The Vascular Endothelium in Chronic Renal Failure

by

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Contents

List of Figures

List of Tables

Table 6.5 Plethysmography : Background characteristics for CsA patients and controls

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Declaration

The experimental design of the work presented in this thesis was that of the author and his supervisors, Dr Alan Jardine and Professor John McMurray. All experimental work was performed by the author except for measurement of homocysteine concentrations (performed by the biochemistry department at Gartnavel General Hospital) and measurement of renin, angiotensin and noradrenaline concentrations (performed by Dr JJ Morton).

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

Scott Morris

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Summary

Patients with chronic renal failure (CRF) have a 20-fold increased risk of cardiovascular death compared to the general population, with sudden death, cardiac failure and atherosclerotic vascular disease being the commonest underlying problems. For many years it has been postulated that accelerated atherosclerosis occurs in uraemia and that this may be secondary to a multitude of "traditional" cardiovascular risk factors including dyslipidaemia, hypertension, smoking and diabetes mellitus, all of which are prevalent in this population. Other authors have suggested that "non-traditional" risk factors are more important including accumulation of endogenous inhibitors of nitric oxide synthase and homocysteine, hyperparathyroidism, increased oxidative stress and other, as yet unidentified, uraemic factors. Whatever the mechanism, these risk factors are likely to act by causing endothelial damage and dysfunction.

The vascular endothelium lies at the interface between the blood and tissues and has several important roles which together prevent atherosclerosis : control of vessel tone, inhibition of platelet aggregation, control of the passage of cells and macromolecules, inhibition of vascular smooth muscle cell proliferation and inhibition of monocyte adhesion and migration. The majority of these anti-atherogenic effects are mediated by endothelium-derived nitric oxide. Dysfunction of the vascular endothelium is thought to be a key initial event in the development of atheroslcerosis and has been demonstrated in a number of conditions/risk factors including diabetes, hypertension,

smoking, dyslipidaemia and ischaemic heart disease. Few studies have examined endothelial function in chronic renal failure.

The work presented in this thesis was performed to examine endothelial function in patients with CRF and in particular to examine endothelium-dependent vasodilatation as this is the easiest aspect of endothelial function to study in humans. Using the *in vivo* method of forearm venous occlusion plethysmography, endothelium-dependent (EDV) and endothelium-independent (EIDV) vasodilatation was assessed in 16 uraemic patients (pre-dialysis and on CAPD) and 18 controls. The order of infusion of drugs (SNP for EIDV and carbachol for EDV) was randomised. Overall there were no differences between the groups but it was apparent that infusion of SNP as the first agent was having a prolonged effect. Thus when 10 patients and controls were examined, in whom carbachol was infused followed by SNP, EIDV was similar but EDV to carbachol was attenuated in the uraemic patients. This pattern suggested endothelial dysfunction in the uraemic patients, with the most likely defect being impaired endothelial NO production, release or effect. No correlates were found in the background laboratory or blood pressure data to predict the response to these agents.

Since many authors have postulated that the most important cause of endothelial dysfunction in uraemia is likely to be the accumulation of circulating factors such as asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, a second *in vitro* study was performed to further examine endothelial function in CRF. Utilising wire myography, subcutaneous resistance artery function was assessed in 12 uraemic patients and 8 controls, with vessels removed from the uraemic milieu. This study again revealed a significantly attenuated EDV in the uraemic group with normal

EIDV to SNP, and the results therefore suggest that endothelial dysfunction in CRF is not readily reversible and is not entirely secondary to a short-lived circulating factor. The study did not completely rule out an effect of ADMA, however, as this agent may accumulate within cells. Together, both studies confirm the presence of endothelial dysfunction in uraemia with the underlying mechanism being unclear. In this myography study, responses to the vasoconstrictors endothelin-1 and noradrenaline were also assessed. Both agents had a tendency to a greater and more long-lasting effect in the uraemic group compared to controls, perhaps reflecting reduced counterbalancing endothelial NO production or increased vessel wall smooth muscle.

As blood pressure in uraemic patients on renal replacement therapy is usually dependent on blood volume a further small study was undertaken to examine the effects of altered fluid balance on endothelial responses in CAPD patients. Recruitment for this study proved difficult and results are presented for just 3 patients. Endothelium-dependent vasodilatation and EIDV were assessed using forearm strain-gauge plethysmography before and after a 2kg weight gain. Results were conflicting and no clear pattern was observed. A larger study is required to assess the hypothesis that increased blood volume is associated with reduced endothelium-dependent vasodilatation and consequent hypertension.

Since the increased cardiovascular risk of patients on dialysis persists following renal transplantation, a final study was performed to examine endothelial function in renal transplant recipients. Using forearm venous plethysmography, EIDV and EDV were assessed in 16 patients with stable renal allograft function and in 12 controls. Overall there was a trend toward attenuated EDV in the transplant patients, but this was only

significant for those patients on cyclosporin-based immunosuppression. Surprisingly, the patients taking prednisolone and azathioprine only (n=7) appeared to have preserved endothelial function, although this may be a result of the small numbers. Thus, the patients taking cyclosporin had impaired stimulated endothelial function evidenced by reduced EDV and preserved EIDV. To assess whether basal endothelial NO production was also abnormal, a further study was performed using L-NMMA as an inhibitor of basal NO production, and noradrenaline as a control vasoconstrictor in 9 cyclosporin-treated transplant recipients and 11 controls. As expected, vasoconstriction to L-NMMA was reduced in the transplant recipients suggesting impaired basal NO production in this group. Surprisingly, some of the patients responded abnormally, with vasodilatation to noradrenaline, and this appeared to be related to age. The reason for this phenomenon is unclear and warrants further study. It is possible that cyclosporin treatment alters adrenergic receptor number or function such that noradrenaline vasodilates via β-adrenoceptors. Overall, the studies in the transplant patients suggested impaired stimulated and basal endothelial NO production, and altered adrenergic responses in those patients treated with cyclosporin, an agent known to play a major role in post-transplant hypertension.

Thus, the studies performed for this thesis demonstrate that there is endothelial dysfunction in patients with CRF including those patients who have been transplanted and are treated with cyclosporin. As the *in vitro* study confirmed these results, it is likely that a short-lived circulating factor is not wholly responsible, although the studies performed do not allow a more detailed assessment of the causation of endothelial dysfunction in uraemia. Further work is required with repeat studies including hypertensive control groups and in the presence of NO synthase inhibitors,

to assess the relative contribution of NO to the defect in endothelial function. A more detailed study of the vascular effects of cyclosporin is also warranted, perhaps infusing cyclosporin locally at plethysmography or by adding cyclosporin to the myography chamber.

The studies confirm endothelial dysfunction in a high-risk cardiovascular group. As endothelial dysfunction is potentially reversible using lifestyle changes, anti-hypertensive agents, statins, anti-oxidants and homocysteine-lowering, these studies provide a platform for future intervention studies using improvements in endothelial function as a surrogate marker for reducing cardiovascular end-points in CRF.

Chapter 1

Introduction

1.1 OVERVIEW OF CARDIOVASCULAR DISEASE IN CHRONIC RENAL FAILURE

The increased availability of dialysis therapy and renal transplantation has unmasked an epidemic of cardiovascular disease in patients with chronic renal failure (CRF). Data from the European Renal Registry reveal that patients receiving renal replacement therapy (RRT) experience a 16-19 fold increased risk of myocardial infarction and ischaemia compared with age and sex-matched populations without renal disease (Raine 1992). The picture is similar in North America - figures from the U.S. Renal Data System (USRDS) confirm that 50% of American RRT patients die of cardiovascular disease (Figure 1.1).

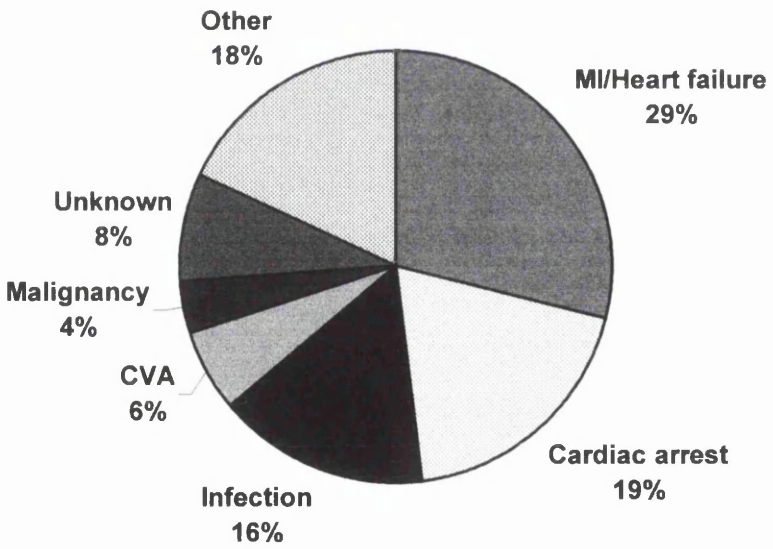


Figure 1.1 Cause of death for ESRD patients aged 45-64, 1991-1993, USRDS 1996.

Many would say that this is not surprising as cardiovascular disease is the commonest cause of death in the general population. However, patients with ESRF die prematurely from cardiovascular disease with, for instance, the CVD mortality rate in dialysis patients aged 25-44 being around 100 times that of the general population (Foley 1998a; Figure 1.2).

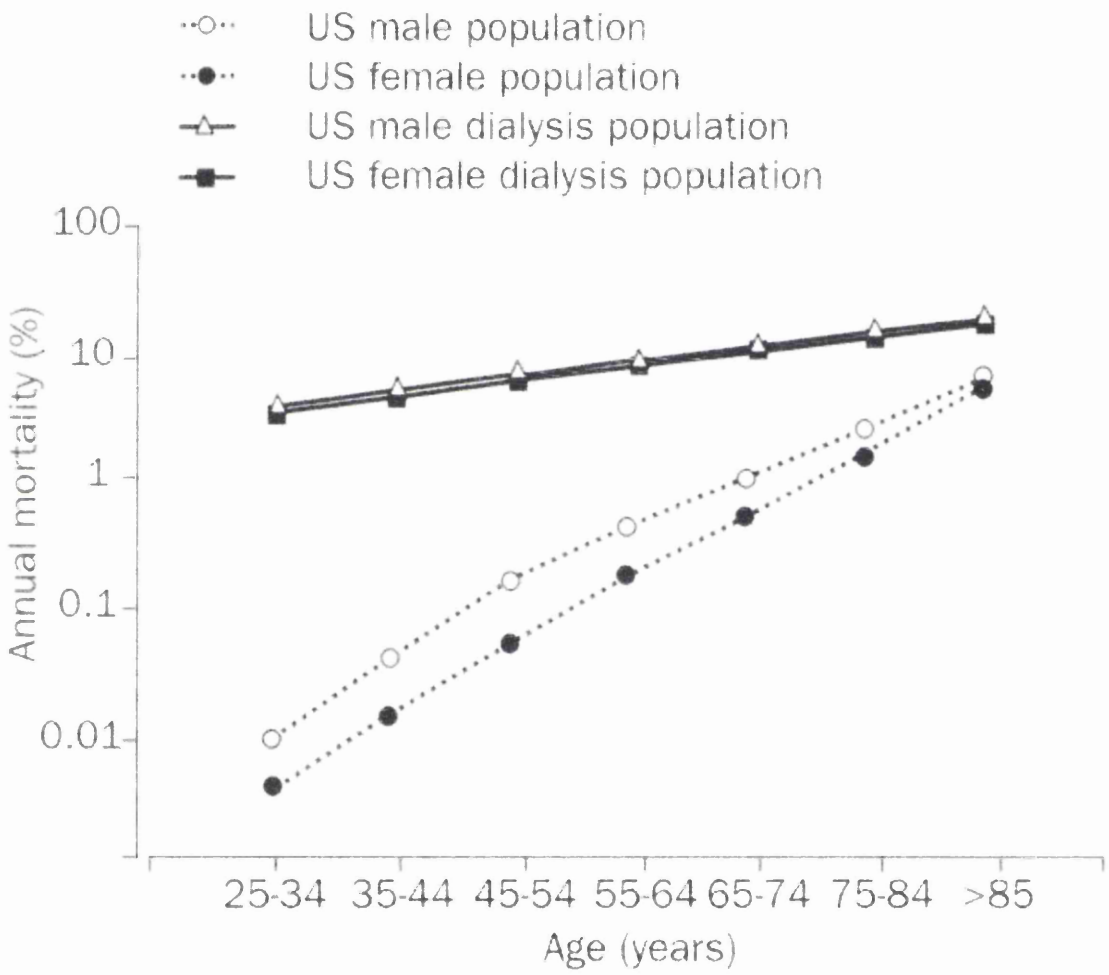


Figure 1.2. Cardiovascular disease mortality by age, race and gender in the general population and in dialysis patients from USRDS 1994-1996. (From Foley 1998a).

The increased risk is seen across populations with different background risks of cardiovascular disease, with a 20-fold increased risk of cardiovascular death in both the UK and Italy (Raine 1992). This compares with the 3-fold increased risk of cardiovascular death in diabetics, a group with widely recognised increased cardiovascular risk. Thus, it seems that we have gone full circle. Before dialysis was available, uraemic patients often died of pericarditis. Now that RRT in its various forms is widely available, patients are again succumbing to cardiovascular disease, and unlike the general population, the incidence of cardiovascular disease in this group is not falling (Raine 1992).

The precise mechanisms underlying the markedly increased risk of cardiovascular death in uraemia are unclear. We do know that while myocardial infarction and atherosclerotic vascular disease are common, this does not afford the entire explanation for the high mortality rate, as patients on long-term dialysis often have symptomatic ischaemic heart disease with patent coronary arteries (Roig 1981). Left ventricular hypertrophy, with its associated risk of sudden arrhythmic death, is very common in this population as a result of years of hypertension. Left ventricular dilatation and heart failure are also common - reflecting chronic fluid overload especially in dialysis patients. As nephrologists we see evidence of atherosclerotic vascular disease on a daily basis with stroke, angina, myocardial infarction and peripheral vascular disease causing great morbidity in our patients. Therefore, it is apparent that the excess of cardiovascular disease in this population reflects changes in both the heart and the vasculature.

1.2 THE HEART IN CHRONIC RENAL DISEASE

There are four main manifestations of cardiac disease in renal failure : ischaemic heart disease (IHD) secondary to coronary atherosclerosis, left ventricular hypertrophy, heart failure and sudden death.

Atherosclerosis is accelerated in uraemia and will be discussed later. While IHD is usually caused by coronary atherosclerosis, other mechanisms may play a role, and in up to a quarter of haemodialysis patients with angina the coronary arteries are not significantly narrowed or occluded at coronary arteriography (Rostand 1984). The symptoms are presumably related to reduced coronary reserve secondary to left ventricular hypertrophy, and microvascular disease with capillary rarefaction and arteriolar wall thickening (Amann 1996).

Left ventricular hypertrophy and failure develop in response to LV pressure and volume overload (Parfrey 1999). Left ventricular pressure overload in uraemia is caused by systemic hypertension, arteriosclerosis (reduced arterial compliance) and (calcific) aortic stenosis, and in turn leads to concentric LV hypertrophy. In contrast, left ventricular volume overload develops in response to salt and water retention, anaemia and increased cardiac output secondary to arteriovenous fistulae (Parfrey 1999), and results in eccentric LV hypertrophy. With both forms of LV hypertrophy the initial changes are beneficial to cardiac function. However, with on-going pressure and volume overload the LV cavity dilates, the density of capillaries is reduced and myocyte death ensues. Heart failure and death follow.

The epidemiology of cardiac disease in uraemia varies with the stage of a patient's "renal life" (Figure 1.3).

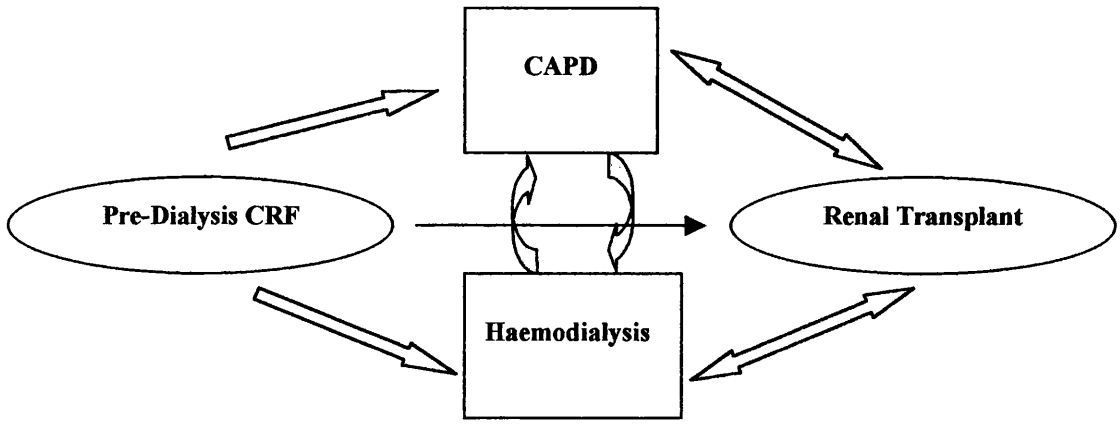


Figure 1.3. A "Renal Life" involves the pre-dialysis phase, followed directly by either pre-emptive transplant, CAPD or haemodialysis. Patients often move from one group to the other.

1.2.1 Pre-dialysis CRF

Data are lacking for this group. We do know that young patients with CRF have increased LV mass and diastolic dysfunction compared to normal controls (Johnstone 1996). In addition, as GFR declines, LV mass rises progressively : in a cross-sectional study, 27% of those with GFR >50ml/min had LV hypertrophy, 31% of those with GFR 25-49ml/min, and 45% of those with GFR <25ml/min had LVH (Levin 1996). Moreover, this progressive rise in LV mass holds true in individual patients with declining renal function (Levin 1999).

Ischaemic heart disease is very common in patients starting dialysis : 41% in the United States and 28% in Canada (Foley 1998a), while heart failure was present in 40% of patients starting haemodialysis in the U.S. (USRDS 1997).

1.2.2 Haemodialysis

One of the largest studies to examine cardiac abnormalities in uraemia was performed by Parfrey et al in Newfoundland, Canada (Parfrey 1996). Using echocardiography, they studied 432 patients who survived for more than 6 months on haemodialysis, and showed that only 16% had a normal heart by echocardiography at the initiation of dialysis therapy. Forty-two percent had concentric left ventricular hypertrophy (LVH), 23% eccentric LVH, 4% left ventricular (LV) dilatation and 16% LV systolic dysfunction. In those patients with systolic dysfunction starting dialysis, median survival was 38 months. The presence of concentric LVH, LV dilatation or LV systolic dysfunction at baseline was associated with a 3-fold increased risk of subsequently developing symptomatic heart failure.

Left ventricular hypertrophy and dilatation progress on haemodialysis. In Parfrey's echocardiography study (Parfrey 1996; Foley 1998b), a subgroup of patients had annual echocardiography whilst on haemodialysis : LV cavity volume was 73ml/m^2 at baseline, increased by 13ml/m^2 at 18 months and by a further 5ml/m^2 at 30 months. Left ventricular mass index rose in a similar way. Silberberg showed that LV mass index correlated with outcome : if LV mass index was >125g/m^2, 5-year mortality was 52%. If LV mass index was <125g/m^2 mortality was 23% over the same period of follow-up. Thus, LVH is an important adverse prognostic factor in patients on RRT.

Ischaemic heart disease carries a worse prognosis in dialysis patients. In a study of 34,189 long-term U.S. dialysis patients the 1 yr survival following myocardial infarction was only 41% with the 5 yr survival being just 10% and the greatest risk being in diabetics and older patients (Herzog 1998).

1.2.3 Peritoneal Dialysis

CAPD may be more "cardio-protective" than haemodialysis, with a more constant blood volume, absence of AV fistulae and less anaemia. LV hypertrophy and dilatation are said to be less common in patients on CAPD, however, there is often a strong selection bias in choosing the dialysis modality with younger, fitter patients opting for CAPD. Despite this, data from the USRDS suggest that the prevalence of IHD and cardiac failure are both around 40% in CAPD patients, and that patients on CAPD have 9.7% annual cardiovascular mortality (c.f. 9.5% for haemodialysis)(Levey 1998).

1.2.4 Renal Transplantation

Left ventricular hypertrophy remains common post-transplantation, reflecting the high incidence of post-transplant hypertension, often cyclosporin-related. McGregor et al performed echocardiography on the eve of transplantation and showed that the average LV mass index was 134 g/m^2 in those who survived compared to 167 g/m^2 in those who died during the 7.5 year follow-up (McGregor 1999).

Cardiac abnormalities are therefore extremely prevalent in chronic renal disease and contribute greatly to the overall cardiovascular mortality and morbidity. Changes in the blood vessels are also common and lead to premature atherosclerosis with resultant IHD, cerebrovascular disease (CVD) and peripheral vascular disease (PVD).

1.3 THE BLOOD VESSELS IN CHRONIC RENAL DISEASE

Blood vessels have two principal functions : conduit and cushioning (London 1997). The conduit function simply involves the transfer of blood from the heart to the tissues with minimal pressure drop, while cushioning refers to the elasticity of major vessels and their ability to smooth out the arterial pressure wave generated by the cardiac contraction, thus maintaining a constant flow of blood to the tissues. Both of these functions may be altered by disease, including renal failure.

Blood flow through a vessel is normally maintained until the lumen is narrowed to 20% or less of its resting diameter (Gould 1976). A reduction in the conduit function is usually caused by luminal narrowing secondary to atherosclerotic plaque formation, and this most commonly occurs at sites of arterial bifurcation or branching, preferential areas being the carotid, coronary, femoral and renal arteries, as well as the infra-renal aorta (Glagov 1988). The mechanisms of atherogenesis and risk factors will be fully discussed in sections 1.7 and 1.8.

Atherosclerosis is a common condition in the general population, but is said to be accelerated in chronic renal failure. The pioneering study in this field was published by Lindner and Charra in the 1970's (Lindner 1974). This group examined outcomes

in the first 39 patients receiving long-term haemodialysis in Seattle from 1960 onwards. Fourteen of the 23 deaths were attributed to cardiovascular disease, confirmed by post-mortem in 19, and this occurred in an era when death from sepsis and technical accidents were still commonplace. Their first patient died from myocardial infarction in 1971 after 11 years of haemodialysis and at post-mortem was found to have "the most severe generalised atherosclerosis" the pathologist had ever seen. This seminal paper therefore described, for the first time, the high cardiovascular mortality in CRF and introduced the concept of accelerated atherosclerosis in uraemia. Subsequent studies confirmed their findings (Lowrie 1974; Ibels 1979; Rostand 1979), although many have questioned whether atherosclerosis is actually accelerated in uraemia or whether we are in fact seeing the cumulative effects of several standard cardiovascular risk factors.

While most research has focussed on changes in the conduit function of blood vessels in CRF, Gerard London and his group have highlighted the importance of changes in cushioning function, and the link between the heart and blood vessels (London 1997). The cushioning function of blood vessels transforms the pulsatile cardiac output into a smooth, continuous blood flow to the tissues such that during systole 50% of the stroke volume is projected forward to the tissues while 50% is "stored" within the aorta and larger vessels. Elastic recoil of these distensible arteries in diastole releases this stored blood to continue the constant blood flow required for organ and tissue function. The efficiency of the process depends on the distensibility (or compliance) of the arterial walls.

While atherosclerosis is the main pathological change affecting conduit function, arteriosclerosis is the principal change affecting cushioning (and has no effect on conduit function at all). Arteriosclerosis occurs primarily in the aorta and larger blood vessels and is characterised by vessel wall dilatation, intimal medial thickening and reduced compliance or stiffening (London 1997). These changes, associated with normal aging, are accelerated in hypertension and CRF and lead to increases in systolic blood pressure and pulse pressure, both known to be linked to increased cardiovascular mortality. In addition, increased arterial stiffness increases afterload and results in compensatory left ventricular hypertrophy. It is partly through this mechanism that changes in vascular endothelial function may ultimately lead to alteration in cardiac structure or function.

Studies have shown that changes in blood flow alter the calibre of blood vessels along with the thickness and structure of their walls through release of endothelial-derived vasoactive substances. Increases in blood flow increase the calibre of vessels and their intimal medial thickness (Kamiya 1980), while decreases in flow have the opposite effect (Langille 1986). Thus, CRF being a state of chronic fluid overload is associated with increased systemic and regional blood flow, and this in turn leads to arteriosclerosis (London 1996). In addition to chronic volume/flow overload, blood vessels in uraemic patients are exposed to high tensile stress accompanying systemic hypertension. Over a long period tensile stress promotes intimal medial hypertrophy with a reduction in lumen diameter (Roman 1992), reduced arterial distensibility and increased pulse pressure.

The vascular endothelium lies at the interface between the blood and the tissues, and plays a key orchestrating role in the pathogenesis of both atherosclerosis and arteriosclerosis.

1.4 STRUCTURE OF THE VASCULAR ENDOTHELIUM IN HEALTH

For many years, scientists have described the vascular endothelium as a simple cellular monolayer which lines the inside of blood vessels and provides a barrier between the blood and tissues. In fact, the endothelium is one of the body's largest and most complex organs containing around $1x10^{12}$ cells, and with a multitude and diversity of functions paralleling those of the liver (Born 1998). To illustrate it's size, Born et al estimated that the total surface area of the vascular endothelium was equivalent to 6 tennis courts.

Around 1960, following the development of electron microscopy, came the realisation that the endothelium was more than just a barrier. Through his descriptions of the passage of macromolecules through the endothelium, Howard Florey's pioneering work laid the foundations for our current understanding of endothelial structure and function (Florey 1960). With a multitude of cell surface receptors, pores and inter-cellular connections, the endothelial cell can sense and respond to mechanical stimuli such as shear stress, to changes in the local milieu such as hypoxia, and to circulating and locally released hormones and transmitters.

Endothelium in culture and *in vivo* is a simple monolayer of cells (figures 1.4 and 1.5) approximately 3 μm thick at the nucleus and thinning to around 0.2 μm at the periphery (Florey 1966). These elongated cells are usually around 30 μm long by 10 μm wide, and are connected to each other by tight intercellular junctions of the fascia occludens type (Burkitt 1993 ed).

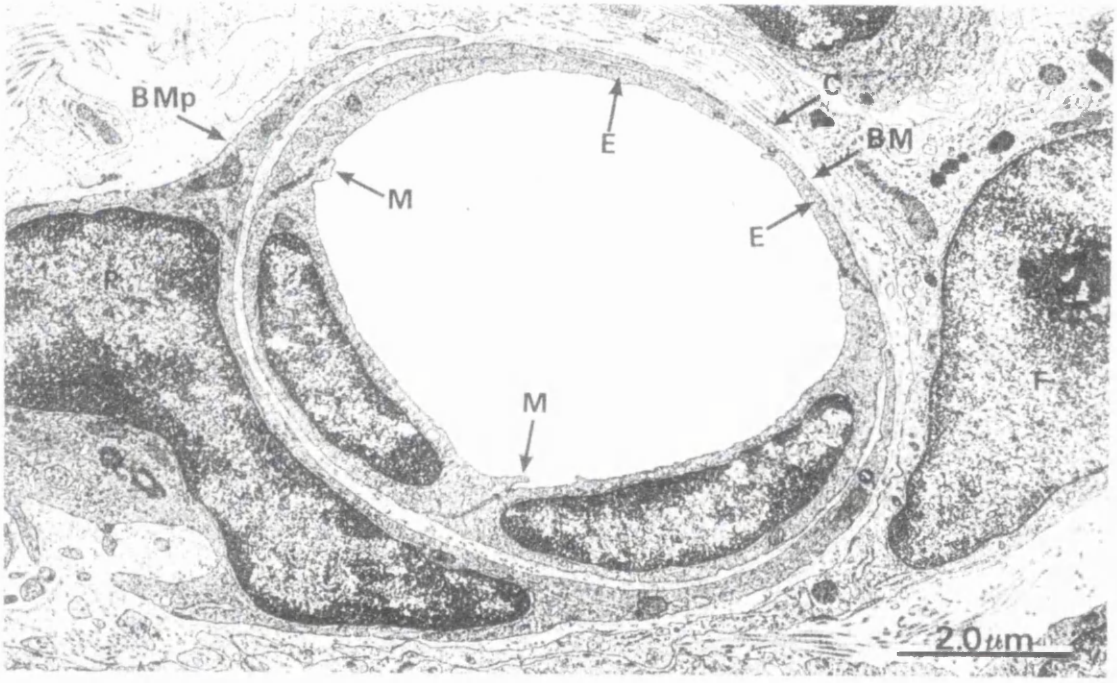


Figure 1.4. Electron micrograph illustrating a capillary of the continuous endothelium type (From : Wheater's Functional Histology 3rd edition).

