-arterial vitamin C on the endothelium independent-dilator GTN.

L-NMMA reduced basal forearm blood flow in pre-dialysis patients (3.0±0.48 ml/100ml forearm/minute before vs. 1.3±0.36 after L-NMMA; P=0.03; n=8) and the dilatation to ACh (fig 5.3; P=0.002 by ANOVA). In the presence of L-NMMA, infusion of vitamin C did not increase the dilator response to ACh (fig 5.3; P>0.05; n=8).
5.3.2 Effect of vitamin C on radial artery endothelial function

In patients with pre-dialysis renal failure, intra-arterial vitamin C did not alter the flow stimulus during reactive hyperaemia of the hand (peak VTI (m) before 0.24±0.1 vs. 0.25±0.1 after vitamin C; P=0.4; flow envelope before 12.9±1.5 ms vs. 12.7±1.7 ms after vitamin C; P=0.6; n=10, fig 5.4a). Vitamin C did not increase radial FMD (6.1±0.8% before and 6.0±1.0% after vitamin C; n=10; ANOVA P=0.8; fig 5.4b) or the area under the dilatation time curve (AUC 640.0±33.1%s before vs. 639.5±39.0 %s after vitamin C; P=0.8; n=10; fig 5.4c). Similarly, the response to GTN was unaffected by vitamin C infusion (8.1±1.5% before vs. 8.5±0.4% after vitamin C; P=0.6; fig 5.4b).
Fig 5.4a)

![Graph showing VTI (m) vs Time (seconds) with ANOVA P=0.6. The graph compares Pre Vitamin C and Post Vitamin C conditions.]

Fig 5.4b)

![Bar chart showing Dilatation (%) with P-values for FMD Pre, FMD Post, GTN Pre, and GTN Post. P=0.8 for FMD and P=0.6 for GTN.]

(caption follows)
5.3.3 Effect of vitamin C on brachial artery endothelial function in patients with dialysis-dependent renal failure

Intravenous vitamin C did not alter the flow stimulus during reactive hyperaemia of the forearm (peak VTI (m) before 0.28±0.02 vs. 0.28±0.03 after vitamin C; P=0.9; flow envelope 14.2±1.2 before and 13.5±1.2 after vitamin C; ANOVA P=0.5; n=17, fig 5.5a). The flow stimuli before vs. after placebo were also similar (peak VTI (m) before 0.32±0.02 vs. 0.34±0.03 after; P=0.1, flow envelope before 15.1±1.2 ms vs. 15.1±1.2 ms after vitamin C; ANOVA P=0.9; n=17, fig 5.5b).
FMD of the brachial artery was unaffected by vitamin C (4.3±0.9% before vs. 3.7±1.0% after vitamin C; P=0.3; n=17; fig 5.5c) or placebo (4.0±1.1% before vs. 3.3±0.9% after; P=0.2; n=17; fig 5.5c). Similarly, the area under the dilatation time curve before and after intravenous vitamin C or placebo remained unchanged (587.4±108.4 %s before vs. 500.1±89.6 %s after vitamin C; P=0.2; n=17; fig 5.5d; 594.2±128.5 %s before vs. 420.1± 71.4 %s after placebo; P=0.2; n=17; fig 5.5e; ANOVA of 4 groups P=0.4).

Dilator response to GTN was also unaffected by either vitamin C or placebo (5.7±1.4% before vs. 5.6±1.4% after vitamin C; 6.5±1.5% before vs. 6.2±1.3% after placebo; ANOVA of 4 groups P=0.5; fig 5.5f).

Fig 5.5a)
Fig 5.5b)

ANOVA P=0.8

- Pre Placebo
- Post Placebo

Fig 5.5c)

ANOVA P=0.3

<table>
<thead>
<tr>
<th></th>
<th>FMD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Vit C</td>
<td>4</td>
</tr>
<tr>
<td>Post Vit C</td>
<td>4</td>
</tr>
<tr>
<td>Pre Placebo</td>
<td>4</td>
</tr>
<tr>
<td>Post Placebo</td>
<td>4</td>
</tr>
</tbody>
</table>
Fig 5.5d)

- Before Vitamin C
- After Vitamin C

P=0.2

Fig 5.5e)