(E=endothelial cell, M=marginal fold, BM=basement membrane, P=pericyte, BMp=basement membrane of pericyte, C=collagen fibrils, F=fibroblast)

In capillaries, the thin endothelial cell layer rests on its basement membrane, and is supported by pericytes, cells which provide a "scaffolding". Around these cells lie the connective tissue comprising fibroblasts and collagen fibrils. In arterioles (Figure 1.5), the endothelial cell layer constitutes the tunica intima and is again supported by a thin basement membrane which separates it from the tunica media. This layer comprises up to 6 concentric layers of vascular smooth muscle cells, supported by the collagen and elastin of the tunica adventitia.

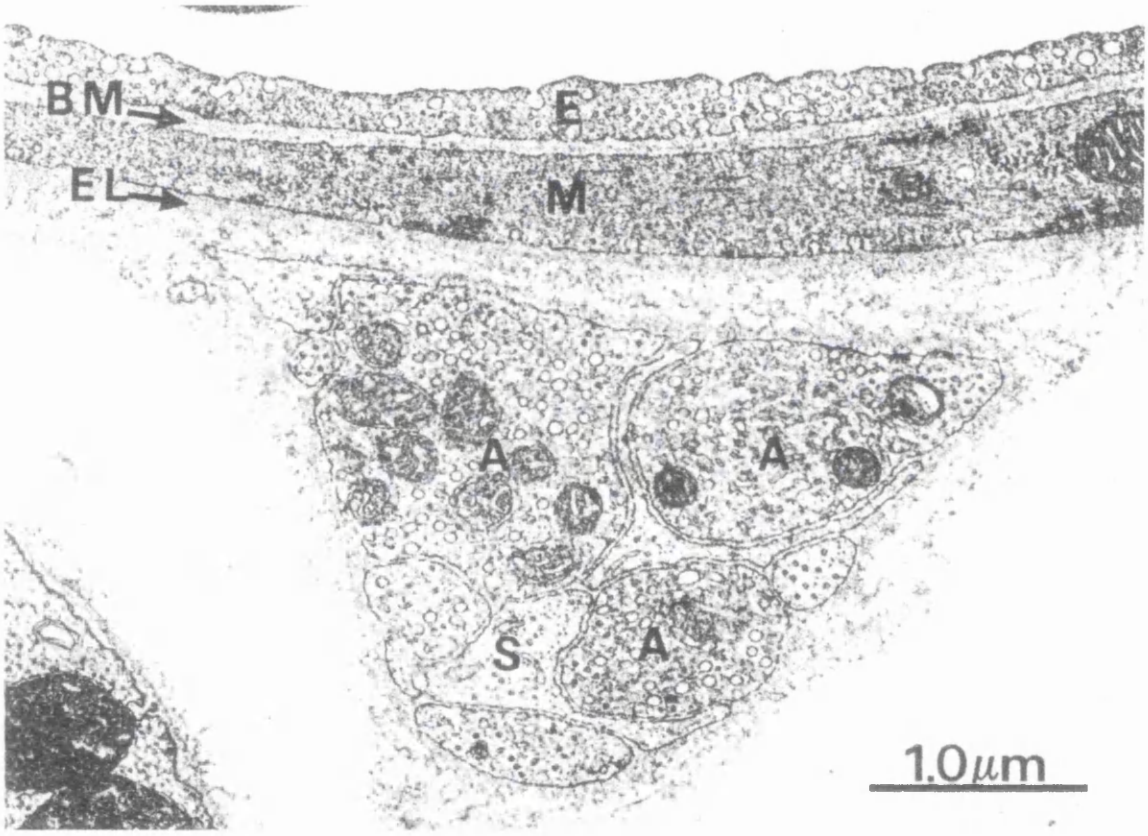


Figure 1.5. Electron micrograph of a small arteriole (From : Wheater's Functional Histology 3rd edition with permission. (E=endothelial cell, BM=basement membrane, M=smooth muscle of tunica media, EL=external lamina, A=axon of sympathetic neuron, S=Schwann cell)

Each endothelial cell contains components of the muscle cell contractile apparatus - actin, myosin, tropomyosin and alpha-actinin – organised in 3 structures : the cortical web, the junction-associated actin filament (JAF) system, and striated myofibril-like stress fibres (see figure 1.6)(Born 1998). The luminal surface of the endothelial cell has occasional microvilli of uncertain function, as well as receptors for numerous vasoactive substances, hormones and neurotransmitters (table 1.1).

ENDOTHELIAL CELL SURFACE RECEPTORS
Endothelin A
Endothelin B
Angiotensin II
Angiotensin I
Acetylcholine (muscarinic M2)
Bradykinin (BK2)
Histamine (H2)
ADP (P2)
Thrombin
5-HT1
Adrenaline (alpha2)
Vasopressin (VP1)
TGFbeta
LDL

Table 1.1 : Receptors present on the vascular endothelial cell surface.

The cortical web serves as an anchor for adhesion molecules such as E-selectin and adherin, and for proteins such as annexin which is thought to play a major role in exocystosis and endocytosis (Born 1998). In addition, the cortical web maintains the structure of the cell. Passage of molecules between cells is controlled by the JAF system, while the striated stress fibres allow the cell to change shape and maintain form and structure when resisting high levels of shear stress.

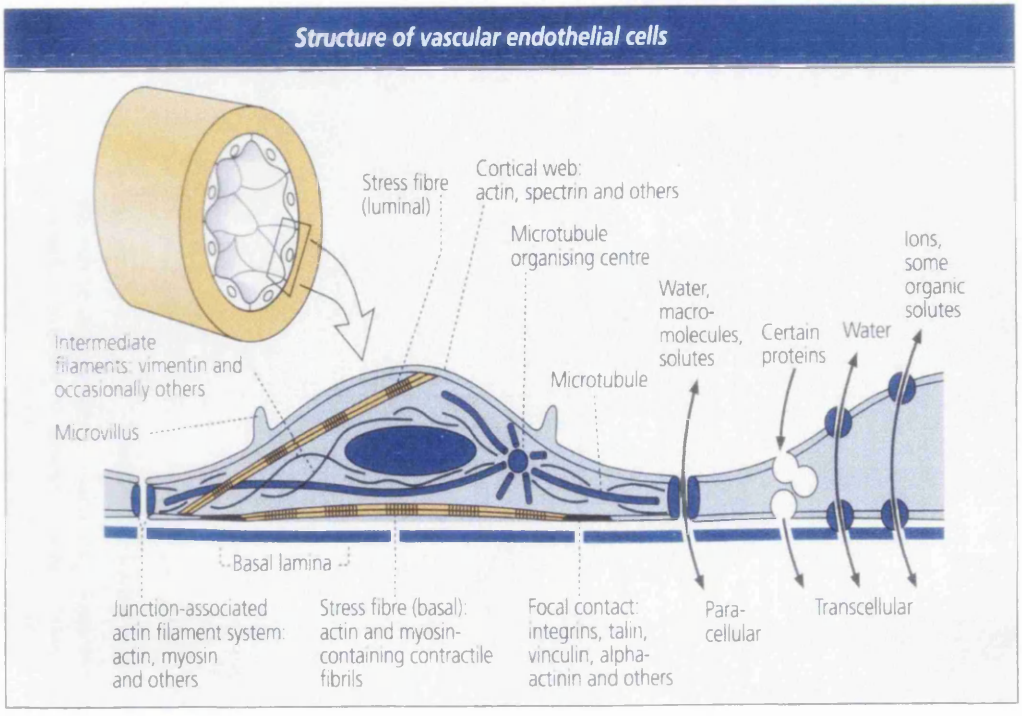


Figure 1.6. Structure of vascular endothelial cells. (From : Clinician's Manual on Endothelium and Cardiovascular Disease, Ed. Born G, Science Press, London, U.K. with permission.)

A further interesting feature of the endothelial cell membrane is the presence of caveoli : vesicular structures rich in lipids, receptors and actin. These structures are involved in the transport of molecules across the cell membrane, and are thought to play a major role in signal transduction.

1.5 FUNCTIONS OF THE VASCULAR ENDOTHELIUM IN HEALTH

1.5.1 Control of vessel tone : Vasodilators

The vascular endothelium responds to changes in blood pressure, shear stress and concentrations of circulating mediators, by releasing both vasoconstrictors and vasodilators. These vasoactive mediators are listed in table 2.

VASODILATORS	VASOCONSTRICTORS
Nitric oxide (NO)	Endothelin
Endothelium-derived hyperpolarizing factor (EDHF)	Angiotensin II
Prostacyclin (PGI_2)	Thromboxane A2
C-type natriuretic factor	Prostaglandin H2
	Superoxide Anion

Table 1.2 Vasoactive substances released by the endothelium.

1.5.1.i Nitric Oxide

The identification of NO as the principal endothelium-derived relaxing factor is one of the major scientific discoveries of the last 2 decades and has opened a new chapter in cardiovascular biology. This discovery was made serendipitously in Robert

Furchgott's laboratory in 1978 during studies of β-adrenoceptor function in rabbit thoracic aortic strips. These strips, mounted in a standard organ bath usually had little response to the addition of acetylcholine to the organ bath. However, on one occasion, a research assistant noticed that a strip of aorta, pre-constricted with noradrenaline, relaxed with the addition of acetylcholine, and thus it was realised that the standard method of preparation of the strips damaged the endothelium. When great care was taken to avoid damage to the endothelial surface, the preparation consistently relaxed (in a dose-dependent manner) to acetylcholine, and other muscarinic agonists (Furchgott 1993; Figure 1.7).

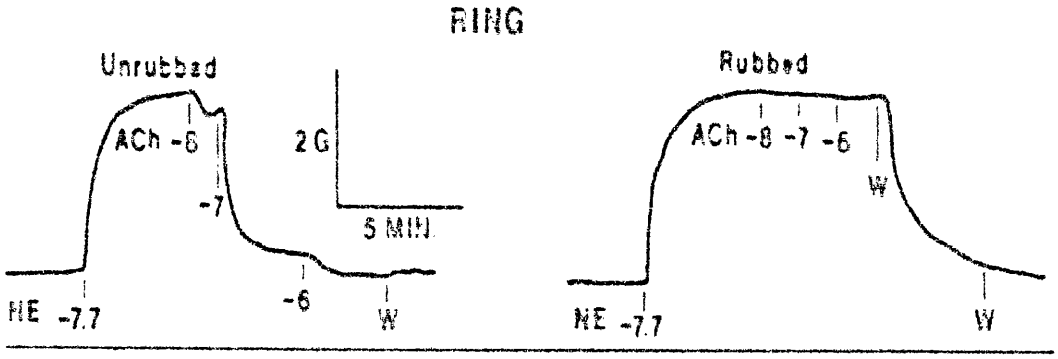


Figure 1.7. Loss of relaxing response of preparations of rabbit aorta to acetylcholine (ACh) after removal of endothelial cells by rubbing. (Furchgott 1980).

Similarly, treatment of the aortic strips with collagenase abolished the endothelium-dependent relaxation. Furchgott went on to perform "sandwich" experiments, in which a strip of aorta denuded of its endothelium was placed next to a strip with intact

endothelium, and made to relax with the addition of acetylcholine. He postulated that a diffusible "relaxing substance" was released from the endothelium of the second strip in the sandwich experiment. Subsequently, the term endothelium-derived relaxing factor or EDRF was coined in 1983 (Cherry 1983).

A multitude of experiments examining endothelium-dependent relaxation rapidly followed. Besides acetylcholine, several agents were shown to produce endothelium-dependent vasodilatation, including 5-HT, bradykinin, histamine, thrombin, ADP, substance P and vasopressin (Furchgott 1989), although there were often species differences and even differences in the response of vessels from different sites within the same species.

The evidence began to accumulate that EDRF may be nitric oxide :

- Endothelium-dependent vasorelaxation was accompanied by a rise in cyclic GMP (Rapoport 1983).
- Relaxation of vascular smooth muscle by nitro-vasodilators was accompanied by a rise in cyclic GMP (Gruetter 1979).
- EDRF was inhibited by methylene blue (an inhibitor of guanylate cyclase) and by haemoglobin (Martin 1985).
- Acidified solutions of sodium nitrite (releases NO and NO_2) produced strong, transient relaxations of rabbit aorta (Furchgott 1988).
- Superoxide anion inactivates EDRF and this effect is blocked by superoxide dismutase (Gryglewski 1986).

- The characteristics of the relaxation of rabbit aorta by acetylcholine-released EDRF and $NaNO_2$-released NO were identical : this led Furchgott and Ignarro (almost simultaneously) to propose that EDRF was in fact NO (Furchgott 1988; Ignarro 1987).
- Moncada and Palmer finally demonstrated using chemiluminescence that the amount of NO released by bradykinin could fully explain the relaxation attributed to EDRF (Palmer 1987).

Since these early experiments much work has been performed to elucidate the biology of NO :

i NO is a colourless, odourless, hydrophobic, free radical gas which diffuses freely through cell membranes.

ii NO has a half-life of 3-5 seconds (Rubanyi 1985).

iii The precursor for NO is L-arginine (Palmer 1988).

iv The production of NO is inhibited by N_G-monomethyl-L-arginine (L-NMMA) and this can be overcome by adding L-arginine (Rees 1989).

v Nitric oxide synthase (NOS), the enzyme responsible for synthesis of NO has 2 main types : constitutive (activity expressed in the absence of external stimulation) and inducible. The constitutive type exists in 2 main forms : neuronal (nNOS; also found in kidney macula densa, pulmonary epithelium and skeletal muscle) and endothelial (eNOS)(Moncada 1999). The conversion of L-arginine to NO by eNOS is a two-step process which yields N-hydroxy-L-arginine as an intermediate product then NO and L-citrulline. Co-factors for eNOS include NADPH, tetrahydrobiopterin (BH4), flavin adenine dinucleotide

(FAD) and flavin adenine mononucleotide (FMN). Constitutive NOS is calcium-dependent, whereas iNOS is not.

vi eNOS can be activated by shear stress and hypoxia, and by receptor-G protein coupling in response to a wide variety of agonists including 5-HT, acetylcholine, substance P and bradykinin (Henderson 1996). The amount of NO produced is in the picomolar range and takes place within seconds (Dattilo 1997).

vii iNOS is found in vascular smooth muscle, hepatocytes, neutrophils and macrophages. It does not produce background NO like eNOS; instead it requires "switching on" by endotoxin, cytokines or shear force within the vascular wall. Production of NO takes 4-6 hours and lasts up to 24 hours, with levels reaching the nanomolar range. This NOS is much more important in pathophysiologic states (Datillo 1997).

viii Nitric oxide produced by nNOS acts as a neuro-modulator or as a neurotransmitter in nitrergic nerves.

ix NO is rapidly oxidised by oxygen and superoxide anion (O_2^-) to nitrite and nitrate. A modified Griess reaction is often utilised to measure nitrite as an indirect measure of NO production. Reaction of NO with O_2^- also leads to the production of peroxynitrite, a toxic free radical, which is rapidly protonated and decomposes to NO_2 and $OH\bullet$, both of which are tissue-damaging agents (Beckman 1990).

Nitric oxide is continuously produced in endothelial cells from L-arginine by eNOS and diffuses readily through cells to act in the vascular compartment and on adjacent vascular smooth muscle cells (VSMCs). The mechanism of vasodilatation involves the binding of NO to the haem moiety of soluble guanylate cyclase, producing a

conformational change in the enzyme that "pulls" the haem component away from the enzyme molecule and allows easier access for the substrate. Activation of guanylate cyclase catalyses the conversion of glutamyl 5'-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Cyclic GMP has several effects that lead to vasorelaxation (Dattilo 1997):

(1) lowering the cytosolic calcium concentration by inhibiting calcium influx and promoting sequestration of calcium in sarcoplasmic reticulum.

(2) hyperpolarizing the cell membrane by increasing K^+ conductance.

(3) activating protein kinases which phosphorylate myosin light chains, preventing the actin/myosin interaction.

NO is produced continually under resting conditions and contributes to basal vascular tone thus infusion of NOS inhibitors such as N_G-monomethyl-L-arginine (L-NMMA) raises blood pressure (Haynes 1993).

1.5.1.ii Endothelium-derived Hyperpolarising Factor (EDHF)

Acetylcholine was first shown to cause endothelium-dependent hyperpolarisation of vascular smooth muscle in 1984 (Bolton 1984). This hyperpolarisation occurs in the presence of indomethacin and L-NMMA, and therefore is not likely to be caused by prostacyclin or NO. While there are occasional membrane-membrane connections between endothelium and vascular smooth muscle cells, there have been several studies demonstrating that a diffusible substance is involved (Vanhoutte 1993). This substance has been termed EDHF and its identity is unknown. The release of EDHF by endothelial cells is calcium and calmodulin-dependent, and unlike NO, is not thought to contribute to resting vascular tone. In addition to acetylcholine, several

agents have been shown to stimulate EDHF release : bradykinin, histamine, ADP, thrombin and substance P (Vanhoutte 1993). Endothelium-derived hyperpolarising factor vasodilates by opening K^+ channels, an effect that can be blocked in some vessels by ouabain. The relative contribution of NO and EDHF to endothelium-dependent vasodilatation is not clear.

1.5.1.iii Prostacyclin

Prostacyclin is a major product of arachidonic acid metabolism in endothelial cells (Moncada 1976; Figure 1.8).

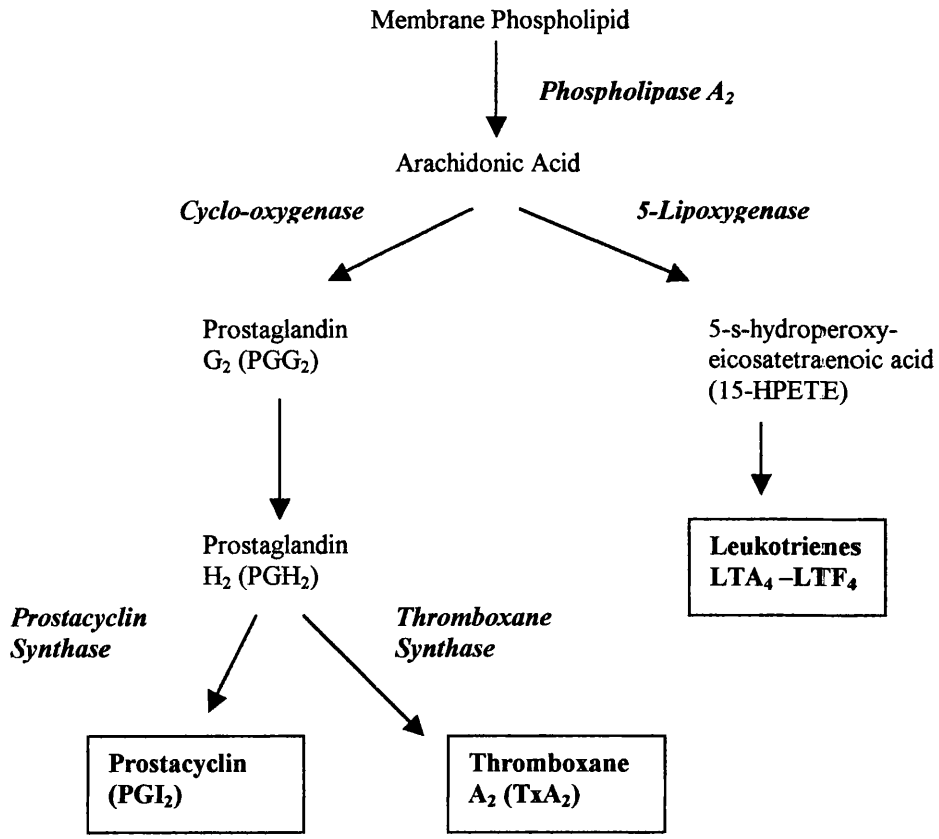


Figure 1.8 : A simplified metabolic pathway for the conversion of arachidonic acid to prostacyclin and thromboxane A2.

Prostacyclin (PGI_2) is produced when the enzyme phospholipase A2 liberates arachidonic acid from membrane phospholipids, and this process is inhibited by glucocorticoids. Arachidonic acid is converted to PGG_2 by cyclo-oxygenase (inhibited by aspirin) which in turn is converted to PGH_2 and to PGI_2 by prostacyclin synthase. The synthesis of PGI_2 is continuous under resting conditions, and can be stimulated by shear stress (Bhagyalakshmi 1989), hypoxia and chemical stimuli such as 5-HT, bradykinin, thrombin, platelet-derived growth factor (PDGF), interleukin-1 and ADP (Epstein 1990). Prostacyclin acts as a local mediator with plasma levels below those required to have a systemic effect (Blair 1982). The half-life is short and the main metabolite (6-keto-prostaglandin F1α) is chemically stable, biologically inactive and excreted in the urine.

Prostacyclin has two principal actions : vasodilatation (Moncada 1979) and inhibition of platelet aggregation (Moncada 1976). The mechanism underlying vasodilatation is activation of adenylate cyclase, causing a rise in intracellular cyclic AMP (cAMP) and lowering of the cytosolic calcium level (Moncada 1979). In most blood vessels the contribution of PGI_2 to endothelium-dependent vasodilatation is negligible, and its effect is essentially additive to that of NO (Vanhoutte 1999). However, PGI_2 is a potent inhibitor of platelet aggregation and adhesion, and its effects are synergistic with those of NO (Moncada 1979). Overall, the effects of PGI_2 are anti-atherogenic. Not surprisingly, the ability of blood vessels to generate prostacyclin declines with advancing age, diabetes and in atherosclerosis (Vane 1990).

1.5.1.iv C-type Natriuretic Peptide

C-type natriuretic peptide (CNP) is a 22-amino acid peptide which is structurally related to atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). CNP immunoreactivity has been found in endothelial cells, plasma and kidney, and this recently described peptide has a specific receptor , the NPR-B receptor (Chen 1998). In the systemic circulation CNP potently induces venodilatation with a resultant reduction in cardiac output and coronary artery dilatation, while its renal effects are minimal. The localisation of NPR-B receptors on vascular smooth muscle cells and CNP within endothelial cells suggests that this peptide may have a significant vasoregulatory role.

1.5.2 Control of Vessel Tone : Vasoconstrictors

The effects of the endothelium-derived relaxing factors are counterbalanced by the production of several constricting factors, some of which are cyclo-oxygenase dependent. The production of these factors is stimulated by increased stretch and pressure, and in conditions of hypoxia.

1.5.2.i Thromboxane A2 and prostaglandin H2

Soon after the discovery of EDRF came the realisation that, in certain vascular beds and circumstances, agonists such as acetylcholine could also stimulate endothelium-dependent vasoconstriction. In veins, cerebral and ophthalmic arteries, acetylcholine,

histamine, 5-HT and arachidonic acid can stimulate endothelium-dependent contractions mediated by thromboxane A2 and prostaglandin H2 (Moncada 1979). These prostaglandins act through the thromboxane receptor on vascular smooth muscle cells, and their contraction is inhibited by indomethacin. In the cerebral circulation, a potent stimulator of endothelium-dependent contraction is rapid stretch. Presumably, this has a role in cerebral autoregulation, in that a rapid rise in blood pressure stretches the vessel and the resulting vasoconstriction reduces cerebral blood flow and pressure towards normal.

1.5.2.ii Superoxide anion

Superoxide anion is an oxygen-derived free radical which can be produced in endothelial cells in circumstances of increased oxidative stress. The first report that this free radical may be an endothelium-derived contracting factor came from work in the canine basilar artery. Katusic et al showed that contractions induced by calcium ionophore A23187 were effectively abolished by superoxide dismutase (Katusic 1989). Although a clear contraction was observed, it is likely that superoxide anion has no direct effect on vascular smooth muscle but rather that it inactivates NO, reducing its effect on basal vascular tone.

1.5.2.iii Angiotensin II

As angiotensin converting enzyme (ACE) is expressed on the endothelial cell surface, these cells are a local source of angiotensin II, the most potent pressor agent known. Angiotensin II also activates AT1 receptors on endothelial cells to stimulate the

production of endothelin, another powerful vasoconstrictor. Previously it was thought that most of the conversion of angiotensin I to II occurred in the lungs. However, the so-called "tissue renin-angiotensin system" is now recognised to play a significant role in cardiovascular regulation.

1.5.2.iv Endothelin

The most powerful vasoconstrictor substances produced by the vascular endothelium are the endothelin family of peptides (Yanagisawa 1988). Three isotypes have been identified containing 21 amino acids : endothelin-1 (ET-1), endothelin-2 and endothelin-3 (Inoue 1989). Endothelin-1 is the most powerful vasoconstrictor and the main isotype found in blood vessels, with major expression of its precursor's mRNA in endothelial cells (Inoue 1989). It is also found in kidney, CNS and cardiac tissue, and in smaller quantities in vascular smooth muscle cells. Endothelin-2 is produced in smaller amounts in endothelial cells, heart and kidney, while endothelin-3 is mainly found in the gut and CNS (Haynes 1998).

The precursor for ET-1, preproendothelin-1 is cleaved to form proendothelin-1, which in turn is cleaved to form big endothelin-1, a 38 amino acid peptide devoid of cardiovascular action. The active peptide, ET-1, is generated by the action of a metalloprotease called endothelin converting enzyme. This enzyme exists in two forms of which ECE-1 is physiologically active. ECE-1a is the subtype responsible for the conversion of big endothelin-1 to ET-1 in the endothelial cell; ECE-1b is expressed in the cell membrane of vascular smooth muscle cells where it converts extracellular big ET-1 to ET-1 (Haynes 1998).

Two endothelin receptor subtypes have been characterised. Endothelin-1 produces its characteristically sustained vasoconstriction by acting on the ET_A receptor which is present on the surface of vascular smooth muscle cells and absent from the endothelium (Hosoda 1991). The ET_B receptor is expressed mainly in endothelial cells where its stimulation leads to production and release of NO and prostacyclin, and thus is involved in a negative feedback or control mechanism. ET_B receptors are also found in vascular smooth muscle cells where they may be involved in vasoconstriction (although to a lesser degree than ET_A receptors)(Haynes 1995). A summary of the 2 receptor types is detailed in table 1.3.

Receptor	ET_A	$ET_{A/B}$	ET_B
Order of potency	ET-1>ET-2>>ET-2		ET-1=ET-2=ET-3
Affinity	ET-1 ~ 10^{-9} mol/l ET-3 ~ 10^{-6} mol/l		ET-1 ~ 10^{-9} mol/l ET-3 ~ 10^{-9} mol/l
Tissue	Vascular smooth muscle		Endothelium Vascular smooth muscle
Vessel type	Conduit and resistance		Endothelium of all vessels. VSMC of resistance and capacitance vessels
Action	Constriction		Dilatation (endothelium) and constriction (VSMC)
Agonists	None	ET-1	ET-3 Sarafotoxin S6c
Prototype antagonists	BQ-123	Bosentan TAK-044	BQ-788

Table 1.3 Endothelin receptors (adapted from Haynes 1998).

Thus, the vascular endothelium orchestrates the control of vascular tone through production of both vasoconstrictor and vasodilator substances, the most important being NO and endothelin-1.

1.5.3 Regulation of vascular smooth muscle cell proliferation

In the presence of an overlying healthy, intact endothelium, vascular smooth muscle cells (VSMCs) remain in a relatively static state. However, when the endothelium is denuded in experimental animals there is a marked proliferation of medial smooth muscle cells to form a neointima (Clowes 1983). In cell culture, endothelial cells produce a number of factors which inhibit VSMC proliferation, including heparin, heparan sulphate and nitric oxide (Nakaki 1990). These inhibitors are counterbalanced by the production of various growth promoters including endothelin, transforming growth factor-β (TGFβ; Kirschenlohr 1995), angiotensin II and platelet derived growth factor (PDGF). It is thought that where endothelial damage occurs, the balance of these endothelium-derived factors is disrupted such that promoters of VSMC proliferation predominate.

1.5.4 Control of endothelial permeability

One of the oldest known functions of the vascular endothelium is its ability to control the passage of water, solute and cells from the vascular compartment to the tissues. Water, ions and small molecules cross the endothelium using a small pore system, while larger molecules and proteins traverse the endothelial cell layer by passing between cells through intercellular junctions, the diameter of which is controlled by

the junction-associated actin filament system (JAF; Born 1998). Several substances, including histamine, bradykinin and platelet activating factor, stimulate contraction of the JAF thereby increasing the size of the intercellular gaps and thus increasing endothelial permeability while nitric oxide stabilises the JAF and reduces permeability (Born 1998).

As part of the normal response to inflammation, leucocytes also traverse the vascular endothelium by passing through the intercellular junctions. Endothelial cells express two major groups of adhesion molecules (Springer 1990) which facilitate binding of leucocytes, the selectin family and members of the immunoglobulin superfamily including ICAM-1 and -2 (intercellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule). The vascular endothelium is therefore not only a simple physical barrier between the blood and the tissues but actively controls the passage of water, molecules and leucocytes by varying the expression of adhesion molecules on the cell surface and changing the size of intercellular pores.

1.5.5 Modulation of Thrombosis and Haemostasis

In health, there is a delicate balance between thrombosis and fibrinolysis, with the vascular endothelium playing a major role. Firstly, endothelial cells provide a non-thrombogenic surface allowing uninterrupted blood flow to the tissues. This is accomplished in part by expression of plasminogen-activating factor, and of glycosaminoglycans which inactivate Factor X and thrombin, and thrombomodulin which binds thrombin and converts it to a protein C activator (Pearson 1993). Second, endothelial cells oppose the effects of platelet-derived thromboxane A_2 by the

production of prostacyclin and NO which both vasodilate and inhibit platelet aggregation (Radomski 1987).

1.5.6 Tissue Growth and Repair

Vascular endothelial cells have a remarkable capacity to adjust their numbers and arrangement to suit local requirements, and the ability of the endothelium to extend and remodel its network of cells allows tissue growth and repair. Embryology research has shown that arteries and veins develop from small vessels comprising only endothelial cells and a basal lamina, with the surrounding smooth muscle and connective tissue being added later where required on the instruction of the controlling endothelial cell (Alberts 1989).

The normal lifespan of a vascular endothelial cell is 6-12 months. However, turnover is much higher at branchpoints of arteries where shear stress is increased. Endothelial cells are therefore capable of cell division and movement, and can be shown to proliferate and cover areas of denuded endothelium in damaged arteries and to migrate over the surface of synthetic grafts inserted by vascular surgeons in a process known as "endothelialisation". In addition to repair, endothelial cells are capable of creating new blood vessels. Angiogenesis is important during growth, remodelling of existing tissues and in repair. One of the most easily visualised examples of this is rubeosis iridis in diabetic retinopathy where new blood vessels grow onto the normally avascular surface of the cornea. In addition, through angiogenesis, endothelial cells are intimately involved in the growth of tumours with important

mediators including vascular endothelial growth factor, basic fibroblast growth factor and nuclear factor-κB.

1.6 ENDOTHELIAL DYSFUNCTION

A healthy vascular endothelium is essential for the maintenance of normal blood flow to the tissues. Endothelial dysfunction occurs in response to damaging insults to the endothelial cell and is usually manifest as a reduction in endothelium-dependent vasodilatation. Most studies suggest a reduced production and/or effect of NO, although production of other endothelium-dependent vasodilators may also be affected. Many authors have described an alteration in the balance of production of vasodilators and vasoconstrictors in favour of the vasoconstrictors. This "see-saw" effect is illustrated in Figure 1.9.

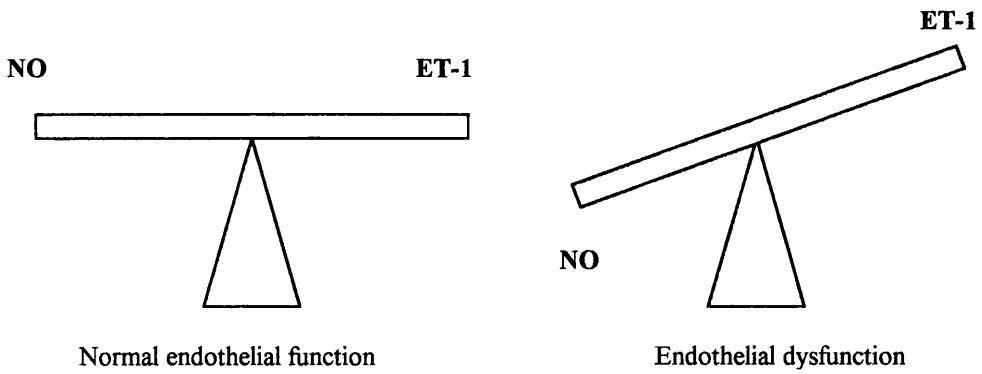


Figure 1.9. Endothelial function and dysfunction

In addition to changes in the production of vasoactive mediators, endothelial dysfunction may involve altered permeability, increased expression of cell surface

adhesion molecules and a loss of control over proliferation of underlying vascular smooth muscle cells. However, abnormal endothelium-dependent vasodilatation is more easily measured and has been documented in many different conditions including hypertension, diabetes mellitus, hypercholesterolaemia and in cigarette smoking. The clinical consequence of endothelial dysfunction is atherosclerosis.

1.7 ATHEROSCLEROSIS

Atherosclerosis, the disease process which leads to occlusive coronary, cerebrovascular and peripheral vascular disease, is responsible for around 50% of all deaths in the general population in the Western world (Ross 1993). Patients with CRF may have accelerated atherosclerosis, first suggested by Lindner in the 1970s, although the exact proportion of deaths in this group which can be directly attributed to atherosclerosis is unclear, as sudden (out of hospital) death is common.

1.7.1 Histology

The earliest visible lesion in the development of the atherosclerotic plaque is the fatty streak, a focal thickening of the vascular intima (Ross 1993). This initially represents a collection of lipid-laden macrophages (foam cells) and T lymphocytes within the intima, and then progresses to an intermediate lesion containing smooth muscle cells and an increased extracellular matrix. Lipid deposition can be both intra- and extra-cellular, recent evidence suggesting that a matrix proteoglycan, biglycan, avidly binds lipoproteins including LDL and apolipoprotein E (O'Brien 1998). Fatty streaks are ubiquitous in the aorta by the early twenties; one large postmortem study of 2876 people who died aged 15-34 of trauma showed that all had evidence of aortic fatty

streaks (Strong 1999). Even younger children, aged 10-14, will have evidence of coronary artery fatty streaks in around 50% (Ross 1993).

The next step in the evolution of the atherosclerotic plaque is the development of a more complex lesion termed a fibrous plaque (Ross 1993). This is characterised by migration and proliferation of smooth muscle cells within the intima, apoptosis of deeper smooth muscle cells with the resulting attraction of further macrophages, and more lipid deposition. Gradually increasing in size, the fibrous plaque begins to project into the arterial lumen and impede the flow of blood to the tissues. An advanced lesion ensues with a thickened fibrous cap and necrotic core. Rupture of this fibrous cap leads to thrombosis and occlusion of the artery.

1.7.2 Pathogenesis

The pathogenesis of atherosclerosis is complex and involves various factors including dyslipidaemia, endothelial dysfunction and inflammation.

1.7.2.i Dyslipidaemia

There are several observations supporting the belief that lipids are intimately involved in the genesis of atherosclerotic plaques :

1. Histological examination of plaques reveals lipid-laden cells within the plaques.
2. Animals fed a cholesterol-rich diet develop lesions of atherosclerosis.
3. In population studies, the incidence of atherosclerosis (and its consequences) is linearly related to serum cholesterol levels.
4. Lipid-lowering trials have reduced the clinical sequelae of atherosclerosis.

Low-density lipoprotein is now known to play a pivotal role in the pathogenesis of atherosclerosis.

- High serum LDL levels are a strong risk factor for the development of atherosclerosis (LaRosa 1990); high serum oxidised LDL levels are associated with coronary artery disease (Holvoet 1998).
- Oxidised LDL is present in lesions of atherosclerosis in humans (Yla-Herttuala 1989). Oxidation of LDL can occur within any of the cells of the artery : endothelium, smooth muscle cells, macrophages and T-lymphocytes. The oxidative process involves a lysine residue of apolipoprotein B (Rosenson 2001).
- Oxidation of LDL is required before uptake by macrophages (via scavenger receptors) to form foam cells (Yla-Herttuala 1989). The most important scavenger receptor is CD36 (Rosensen 2001)
- Radiolabelled oxidised LDL rapidly accumulates in the foam cells of atherosclerotic plaques (Iuliano 2000).
- Oxidised LDL can cause reduced endothelium-dependent vasodilatation (Anderson 1996) and reduced NO production through the inhibition of L-arginine uptake by the endothelial cell (Vergnani 2000).
- Oxidised LDL is chemotactic for monocytes and can up-regulate the genes for macrophage colony-stimulating factor and monocyte chemotactic protein derived from endothelial cells (Ross 1999).
- Foam cells may rupture with release of oxidised LDL, other free radicals and enzymes that can further damage the vessel wall (Ross 1999).

While LDL is clearly the most important lipid involved in atherogenesis, other lipoproteins and triglyceride may play a role :

- Lipoprotein(a) : may be involved through inhibition of conversion of plasminogen to plasmin, thereby promoting thrombus formation, or though chemo-attraction of monocytes and promotion of monocyte binding (Rosenson 2001).
- Triglycerides/VLDL : role uncertain as high levels usually associated with low HDL levels. However, hypertriglyceridaemia is associated with hypercoagulability and increased blood viscosity which may promote atherogenesis.
- High density lipoprotein (HDL) : anti-atherogenic actions include reverse cholesterol transport, maintenance of endothelial function and low blood viscosity. Therefore, low HDL levels are associated with atherosclerosis (Rosenson 2001).

1.7.2.ii Endothelial Dysfunction and Atherosclerosis

An intact and healthy vascular endothelium is necessary to prevent the development of atherosclerosis. Many factors are known to damage the endothelial cell including excessive shear stress in hypertension, oxidised LDL and factors contained in cigarette smoke. Impaired endothelium-dependent vasodilatation has been documented in patients with many different risk factors for atherosclerotic vascular disease including diabetes mellitus, +ve family history, cigarette smoking hypertension, hyperhomocysteinaemia and hypercholesterolaemia. The mechanisms by which endothelial dysfunction promotes/is involved in atherogenesis include :

- reduced production of NO with resultant loss of inhibitory effects on smooth muscle proliferation and platelet aggregation.
- enhanced endothelin-1 production plus reduced NO production will cause abnormal vasoconstriction.
- oxidised LDL can induce expression of cell surface adhesion molecules on endothelial cells (Ross 1993) with increased binding of platelets and monocytes; in addition, circulating levels of adhesion molecules are raised in acute coronary syndromes.
- damaged endothelium may be more permeable to lipoproteins, and to monocytes through the effects of oxidised LDL and through increased production and release of monocyte chemotactic protein-1, osteopontin, platelet-derived growth factor(PDGF) and macrophage colony stimulating factor (M-CSF) (Ross 1999).
- damaged endothelium may synthesize and release powerful mitogens for smooth muscle proliferation including fibroblast growth factor, PDGF and transforming growth factor β (TGF-β), as well as activating factors for underlying macrophages such as M-CSF, oxidised LDL and granulocyte-macrophage colony stimulating factor (GM-CSF) (Ross 1993).

To summarise, dysfunction of the endothelium leads to the development of atherosclerosis through increased stickiness for cells, influx of lipoproteins and cells into the intima, vasoconstriction and promotion of underlying VSMC proliferation.

1.7.2.iii Inflammation and atherosclerosis

Atherosclerosis, like glomerulosclerosis, is an inflammatory process and involves humoral and cell-mediated pathways. Indirect evidence for the role of inflammation comes from the observation that C-reactive protein levels are elevated in patients with angina and further still in those with myocardial infarction (Anderson 1998). CRP also predicts vascular events in apparently healthy people and may therefore be a surrogate marker for atherosclerosis (Ridker 1998a).

Macrophages, platelets, endothelial cells and smooth muscle cells release a variety of cytokines, growth factors and inflammatory substances, all of which may play a role in the development of the atherosclerotic plaque. These factors include :

1. Interleukin-1 (IL-1), IL-3 and tumour necrosis factor (TNF)-α induce the expression of cell surface adhesion molecules and promote smooth muscle proliferation (Ross 1999). TNF-α also promotes macrophage differentiation and foam cell development, and increases endothelial permeability.
2. M-CSF and GM-CSF are produced when monocytes come into contact with the endothelium and promote the differentiation of monocytes into macrophages (Rosenson 2001)
3. Monocyte chemotactic protein-1 promotes adhesion and transgression of monocytes to and through the endothelium.
4. The chemokine RANTES (regulated on activation normally T-cell expressed and secreted) is laid down by platelets on the surface of damaged endothelial

cells and induce "monocyte arrest" on the damaged surface (von Hundelshausen 2001).

5. Activated neutrophils release alpha-defensins which stimulate the binding of LDL to endothelial cells (Rosenson 2001).

1.7.2.iv Other factors which may play a role in the development of atherosclerosis

a) **Angiotensin II** : in addition to being a potent vasoconstrictor, angiotensin II may promote atherosclerosis. Macrophages in atherosclerotic plaques contain angiotensin II; release of this may be involved in regulation of VSMC proliferation and extracellular matrix production (Potter 1998). Interestingly, the angiotensin receptor antagonist losartan, when given to hypercholesterolaemic monkeys reduced the formation of fatty streaks by 50% through a mechanism that appeared to involve reduced oxidation of LDL and chemotaxis of monocytes (Strawn 2000). In addition, angiotensin II can inhibit NOS and induce oxidative stress through the activation of nicotinamide adenine dinucleotide oxidase (Taddei 1998). The beneficial effects of ACE-inhibitors will be discussed in Chapter 7.

b) **Tissue factor** : present in advanced plaques and directly responsible for activation of coagulation upon plaque rupture.

c) **Endothelin-1** : oxidised LDL increases the production and release of ET-1 from the endothelium. ET-1 is both a powerful vasoconstrictor and VSMC mitogen.

d) **Infections** : infection may theoretically promote atherogenesis in two ways – direct damage to the endothelium by an infectious agent such as a virus, or a chronic low grade systemic inflammatory response to a chronic infection. Some studies have shown an association between the severity of coronary atherosclerosis and the number of antibodies to different pathogens (Zhu 2000) which would back up the second theory. There is certainly some evidence of a link between atherosclerosis and Chlamydia pneumoniae, Helicobacter pylori and Cytomegalovirus infections, although data are often conflicting and no studies in humans have shown a benefit of antibiotic treatment in this situation (Rackley 2001).

1.7.3 Clinical Consequences

Atherosclerosis has few clinical effects in its earliest and intermediate stages. However, when the vessel lumen is narrowed by 70% or more, distal ischaemia ensues : this may present as stable exertional angina, intermittent claudication or occasionally, transient ischaemic attacks. Rupture of the atherosclerotic plaque often brings catastrophic consequences : myocardial infarction, sudden death, gangrene of an extremity or stroke.

1.8 "TRADITIONAL" CARDIOVASCULAR RISK FACTORS IN CRF

1.8.1 Dyslipidaemia

The following lipid abnormalities are established risk factors for atherosclerotic coronary heart disease in the general population :

- Raised total serum cholesterol (Stamler 1986)
- Rasied LDL cholesterol (LaRosa 1990).
- Raised small dense LDL cholesterol (Gardner 1996).
- Low HDL cholesterol (Gordon 1977).
- Raised total/HDL cholesterol ratio (Kinosian 1994).
- Raised lipoprotein(a) (Danesh 2000).

Confusion remains over hypertriglyceridaemia : some studies show a link with CHD but this may be through the association of hypertriglyceridaemia with low HDL and raised small dense LDL (Avins 2000).

Dyslipidaemia is common in renal disease, however the pattern varies between different groups of patients (Table 1.4). In contrast with the general population, dyslipidaemia is less clearly associated with adverse cardiovascular risk in patients with renal disease. In fact, one of the first studies to question the importance of dyslipidaemia showed an increased mortality in haemodialysis patients with low total serum cholesterols (Lowrie 1990). While this goes against most data from the general population, it does not necessarily mean that high cholesterols are good for HD

patients, nor that lowering cholesterol is dangerous, but more likely reflects malnutrition in sicker HD patients.

In terms of dyslipidaemia predicting adverse cardiovascular outcome in renal disease the only known associations are :

- Increased lipoprotein(a) is associated with CV events in haemodialysis patients (Cressman 1992, Kronenberg 1999).
- In diabetic patients on haemodialysis, raised total cholesterol, LDL cholesterol, LDL/HDL ratio and apolipoprotein B predicted cardiac death (Tschope 1993).
- In pre-dialysis CRF with creatinine clearance between 20 and 50 ml/min/1.73m2 BSA, the only lipid-related correlate of MI was low HDL (Jungers 1997).
- Increased risk of MI and CV death in nephrotic syndrome (Ordonez 1993); although there are no prospective studies linking dyslipidaemia in nephrotics to increased CV risk.
- Raised intermediate density lipoprotein (IDL) is correlated with carotid atherosclerosis in haemodialysis patients (Shoij 1998).

Patient Group	*Total chol*	*LDL*	*Small dense LDL*	*HDL*	*Triglycerides*	*Lp(a)*
Nephrotic syndrome	↑↑↑	↑↑	↑	↓	↑	↑↑
Pre-dialysis CRF	→	→	↑	↓	↑↑	↑
CAPD	↑	↑	↑	↓	↑	↑
Haemodialysis	→	→	↑	↓	↑↑	↑
Transplant	↑↑	↑		→	↑	↑

Table 1.4. Patterns of dyslipidaemia in renal disease. (Data adapted from Kasiske 1998, Wheeler 1994, Deighan 2000).

1.8.2 Hypertension

In the general population, both systolic and diastolic hypertension are well-established risk factors for chronic renal failure (Klag 1996), stroke (MacMahon 1990), coronary heart disease (Kannel 1971) and left ventricular hypertrophy (Frohlich 1992). In the MRC Mild Hypertension Trial, pulse pressure was also a powerful predictor of coronary events (Millar 1999).

While hypertension is an almost invariable accompaniment of CRF and is clearly associated with progression of CRF, the evidence linking hypertension with adverse cardiovascular outcome in patients with end-stage renal failure is conflicting. In fact, the picture is similar to that seen in hyperlipidaemia with increased mortality in dialysis patients with lower systolic blood pressures (Port 1999). A recent study has confirmed this, and has demonstrated a U-shaped curve in haemodialysis patients for pre-dialysis SBP (Mazzuchi 2000). It is now recognised that low blood pressure in haemodialysis patients is often indicative of cardiac failure. Thus, high blood pressure (at least post-dialysis) is likely to be a significant risk factor for adverse cardiovascular outcome in haemodialysis patients. To date, there is little evidence linking hypertension to cardiovascular events in CAPD patients (Cheigh 1999) and renal transplant recipients (Kasiske 1996). The prevalence and aetiology of post-transplant hypertension will be discussed fully in chapter 4.

1.8.3 Tobacco Smoking

The American Heart Association has stated that "smoking is the single most alterable risk factor contributing to premature morbidity and mortality in the United States, accounting for more than 400,000 deaths annually" (Ockene 1997). The following general information is now accepted regarding the cardiovascular risks of tobacco smoking in the "non-renal" population :

- Smoking increases all-cause mortality by 62% and cardiovascular mortality by 63% (Qiao 2000).
- The incidence of MI is increased 6x in women and 3x in men who smoke at least 20 cigarettes a day (Prescott 1998).

- Smoking doubles the risk of stroke (Kawachi 1993).
- The increased cardiovascular risk associated with smoking is worse in those who drink no alcohol (Foody 2001), perhaps explaining the "French paradox".

Through its atherogenic effect, cigarette smoking is likely to play a prominent role in the genesis of renovascular disease and ischaemic nephropathy. In addition, newer data now suggest that smoking increases the chance of developing microalbuminuria in type 1 diabetes mellitus, increases the rate of decline of GFR in type 1 and 2 diabetes and increases the risk of developing ESRF in a number of primary renal diseases (Orth 2000).

In patients with ESRF :

- Smoking increases the mortality rate in haemodialysis patients (USRDS 2000).
- Smoking is associated with a doubling of mortality in diabetic dialysis patients (McMillan 1990).
- Smoking increases the risk of renal transplant loss, through death from cardiovascular disease with a functioning graft (Kasiske 2000).
- In our own renal transplant population, smoking was associated with a hazard ratio of 1.81 for survival (Woo 2002).

1.8.4 Diabetes Mellitus

Coronary heart disease, stroke and peripheral vascular disease are major causes of morbidity and mortality in patients with diabetes mellitus. In the Framingham Study,

types 1 and 2 diabetes mellitus, as well as glucose intolerance, were strong independent risk factors for coronary death (Kannel 1979). More recently, type 2 diabetes mellitus has been viewed as predominantly a vascular disease with patients exhibiting a triad of insulin resistance, dyslipidaemia and hypertension. Atherosclerotic vascular events are particularly common in these patients, and ESRF secondary to type II diabetes mellitus is becoming increasingly prevalent.

In the U.K., around 20% of patients under 65yrs of age starting dialysis have diabetic nephropathy, while the figure is 12% in those over 65. In the USA, 45% of prevalent RRT patients have diabetic nephropathy (USRDS 2000). The combination of diabetes mellitus and chronic renal failure confers a particularly high cardiovascular risk, and it is widely accepted that diabetes mellitus is an independent risk factor for atherosclerotic CVD in patients with CRF (Foley 1994). Data from the USRDS and ERA show that survival in haemodialysis patients is considerably worse in those patients whose primary renal disease is diabetic nephropathy (USRDS 1998; ERR 2000), with the majority of deaths being cardiovascular. Brown et al assessed comparative mortality rates in haemodialysis patients in Manchester Royal Infirmary and showed that mortality from CVD was 10 times higher in dialysis patients compared to the general population and 44 times higher in those dialysis patients who were diabetic (Brown 1994). In the renal transplant population, Kasiske showed that diabetes carries a relative risk of ischaemic heart disease of 3.25 and of peripheral vascular disease of 28.18 (Kasiske 1996).

1.8.5 Family History

A positive family history is a significant independent risk factor for myocardial infarction in the general population. The GISSI investigators showed that the relative risk for MI in an asymptomatic individual was 2.0 if one parent affected, 3.4 if a sibling affected and 20.0 if two or more relatives had an MI before the age of 50 (Roncaglioni 1992). There are no studies to suggest that the effect of a positive family history would be different in those with renal disease.

1.8.6 Other "Traditional" Risk Factors in CRF

There are several other risk factors for CHD for which there is some evidence in the general population, but none in the CRF population. These include obesity, lack of physical exercise, diet deficient in fresh fruit and vegetables and abstinence from alcohol consumption.

1.9 "NON-TRADITIONAL" RISK FACTORS FOR CHD IN CRF

While patients with ESRF often have one or more of the risk factors detailed above, there are a number of less traditional risk factors for CHD in this group.

1.9.1 Anaemia

Anaemia secondary to erythropoietin deficiency almost always accompanies end-stage renal failure, and is clearly associated with increased morbidity and mortality in

haemodialysis patients (Ma 1999). A considerable body of evidence now exists linking anaemia in CRF with cardiac structural and functional changes, most commonly left ventricular hypertrophy and dilatation (Parfrey 1999). In the pre-dialysis phase, the haemoglobin begins to fall when GFR is between 25 and 50 ml/min. Levin showed that in a cohort of pre-dialysis patients, each 0.5g/dl fall in haemoglobin was associated with increased left ventricular growth (Levin 1999). In the dialysis cohort followed by Parfrey and Foley, anaemia was independently associated with progressive LV dilatation on echocardiography, the development of de novo cardiac failure and overall mortality (Foley 1996a). The mechanism linking anaemia to LV hypertrophy and dilatation is complex but principally involves volume overload - lower blood viscosity plus arterial and venous dilatation lead to reduced peripheral resistance and in turn, to increased venous return.

Correction of anaemia with erythropoietin has been shown in some studies to partially reverse left ventricular hypertrophy (Silberberg 1990). However, the target haemoglobin or haematocrit is a subject of debate and on-going research, one recent study being terminated early as the high haematocrit (Hct 42% v 30%) group had a slightly increased number of cardiovascular events and vascular access thromboses (Besarab 1998). Many believe that the problem with such trials is that we are intervening too late when LVH and heart failure are well-established, and that EPO should be used at an earlier, pre-dialysis, stage to maintain a near-normal haemoglobin throughout a patient's renal life.

Are all the cardiovascular changes related to anaemia in CRF limited to the heart? Certainly, there is no evidence in the general or uraemic populations that anaemia

promotes atherosclerosis. Theoretically, however, one could postulate that as haemoglobin "mops up" nitric oxide, then anaemia would be associated with a relative increase in the bioavailability of this anti-atherogenic molecule with a reduction in atherosclerosis. Enhanced NO bioavailability has already been postulated as the mechanism linking anaemia with a "hyperdynamic circulation" (Anand 1993). While endothelial dysfunction and atherosclerosis have not been directly linked to renal anaemia, arterial remodelling has. Gerard London's group have shown that vessels such as the common carotid artery have an increased internal diameter and intima-media thickness in anaemic ESRD patients and that these measurements correlate inversely with haematocrit. These changes are thought to occur in response to increased cardiac output, blood flow and shear stress in the central arteries (Metivier 2000).

1.9.2 Hyperparathyroidism

In common with anaemia, secondary hyperparathyroidism is one of the hallmarks of chronic renal failure, driven by chronic hyperphosphataemia and hypocalcaemia. Elevated calcium-phosphate product plays a major role in metastatic calcification of organs and tissues including the blood vessels. Despite great improvements in dialysis adequacy and management of anaemia, nephrologists have made little impact on phosphate control over the last decade, and it is only recently that newer calcium-free and aluminium-free phosphate binders have become available. Large registry data demonstrate that phosphate is an important independent predictor of outcome on dialysis (perhaps simply reflecting dialysis adequacy). The importance of deranged calcium-phosphate homeostasis in the advanced atherosclerosis seen in uraemia is

demonstrated by the high calcium content of atherosclerotic plaques from patients with ESRF (Schwarz 2000). When the concept of accelerated atherosclerosis was first put forward, PTH was considered by many to be a prime candidate for the unknown "uraemic factor" that promoted atherosclerosis. This remains unproven, but certain facts are now known about hyperparathyroidism and altered calcium-phosphate homeostasis with regard to their adverse effects on cardiovascular function :

- As PTH is a calcium ionophore at a cellular level, chronic exposure to high PTH levels leads to intracellular hypercalcaemia which in turn results in cellular and organ dysfunction (Block 2000).
- PTH promotes cardiac interstitial fibrosis and cardiac arteriolar thickening in uraemic rats (Amann 1995).
- PTH may inhibit hepatic triglyceride lipase and lipoprotein lipase, causing hypertriglyceridaemia, raised LDL and reduced HDL levels (Block 2000).
- Raised calcium-phosphate product may lead to cardiac valve calcification and dysfunction (Block 2000).
- Raised PTH and calcium phosphate product is associated with vascular calcification. Electron-beam computed tomography detected much greater coronary artery calcification in haemodialysis patients compared to control, with the highest calcification scores in those with highest PTH values (Braun 1996).
- In dialysis patients, elevated serum phosphate is independently associated with left ventricular dilatation (Block 2000), all cause mortality and cardiovascular mortality (Block 1998).

- Calcium-phosphate deposition within the myocardium may be associated with cardiac failure (Rostand 1988).
- Using high resolution ultrasound, serum phosphate and PTH levels have been associated with both carotid and femoral atherosclerosis (Kawagishi 1995).

Thus, there is now a body of evidence supporting a role of increased serum phosphate, calcium-phosphate product and PTH in the excessive cardiovascular disease burden of uraemic patients.