- Before Placebo
- After Placebo

P=0.2
5.3.4 The effect of vitamin C on markers of oxidative stress

Intrarterial infusion of 25 mg/min vitamin C resulted in a 100-fold increase in plasma vitamin C in the venous effluent of the infused arm (25.6±3.3 μmol/l before vs. 2910.5±303.9 μmol/l after; n=10; P=0.00001; table 5.5) and total antioxidant capacity (459.5±34.1 μmol/l before vs. 5582.1 ±495.5 μmol/l Trolox Eq after; P<0.0001; n=10; table 5.5). Whole blood glutathione levels were, however, unaffected (533.3±74.3 μmol/l before and 532.2±75.4 μmol/l after; P=0.6; n=10; table 5.5).
Intravenous infusion of 3g of vitamin C resulted in an approximately 4.5 fold increase in plasma concentration (44.1±4.5 μmol/l before vs. 201±20.1 μmol/l after; n=17; P<0.0001; table 5.6). There were similar increases in total antioxidant capacity (496.4±20.4μmol/L before vs. 1223.5±75.9 μmol/l Trolox Eq after vitamin C; n=17; P<0.0001; table 5.6). Whole blood and plasma glutathione levels were unaffected by intravenous vitamin C, (whole blood oxidized glutathione: 404.8±25.8 μmol/l before vs. 391.3±77.1 μmol/l after; n=17; P=0.5; table 5.6) (plasma oxidized glutathione: 0.31±0.19μmol/L before and 0.24±0.08 μmol/l after; n=17; P=0.2; table 5.6). In contrast, vitamin C increased lipid hydroperoxides (11.0±1.7 μmol/l before vs. 14.7±1.8 μmol/l after vitamin C; P=0.01; n=17; table 5.6). These indices of oxidant stress were unaffected by placebo (table 5.6).

*Table 5.5 The effect of parenteral vitamin C on biochemical measures of oxidant stress in predialysis patients*

<table>
<thead>
<tr>
<th>Predialysis renal failure (protocol 5.1, 5.3, 5.4)</th>
<th>Pre- Vitamin C</th>
<th>Post- vitamin C</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Antioxidant capacity TAC (μmol/l, Trolox eq)</td>
<td>459.5±34.1</td>
<td>5582.1±495.5</td>
<td>5x10^6</td>
</tr>
<tr>
<td>Vitamin C (μmol/l)</td>
<td>25.6±3.3</td>
<td>2910.5±303.9</td>
<td>10^5</td>
</tr>
<tr>
<td>Whole blood glutathione (pmol/ml)</td>
<td>533.3±74.3</td>
<td>532.2±75.4</td>
<td>6x10^1</td>
</tr>
</tbody>
</table>
Table 5.6 The effect of parenteral vitamin C on biochemical measures of oxidant stress in haemodialysis patients.

<table>
<thead>
<tr>
<th></th>
<th>Intravenous Vitamin C, ESRF n=17 (protocol 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Vitamin C</td>
</tr>
<tr>
<td>Lipid hydroperoxides (μmol/l)</td>
<td>11.0±1.7</td>
</tr>
<tr>
<td>Total Antioxidant capacity TAC (μmol/l, Trolox eq)</td>
<td>496.4±28.4</td>
</tr>
<tr>
<td>Vitamin C (μmol/l)</td>
<td>44.1±4.5</td>
</tr>
<tr>
<td>Plasma glutathione (pmol/ml)</td>
<td>0.31±0.19</td>
</tr>
<tr>
<td>Whole blood glutathione (pmol/ml)</td>
<td>404.8±25.8</td>
</tr>
</tbody>
</table>
5.4 Discussion

This study demonstrates that acute administration of vitamin C increased endothelium-dependent dilatation in the resistance vasculature of patients with pre-dialysis renal failure. This effect is NO-dependent because it disappeared in the presence of the NOS inhibitor L-NMMA. In contrast, vitamin C did not alter endothelium-dependent dilatation in peripheral conduit vessels of either pre-dialysis patients or patients with dialysis-dependent renal failure. Vitamin C had no effect on endothelium-independent dilatation to GTN or SNP. These observations suggest that, in renal failure, oxidative stress is a short-term determinant of resistance (but not conduit) artery endothelial function through modulation of the activity of the NO pathway.

Endothelial dysfunction has been consistently demonstrated in conduit vessels of patients with renal failure (227;228;230;231;231;301), with a smaller defect seen in the resistance vasculature (223;225). Increased oxidative stress may contribute to endothelial dysfunction in these patients. Sources of oxidative stress in renal failure include hyperhomocysteinaemia (85), anaemia (and treatment with intravenous iron or erythropoietin (99)) and dialysis membrane bio-incompatibility (causing recurrent leukocyte activation), (see section 1.2.2.5). In addition to increased production of ROS, antioxidant defence systems are deficient in renal disease, with low plasma concentrations of antioxidant vitamins (due to low dietary intake and increased plasma clearance during dialysis) (302), reduced glutathione (303), and reduced activity of the anti-oxidant enzymes superoxide dismutase, catalase and glutathione peroxidase (100). Consistent with a role for oxidative stress in endothelial dysfunction and increased cardiovascular risk in renal failure (304), administration of anti-oxidants in
haemodialysis patients with pre-existing cardiovascular disease improves cardiovascular outcome (95).