1.9.3 Hypoalbuminaemia

In Parfrey and Foley's cohort of haemodialysis patients, falling serum albumin levels were highly predictive of the development of cardiac failure, ischaemic heart disease, cardiac mortality and overall mortality (Foley 1996b). Similarly, they found that in peritoneal dialysis patients, hypoalbuminaemia was associated with LV dilatation, de novo cardiac failure and all-cause mortality (Foley 1996b). It is unclear how one should interpret these data as hypoalbuminaemia is a marker for under-dialysis, chronic sepsis, inflammation and malnutrition, factors which in themselves may be related to cardiac disease. The role (if any) of hypoalbuminaemia in promoting atherosclerosis in CRF is unclear.

1.9.4 Oxidative Stress

Reactive oxygen species, including peroxynitrite, hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl ($OH^\cdot$) are continuously formed *in vivo* by many cell

types including macrophages, endothelium and vascular smooth muscle cells. Production of these molecules is counterbalanced by the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. Superoxide is converted to H_2O_2 by the activity of SOD, while in conditions of low SOD activity, O_2^- reacts with NO to form peroxynitrite ($ONOO^-$). Peroxynitrite is relatively stable, but exists in equilibrium with its acid HOONO which rearranges to form nitrate and $OH^\cdot$ which is highly reactive. What are the consequences for vascular biology of accumulation of these reactive oxygen species?

- When excess O_2^- is produced or SOD activity is low, NO is scavenged and its protective, "anti-atherogenic", properties are negated.
- Reactive oxygen species are involved in the oxidation of LDL to a form that is toxic to endothelial cells, is more easily taken up by macrophages to form foam cells, and is involved in recruitment of monocytes (Becker 1997)
- Lp(a) is also oxidised to a form which is again toxic to endothelium and is more readily taken up by macrophages to form foam cells (Becker 1997).

Studies of the cardiovascular effects of increased oxidative stress are hampered by the lack of a standardised test. In the general population there is some evidence from large population studies that diets high in beta-carotene and vitamin E (both anti-oxidants) may be protective against myocardial infarction (Rimm 1993). A direct causative effect is difficult to show however as such diets (high in fresh fruit and vegetables) are also associated with higher social class, increased exercise and non-smoking. Oxidised LDL clearly plays a major role in atherogenesis, and there is evidence that titres of anti-oxidised LDL antibodies correlate with the risk of

progressive atherosclerosis in non-uraemic subjects (Becker 1996). To date, no study has proven that oxidative stress is a major factor in the development of atherosclerotic vascular disease in the general population.

In CRF, the antioxidant capacity of uraemic patients is low (Westhuyzen 1995) perhaps reflecting depletion of antioxidants such as vitamin C and E during dialysis, and there is enhanced oxidation of LDL and raised anti-oxidised-LDL antibody levels, suggesting increased oxidative stress (Maggi 1994). A recent study has shown an eight-fold increase in oxidised LDL levels in uraemic patients compared to controls (Itabe 1996). In addition, Miyata et al have demonstrated that in uraemia there is accumulation of advanced glycation end products (AGE), more usually seen in diabetes (Miyata 1999). These AGEs are not related to hyperglycaemia, but rather reflect "carbonyl stress", with accumulation of small carbonyl precursors of AGEs produced through oxidation of carbohydrates and lipids. The mechanisms underlying increased oxidative stress in uraemia remain unclear. Uraemic toxins may be important, as may intravenous iron, leptin, and haemodialysis, particularly using bio-incompatible membranes, which increases the generation of oxygen free radicals (Westhuyzen 1995; Haklar 1995).

1.9.5 CRP and Inflammation

C-reactive protein (CRP) is a hepatic-derived acute phase protein which rises in a multitude of acute and chronic situations including infection, inflammation, trauma, infarction and some malignancies. The acute phase response is driven by cytokines including interleukin-6, IL-1, interferon gamma, TNF-α and TGF-β, and besides CRP

includes serum amyloid A protein, fibrinogen, lipoprotein(a), caeruloplasmin, ferritin and alpha-1 antitrypsin. Recent evidence has pointed to a link between atherosclerotic cardiovascular disease and CRP. In healthy men and women, elevated CRP is a predictor for future cardiovascular events (Koenig 1999; Ridker 1998b), while in patients with unstable angina it predicts short- and long-term outcome (Liuzzo 1994). The predictive effects of CRP appear to be additive to those of established coronary risk factors such as increased total:HDL cholesterol ratio. Several investigators are cynical believing that CRP is simply a marker, for example of the size of the ischaemic area in unstable angina, of the effects of other risk factors such as smoking and dyslipidaemia or a marker of the inflammatory nature of atherosclerosis. Nevertheless, there are several pieces of evidence that point to a more direct atherogenic role for CRP :

- CRP has been found within atherosclerotic plaques (Podrid 2001).
- CRP binds to LDL and allows uptake by macrophages without the need for oxidation (Zwaka 2001).
- CRP induces adhesion molecule expression on endothelial cells (Pasceri 2000).

CRP levels tend to be higher in patients with ESRF, and again, most of the studies with a relatively long follow-up have shown that CRP is predictive of cardiovascular mortality in both haemodialysis and CAPD patients (Yeun 2000). The role of CRP in renal transplant recipients is not yet known. Postulated reasons for elevated CRP in ESRF include (Yeun 2000) :

- A direct inflammatory effect of uraemia.
- Bio-incompatibility of haemodialysis membranes and CAPD fluids.
- Chronic infection of vascular access.

- Dialysis water impurities.

The evidence linking CRP with adverse cardiovascular outcome in the general and ESRF populations appears moderately strong, but it remains unclear if the relationship is causal.

1.9.6 Hyperhomocysteinaemia

Homocysteine is a sulphur-containing amino acid that is formed during the conversion of methionine to cysteine, and that accumulates in homocystinuria, an autosomal recessive condition marked by premature cardiovascular death. It was this observation by McCully in 1969 that led to the concept of homocysteine being atherogenic. Mild elevations in serum homocysteine are seen in 5-7% of the general population where observational studies have clearly shown an association between hyperhomocysteinaemia and atherosclerotic vascular disease and recurrent venous thromboembolism (Welch 1998, Bostom 1999a). Meta-analyses have suggested that the relative risk of CV events (after adjustment for standard risk factors) associated with a fasting tHcy of >15μmol/l is 1.4 (Bostom 1999a).

Hyperhomocysteinaemia can occur in a number of ways. Normal metabolism of this amino acid is by one of 2 pathways : transsulfuration to cysteine (requires vitamin B6) or remethylation to methionine (requires vitamin B12 and folate). Deficiencies of the vitamins, mutations of the enzymes involved, high methionine intake, liver disease, CRF and drugs may all cause elevation in plasma homocysteine (tHcy) levels (Welch 1998). The mechanism in CRF is debated : despite their being a linear inverse

relationship between tHcy levels and GFR, tHcy excretion in urine is not thought to be significant in normal states (Yeun 2000). Studies now suggest that the defect in CRF is in the remethylation pathway (van Guldener 1999). The result is that hyperhomocysteinaemia (fasting tHcy >13.9 μmol/l) is around 100 times more common in dialysis patients and around 18 times more common in stable renal transplant recipients compared to matched controls without renal disease (Bostom 1999).

In keeping with the data for the general population, hyperhomocysteinaemia has been independently associated with increased cardiovascular mortality in patients with ESRD (Bostom 1999b). The pooled data from three prospective studies (two in dialysis patients and one in pre-dialysis patients) reveal a relative risk of a cardiovascular event being 2.8 times greater in the group with the highest tertile of homocysteine (C.I. 1.6 to 5.0) (Bostom 1999b). Moreover, in a recent prospective study of 84 haemodialysis patients, hyperhomocysteinaemia was associated with vascular access thrombosis, with each 1μmol/l increase in fasting tHcy being associated with a 4% increased risk of access thrombosis (Shemin 1999).

The mechanism whereby homocysteine promotes atherosclerosis and thrombosis is not yet fully understood. Some authors have suggested reverse causality i.e. the elevated homocysteine is simply a marker of the underlying atherosclerosis. This is unlikely as children with homocystinuria develop atherosclerosis at an early age and this can be partially prevented by lowering homocysteine. Similarly, acute hyperhomocysteinaemia (induced by oral methionine loading) leads to impaired endothelium-dependent vasodilatation in otherwise healthy individuals (Chambers

1999). Several studies have now shown impaired endothelium-dependent vasodilatation in hyperhomocysteinaemia (Tawakol 1997; Kanani 1999; Chambers 1999) and that this is reversible with vitamin C (Chambers 1999). These data lend weight to the theory that homocysteine's principal atherogenic effects are mediated through endothelial dysfunction with the mechanism involving increased oxidative stress. Additional factors may play a role :

- There is *in vitro* evidence that homocysteine is directly toxic to vascular endothelium (Blundell 1996).
- Homocysteine enhances LDL oxidation (Bostom 1999b).
- Homocystinuria is associated with increased thromboxane-mediated platelet aggregation (Di Minno 1993).
- Homocysteine stimulates vascular smooth muscle proliferation (Tsai 1994).
- Homocysteine enhances binding of lipoprotein(a) to fibrin (Harpel 1992).

Several studies have looked at lowering homocysteine and these will be discussed further in chapter 7.

1.9.7 Endogenous inhibitors of NO Synthase

The synthesis of NO by NO synthase may be competitively inhibited by naturally-occurring analogues of L-arginine such as N^G-monomethyl-L-arginine (L-NMMA), symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA), substances derived from the breakdown of proteins containing methylated arginine residues. Accumulation of ADMA (either from reduced excretion &/or metabolism) has been shown to occur in CRF with the original paper reporting an 8-fold rise in

plasma ADMA concentrations (to 4μmol/l) compared to healthy controls (Vallance 1992). This group were able to demonstrate that 5μmol/l ADMA inhibited NO production *in vitro* by around 17% in a cytosolic preparation of macrophage NO synthase, and that ADMA vasoconstricted rat aortic rings with the EC_{50} for ADMA being 26μmol/l. They also demonstrated that infusion of ADMA in guinea pigs to achieve a plasma concentration of 9μmol/l raised systolic blood pressure by 15%. It was concluded that accumulation of ADMA in ESRF may reach physiologically significant levels, and that the resultant inhibition of NO production may be involved in the pathogenesis of hypertension and immune dysfunction associated with ESRF.

However, a subsequent paper by MacAllister et al showed that ADMA concentrations only rose to around 0.9 μmol/l in 10 uraemic subjects, a 3-fold increase over control, and the clinical relevance of ADMA in uraemia was therefore disputed (MacAllister 1996). More recently, however, this issue has been examined again in a larger group of dialysis patients. Kielstein et al found that in 43 haemodialysis patients, ADMA concentrations were 6.0 μmol/l pre-dialysis, a 6-fold increase over control, while the concentration was normal in 37 peritoneal dialysis patients (Kielstein 1999). The difference related to treatment modality was thought to be a result of differential dialytic clearance or metabolism of ADMA. The same group also found that L-arginine levels were unchanged in ESRF contrary to previous reports. It should be noted that each of the studies reported above give different "normal" values for ADMA (0.3 - 1.0 μmol/l), reflecting differences in assays and laboratory technique.

Raised serum dimethylarginine levels have also been reported in other conditions including hypercholesterolaemia, cardiac failure, peripheral vascular disease and

essential hypertension (Cooke 2000). In these situations (and possibly also in CRF) accumulation of ADMA, L-NMMA and SDMA is thought to be secondary to dysfunction of the enzyme dimethylarginine dimethylaminohydrolase (DDAH), an enzyme responsible for the breakdown of ADMA to citrulline (Cooke 2000). The activity of the enzyme may be directly inhibited by hypercholesterolaemia, hyperglycaemia and oxidative stress.

Thus, accumulation of ADMA certainly occurs in renal failure, the degree varying considerably between studies, and also with treatment modality. Whether the levels reached are physiologically significant has been a matter of debate until recently, although the intracellular ADMA concentrations may be many fold higher than the measured plasma levels (MacAllister 1994). Recent publications by Zoccali have thrown further light onto the clinical relevance of ADMA accumulation in ESRF. In a cohort of 225 haemodialysis patients followed for around 3 years, ADMA levels were independently predictive of overall and cardiovascular mortality suggesting that significant inhibition of NO synthase occurs *in vivo* (Zoccali 2001). A further study in 90 haemodialysis patients revealed a strong link between ADMA levels and CRP, and the interaction between these factors was the most important predictor of progression of carotid intimal lesions (Zoccali 2002). Thus ADMA may be one of the most important "non-traditional" risk factors for atherosclerotic vascular disease in CRF.

In summary then, CRF is associated with many risk factors for atherosclerotic vascular disease, some traditional, others not (Figure 1.10). Many of the risk factors have been directly linked to atherosclerosis or adverse cardiovascular outcome in

CRF. Some are associated with endothelial dysfunction and this will be discussed further in Chapters 3-6, and in the general discussion.

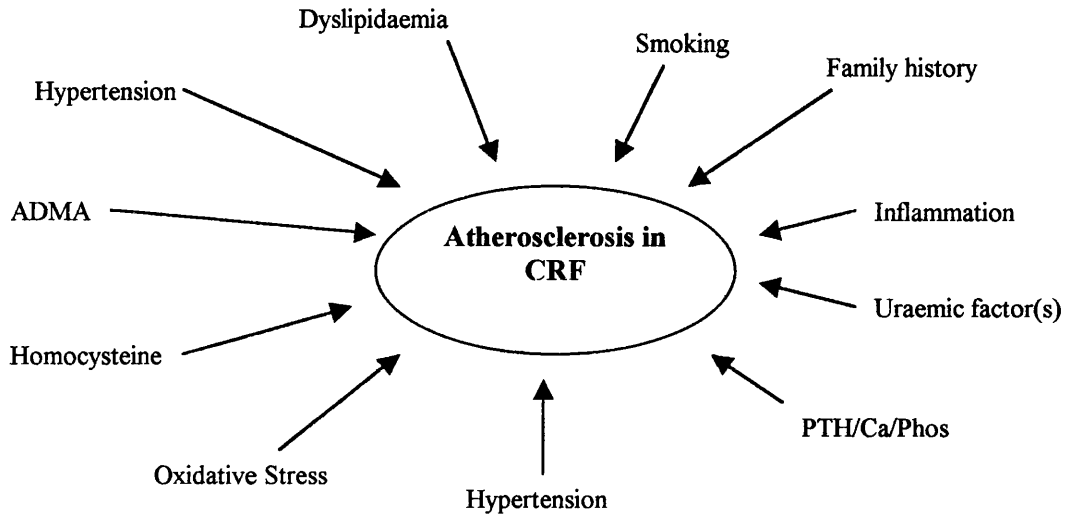


Fig. 1.10 Risk factors for atherosclerosis in uraemia.

1.10 Endothelial dysfunction in renal disease : background

As uraemic patients often have several risk factors for atherosclerotic vascular disease, and as many of these are associated with endothelial dysfunction, one would expect endothelial dysfunction to be present in CRF. Several groups have looked at markers of endothelial function in renal failure, with the majority of work being on the L-arginine/NO pathway. It has been known for many years that uraemic serum

inhibits growth or is cytotoxic to cell lines in culture (Delaporte 1982; Wessel-Aas 1981). A recent study of the effects of pooled uraemic serum on cultured human umbilical vein endothelial cells (HUVECs) demonstrated that while the uraemic serum had no appreciable effect on HUVEC cell number or viability, the cells were less adhesive to gelatin-coated cover-slips, and the extracellular matrix (ECM) generated by the HUVECs was reduced in quantity and the fibrils disorganised (Aznar-Salatti 1995). It was concluded that the uraemic serum adversely affects endothelial ECM production and attachment of the endothelial cells to the subendothelium. Arese et al (Arese 1995) also demonstrated that uraemic serum inhibits NO synthesis (iNOS) in murine macrophage and endothelial cell lines, but not in HUVECs. However, it is still unclear whether NO production is reduced or elevated in uraemia, one reason being that nitric oxide is extremely difficult to measure in plasma owing to its short half-life. The stable metabolites of NO, nitrite and nitrate, are often measured as an indirect indication of NO production, although these results may be confounded by nutritional status. The observation that the prolonged bleeding time in uraemic rats may be corrected by L-NMMA, suggests that NO production may be enhanced (Remuzzi 1990). Noris et al (Noris 1993) examined this issue in humans and demonstrated enhanced NO production by uraemic platelets and by HUVECs exposed to uraemic plasma. The clinical relevance of enhanced NO production by platelets in uraemia may be dialysis hypotension and the uraemic bleeding tendency. It is quite possible that while endothelial NO production is reduced, platelet NO production is enhanced; this would reconcile the expected finding of endothelial dysfunction in uraemia and the observed increased NO production by uraemic platelets. Many questions remain unanswered.

Some authors have shown that there are markers of endothelial damage in uraemia. The vascular endothelium is the main site of production of the glycoproteins, von Willebrand factor (vWf), and tissue-type plasminogen activator (t-PA). Levels of these glycoproteins have been shown to rise in uraemia (Haaber 1995, Gris 1994) and are thought to reflect underlying endothelial dysfunction. In addition, two groups (Gris 1994, Nakayama 1994) have demonstrated raised soluble thrombomodulin in uraemia, a further marker of endothelial injury (Kazama 1991), and a reduced ability of the endothelium to release t-PA upon stimulation with desmopressin, a defect which may reflect general endothelial dysfunction and predispose to thrombosis.

All of the above studies use surrogate markers of endothelial function. In patients with nephrotic range proteinuria, Stroes et al demonstrated impaired stimulated but preserved basal endothelial NO production (Stroes 1995a) using venous occlusion plethysmography, an invasive technique which allows measurement of forearm blood flow and the effects of endothelium-dependent and -independent vasodilators. At the beginning of this research project in 1996 there was little published on endothelial function *in vivo*, and in particular on endothelium-dependent vasodilatation in uraemia. The studies performed for this thesis were designed to examine endothelial function in adult patients with CRF.

1.11 Aims of this project

The principal aims of this work were :

i To establish the presence or absence of endothelial dysfunction in patients with CRF including renal transplant recipients.

ii To identify correlates of endothelial dysfunction.

iii By using a combination of in vitro and in vivo techniques to determine the influence of circulating factors on endothelial function in CRF.

Secondary aims were :

iv To examine the effects of fluid balance on endothelial function in CAPD patients using forearm plethysmography.

v To assess the effects of imunosuppressive drug therapy on endothelial function in renal transplant recipients

1.12 Hypothesis

The studies performed are designed to test the hypothesis that patients with CRF including those on renal replacement therapy and following renal transplantation have endothelial dysfunction and that this is due to the presence of a circulating factor that may be unique to uraemia

1.13 Outline of the studies contained in this thesis

Four principal studies were performed and are detailed in Chapters 3-6 :

Chapter 3 : An *in vivo* study of endothelial function in CRF

Chapter 4 : An *in vitro* study of endothelial function in CRF

Chapter 5 : A study of endothelial function and fluid balance in CAPD patients

Chapter 6 : An *in vivo* study of endothelial function in renal transplant recipients

Chapter 2

Materials and Methods

2.1 Introduction

The principal techniques used for this thesis were forearm venous occlusion plethysmography and wire myography. Chapters 3, 4 and 5 detail the forearm plethysmography studies while chapter 6 focuses on wire myography. Within each individual chapter, a description of methods particular to that study will be given. In this present chapter are described the materials, apparatus, experimental technique and physiological basis behind each of the main techniques used in the studies.

2.2 Ethics Committee Approval

The studies contained within this thesis were approved by the West Glasgow Hospitals University NHS Trust Ethics Committee. All subjects gave written informed consent, the forms being approved by the Ethics Committee.

2.3 Forearm Venous Occlusion Plethysmography

2.3.1 Plethysmography : History

Plethysmography is the measurement of variation in the volume of a part of the body. Venous occlusion plethysmography, first described by Brodie and Russell in 1905 (Brodie 1905) has become the standard method for estimating the peripheral blood flow of limbs in man. This technique works by temporarily arresting venous return (without affecting arterial inflow), with recording of the subsequent change in volume

of the limb under study. If arterial blood pressure remains constant, then the rate of swelling of the forearm equates to the rate of arterial inflow (Whitney 1953).

The historical studies measured absolute changes in volume of a body part by enclosing the part under study in a sealed jacket filled with air or water, and measuring the amount of air or water displaced (Whitney 1953, Brodie 1905, Hewlett 1909). As this method was cumbersome, Whitney devised the strain-gauge plethysmograph in 1949 (Whitney 1949), and this was subsequently validated in direct comparisons with air-filled (Eickhoff 1980) and water-filled plethysmographs on several occasions (Whitney 1953; Dahn 1970, Clarke 1957). With in situ electrical calibration (Whitney 1953), the mercury-in-rubber strain-gauge plethysmograph has become the preferred method for the measurement of absolute forearm blood flow. This technique has been widely utilised to examine the effects of particular drugs on blood flow, and also to investigate vascular function in a wide range of clinical conditions compared to control subjects. It has the advantage of allowing the study of vessels and their responses to pharmacological manipulation within their normal physiological environment. There are now over 300 human forearm strain-gauge plethysmography studies published.

2.3.2 Plethysmography : Principles of the technique

The underlying principle of venous occlusion plethysmography is straightforward : "if venous return from the arm is obstructed and arterial inflow continues unimpeded, the forearm swells at a rate proportional to the rate of arterial inflow" (Benjamin 1995). Temporary venous occlusion is achieved by inflation of a pneumatic "collecting" cuff

placed over the upper arm. Studies by Patterson and Greenfield in the 1950s confirmed that inflation of a collecting cuff to sub-diastolic pressures did not affect arterial inflow while the forearm volume increased by up to 2% (Greenfield 1954). As blood flow in the hand is mainly through skin, with a high proportion of arterio-venous shunts (Benjamin 1995), the hand is excluded from the system by inflation of a wrist cuff to supra-systolic pressures. Venous occlusion plethysmography therefore measures blood flow in the forearm alone, with 50-70% of this being through skeletal muscle and the remainder through skin (Cooper 1995). Forearm arterial inflow becomes compromised when the forearm veins are fully distended and venous pressure reaches 40mmHg (Wilkins 1946). To prevent this, the venous collecting cuff is inflated for 10secs during each 15secs, and the forearm is placed above the level of the right atrium, allowing rapid emptying of the forearm veins when the cuffs are deflated.

The strain-gauge method devised by Whitney measures percentage change in circumference of the forearm. Each gauge is a fine-bore rubber/silastic tube, filled with mercury and attached by copper plugs to a modified Wheatstone bridge. If the gauge is initially slightly extended, then minor changes in the length of the gauge are recorded as changes in resistance of the mercury. If the forearm is considered as a cylinder, and the length obviously remains unaltered during a study, then the percentage change in volume is approximately equal to twice the percentage change in circumference (Whitney 1953). This percentage change in volume is simply expressed as a blood flow of x ml/100ml of forearm volume/time recorded.

A sample tracing from a plethysmography study is given in Figure 2.1. One can see that there is a pulsatile nature to the trace corresponding to the arterial waveform, and that there is a 1% calibration mark, which is produced by the in-built calibration on the plethysmograph. The measurements are taken from the linear section of the trace before any flattening occurs.

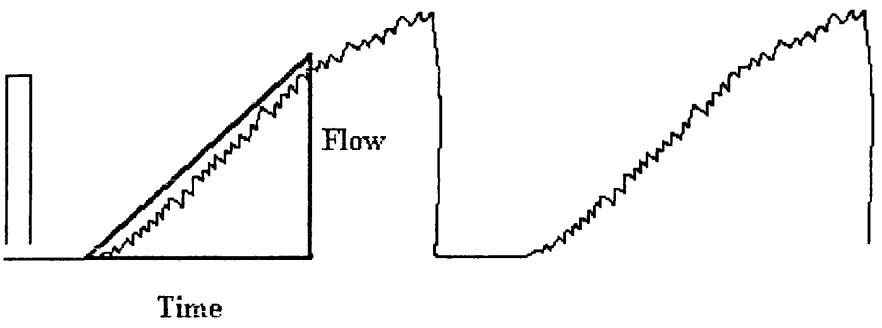


Figure 2.1 A sample plethysmography trace demonstrating the pulsatile rise of the pen tracing in response to distension of the forearm. Flow is measured from the linear section of each curve.

2.3.3 Plethysmography : Equipment and Technique

All forearm studies were performed in a sealed, soundproofed, vascular research laboratory maintained at 24-26°C. Subjects lay supine with arms supported above heart level on foam blocks. Bilateral plethysmography was performed with forearm blood flow measurements being made in both arms simultaneously. Paediatric cuffs (Hokanson TMC-7, PMS Instruments, Maidenhead, England) were placed around the wrists, and inflated to at least 40mmHg above systolic blood pressure for three minutes during each recording, to exclude the hand circulation from the system. Collecting cuffs (Hokanson SC10) were placed around the upper arms and inflated (40mmHg) and deflated in a 15 second cycle to occlude venous return and allow distension of the forearms. Rapid cuff inflation was achieved using a Hokanson AG101 air source, linked to two Hokanson E20 rapid cuff inflators.

Blood pressure was measured at the end of each blood flow recording, using a semiautomatic oscillometric sphygmomanometer (Critikon Dinamap Plus, Florida) placed around the dominant arm, over the collecting cuff. Prior to each study, the maximal circumference of the forearm was measured and a mercury-in-silastic strain gauge (Hokansen forearm set) chosen 2cm shorter than the circumference. Strain gauges were calibrated electrically while in position using a built-in calibration method.

Under local anaesthesia (1ml 1% lignocaine), a sterile 27 gauge dental needle (Terumo, Japan) was inserted into the brachial artery of the non-dominant forearm. (Many authors report the use of 18 gauge brachial artery cannulae to allow the

continuous measurement of intra-arterial blood pressure. However, this carries a greater risk of damage to the brachial artery and is only useful if measurement of forearm vascular resistance is required. Benjamin et al (Benjamin 1995) argue that such readings are not helpful and ethically difficult to justify). The needle was connected to an IVAC infusion pump using a modified 16-gauge epidural giving set (Portex, Hythe, England) with the connection being sealed with dental wax. Drugs or 0.9% saline (Baxter) were infused throughout the study at 1ml/min. Baseline measurements of FBF were obtained at 10 minute intervals for 30 minutes to allow acclimatisation to inflation and deflation of the cuffs. The final baseline reading was used as the baseline FBF. The equipment and technique is illustrated in Figures 2.2-2.4.

2.3.4 Plethysmography : Infusion Protocols

Details of infusion protocols for individual studies are given in Chapters 3-6. In all cases, following insertion of the needle, 0.9% saline was infused at 1ml/min while preliminary FBF readings were measured for 30 minutes. This allowed the subject to become acclimatised to the vascular laboratory and the routine of cuff inflation and deflation.

During this initial period any problems with the needle, cuff or patient position were identified and rectified if possible. Following the 30-minute acclimatisation period, baseline readings of FBF were made. Then, drug 1 was infused in incremental doses with FBF recordings being made at regular intervals, followed by a washout period of

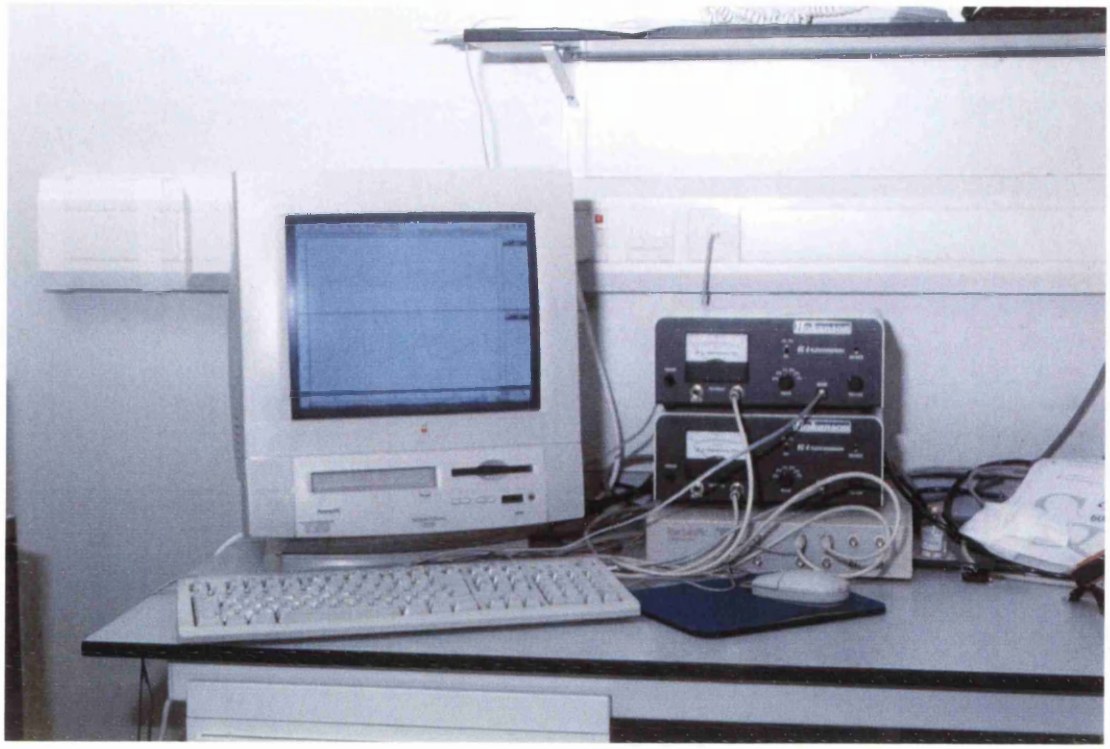


Figure 2.2 Photograph of the forearm plethysmography set-up. This demonstrates a sample tracing on the monitor, and two Hokansen transducers.

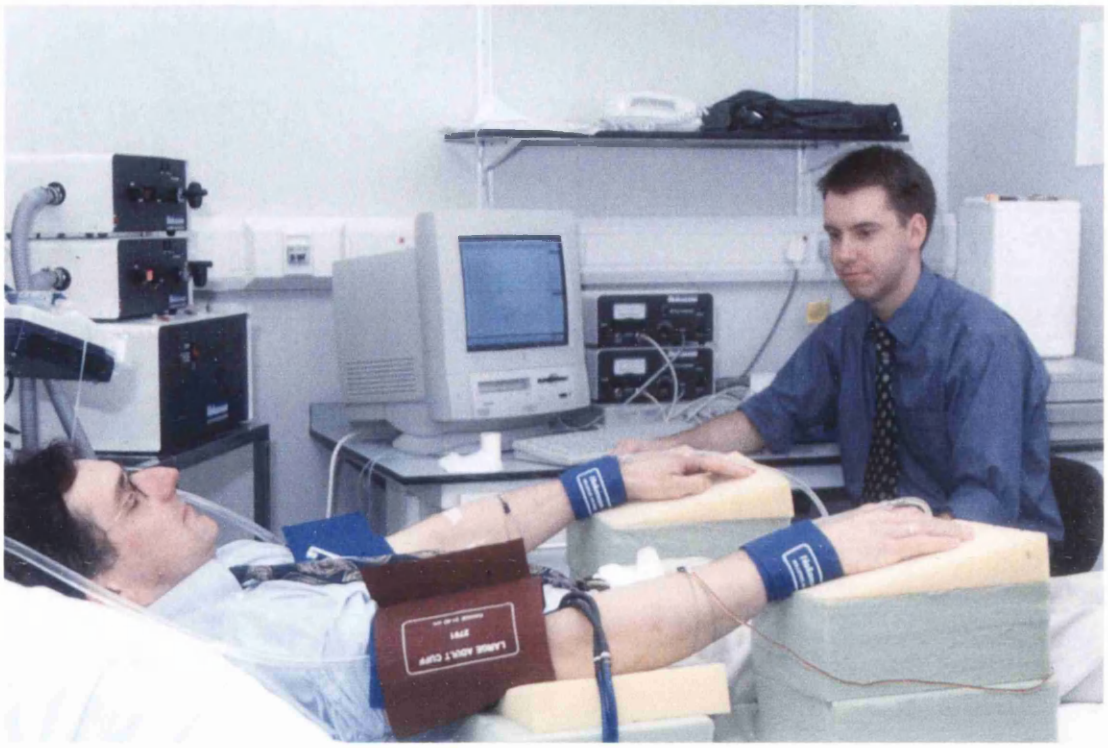


Figure 2.3. Photograph of a subject undergoing forearm plethysmography. This illustrates the upper arm and wrist cuffs, the mercury-in-silastic strain gauge in situ and the subject's position.

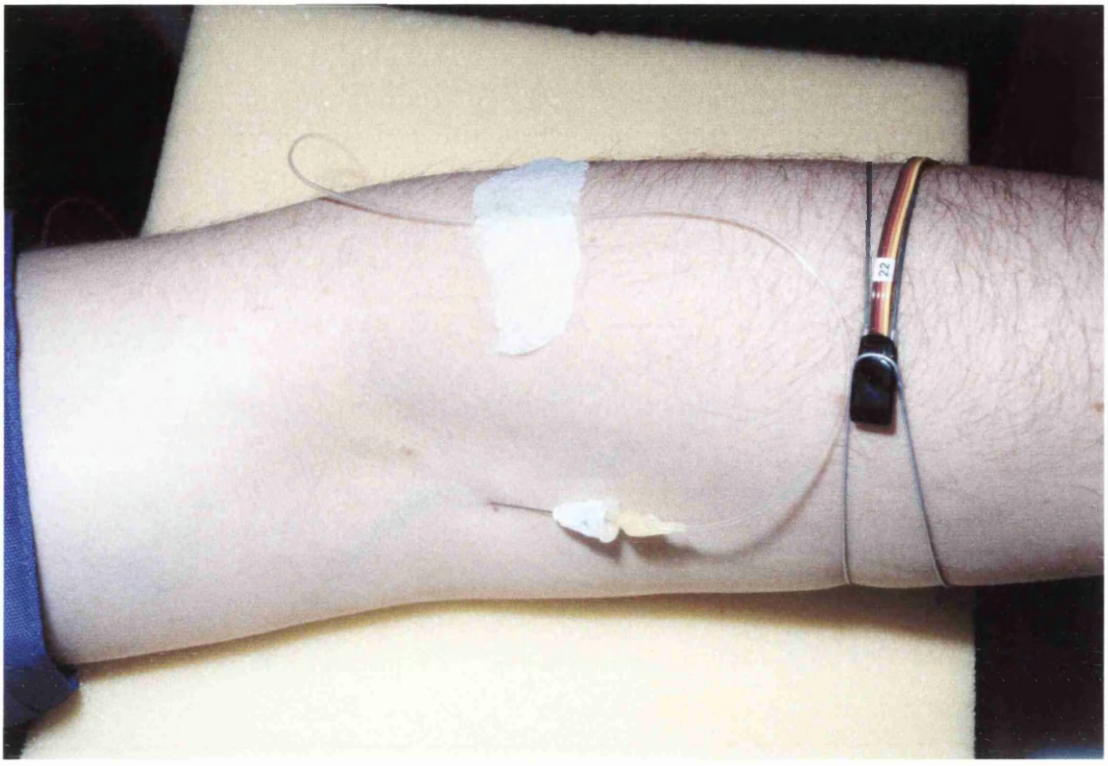


Figure 2.4. Photograph of the 27g dental needle in the brachial artery and the mercury-in-silastic strain gauge.

20minutes when 0.9% saline was again infused at 1ml/min. Further baseline readings of FBF were measured and drug 2 infused, again in incremental doses with regular FBF measurements being made at each dose level. In some studies, there was a further washout period followed by infusion of a third drug. A typical infusion protocol is illustrated in Figure 3.1.

All drugs were diluted in 0.9% saline and were supplied in syringes by our pharmacy sterile productions unit. For simplicity, we opted for fixed doses of drugs and details are given in the individual chapters. Some authors have used doses corrected for forearm volume and/or body mass index (BMI), thereby using a fixed concentration of the drug within the forearm. However, the calculations involved are cumbersome and Benjamin et al in their review of the technique felt that a fixed dose was more valid. To avoid widely differing forearm sizes between groups, we have used groups with a similar age and sex distribution, and similar BMIs. In addition, basal FBF was similar between groups in each study (widely varying basal FBF would mean considerable differences in drug concentration within the forearm).

2.3.5 Plethysmography : Statistical Analysis

For each dose of drug infused, several FBF measurements were made over 3 minutes. The mean FBF of the final five readings was used as the FBF for that drug dose. For each subject, dose response curves were constructed with drug dose on the X-axis and percentage change in FBF ratio (infused:non-infused) from baseline on the Y-axis. For each group, dose response curves were constructed with mean ± sem values, allowing easy visual comparisons. However, the best method for the comparison of

results between groups is debated (Chin-Dusting 1999) : the most frequently utilised options include repeated measures analysis of variance, simple comparisons at each dose using t test or Mann-Whitney test or the calculation of area under the curve (AUC) giving a single summary measure for each subject. The calculation of EC50 for plethysmography curves is not often used, as one would need several more drug doses to allow attainment of the plateau phase of the dose-response curve. Matthews et al suggest that area under the curve is probably the best method available for analysis of serial measurements (Matthews 1990). In the plethysmography studies comprising this thesis, a variety of statistical methods of analysis are used : details are given within each chapter. Statistical comparisons and graphs were performed using Microsoft Excel 1997 or SPSS statistical package for Windows Versions 8.0 and 9.0.

Power calculations are difficult for these studies as the expected difference is not easy to estimate. However, we estimated that 12 patients per group would give an 85% likelihood of detecting a difference at a 5% level with a δ of 1.25. To allow analysis of factors that might influence endothelial responses, it was planned that 20 patients would be required for correlation analysis in the uraemic and transplant studies

2.3.6 Plethysmography : Reproducibility

Within individuals, changes in FBF occur in response to alterations in circulating hormones, sympathetic tone and time of day. Thus, changes in blood pressure, room temperature, light levels, noise and mental arousal may all produce large changes in FBF. Several factors within the methodology help reduce this variability (Benjamin 1995; Chin-Dusting 1999) :

- Local infusion of drugs avoids changes in limb perfusion pressure. The drug dose required for the forearm is often 100-1000 times smaller than a systemically effective dose.
- Maintenance of a quiet environment at fixed temperature.
- Measurement of FBF in both limbs simultaneously and expression of the result as a ratio between infused:non-infused arm reduces the effect of small changes in systemic blood pressure and sympathetic tone.

The use of the contralateral arm as a control was first described by Greenfield and Patterson in 1954. Work from our own centre has subsequently shown that bilateral forearm plethysmography is superior to unilateral plethysmography. Petrie et al measured unilateral FBF and bilateral FBF ratios in 9 healthy male volunteers on 3 separate occasions (Petrie 1998). The coefficient of variation for the unilateral FBF measurements was 31-39%, while the coefficient of variation for FBF ratio was 19%. They concluded that bilateral forearm plethysmography was more reproducible, and in a subsequent "simulation analysis" have shown that the difference in reproducibility was statistically significant (Petrie 2000).

Accepting that the coefficient of variation for FBF ratio within individuals is around 19% in our laboratory, no further reproducibility studies were performed for this thesis.

2.4 Wire Myography

2.4.1 Myography : History

Prior to the 1970's, researchers in the field of vascular physiology and pharmacology utilised ring preparations of arteries in standard organ bath experiments. The smallest vessel which had been studied with these techniques was the rat tail artery. In 1972, Bevan & Osher described the first wire myograph, a revolutionary technique that allowed the mounting of 200μm arterial rings on two wires (fixed at one end) and the recording of isometric responses to various agonists (Bevan 1972). The problem with this, and other similar set-ups (Hogestatt 1983; Nielsen-Kudsk 1986), was that the wires had to be reasonably thick to allow isometric contraction. This necessitated the use of larger vessels and mounting was often traumatic with resultant vessel damage.

Mulvany took the technique one step further when he developed a wire myograph using finer wires that were fixed at both ends (Mulvany 1976; Mulvany 1977), and allowed the isometric study of vessels down to 100μm internal diameter. Using this technique vessel rings, approximately 2mm in length, are mounted on two 40 μm stainless steel wires, with one wire being connected to a force transducer and one wire to a micrometer. This arrangement allows the wall tension to be measured at a pre-determined internal circumference. A diagrammatic representation is shown in Figure 2.5 with a photograph of a vessel mounted in the myograph shown in Figure 2.6.

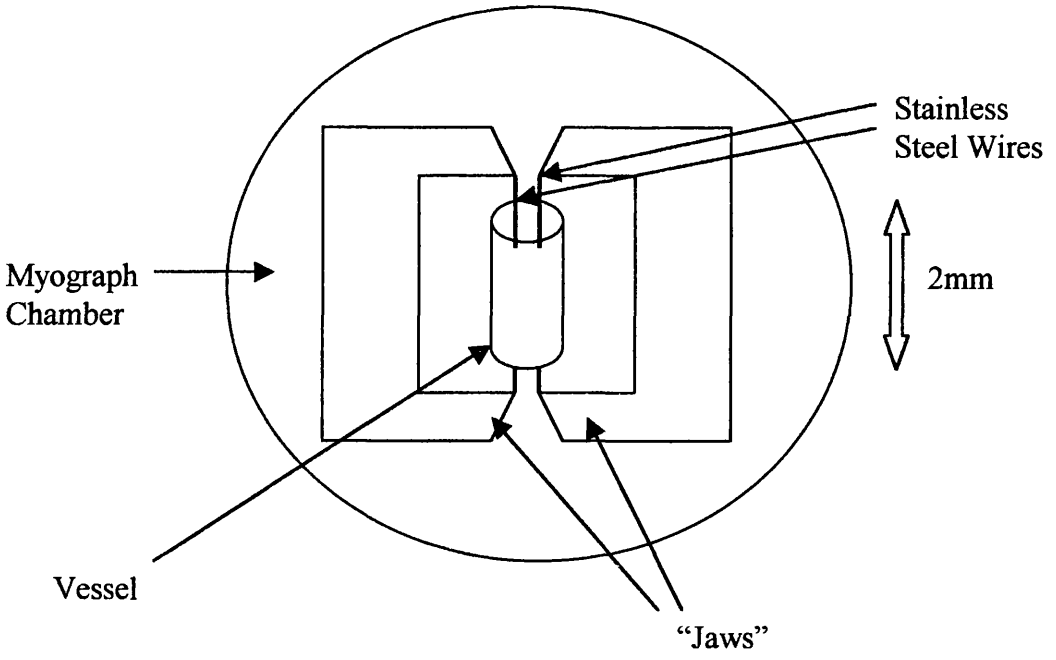


Figure 2.5 Diagrammatic representation of a myograph chamber with mounted vessel. The jaws are connected to the micrometer screw and a force transducer.

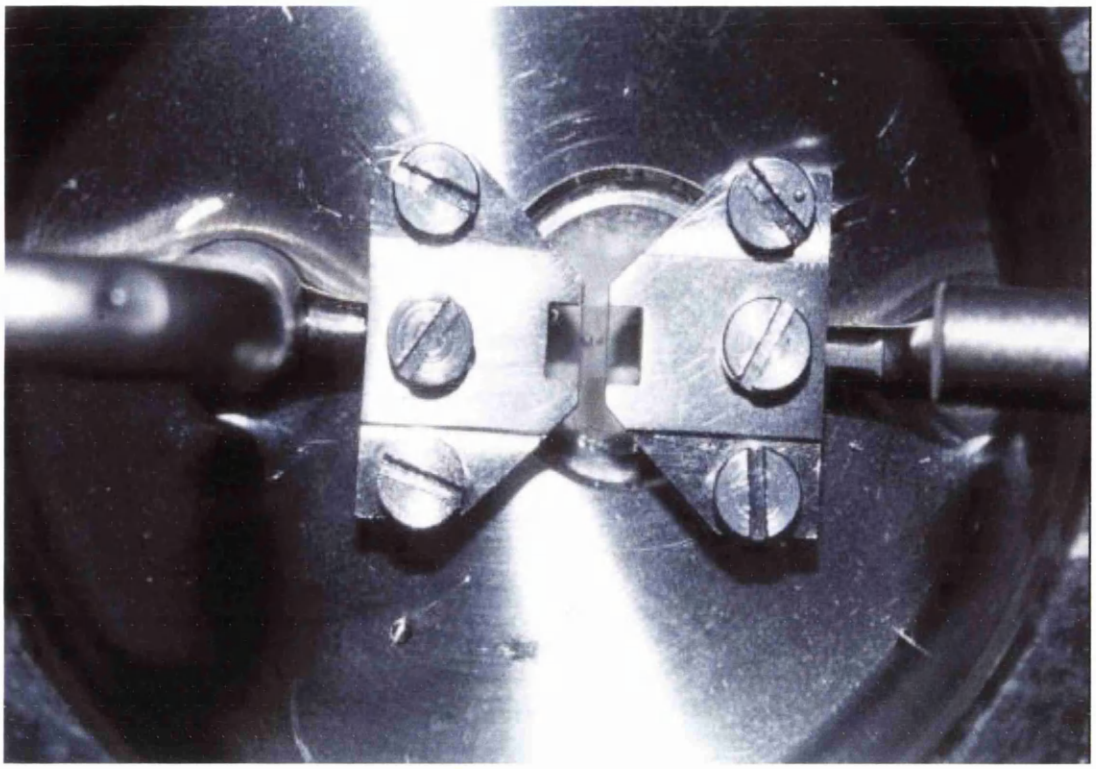


Figure 2.6 Photograph of myography chamber with mounted vessel.

The original studies were performed on rat mesenteric resistance arteries (Mulvany 1976; Mulvany 1977) with the first human vessels being studied in 1981 (Aalkjaer 1981). Using wire myography it has now been possible to study human resistance vessels in uraemia (Aalkjaer 1986), cardiac failure (Stephens 1998), polycystic kidney disease (Wang 2000), essential hypertension (Aalkjaer 1987) and pre-eclampsia (Aalkjaer 1985).

2.4.2 Myography : Resistance Vessels

The body controls the delivery of blood to the tissues through the resistance vasculature. While it was originally assumed that the resistance vasculature consisted solely of arterioles (internal diameter $\leq$ 80μm), we now know that arterioles contribute only around 50% of this resistance and that all small arteries with an internal diameter $\leq$ 500μm are involved in the control of peripheral resistance (Mulvany 1990).

Wire myography principally looks at the function of pre-arteriolar arteries with internal diameters of 100-500 μm. These vessels control blood flow to the tissues through changes in their tone, mediated either locally (tissue metabolites, autocrine factors and physical stimuli) or via neurohumoral mechanisms. Physical stimuli involved in changes in resistance artery tone include shear stress (the force per unit area acting in the direction of blood flow at the endothelium, and thus endothelium-dependent) and increased intravascular pressure, mediated by an endothelium-independent stretch response and an endothelium-dependent pressure response (Harder 1987). Resistance arteries are exposed to circulating humoral factors

including histamine, noradrenaline and adrenaline, endothelin, vasopressin and angiotensin II. The principal innervation of these arteries comes from small sympathetic neurones (releasing noradrenaline) and parasympathetic neurones (releasing acetylcholine) lying within the adventitia of the vessel.

2.4.3 Myography : Retrieval and preparation of resistance arteries

Subcutaneous fat was obtained from patients and controls undergoing abdominal surgery. Further details are given in Chapter 6. At the time of operation, and prior to the use of diathermy, an ellipse of skin approximately 2cm x 1cm with adherent fat was removed to a depth of 2cm following the method of Aalkjaer (Aalkjaer 1986). The tissue was immediately placed into chilled 0.9% sodium chloride solution and transported to the laboratory where it was placed into physiological salt solution (PSS), composition in mmol/l : NaCl 118.4, KCl 4.7, $MgSO_4.H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, glucose 11.1 and EDTA 0.023. When aerated with a 5% CO_2/95% O_2 mixture, the solution had a pH of 7.4.

All dissection was carried out at room temperature in un-oxygenated PSS (Aalkjaer 1986). The tissue was initially examined in a Petri dish containing PSS using a dissecting microscope (Zeiss Stemi 2000). Resistance arteries were often found just below the skin with visualisation being aided by pinning the tissue down onto the Petri dish. The vessels were carefully dissected (along with some surrounding fat) from the block of tissue using micro-dissecting instruments (Vicarey, Davidson & Co., Glasgow). Once a resistance artery was freed from the tissue, it was transferred to a second Petri dish containing fresh PSS, and cleaned of surrounding fat and

connective tissue using ocular dissection scissors while taking care not to handle the vessel directly. The artery was then stored in a small glass vial containing PSS overnight in the refrigerator at 4°C after first gently removing any blood from the lumen by careful manipulation using forceps. Where possible, several sections of resistance artery were removed from each block of tissue.

All myography experiments were performed on a 4-chamber myograph (Model 600) obtained from Myotech, Denmark (Figure 2.7). Experiments were carried out on the morning following dissection as studies within our department have shown that vessels are viable up to 24 hours after dissection. The resistance arteries were again placed in a Petri dish containing PSS and where possible, 4 segments of artery approximately 2mm in length were identified. Forty μm stainless steel wire was cut into lengths of 2-3 cm. Using micro-forceps to steady the vessel, each segment of artery was then threaded carefully onto a piece of stainless steel wire. The wire myograph chambers were filled with PSS and aerated with a 95%O_2/5%CO_2 mixture. Each wire in turn was placed in a myograph chamber with the resistance artery segment between the jaws such that the wire was clamped but the vessel untouched. The ends of the wire were then gently placed under the fixing screws on one side of the myograph, the screws tightened to secure the wire and the jaws opened again. A second wire was then gently threaded upwards through the artery from the proximal end, the wire sliding under the first wire. Again the jaws were closed, this time to secure the second wire, which was then secured under the fixing screws on the opposite side of the myograph, and the jaws opened so that the vessel was very slightly stretched (Figure 2.6). The wires at this point were levelled so that the plane

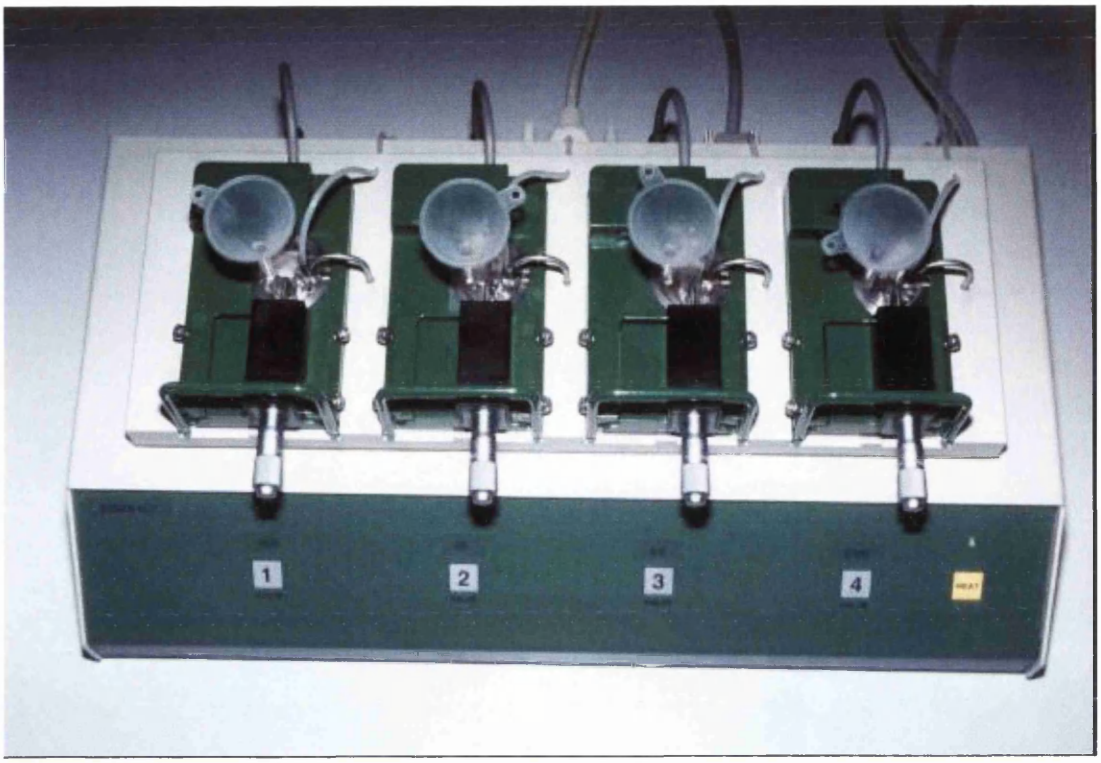


Figure 2.7. Photograph of 4-chamber Mulvany-Halpern myograph. This demonstrates 4 individual chambers with oxygen and suction tubing, funnels to allow addition of drugs and solutions, and micrometer screws.

containing the wires was horizontal. Any part of the vessel protruding from the jaw of the myograph was cut away.

The length of the vessel segment was then carefully measured using a micrometer eyepiece (attached to the Zeiss microscope) which had previously been calibrated using a graticule. Using the micrometer, the wires were then moved together until just touching and the micrometer reading recorded at that point (x_0).

2.4.4 Myography : normalisation

Mulvany's original work on rabbit vessels led to the development of a standardised normalisation procedure (Mulvany 1976/1977). This is required for 3 reasons :

i the size of an elastic vessel is influenced by transmural pressure and this needs to be defined.

ii the active response of a vessel is dependent on the degree of stretch it is under.

iii the sensitivity of a vessel to pharmacological agents is also dependent on the degree of stretch it is under.

After a 30-minute acclimatisation period, the vessel was normalised at L_{100}. This is the internal circumference that the vessel would have if it were relaxed and under a transmural pressure of 100mmHg (Mulvany 1976). Normalisation involves the stepwise stretching of the vessel with simultaneous recording of the micrometer reading on the myograph and the force measured by the transducer connected to the pen recorder (as dictated by the active tension-internal circumference relationship). The length of the vessel was measured using a calibrated eyepiece on the dissecting microscope; the wall length is equal to twice the segment length (as there is an upper

and lower wall to the vessel). The internal circumference was calculated from the known diameter of the mounting wires and the distance between the 2 wires as measured on the micrometer. The internal radius = internal circumference/2π. Thus, wall tension is the measured force divided by the vessel length, and using the law of Laplace :

effective pressure = wall tension/internal radius (or **P=T/R**).

Using a computer programme (devised in-house), after each stepwise stretch of the vessel, the micrometer reading (from this was calculated the internal circumference) and force (from this was calculated effective pressure) were entered, subtracting **x0** from the micrometer reading with each successive stretch. The force was measured from the displacement of the pen on the pen recorder; this was previously calibrated against a force of known magnitude. The stretching was carried out at one-minute intervals to allow for "stress relaxation" and stopped when the effective pressure reached 13.3kPa (100mmHg). Using the computer, an exponential curve was then fitted to the circumference/pressure data. From this curve, the circumference corresponding to 100mmHg was determined (L_{100}) and the vessel circumference set at 90% of this value (L_1), as previous work by Halpern and Mulvany has shown that the active force generated by the vessel is maximal at this internal circumference (Aalkjaer 1986). The aim was to study arteries in the range of L1=250-300 um, but they were not discarded unless L1 was greater than 500um.

2.4.5 Myography : "wake up" protocol

Following normalisation, the arteries were exposed twice to KPSS (PSS with KCl substituted for NaCl on an equimolar basis) and once to noradrenaline 10 μmol/l as a "wake up" procedure (Aalkjaer 1981). The myograph chambers were carefully rinsed with PSS between each contraction. After a plateau had been reached with noradrenaline, 3 μmol/l acetylcholine was added to assess endothelium-dependent vasodilatation and thus the presence of an intact endothelium. Vessels which failed to contract to KPSS or noradrenaline, or which failed to relax >50% to acetylcholine were discarded. Occasionally, if time and vessels permitted, it was possible at this point to mount a replacement resistance vessel.

Following the wake-up procedure, the myograph chambers were rinsed out several times with fresh PSS. The vessels were allowed to "rest" for 60 minutes prior to the commencement of the drug protocols : these are detailed in Chapter 6.