Vitamin C has been widely used as an experimental tool in the investigation of the functional consequences of oxidant stress in the human cardiovascular system. In the present study, administration of vitamin C into the forearm of pre-dialysis patients increased dilatation to the endothelium-dependent dilator ACh, but had no effect on the response to either GTN or SNP indicating a specific effect on the vascular endothelium. L-NMMA reduced dilatation to ACh in patients with pre-dialysis renal failure, implicating NO in the dilator mechanism of ACh in patients with renal failure. Furthermore, L-NMMA abolished the effect of vitamin C to augment the response to ACh, indicating that its action is NO-dependent. This finding suggests that the action of vitamin C is to promote the bioactivity of endogenous NO rather than an action on other endothelium-derived mediators (such as prostacyclin or EDHF). These results are consistent with studies in other cardiovascular risk groups in which infusions of vitamin C have increased dilatation of the resistance vasculature to ACh (193;195;196). Vitamin C had no effect in healthy controls, which implies that under physiological conditions, oxidative stress has little role in determining NO bioavailability (either NO synthesis and release, or stability of NO post-synthesis).

In contrast, vitamin C (despite 100-fold elevations in local concentration) had little effect on conduit artery function in either dialysis or predialysis patients despite the presence of high levels of oxidative stress in this group (303;305). In dialysis patients, we chose to administer vitamin C intravenously using a protocol that has been demonstrated both to reduce measures of oxidative stress and improve conduit artery endothelial function (185), in hyperhomocysteinaemia (87), hypertension (190) and hypercholesterolaemia (195).
It is not clear why conduit and resistance vessels should differ in their response to vitamin C. Anti-oxidants including vitamin C, may also have pro-oxidant properties (306-308), particularly in the presence of transition metal ions. This interaction between high doses of ascorbic acid and redox active metal ions such as iron might offset any effect to promote NO activity, and this balance might be different in conduit and resistance vessels. Our biochemical measures of oxidative stress indicated a complex picture after administration of vitamin C. Total anti-oxidant activity and vitamin C concentration were elevated after vitamin C administration, whole blood and plasma glutathione levels were unaffected, but lipid hydroperoxides increased. Whether this accounts for the disparity between the effects of vitamin C in conduit and resistance vessels or not remains to be determined. It is possible that the apparent increase in lipid hydroperoxides resulted from a non-specific effect that relates to the high concentrations of vitamin C in the FOX 2 assay.

The mechanism by which Vitamin C improves vascular endothelial function or cardiovascular outcome remains contentious. Vitamin C might increase NO bioactivity by a variety of mechanisms, including increased NO production (secondary to increased provision of tetrahydrobiopterin, a cofactor for NO synthesis (309-311)), release of NO from endogenous NO-thiols, or protection of NO from premature deactivation by combination with superoxide. In addition, vitamin C might improve endothelial function by limiting the oxidation of LDL and preserving intracellular glutathione (312). There is, however, other evidence of a dissociation between the effect of vitamin C on oxidative stress and vascular function. In a study of 55 subjects with congestive cardiac failure, the lack of correlation between improvement in endothelial function and oxidative stress after intervention with vitamin C implied possible additional non-antioxidant effects of vitamin C (185). The effect of dose and duration of treatment with antioxidants may also have an impact on vascular endothelial function. Sherman et al.
demonstrated that, in hypertensives, the beneficial effects of intra-arterial vitamin C on resistance vasculature was only elicited at supraphysiological doses (193). In smokers, Raitikari et al. demonstrated an initial acute improvement in endothelial dilator function in response to vitamin C, which was not sustained after 8 weeks of chronic oral therapy despite a persistent increase in both vitamin C levels and antioxidant capacity (192).