2.4.6 Myography : Evidence for the viability of mounted vessels

From the early work of Mulvany and Halpern there have been several observations that suggest that the mounted vessels are indeed viable :

1. The pressure response of vessels from normotensive individuals is ≥ 20 kPa : at least twice the pressure achieved in these vessels *in vivo* (Halpern 1978).
2. The contractile response of the vessels correlates roughly with the amount of smooth muscle present in the arterial wall (Halpern 1978).

3. The cell membrane potential and sodium efflux rate of rat mesenteric vessels mounted in the myograph are similar to values obtained from larger vascular smooth muscle preparations (Mulvany 1996).
4. Similar agonist threshold concentrations are found for vessels mounted in the myograph compared to those in perfusion experiments (Mulvany 1996).

2.4.7 Myography : Measurement of responses

The response of a vessel to an agonist is presented as an increase in active effective pressure from baseline (ΔP). To calculate this it is first necessary to measure the resting force ($F_{resting}$) and the force achieved with the agonist ($F_{agonist}$) :

$$F_{resting} = \text{alpha x } (\text{reading}_{resting} - \text{reading}_{baseline})$$

$$F_{agonist} = \text{alpha x } (\text{reading}_{agonist} - \text{reading}_{baseline})$$

where alpha is the force transducer calibration and reading is the reading on the pen recorder.

The increase in force (ΔF) is $F_{agonist} - F_{resting}$ which is therefore alpha x ($\text{reading}_{agonist} - \text{reading}_{resting}$).

The increase in wall tension (ΔT) is thus :

$$\Delta T = \Delta F/\text{vessel length}$$

Using the Law of LaPlace, the increase in active effective pressure is thus :

$$\Delta P = \Delta T/(L_1/2)$$

Responses for the vasodilators (acetylcholine and SNP) were calculated in a similar manner with the baseline being taken as the pre-constricted force after addition of noradrenaline, and the change in pressure calculated as above.

2.4.8 Myography : Data presentation & statistical analysis

Concentration-response curves were constructed for each drug with response on the y axis being denoted as ΔP. Where a complete concentration-response curve was not possible for technical reasons, then the data for that vessel were discarded.

For each agonist, responses were measured as detailed above. Agonist potency was expressed as the maximum response obtained and EC_{50} value, that being the concentration required to produce 50% of the maximum response. For the vasodilators, EC_{50} values were calculated and potency was also expressed as maximum % relaxation from baseline. Comparison between groups was by Mann-Whitney U test with significance defined at the 5% level. Graphs were produced using GraphPad Prism v2.01, and statistical analyses were performed with SPSS for Windows v7.5.

2.4.9 Myography : Cleaning, calibration and maintenance of myograph

The myograph was calibrated on a regular basis using Mulvany's method (Mulvany 1996), and maintained by technical staff from the University of Glasgow Department of Physiology. Cleaning was performed after every protocol using 8% acetic acid to remove calcium deposits and deionised water to rinse thoroughly.

Chapter 3

An *in vivo* study of endothelial function in uraemia

3.1 INTRODUCTION

I have already outlined the burden of cardiovascular morbidity and mortality in uraemia in Chapter 1. Patients with CRF have many traditional risk factors for atherosclerotic cardiovascular disease and many "newer" risk factors, some specific to uraemia. Endothelial dysfunction is a key initial event in the development of atherosclerosis, and has been demonstrated in several of the cardiovascular risk factors detailed in chapter 1. It would seem logical that uraemia would be characterised by endothelial dysfunction, perhaps severe, reflecting the number of cardiovascular risk factors present. At the inception of this project there were no published data using forearm plethysmography in this group, although some studies had shown that there were circulating markers of endothelial damage in uraemia.

This study was therefore designed to examine endothelium-dependent and independent vasodilatation in adult uraemic patients compared to controls using bilateral forearm strain-gauge plethysmography. The initial plan was to examine 3 groups : pre-dialysis patients with advanced CRF, patients on continuous ambulatory peritoneal dialysis (CAPD) and controls. However, as recruitment proved difficult, the first two groups were combined to create a "uraemic group".

3.2 METHODS

3.2.1 Subjects

Patients with advanced renal failure (median creatinine 786μmol/l) were recruited from the pre-dialysis (n=10) and CAPD (n=6) clinics at the Western Infirmary,

Glasgow. The recruitment process was random; one investigator (SM) attended the above clinics whenever possible for 1 year and asked all suitable patients if they were willing to participate. In addition to the 16 patients for whom entire studies were performed, another 10 patients attended but the studies were abandoned owing to problems including : need for micturition during study, inability to lie still for 2 hours and movement causing dislodgment of the brachial artery cannula (if the cannula was not easily re-manipulated back into position, the study was abandoned). Controls without renal disease were obtained from hospital staff and by advertisement within the hospital and University grounds. Those obtained by advertisement were paid £50 to cover expenses and time away from work. All subjects gave written informed consent, and the protocol (including re-imbursement of controls) was approved by the local ethics committee.

The patient and control groups were comparable for age, sex and body mass index, and had similar smoking habits and total serum cholesterols. Background characteristics for patients and controls are detailed in Table 3.1. The primary renal disease was : unknown (2), chronic glomerulonephritis (6), adult polycystic kidney disease (2), reflux/chronic pyelonephritis (4), calculi (1) and medullary cystic disease (1). Patients were excluded if :

- they were anticoagulated
- had diabetes mellitus
- had a systemic vasculitis
- were taking statins or ACE inhibitors
- were taking anti-anginal medication

Patients were also excluded if they had severe hypertension such that anti-hypertensive drugs could not be withheld prior to the study, or if they had previously been treated by haemodialysis and had functioning AV fistulae. For those patients on CAPD the median (range) time on dialysis was 11.5 (7,32) months. Nine of the 16 patients were taking anti-hypertensive drugs : 7 were taking a calcium channel blocker, 6 were taking a β-blocker and one patient was on doxazosin. No patients or controls were taking aspirin.

	CONTROL	**URAEMIC**	**p value**
number	18	16	-
male	14	11	>0.5
smoker (no.)	9	5	>0.2
age (years)	38 (25,51)	40 (21,59)	0.463
creatinine (μmol/l)	84 (57,108)	786 (380,1197)	<0.001
glucose (mmol/l)	4.8 (3.9,5.6)	4.7 (3.8,5.7)	0.551
cholesterol (mmol/l)	5.2 (3.1,7.0)	5.0 (2.8,8.8)	0.984
triglyceride (mmol/l)	0.9 (0.4,3.1)	1.8 (0.6,3.9)	0.005
haemoglobin (g/dL)	14.2 (12.4,15.6)	10.5 (8.9,15.8)	<0.001
S.B.P. (mmHg)	123 (104,158)	140 (106,197)	0.007
D.B.P. (mmHg)	67 (50,78)	78 (62,101)	0.001
F.B.F. (ml $100ml^{-1}$)	2.85 (1.55,5.14)	3.52 (1.85,8.22)	0.081
Body mass index	24.2 (22.0,34.7)	25.2 (19.9,32.5)	0.597

Table 3.1 Background characteristics for uraemic patients and controls.

Results are expressed as median (range). Comparison is by Mann-Whitney or Chi-Square test.

All subjects were studied in the morning after an overnight fast, and were asked to omit anti-hypertensive medication for at least 48 hours before the study. Subjects were asked to avoid caffeine and alcohol, and to refrain from smoking, for at least 12 hours prior to the study.

3.2.2 Blood Sampling

Prior to application of the plethysmography cuffs, 30 ml of venous blood was drawn and analysed in our hospital biochemistry and haematology laboratories (using standard analysers) for electrolytes, cholesterol, urea, creatinine, glucose and haemoglobin. A further sample of fasting blood was centrifuged and frozen within 20 minutes of collection to –70°C. Plasma total homocysteine was measured by ion-paired, reverse-phase high performance liquid chromatography with electrochemical detection (Martin 1998).

3.2.3 Drugs and solutions

Throughout each plethysmography session, 0.9% sodium chloride solution (Baxter, U.K.) or drug solutions were infused at 1ml/min. The following drugs (supplier) were stored and diluted to the appropriate concentration by the sterile pharmacy production unit at the Western Infirmary, Glasgow : sodium nitroprusside (Faulding DBL, England), carbachol (Martindale Pharmaceuticals, England) and L-NMMA (ICN, England).

Sodium nitroprusside was chosen as the endothelium-dependent vasodilator as it is a readily available NO donor. Carbachol is a muscarinic agonist and was chosen as the endothelium-dependent vasodilator as it is more stable than acetylcholine and is not broken down by cholinesterase. There is a significant degree of inter-individual variation in the degree and rate of metabolism of acetylcholine by cholinesterase. The NO synthase inhibitor, L-NMMA, was chosen to assess basal NO production as previously published forearm plethysmography studies had used this agent (Calver 1992).

3.2.4 Plethysmography : Infusion protocol

All forearm studies were performed in a sealed, sound-proofed, vascular research laboratory maintained at 24-26°C following the method of Petrie (Petrie 1998). Details of the set-up are given in Chapter 2. Baseline measurements of FBF were obtained at 10 minute intervals for 30 minutes to allow acclimatisation to inflation and deflation of the cuffs. The final baseline reading was used as the baseline FBF.

After an acclimatisation period of 30 minutes with infusion of 0.9% saline, incremental infusions of carbachol (0.1, 0.3, 1.0, 3.0 μg/min) and sodium nitroprusside (0.3, 1.0, 3.0, 10.0 μg/min) were commenced. There was a 30 minute washout period with 0.9% saline between drug infusions and the order of drugs infused was randomised between subjects in blocks of ten. The protocol is illustrated in Figure 3.1. If subjects could tolerate the extra time required then after a further 30 minutes washout, a single dose of L-NMMA (8μmol/min) was infused. All drug

solutions were prepared in 0.9% saline in the sterile pharmacy productions unit in the hospital. Each drug was infused for 7 minutes with FBF measurements taken from minutes 3-6, and blood pressure recorded in the seventh minute. During each drug infusion, several FBF measurements were made; the mean of the final five readings was taken as the FBF achieved for each dose of drug.

3.2.5 Statistical analysis

Results are expressed as median (range) unless otherwise specified. For each dose response curve, area under the curve (AUC) was calculated using the trapezoid rule (Matthews 1990). Comparison between groups is by Mann-Whitney test with statistical significance defined at the 5% level.

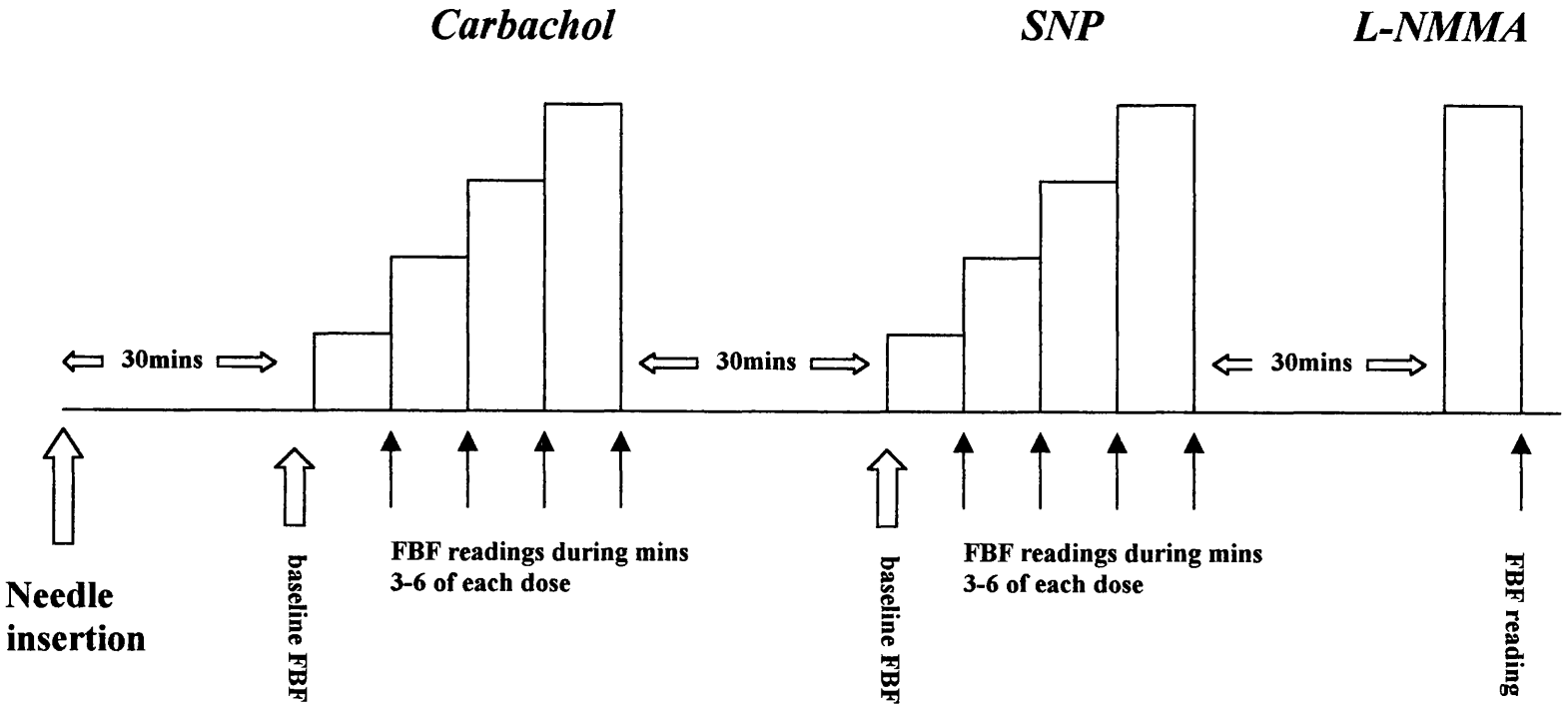


Figure 3.1 Illustration of the plethysmography protocol

3.3 RESULTS

The two groups had a similar age and sex distribution, and there were a similar number of cigarette smokers in each group (Table 3.1). Total serum cholesterol was similar in patients and controls, although the fasting serum triglyceride level was significantly greater in patients. Both systolic and diastolic blood pressure were higher in uraemic patients, and the uraemic patients had lower haemoglobin concentrations, as would be expected.

3.3.1 Effect of infusion of carbachol and SNP in all subjects

Overall there was no difference in response of the two groups to infusion of carbachol or SNP. Infusion of carbachol resulted in a (median(range)) AUC for "uraemics" of 487.9 (150.8,1180.3) compared to 598.4 (191.5,1576.6) for controls, p=0.463 (Figure 3.2). Infusion of SNP resulted in an AUC of 1867.4 (216.6,3521.4) for "uraemics" compared to 1734.6 (857.8,4717.1) for controls, p=0.986 (Figure 3.3). However, when performing the studies it became clear that there was a drug order effect which was introducing error. Despite a 30 minute wash-out period, infusion of SNP as the first drug appeared to have an unusually prolonged effect, such that the second baseline was rarely equivalent to the first. The infusion of SNP as the first drug therefore blunted the subsequent response to carbachol. This is illustrated in Figure 3.4, where patients and controls combined as a group are split into those who received carbachol as the first or second drug. It can clearly be seen that the response to carbachol is markedly attenuated if it is infused after SNP.

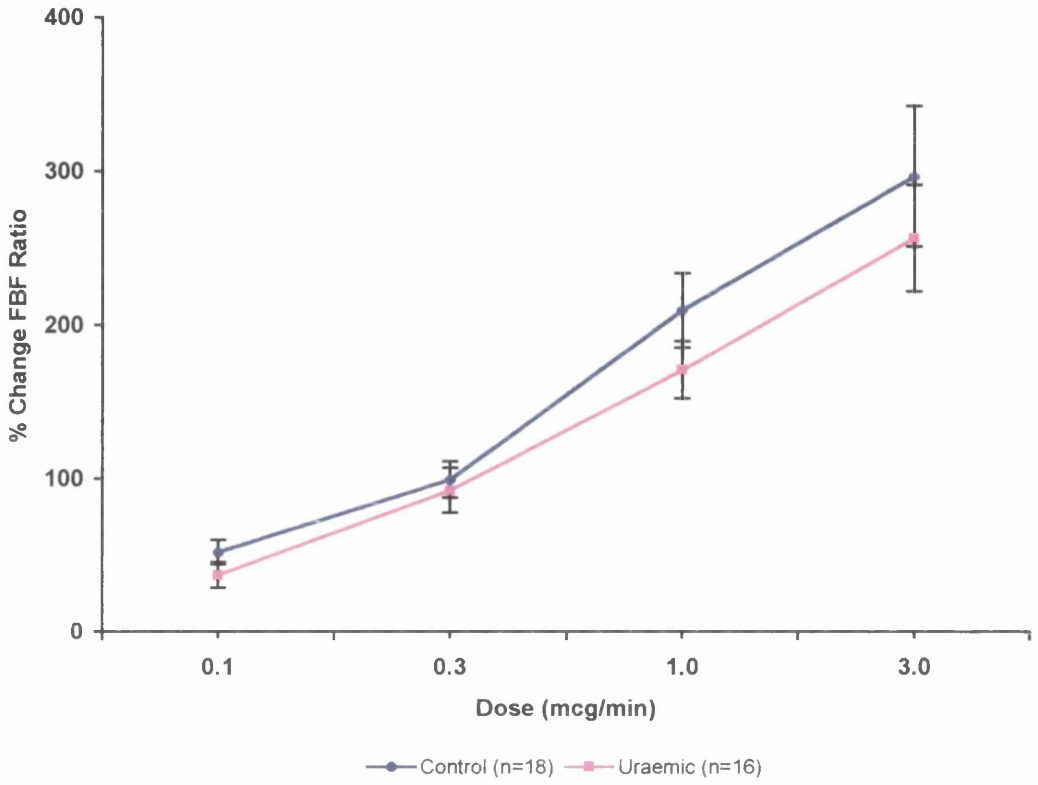


Figure 3.2 Dose response curves for infusion of carbachol in all patients. Results are expressed as mean±sem. There was no significant difference for AUC between groups by Mann-Whitney test.

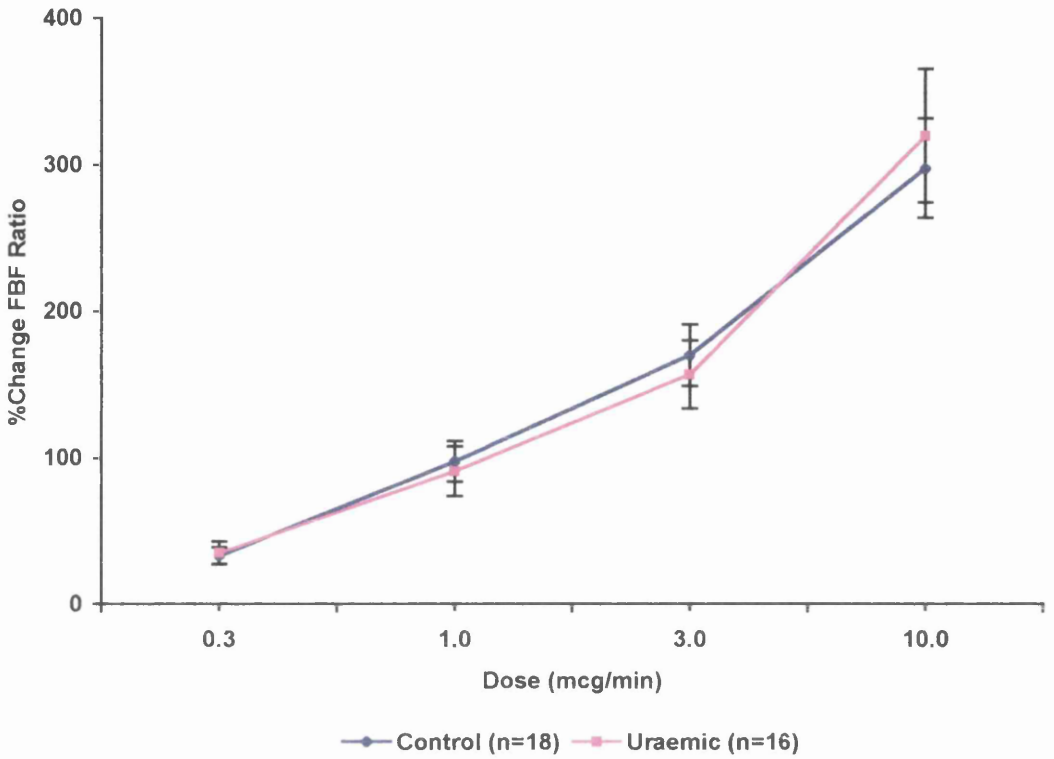


Figure 3.3 Dose response curves for SNP in all patients. Results are expressed as mean±sem. There was no significant difference for AUC between groups by Mann-Whitney test.

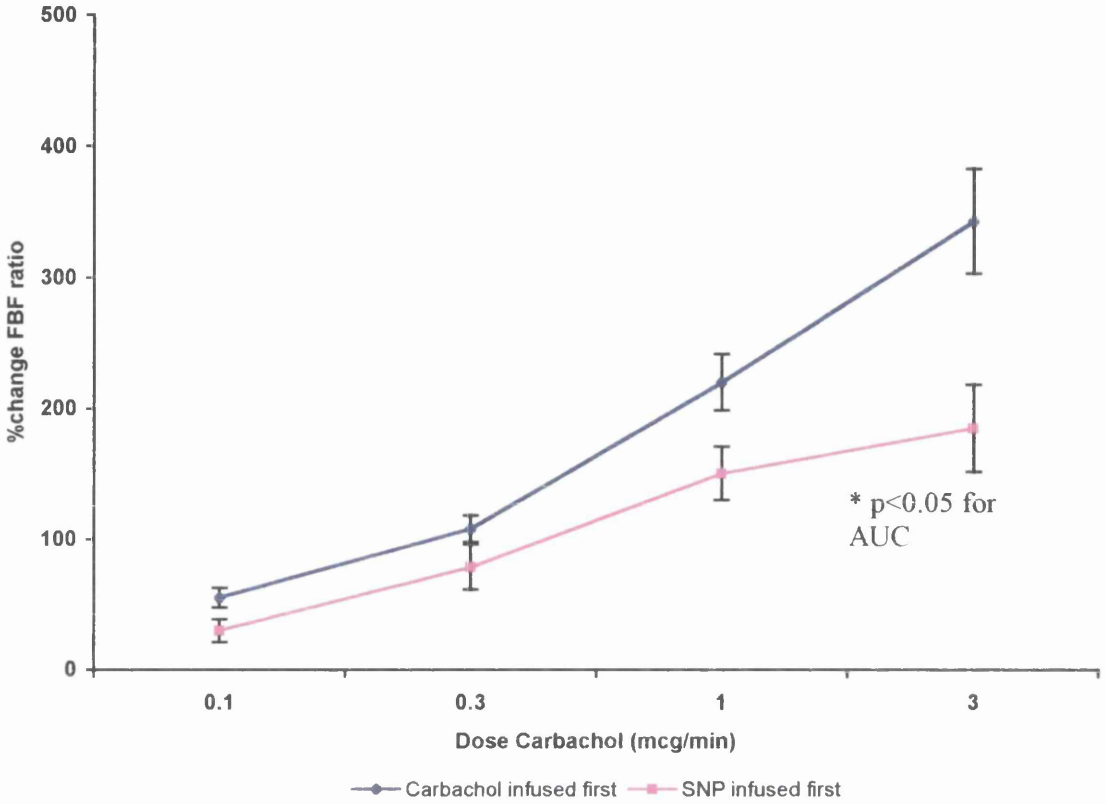


Figure 3.4 Dose-response curves for infusion of carbachol in all subjects, divided into those who received carbachol as first drug infused and those who received carbachol second, after infusion of SNP. Results expressed as mean±s.e.m. with comparison of AUC by Mann-Whitney test.

3.3.2 Effect of infusion of carbachol and SNP in subgroup of subjects in whom SNP was infused last

When those patients (n=10) and controls (n=10) who received carbachol first followed by SNP are examined as a subgroup, results are clearly different. The background characteristics of this subgroup are detailed in Table 3.2; patients and controls are again matched for age, sex, smoking habits and serum cholesterol.

	CONTROL	URAEMIC	p value
number	10	10	-
male	6	8	>0.2
smoker (no.)	6	4	>0.2
age (years)	38 (25,50)	40 (21,59)	0.684
creatinine (μmol/l)	80 (57,101)	821 (525,1197)	<0.001
glucose (mmol/l)	4.9 (4.4,5.6)	5.0 (4.0,5.7)	0.905
cholesterol (mmol/l)	4.7 (3.5,6.6)	4.7 (2.8,8.8)	0.863
triglyceride (mmol/l)	1.1 (0.3,3.1)	1.9 (0.6,3.9)	0.190
haemoglobin (g/dL)	14.2 (12.4,15.6)	10.1 (8.9,12.5)	<0.001
S.B.P. (mmHg)	114 (104,140)	140 (113,168)	0.013
D.B.P. (mmHg)	65 (50,75)	81 (62,101)	0.006
Body mass index	24.1 (22.8,34.7)	23.5 (19.9,32.0)	0.353
F.B.F. (ml $100ml^{-1}$)	2.74 (1.68,4.98)	3.15 (1.85,8.22)	0.315

Table 3.2. Background characteristics for subgroup of uraemic patients and controls in whom SNP was infused last. Results are expressed as median (range). Comparison is by Mann-Whitney or Chi-Square test.

The response to infusion of carbachol is depicted in Figure 3.5 and to SNP in Figure 3.6. The AUC for carbachol was significantly reduced in uraemic patients (529.0 (150.9,834.7)) compared to controls (703.9 (583.5,1576.6); p=0.028), while the AUC for SNP did not differ significantly between uraemics (1328.1 (216.6,3311.4)) and controls (1475.0 (857.8,4717.1); p=0.545).

Were responses different between pre-dialysis and CAPD patients?

Owing to difficulties recruiting patients for this study, there were insufficient numbers to formally compare responses between the 2 uraemic groups. Figures 3.7 and 3.8 show the dose response curves for carbachol and SNP respectively, splitting the uraemic group into pre-dialysis and CAPD. Statistical comparison was not performed as the numbers were small. Similarly, I did not examine the order effect in the CAPD and pre-dialysis groups as the numbers were small.

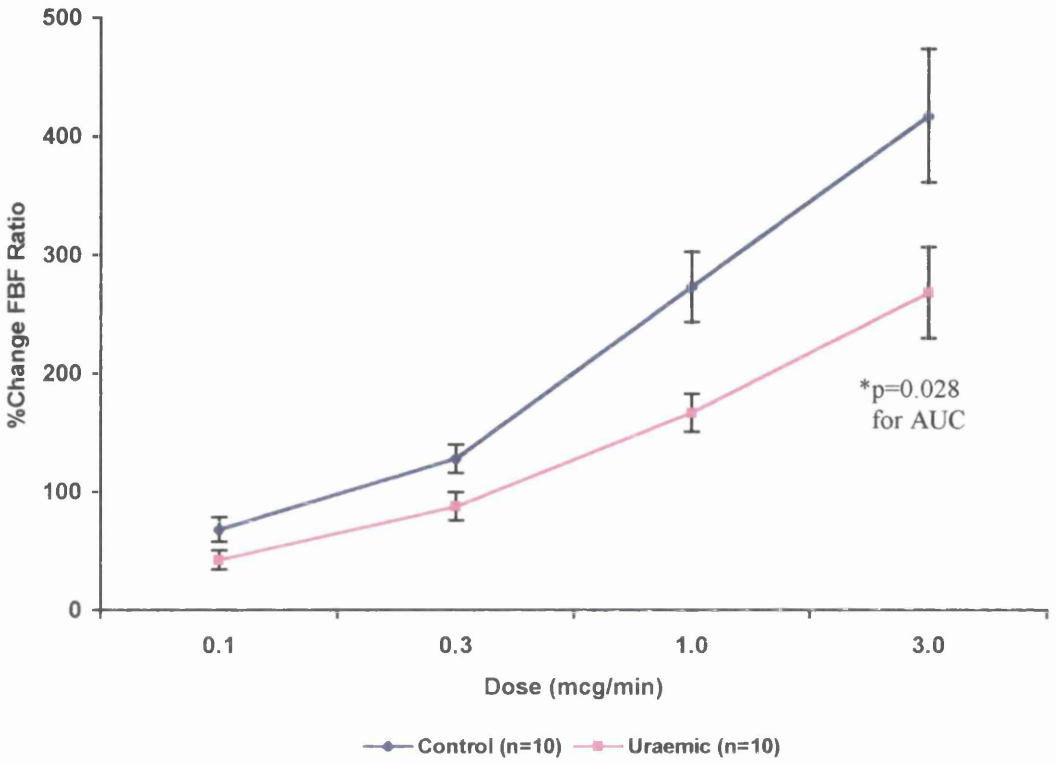


Figure 3.5 Dose response curves for infusion of carbachol in the subgroup of 20 subjects who received carbachol followed by SNP. Results are mean±sem, and comparison of AUC between groups is by Mann-Whitney test.

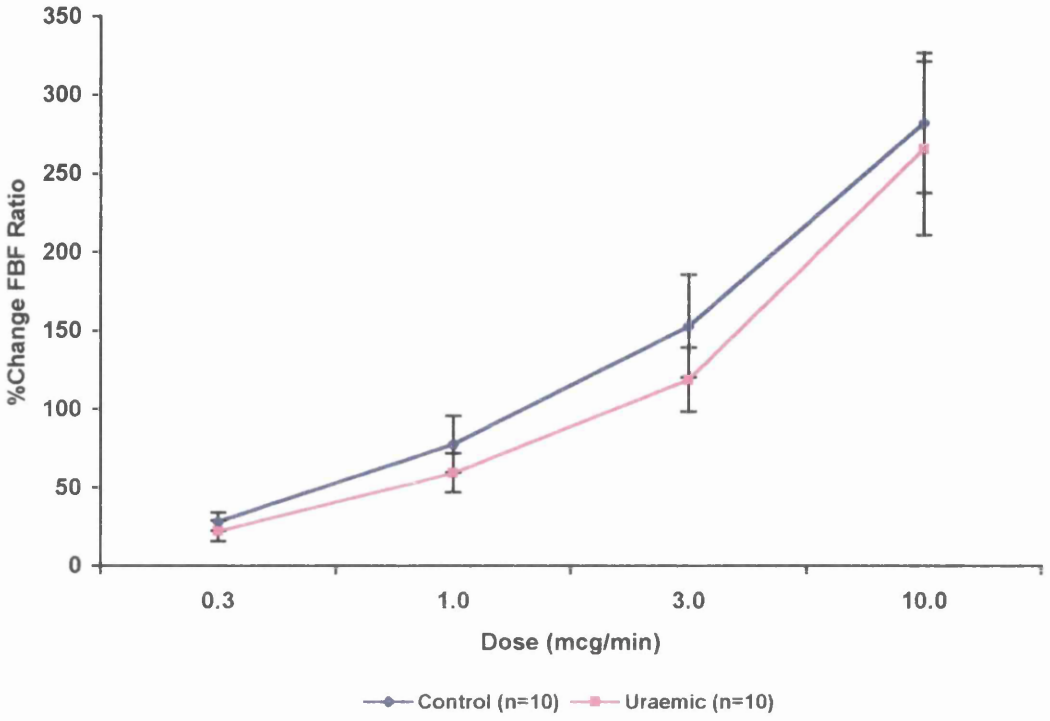


Figure 3.6 Dose response curves for infusion of SNP in the subgroup of 20 subjects who received carbachol followed by SNP. Results are mean±sem and comparison of AUC by Mann-Whitney test was not significant.

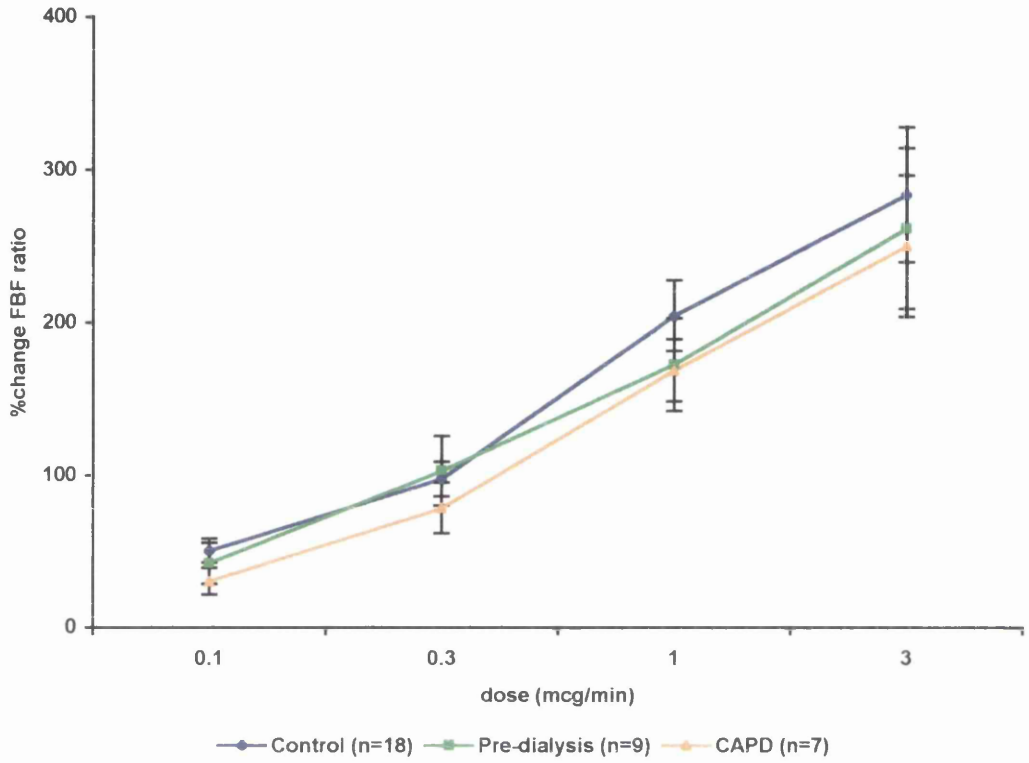


Figure 3.7 Dose response curves for carbachol in all subjects with uraemic group split into pre-dialysis and CAPD. Results are mean±sem.

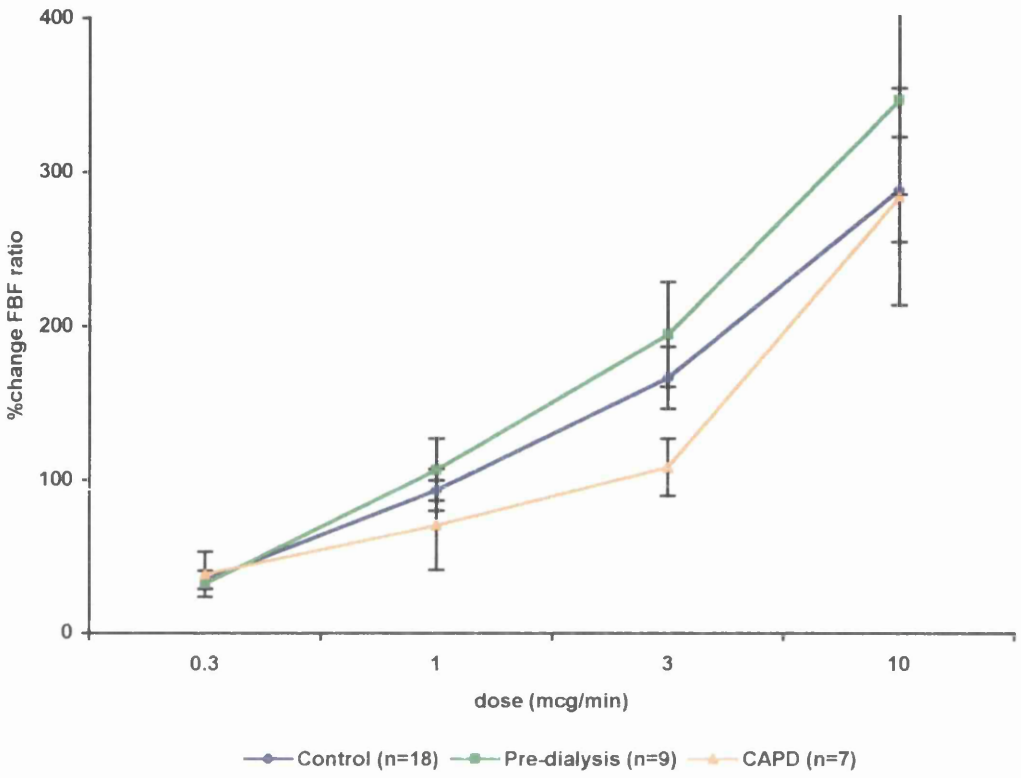


Figure 3.8 Dose response curves for SNP in all subjects with uraemic group split into pre-dialysis and CAPD. Results are mean±sem.

3.3.3 Blood pressure

As the doses of drugs were chosen to act only on the forearm, blood pressure did not change significantly throughout the studies (Tables 3.3 and 3.4) except for a small decrease in DBP after infusion of SNP in controls.

There was no significant difference in response to carbachol or SNP between those subjects taking calcium antagonists and those who were not.

	Before Carbachol	**After Carbachol**	**p value**
SBP control	116.7±11.7	120.3±16.6	0.057
DBP control	64.8±8.7	63.7±8.7	0.503
SBP uraemic	149.1±23.6	149.7±26.5	0.860
DBP uraemic	83.1±14.8	81.6±15.8	0.309

Table 3.3 Blood pressure values before and after infusion of carbachol. Results are expressed as mean±s.d., with comparison by paired t test.

	Before SNP	**After SNP**	**p value**
SBP control	121.3±13.3	120.1±15.0	0.529
DBP control	66.8±8.6	64.8±7.3	0.033
SBP uraemic	149.6±27.0	150.3±22.7	0.785
DBP uraemic	82.4±16.3	81.4±15.5	0.648

Table 3.4 Blood pressure values before and after infusion of SNP. Results are expressed as mean±s.d., with comparison by paired t test.

3.3.4 Homocysteine

Median (range) fasting total homocysteine (tHcy) levels were considerably higher in uraemic patients compared to controls : 28.4 μmol/l (17.8,90.5) v 10.5 μmol/l (6.9,35.3), $p<0.001$, in keeping with other published work for this group. Previous studies have suggested an upper limit of normal for homocysteine in the general population of around 13.9 μmol/l (Bostom 1999); all of our uraemic group therefore had hyperhomocysteinaemia, compared to 4 of our control group.

3.3.5 Factors correlating with responses to carbachol and SNP

To assess whether the response to carbachol or SNP was associated with any particular variable from the background characteristics or laboratory data a further correlation analysis was performed using the results of those 20 subjects who received carbachol first followed by SNP.

Bivariate Spearmann correlation coefficients were calculated for AUC carbachol, AUC SNP, haemoglobin, SBP, DBP, homocysteine, smoking status, age, cholesterol and triglyceride.

The only significant correlation was between AUC carbachol and triglyceride concentration : Spearmann's correlation coefficient –0.572, $p=0.013$. While this may be a spurious result in view of small numbers, it suggests a poorer endothelial response with higher triglyceride levels. In particular, there was no relationship between SBP, DBP, homocysteine or cholesterol and endothelial responses, perhaps reflecting small numbers.

3.3.6 Responses to L-NMMA 8μmol/l

Following the concentration-response curves for carbachol and SNP, a single dose of L-NMMA 8μmol/l was infused after a washout period of 20minutes. Three controls and 2 uraemic patients were unable to tolerate the extra time required to complete this section of the protocol, therefore data are available for 15 controls and 14 controls.

Results are depicted in Figure 3.9.

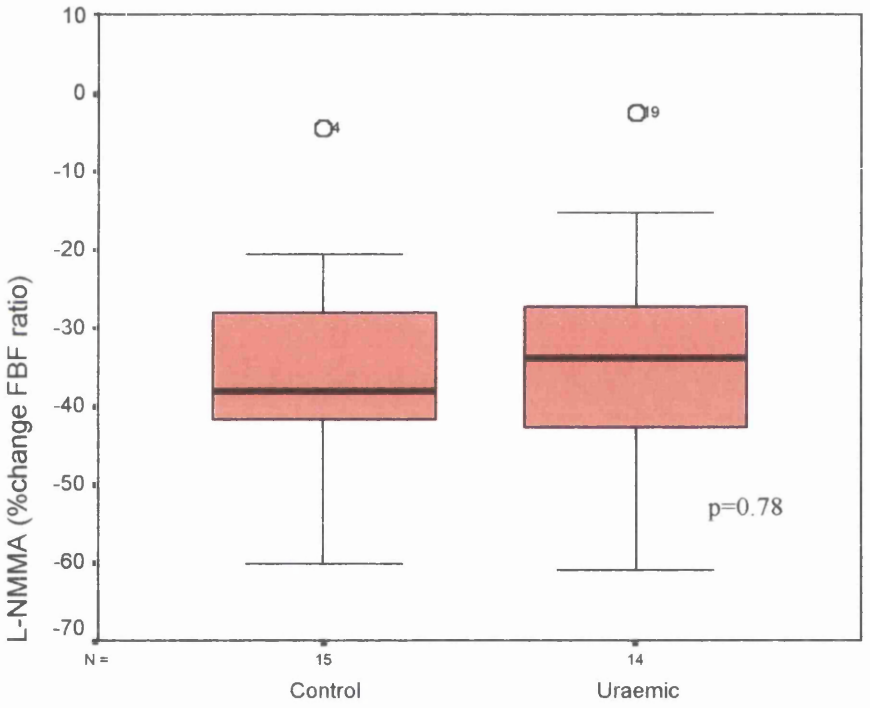


Figure 3.9 Stem and leaf plots for response to L-NMMA 8μmol/l in controls and uraemic patients. The response is measured as % change FBF ratio from baseline. The plots show median, range and inter-quartile range, with outlying values. p=0.78 by Mann-Whitney U test.

There was no significant difference in median (range) % change FBF ratio between controls [-38.1 (-60.2,-4.5)] and uraemic patients [–34.0 (-60.9,-2.5)]; p=0.78. In addition, no significant correlation was found between the response to L-NMMA and background laboratory or blood pressure data, or indeed with responses to carbachol.

3.4 DISCUSSION

3.4.1 Results of these studies

The vascular endothelium provides a strategic barrier between the circulating blood and the tissues. Endothelial dysfunction is thought to be a key initial event in the development of atherosclerosis, principally through a loss or reduction in effect of the anti-atherogenic molecule nitric oxide. This study was designed to examine endothelium-dependent vasodilatation, and thus stimulated NO production, in adult patients with advanced uraemia.

Results from all subjects demonstrated similar vasodilatation to both carbachol (endothelium-dependent vasodilator) and SNP (endothelium-independent vasodilator) in both uraemic patients and controls without renal disease. However, the infusion of SNP first appeared to blunt the subsequent response to carbachol, perhaps owing to a more prolonged effect of SNP. This feature has not previously been described in plethysmography (although has been commented upon by other local workers in the field), and is important when planning future studies. We therefore excluded those patients and controls who received SNP first from subsequent analysis. This left ten

patients and controls in whom carbachol was infused first followed by SNP, and these groups were again comparable in age, sex, cholesterol and smoking habits. In this substudy the pattern of normal vasodilatation to SNP but impaired vasodilatation to carbachol indicates endothelial dysfunction. The main defect is likely to be a reduced ability of the endothelium in the uraemic group to produce and release NO on stimulation, but defects in the production and release of other endothelium-dependent vasodilators may also play a role.

To assess basal NO production, a single dose of L-NMMA 8μmol/l was infused at the end of the protocol. This vasoconstricted all subjects, reducing FBF ratio to a similar degree in both uraemic and control groups. While this does suggest that basal NO production is unaltered in uraemia, most subjects found the long protocol difficult and therefore other factors may be playing a role 2 hours into each study such as increased sympathetic tone. In addition, a control vasoconstrictor was not used owing to time constraints. Thus, in order to accurately assess basal NO production in CRF, a further study is required to construct dose-response curves to L-NMMA with noradrenaline as a control vasoconstrictor.

A similar pattern of endothelial dysfunction has previously been described in hypercholesterolaemia (Stroes 1995), smoking (Celermajer 1993), types 1 and 2 diabetes mellitus (Johnstone 1993, Williams 1996) and with normal aging (Gerhard 1996) and is thought to predispose to the development of atherosclerosis. In our study, diabetics were excluded and patients had similar ages, smoking habits and serum cholesterol levels to controls. However, blood pressure was significantly higher in the

uraemic patients as would be expected, although the median blood pressure in this group was only 140/81.

It is widely believed that hypertension is associated with endothelial dysfunction, although two early forearm plethysmography studies in this field gave conflicting results. Panza et al (Panza 1990) examined the response to intra-brachial infusion of acetylcholine and SNP in 18 hypertensive patients (mean age 50.7yrs) who had been treated with anti-hypertensive agents for at least 5 years, and 18 controls of a similar age with normal blood pressure. Mean arterial pressure was 117 in the hypertensive group. This study showed that while responses to SNP were similar between groups, there was a large, statistically significant, reduction in the vasodilatation produced by the highest dose of acetylcholine. They concluded that the pattern was in keeping with endothelial dysfunction in essential hypertension.

Four years later, Cockcroft's group in London published a similar study : they compared the responses to intra-brachial acetylcholine, carbachol and SNP in 58 patients with essential hypertension (MAP 114) and 37 normotensive controls (Cockcroft 1994). An initial study examined untreated hypertensives and a follow-on study looked at both treated and untreated hypertensives. Surprisingly, there were no differences between groups for the vasodilatation produced by these agents and they concluded that essential hypertension was not characterised by endothelial dysfunction. Key differences between Panza and Cockcroft's studies were :

- the patients in Cockcroft's study had newly diagnosed hypertension
- the patients in Cockcroft's study were untreated hypertensives
- the patients in Cockcroft's study were younger (mean age 41yrs)

- lower doses of acetylcholine were used by Cockcroft
- the patients in Cockcroft's study were heavier and had slightly higher BMIs than the controls (effectively meaning a slightly lower dose of drug per kg or unit of forearm volume). In Panza's study, patients were lighter. However, none of these differences were statistically significant and their influence on the results is unclear

The two groups were unable to reconcile their differing results but it was suggested that the above factors might have been involved i.e. that Cockcroft's study population was different.

However, there is now a large body of evidence, both *in vivo* and *in vitro*, supporting the results obtained by Panza et al and it is generally accepted that endothelium-dependent vasodilatation is impaired in essential hypertension (Taddei 1998, Ferro 1997). Furthermore, studies have shown that basal NO production in essential hypertension is reduced (Calver 1992, Forte 1997). The mechanism underlying impaired endothelium-dependent vasodilatation in hypertension is sensitive to inhibition by indomethacin but not affected by L-arginine or L-NMMA. Thus, current opinion suggests that hypertension is characterised by over-production of oxygen-derived free radicals (which mop up NO) and/or endothelium-derived vasoconstricting prostanoids (Taddei 1998).

The predisposition to endothelial dysfunction in hypertension is at least partly genetically determined, as impaired endothelium-dependent vasodilatation has been demonstrated in normotensive children of hypertensive adults (Taddei 1992). That essential hypertension is not entirely attributable to endothelial dysfunction is

supported by the observations that endothelial dysfunction occurs in secondary forms of hypertension, and that endothelial dysfunction improves with anti-hypertensive treatment.

Unfortunately, we were unable in this present study to match patients and controls for blood pressure, and further larger studies are warranted to compare endothelial function in uraemic and essential hypertensive patients.

Our patient and control groups also differed with respect to triglyceride and haemoglobin levels. Hypertriglyceridaemia, while being an independent cardiovascular risk factor, is not thought to adversely affect endothelial function (Chowienczyk 1997). In contrast, as NO binds to the haem moiety of haemoglobin, changes in haemoglobin concentration would be expected to affect the response to endothelium-dependent vasodilators. Indeed, enhanced bioavailability of NO as a result of anaemia has been postulated as the mechanism underlying the hyperdynamic circulation seen with anaemia (Anand 1993), and recent animal work has demonstrated upregulation of NOS in iron-deficient rats (Ni 1997). In our study the uraemic patients had lower haemoglobin concentrations and would therefore be expected to have an exaggerated response to carbachol as NO would theoretically have a longer half-life. This was not seen, and the effects of anaemia on endothelial function in this situation are therefore not clear.

The pattern of endothelial dysfunction demonstrated by this technique in uraemic patients may reflect a direct inhibitory effect of circulating factors such as ADMA on NO synthase, supporting the findings of Zoccali that ADMA levels are correlated

with cardiovascular outcomes (Zoccali 2001). It is also possible that the impaired endothelium-dependent vasodilatation is secondary to the damaging effects of increased oxidative stress in keeping with recently published data linking oxidative stress and endothelial function in uraemia (Annuk 2001a).

Another potential atherogenic "uraemic factor" may be homocysteine. Hyperhomocysteinaemia is associated with increased cardiovascular mortality in the general and CRF populations, and hyperhomocysteinaemia has been linked to impaired endothelium-dependent vasodilatation in uraemic (van Guldener 1998) and general populations (Tawakol 1997). Given that there is a close correlation between serum creatinine and homocysteine level, it would be extremely difficult, if not impossible, to perform a plethysmography study examining endothelial function in uraemia and controlling for all the other associated risk factors, and in particular, controlling for homocysteine. In this study there appeared to be no correlation between drug responses and homocysteine levels.

3.4.2 Other studies of endothelial function in uraemia

There are now a number of published studies looking at endothelial function in chronic renal disease. They fall into several broad groups :

a) Studies examining NO production/excretion

b) Studies measuring circulating markers of endothelial dysfunction

c) Cell culture studies with uraemic serum

d) Studies examining vascular reactivity in CRF

I will briefly outline the findings of studies in the first 3 groups and then discuss in more detail the results of studies examining vascular reactivity, most of which are recent *in vivo* studies.

3.4.2.i Studies examining NO production/excretion in uraemia

- Remuzzi first showed in 1990 that the bleeding tendency of uraemic rats was reversed by L-NMMA suggesting that over-production of NO was responsible for the uraemic bleeding tendency (Remuzzi 1990).
- In 1993, Noris et al demonstrated that platelets from uraemic patients produced more NO than platelets from controls, and that uraemic plasma induced NO synthesis in cultured human endothelial cells (HUVECs) (Noris 1993).
- In 1997, Remuzzi's group again examined this issue. In rats undergoing extensive renal mass reduction (RMR), urinary nitrite/nitrate excretion was reduced, indirectly indicating reduced NO synthesis. Furthermore, they showed reduced renal NOS activity and iNOS immunostaining, but normal eNOS staining. However, plasma nitrite/nitrate levels were elevated post RMR, and aortic tissue showed higher NOS activity, iNOS and eNOS immunostaining. They concluded that vascular NO production was enhanced in uraemia, while renal NO production was reduced (Aiello 1997).
- Similarly, a Japanese group demonstrated enhanced NO content in exhaled air and increased plasma nitrite/nitrate levels in dialysis patients compared to healthy controls (Matsumoto 1999).

- Schmidt et al examined plasma levels and urinary excretion of nitrite and nitrate in haemodialysis patients and showed reduced NO output compared to controls (Schmidt 1999).
- A further method of analysing NO production indirectly is to measure the conversion of [$^{15}N_2$]-arginine to [$^{15}N_2$]-citrulline in plasma. Rabelink's group used this method to show that basal NO production is reduced in CRF (Wever 1999).

This field is therefore extremely confusing, partly a result of the great difficulty in measuring plasma NO concentration *in vivo* because NO has a very short half-life. One must try to reconcile 2 concepts : accelerated atherosclerosis in this group suggests reduced bioavailability of NO, while the uraemic bleeding tendency, and perhaps dialysis hypotension, suggest increased NO production. There may be a complex change in uraemia with downregulation of endothelial eNOS and concomitant upregulation of platelet iNOS.

3.4.2.ii Studies measuring circulating markers of endothelial dysfunction

- Several groups have now demonstrated raised circulating von Willebrand factor (vWf) levels in predialysis (Haaber 1995, Thambyrajah 2000a) and dialysis (Gris 1994, Bolton 2001) patients compared to controls. Von Willebrand factor is an endothelium-derived protein; raised levels have been

associated with endothelial damage and cardiovascular events in the general population (Boneu 1975, Meade 1994).

- Further endothelium-derived proteins that are elevated in uraemia include soluble thrombomodulin (Nakayama 1994, Gris 1994) and tissue-type plasminogen activator (Gris 1994).

3.4.2.iii Cell culture studies with uraemic serum

- The first study in this area demonstrated that uraemic serum inhibited proliferation of lymphocytes and a malignant cell line in culture (Wessel-Aas 1981).
- Delaporte et al showed that uraemic plasma and haemodialysis ultrafiltrate inhibited cell proliferation *in vitro* (Delaporte 1982).
- iNOS but not eNOS was inhibited in cell culture by uraemic serum (Arese 1995).
- Finally, Aznar-Salatti et al found that uraemic serum affected human endothelial cells in culture, in that the cells produced a deficient matrix and were less firmly attached to the subendothelium (Aznar-Salatti 1995).

3.4.2.iv Studies examining vascular reactivity in CRF

The first, and in many ways, best study in this field was published in 1997 by Kari et al (after the work for this thesis commenced). This group controlled for blood pressure, cholesterol, smoking and anti-hypertensive drugs by examining 23 normotensive, pre-dialysis children with CRF (mean GFR 17.5ml/min/1.73m2) who

were on no vasoactive drugs and who had normal serum cholesterol levels (Kari 1997). Using high resolution ultrasound examination of the radial artery they showed that flow-mediated (endothelium-dependent) vasodilation was impaired in the uraemic children compared to controls, while vasodilatation to sublingual GTN (endothelium-independent) was similar in the two groups. The authors concluded that uraemia was associated with endothelial dysfunction and that this may predispose to accelerated atherosclerosis.

In keeping with our results in uraemic adults, several groups have also found impaired endothelium-dependent vasodilatation in CRF :

- Joannides et al 1997 – non-invasive study using ultrasound examination of radial artery. Showed reduced flow-dependent vasodilatation but also slight reduction in GTN-mediated dilatation.
- Hand et al 1998 – non-invasive study using dorsal hand vein diameter. They showed that venodilatation to acetylcholine was impaired in haemodialysis patients compared to controls, while venodilatation to GTN was similar. Haemodialysis and L-arginine (but not D-arginine) corrected the impaired response in the uraemic patients. They concluded that the results were in keeping with endothelial dysfunction caused by accumulation of NOS inhibitors that are cleared by dialysis.
- Thambyrajah et al 2000a – non-invasive study using ultrasound of brachial artery. They showed impaired flow-mediated dilatation but preserved GTN-mediated dilatation in 80 uraemic (pre-dialysis) patients compared to controls, and found no difference in response between those patients with mild CRF

and those with more advanced CRF, concluding that endothelial dysfunction is present early in uraemia.

- Annuk et al 2001b – large forearm plethysmography study of 56 patients with moderate CRF compared with 56 controls. They showed impaired vasodilatation to methacholine (endothelium-dependent) with preserved responses to SNP. They controlled for blood pressure in multivariate analysis and found that the results were still valid.
- Bolton et al 2001 – non-invasive study using brachial artery ultrasound. They showed impaired flow-mediated vasodilatation with preserved GTN-mediated dilatation in pre-dialysis and dialysis patients. This group also looked at oxidised LDL and pro-inflammatory cytokines (IL-6 and TNF α) and found no correlation between oxLDL levels and endothelial responses, but a significant negative correlation between the pro-inflammatory cytokines and endothelium-dependent vasodilatation.

3.5 Conclusion

In summary, this study has demonstrated endothelial dysfunction in uraemic patients compared to controls, as assessed by endothelium-dependent vasodilatation. Indirectly this suggests a reduced ability of the vascular endothelium in uraemia to produce and release NO (or enhanced NO breakdown/scavenging). Our results are in keeping with those of other methodologically-similar studies, and with those of a number of non-invasive studies. For the first time we showed that drug order should probably not be

randomised in plethysmography as SNP appears to affect the subsequent response to carbachol.