5.5 Conclusions

In conclusion, these results show that acute administration of vitamin C reduces the levels of oxidant stress in renal failure and improves NO-mediated resistance vessel dilatation. Endothelium-dependent dilatation of the conduit vasculature was, however, unaffected. This is in keeping with the results of intervention trials with anti-oxidants in cardiovascular risk groups which have produced inconsistent evidence of reduction in cardiovascular events related to large vessel atherosclerosis (92;94). The beneficial effects of antioxidant therapy are likely to be most marked in conditions associated with high oxidant stress such as renal failure (97). These results suggest a need for further studies to characterize the effects of different anti-oxidant strategies in this condition.
Chapter 6

Conclusions
6.1 Introduction

Since NO inhibits key processes in atherogenesis, any reduction in the availability of NO might tend to promote the development of atherosclerosis. Indeed, clinical studies have confirmed impaired NO bioactivity in patients with atherogenic risk factors, established atherosclerosis and renal failure. Dysfunction of the endothelial NOS pathway may be due to impaired signal transduction, reduced e-NOS activity, reduced intracellular availability of the substrate L-arginine, increased inactivation of NO by free radicals, or a combination of these mechanisms. In these studies, I have used strategies aimed at improving endothelial function, including acute removal of L-arginine analogues during conventional dialysis, exogenous administration of L-arginine, and the use of antioxidants.

6.2 Manipulating Circulating Uraemic Toxins and the Role of Competitive Inhibition on the Production of NO in Chronic Renal Failure

In the first series of experiments (chapter 3), I demonstrated that HD reduces the concentration of circulating uraemic toxins, and that this is associated with an acute improvement in endothelial function. This effect is lost within 24 hours of completing a haemodialysis treatment. In contrast, APD is not associated with either acute reduction in uraemic toxins or improvement in endothelial function, but does appear to have a beneficial effect on background endothelial function. Although these studies were inadequate to prove this point conclusively, this observation is in line with published data. What is evident from this study is that HD is associated with greater swings in dialysable endothelial toxins and endothelial function than APD.
To establish whether these observations resulted from competitive inhibition of NOS by L-arginine adducts, I performed a second group of experiments aimed at normalising, or indeed exceeding, the ratio of L-arginine to its methyl analogues present in plasma. This strategy failed to reverse endothelial dysfunction in either conduit or resistance arteries in subjects with varying degrees of renal impairment despite employing a broad concentration range for L-arginine. I concluded that the mechanism for impaired endothelial dysfunction is not simply the result of competitive inhibition of NOS by L-arginine adducts.

6.3 Areas of Uncertainty that Arise from the Work on Arginine and its Methyl Analogues

Haemodialysis is associated with an acute improvement in endothelial-dependent vasodilatation that is unlikely to be related to acute alterations in plasma volume or haematocrit. It therefore follows that there may be a dialysable molecule that accumulates in renal failure, and is removed by the process of HD. In support of this hypothesis, Bradley et al. demonstrated that dialysis-induced hypotension is not observed during ultrafiltration alone, suggesting that there may be a circulating vasoactive agent removed during HD (313). Although ADMA and homocysteine seemed likely candidates, the absence of any correlation between the plasma concentration of these substances and FMD in these studies suggests that they do not play an important role in the acute variations observed.

The failure to improve endothelial function after L-arginine supplementation could be due to the compartmentalisation of NOS in intracellular caveolae such that acute changes in plasma arginine are not rapidly conveyed to the site of enzyme activity. Alternatively, as yet unidentified analogues of L-arginine may bind irreversibly to NOS
in uraemia and prevent the rapid reversal of inhibition as tested in this experimental protocol. These acute studies therefore do not exclude a beneficial effect of long-term arginine supplementation in this patient group.

Finally, these experiments were limited to subjects with advanced renal impairment, and it remains possible that similar studies conducted at an earlier stage in the natural history of this disease might have a different outcome.

6.4 The Role of Oxidant Stress in Uraemic Endothelial Dysfunction

In the final group of experiments, I have shown that vitamin C is effective in reversing the imbalance between pro and antioxidants in subjects with renal failure. This biochemical effect is associated with improved endothelial cell function in resistance, but not conduit arteries. This suggests that endothelial dysfunction of resistance vessels is, at least in part, mediated by increased oxidant stress. The dependence of this phenomenon upon NO rather than another endothelium-derived mediator, is supported by the observation that this amelioration is abolished by the co-infusion of the NO inhibitor, L-NMMA.

6.5 Areas of Uncertainty that Arise from the Work on Antioxidants in Renal Failure

Vitamin C improved endothelial dependent vaso-dilatation in resistance, but not conduit vessels. Reasons for this discrepancy may relate to the differences in the biology of arteries of varying calibre. Although similar in morphology, conduit and resistance vessels differ importantly in size, function and local environment. There are precedents
for different vascular beds to respond distinctly to manipulation of the NOS pathway. In animal studies, whereas conduit arteries develop hypertrophy after chronic experimental NOS blockade, resistance arteries remodel without hypertrophy (314). Dilator responses to ACh were found to be impaired in conduit arteries, but preserved in resistance arteries in deoxycorticosterone-salt hypertensive rats (315), and the major mediator of endothelium-dependent vasodilatation differed between conduit and resistance vessels. Similarly, in humans, Houghton et al. has demonstrated heterogeneity in the response to ACh in epicardial and resistance coronary vasculature in hypertensive subjects with chest pain (316).

The exact mechanism by which vitamin C improves dilator function is not established by this study, and there are a number of possibilities. Ascorbic acid has been shown to improve impaired endothelium-dependent vasodilation in patients with atherosclerosis. The mechanism for this may involve the protection of NO from inactivation by free oxygen radicals. The activity of NOS is highly sensitive to the local concentration of various free radicals capable of premature denaturation, and measures designed to minimise free radical concentrations, such as addition of superoxide-dismutase, are associated with increased NO bioactivity. Consistent with this, ascorbic acid also scavenges superoxide. However, many of the clinical studies in high risk factor groups achieve extracellular concentrations of vitamin C that are inadequate (~100 μM) pharmacokinetically to account for the increased bioavailability of NO observed (317). This suggests that another mechanism, distinct from its quenching effect, may be responsible. Intracellular vitamin C enhances NO synthesis in endothelial cells by an increase in the Vmax of NOS with no effect on L-arginine binding (Km), suggesting that the mechanism for increased NO bioavailability is independent of both L-arginine and quenching by free radicals (309;310). These authors postulated that the vitamin C effect was mediated by increased intracellular tetrahydrobiopterin.
To complicate matters further, there is some evidence that administration of vitamin C may have a paradoxical pro-oxidant effect (306-308), particularly in the presence of transition metal ions. Consistent with this, in this study I observed elevated lipid hydroperoxides and no alteration in intracellular or plasma levels of glutathione after infusion of supraphysiological doses of vitamin C.

This study does not exclude the possibility that the chronic application of antioxidants may improve conduit artery endothelial function nor does it provide information about the possible long term beneficial effect of antioxidant therapy in this patient group, though the experimental observations are consistent with the interventional trial data available (95).

6.6 Future Work

6.6.1 Chronic supplementation studies

To assess the effects of chronic supplementation on vascular endothelial function, a randomised double blind crossover trial of chronic oral L-arginine or antioxidants with a suitable washout period in between study periods is required.

6.6.2 The effect of tetrahydrobiopterin on endothelial function in chronic renal failure

Tetrahydrobiopterin (BH₄) is a critical cofactor for the activity of NOS, which is involved in the binding of the enzyme to its physiological substrate, L-arginine. BH₄ is highly redox reactive with both anti-oxidant and pro-oxidant properties. In the absence
of BH₄, NOS transfers electrons to molecular oxygen resulting in the formation of superoxide anions (318). Vitamin C is capable of affecting cellular NO synthesis directly through a tetrahydrobiopterin-dependent mechanism. (309). These authors suggested that NO formation was influenced by the intracellular concentration of NOS co-factors such as FAD, calmodulin, FMN and BH₄, and demonstrated that the vitamin C effect was abolished in the absence of BH₄. These data suggest that ascorbic acid acts, at least in part, through an effect on the availability of BH₄, or by enhancing the affinity of this cofactor for e-NOS. In certain cardiovascular risk groups, for example hypercholesterolaemia, diabetes mellitus, and smokers, BH₄ levels are reduced, and provision of exogenous BH₄ is associated with improved endothelial function (175) (319). To date, no studies have been carried out looking at either plasma levels or the effect of BH₄ on endothelial function in chronic renal failure. This may be a fruitful area of future work.

6.6.3 Endothelial function and cardiovascular outcome studies

Cardiovascular interventions that have a beneficial effect on cardiovascular outcome in the general population such as antihypertensives (159), lipid lowering, and ACE inhibitors (320;321) are in turn associated with improved endothelial function. A relationship has also been established between vascular endothelial dysfunction and cardiovascular morbidity and mortality for both resistance (198) and conduit vessels (264). Beyond these associations, however, it has been difficult to establish a causal link between endothelial dysfunction and cardiovascular outcome. There is a paucity of information about the relationship between impaired coronary / brachial artery endothelial function and cardiovascular outcome. The data that exist suggests that this
surrogate marker of early vascular disease could prove to be a useful predictor of coronary events (198-200).

The identification of a clear association between a specific measure or measures of endothelial function and eventual cardiovascular outcome could allow the development of a clinically useful diagnostic and prognostic tool to test the effectiveness of clinical interventions earlier in the natural history of cardiovascular disease. However, to establish this relationship, a large study is required, where vascular function is described accurately in a specific patient group who can then be followed until there is a cardiovascular outcome. Given their high event rate, subjects with renal failure are an ideal patient population for this kind of study.
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