The mechanism underlying endothelial dysfunction in uraemia remains unclear. The only factor that appeared to correlate with the response to carbachol in this study was triglyceride level, and while this may be significant, a previous plethysmography study in non-uraemic patients showed no effect of severe hypertriglyceridaemia on endothelial responses (Chowienczyk 1997). As circulating factors may play a major role, a further study was performed examining vessels from patients with CRF removed from the uraemic milieu.

Chapter 4

An *in vitro* study of endothelial function in uraemia

4.1 INTRODUCTION

The results of the forearm plethysmography study detailed in Chapter 3 indicate impaired endothelium-dependent vasodilatation in adult uraemic patients. Several other studies using forearm plethysmography or high-resolution ultrasound confirm these findings. The mechanism, however, remains unclear.

One postulated mechanism for uraemic endothelial dysfunction is the accumulation of endogenous uraemic toxins including ADMA, PTH, homocysteine and other unidentified factors that may directly harm the endothelium or alter its function.

This present study was designed to examine endothelial function *in vitro*, using uraemic vessels in an environment removed from the uraemic milieu. Wire myography was utilised to examine resistance vessels from patients with CRF either pre-dialysis or already undergoing dialysis. This technique has been in use for over 20 years and is a powerful means of looking at vascular responses in vessels that control the peripheral resistance. Details of this method have been described in chapter 3.

4.2 METHODS

4.2.1 Materials

Acetylcholine, sodium nitroprusside and noradrenaline were purchased from Sigma-Aldrich, UK. Human endothelin-1 was purchased from ICN (U.K.). Experiments

were performed on a Mulvany-Halpern 4-channel myograph (JP Trading), connected to a Linseis pen recorder.

4.2.2 Subjects

Twelve patients with chronic renal failure agreed to have a subcutaneous fat biopsy at the time of CAPD catheter insertion (n=5) or renal transplantation (n=7) under general anaesthetic. Control tissue was obtained from 8 volunteers without known renal disease undergoing abdominal surgery or hernia repair. All patients and controls that were eligible over a 9-month period were asked to consent, and as there was a learning curve for the technique, the initial data from around 10-15 subjects was not included. Owing to technical problems, results from this initial period were incomplete. All subjects gave written informed consent and the protocol was approved by the local ethics committee.

The background characteristics of patients and controls are detailed in Table 4.1. The underlying renal disease was : chronic glomerulonephritis (6), unknown (4), obstructive uropathy (1) and hereditary nephritis (1). For the patients who had previously been dialysed the median (range) duration of ESRF was 27 (5-69) months. Exclusion criteria included known cardiovascular disease (angina pectoris, previous myocardial infarction or CABG), diabetes mellitus or systemic vasculitis.

	Control (n=8)	Uraemic (n=12)	P Value
Male	5	8	ns
Smoker	3	4	ns
Age (yrs)	42 (31,73)	46 (20,77)	0.910
Urea (mmol/l)	5.5 (2.7,10.2)	21.8 (7.2,35.1)	<0.001
Creatinine (μmol/l)	85 (66,123)	735 (476,1041)	<0.001
Glucose (mmol/l)	4.7 (4.3,6.0)	5.2 (3.4,6.8)	0.913
Cholesterol (mmol/l)	4.6 (2.8,7.4)	4.5 (3.7,5.3)	0.408
Triglyceride (mmol/l)	0.9 (0.7,3.2)	1.4 (0.8,2.0)	0.902
Haemoglobin (g/dL)	13.2 (11.8,14.5)	9.8 (7.9,11.1)	<0.001
SBP (mmHg)	124 (106,152)	149 (112,180)	0.043
DBP (mmHg)	78 (68,88)	82.5 (52,110)	0.601
BMI	23.6 (16.8,28.3)	25.2 (19.8,30.8)	0.315

Table 4.1 Background characteristics for uraemic patients and controls expressed as median (range) with comparison by Chi-Square or Mann-Whitney U tests. (SBP=systolic blood pressure; DBP=diastolic blood pressure; BMI=body mass index)

4.2.3 Human subcutaneous resistance arteries

At the time of operation, an ellipse of skin approximately 2cm x 1cm with adherent fat was removed from the anterior abdominal wall to a depth of 2cm. Resistance arteries were dissected from this tissue and mounted on a 4-chamber myograph, as described in Chapter 2.

4.2.4 Protocol

After a 30-minute acclimatisation period, the vessel was normalised at internal diameter L_0 (Aalkjaer 1986), the arteries were then exposed twice to KPSS (PSS with KCl substituted for NaCl on an equimolar basis), and once to noradrenaline 10 μmol/l. After a plateau contraction had been reached to noradrenaline, acetylcholine 3 μmol/l was added to assess endothelium-dependent vasodilatation and thus the presence of an intact endothelium. Vessels which failed to contract to KPSS or noradrenaline, or which failed to relax >50% to acetylcholine were discarded.

Two separate protocols were then carried out. A cumulative concentration-response curve to noradrenaline (10^{-9}M to $3x10^{-4}$M) was constructed with two of the vessels involving exposure to each dose of noradrenaline for 4 minutes or until a peak response at that concentration was reached. Following a washout period of 30 minutes and re-establishment of baseline, a cumulative concentration-response curve to endothelin-1 (10^{-12}M to $3x10^{-6}$M) was constructed, with arteries exposed to each concentration of endothelin-1 for 10 minutes, or until a peak was reached.

With the two remaining vessels, after preconstriction with noradrenaline ($3x10^{-6}$M), a concentration response curve to acetylcholine (10^{-9} to $3x10^{-4}$ M) was constructed involving exposure to each concentration of acetylcholine for 2 minutes or until the maximum relaxation was achieved. Again, following a washout period of 30 minutes, the arteries were preconstricted with noradrenaline ($3x10^{-6}$M) and a concentration-response curve was performed for SNP (10^{-9} to $3x10^{-4}$ M) with arteries being in

contact with each concentration of SNP for 2 minutes or until a plateau had been reached.

When a complete concentration-response curve was not possible for technical reasons the vessel was discarded. The number of vessels studied for each drug, and the corresponding size of these vessels, is detailed in Table 4.2.

		Control	Uraemic	P value
Noradrenaline	**number**	17	22	
	L_0	395.8(212.2,500.9)	360.9(241.2,536.4)	0.377
Endothelin-1	**number**	14	16	
	L_0	404.5(240.2,500.9)	374.6(262.8,530.5)	0.697
Acetylcholine	**number**	19	23	
	L_0	413.3(212.2,500.9)	345.6(183.2,536.4)	0.114
Sodium nitro-prusside	**number**	12	20	
	L_0	358.6(212.2,474.4)	363.7(183.2,536.4)	0.833

Table 4.2. Background data for vessels. Number=number of vessels in which a complete concentration-response curve was obtained for that particular drug. L_0=normalized internal diameter of vessels. Comparison is by Mann-Whitney U test.

4.2.5 Statistical analysis

Contractile responses were expressed as an increase in active effective pressure (kPa), calculated as an increase in isometric tension above resting divided by the normalised

internal radius. Agonist potency was expressed in terms of maximum response obtained and EC_{50} value, this being the concentration required to produce 50% of the maximum response. For SNP and acetylcholine, the response was expressed as maximum % relaxation from baseline. Comparison between groups was by Mann-Whitney *U* test. All analyses were performed using SPSS for Windows (version 7.5), with significance at a level of $P<0.05$. Concentration-response curves were generated using GraphPad Prism v 2.01.

4.3 RESULTS

Patients and controls were of a similar age and sex distribution, and had similar total serum cholesterols and smoking habits (Table 4.1). Haemoglobin was significantly lower in the uraemic patients and systolic blood pressure was significantly higher as expected. Table 4.2 details the number and size of the vessels used for each drug. There was no significant difference in vessel size between groups.

The results for the vasoconstrictors noradrenaline and endothelin-1 are shown in Table 4.3 and in Figures 4.1 and 4.2. The potency of noradrenaline and endothelin-1 were similar in uraemic and control groups (no significant difference in EC_{50}). However, from the concentration response curves it can be seen that there is a tendency for a more sustained contraction to these agents with the response (kPa) at the highest dose of norepinephrine being significantly greater in the uraemic group [16.89 (1.7,30.6) uraemic v 6.96 (0,25.9) control; p=0.001] and the response to the highest dose of endothelin-1 being significantly greater in the uraemic group [19.08(8.9,37.8) uraemic v 13.44(2.2,22.2) control; p=0.006].

	Control	Uraemic	P value
NA EC_{50}	$2.23x10^{-7}$ ($0.72x10^{-7}$, $21.30x10^{-7}$)	$6.78x10^{-7}$ ($0.23x10^{-7}$, $58.10x10^{-7}$)	0.163
ET-1 EC_{50}	$6.01x10^{-10}$ ($0.15x10^{-10}$, $13.50x10^{-10}$)	$9.50x10^{-10}$ ($2.69x10^{-10}$, $71.04x10^{-10}$)	0.208
Ach EC_{50}	$4.81x10^{-8}$ ($0.69x10^{-8}$, $59.51x10^{-8}$)	$7.35x10^{-8}$ ($0.74x10^{-8}$, $37.73x10^{-8}$)	0.604
SNP EC_{50}	$5.40x10^{-8}$ ($0.58x10^{-8}$, $87.12x10^{-8}$)	$5.43x10^{-8}$ ($0.40x10^{-8}$, $19.05x10^{-8}$)	0.716
Ach max % relaxation	98(78,100)	77(41,97)	<0.001
SNP max % relaxation	94(71,100)	95(63,100)	0.751

Table 4.3 Potency (EC_{50} (Molar)) of noradrenaline, endothelin-1, acetylcholine and sodium nitroprusside in uraemic and control vessels expressed as median (range) with comparison by Mann-Whitney U test. (NA = norepinephrine, ET-1 = endothelin-1, ACh = acetylcholine, SNP = sodium nitroprusside). Maximum % relaxation from baseline of acetylcholine and sodium nitroprusside expressed as median (range).

The results for the endothelium-independent vasodilator SNP are detailed in Table 4.3 and Figure 4.3. Again, the potency of this agent was similar in uraemic and control groups, and the same maximum relaxation was obtained in the two groups. However, when we examine the results for the endothelium-dependent vasodilator acetylcholine (Table 4.3 and Figure 4.4), the maximum relaxation obtained in uraemic patients (77% (41,97)) was significantly lower than that obtained in controls (98% (78,100); $p<0.001$).

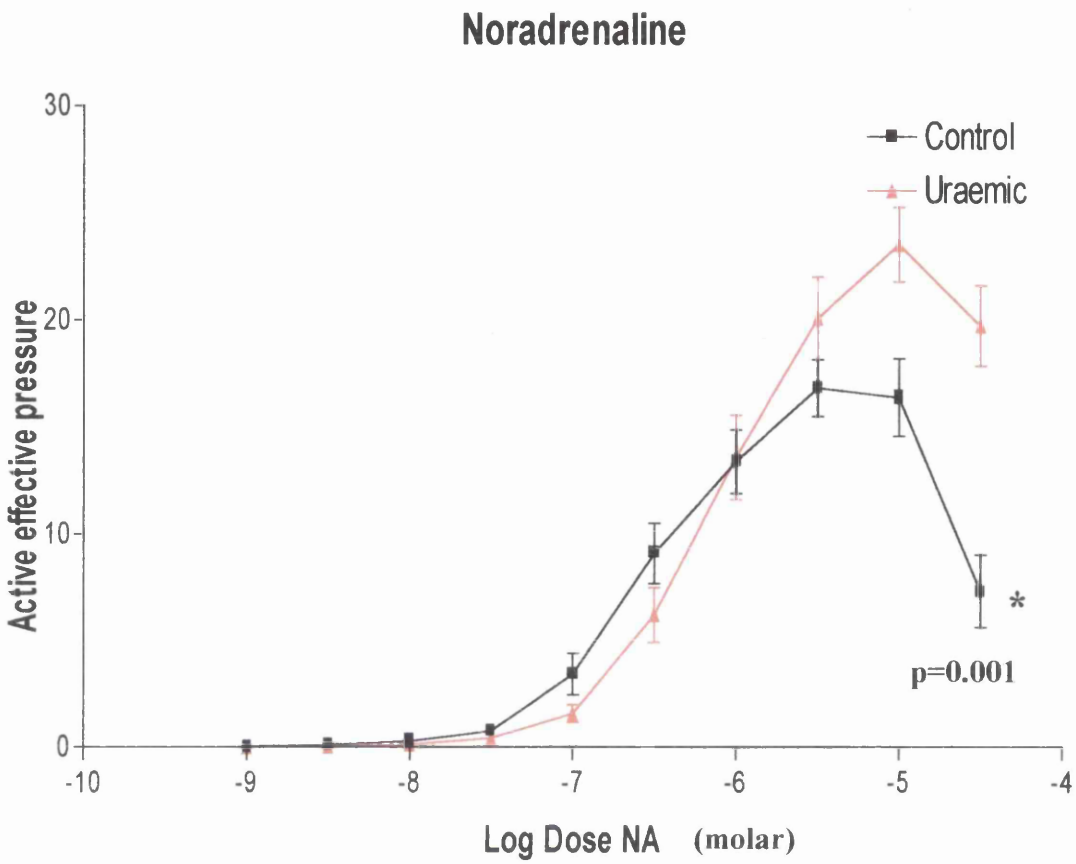


Figure 4.1 Concentration response curves for the effect of noradrenaline on uraemic and control resistance arteries. Response measured as active effective pressure (kPa) and expressed as mean ± sem. Comparison of active effective pressure at highest concentration of noradrenaline is by Mann-Whitney U test.

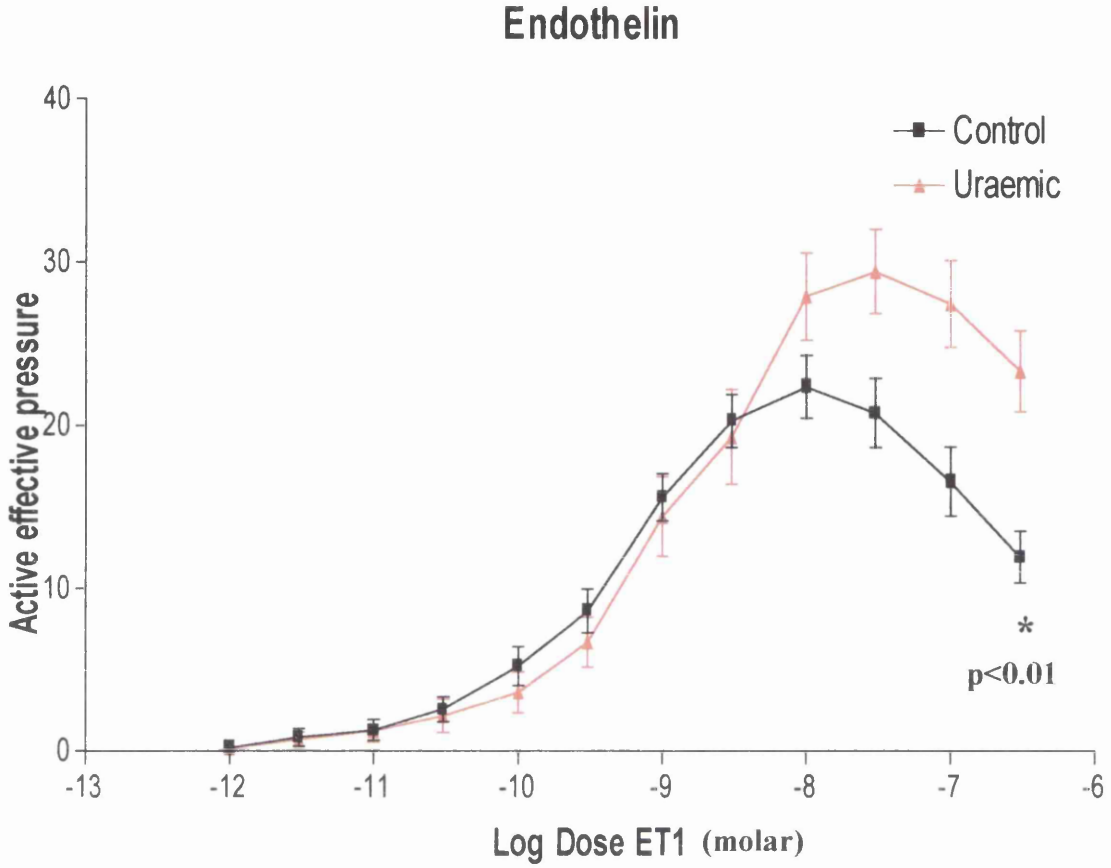


Figure 4.2 Concentration-response curves for the effect of endothelin-1 on uraemic and control resistance arteries. Response measured as active effective pressure (kPa) and expressed as mean ± sem. Comparison of active effective pressure at highest concentration of endothelin-1 is by Mann-Whitney U test.

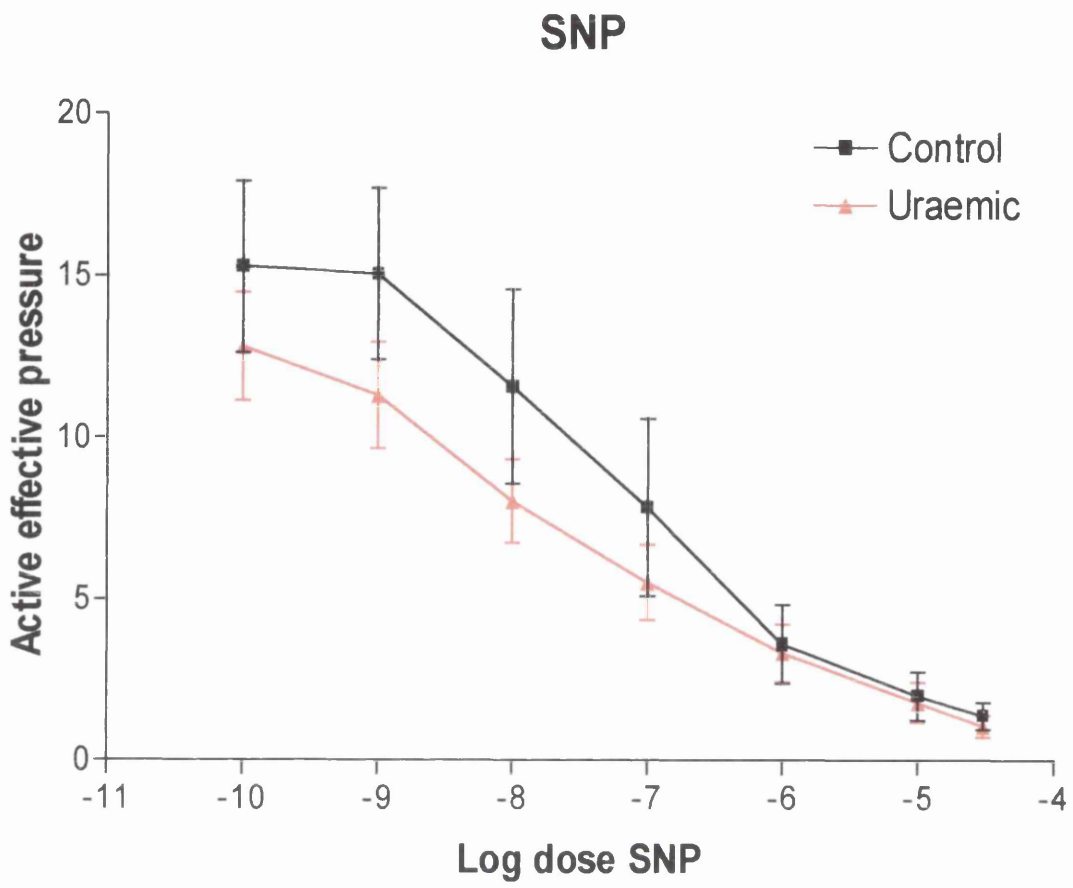


Figure 4.3 Concentration-response curves for the effect of sodium nitroprusside on uraemic and control resistance arteries. Vessels were pre-constricted with noradrenaline ($3x10^{-6}$M). Response measured as active effective pressure (kPa) and expressed as mean ± sem. Comparison by Mann-Whitney U test at each concentration not significant.

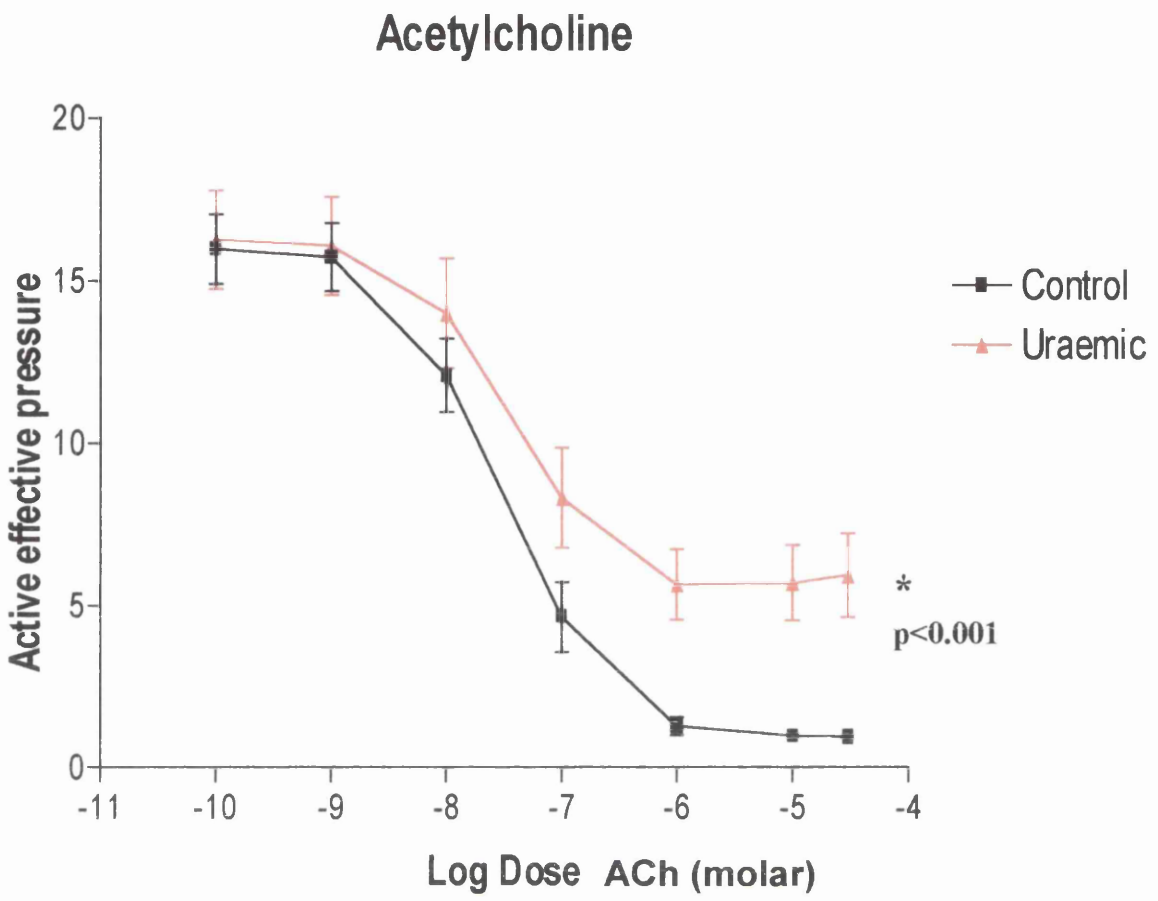


Figure 4.4 Concentration-response curves for the effect of acetylcholine on uraemic and control resistance arteries. Vessels were pre-constricted with noradrenaline ($3x10^{-6}$M). Response measured as active effective pressure (kPa) and expressed as mean ± sem. Comparison of maximum % vasodilatation by Mann-Whitney U test.

The factors influencing endothelium-dependent vasodilatation were further examined in all subjects. There was no significant difference between the median (range) maximum % relaxation to ACh in male subjects (82.7 (45.0,99.3)) compared to female subjects (89.0 (48.0,95.0); p=0.938 by Mann-Whitney test). In addition there was no significant difference for ACh response in smokers (85.5 (91.0,100)) compared to non-smokers (87.7 (45.0,99.3); p=0.817).

Spearman correlation coefficients were determined for maximum % relaxation to ACh, SBP, DBP, cholesterol, triglycerides, haemoglobin, age and glucose. None were statistically significant. The only factor approaching a statistically significant correlation with ACh response was triglyceride : Spearmann coefficient 0.532, p=0.05, however, this was a positive correlation which is at odds with the results for plethysmography and thus difficult to explain.

A similar analysis showed no significant difference in response to SNP, noradrenaline or endothelin-1 between males and females, and between smokers and non-smokers. Spearmann correlation coefficients were determined for maximum response to NA, maximum response to ET-1, EC_{50} for NA, EC_{50} for ET-1, urea, creatinine, glucose, age, cholesterol, haemoglobin and triglycerides. The following significant correlations were noted :

- max NA and cholesterol : Spearmanns coefficient 0.648, p=0.017
- EC_{50} NA and diastolic BP : Spearmanns coefficient 0.576, p=0.031
- EC_{50} ET-1 and creatinine : Spearmanns coefficient 0.644, p=0.007

4.4 DISCUSSION

This *in vitro* study has demonstrated for the first time that there is impaired endothelium-dependent vasodilatation in isolated human uraemic resistance arteries. Responses to the endothelium-independent vasodilator SNP were preserved, thus the endothelium in these vessels is less able to produce and release endothelium-dependent vasodilators, predominantly NO, upon stimulation. These findings are in keeping with the results of the *in vivo* study detailed in chapter 2, and again are consistent with the published literature of forearm plethysmography and ultrasound studies in CRF.

In this study, we have shown that there is endothelial dysfunction in human uraemic vessels *in vitro*, when vessels are removed from the uraemic milieu, and therefore, in the absence of circulating factors such as ADMA or homocysteine. This suggests one of several possibilities :

- that a short-lived circulating factor such as ADMA is not involved in causing endothelial dysfunction in uraemia (although recent studies suggest that ADMA may accumulate intracellularly (Al Baanchbouchi 2000))
- that storage of the uraemic resistance arteries overnight in cooled physiological saline solution does not remove circulating factors from the endothelium
- that the endothelial damage in uraemia is not easily reversible, and possibly permanent
- that there may be down-regulation of eNOS which takes some time to resolve

Our results are in direct contrast to those of a recently published study that found normal endothelium-dependent vasodilatation in resistance vessels from uraemic rats (Thuraisingham 1999). This group examined the effects of acetylcholine and SNP on preconstricted mesenteric resistance arteries from control and spontaneously hypertensive rats which had undergone 5/6 or sham nephrectomy 4 weeks previously. While there was no statistically significant difference in EC50 or maximum % relaxation for acetylcholine, their results do suggest a trend toward a reduced maximum % relaxation in the uraemic rats compared to control rats. Of course, this study used an animal model where the duration of uraemia was short and thus its limitations are clear. Interestingly, one major difference was that all experiments were performed in the presence of indomethacin : it is therefore possible that at least some of the differences seen in our study are not NO-dependent but rather related to changes in the production of a prostaglandin.

A similar study in rats with autosomal dominant polycystic kidney disease demonstrated (after sacrifice at 8 weeks, and therefore not uraemic) impaired endothelium-dependent vasodilatation that was attenuated with L-NAME, suggesting a defect in NO production &/or release early in renal disease (Wang 1999). They also showed a greater response to noradrenaline and a higher media/lumen ratio in the PKD rats. Following this study they examined resistance vessels from humans with adult polycystic kidney disease, normal blood pressure and normal serum creatinine levels (Wang 2000). Endothelium-dependent vasodilatation was again impaired in the APKD vessels and unaffected by L-arginine or L-NAME, suggesting an impairment of NO synthase in APKD.

The only previously published wire myography study of human resistance artery function in CRF was by Mulvany's group in 1986. Using a similar technique they examined anterior abdominal wall subcutaneous resistance arteries from 20 patients on haemodialysis and 11 controls, and found no changes in vascular morphology or sensitivity to noradrenaline, angiotensin II, potassium or calcium (Aalkjaer 1986). Endothelium-dependent and independent vasodilatation were not assessed. Their results were surprising and not easily explained, as the patients were of a similar age to ours and had been uraemic for a considerable period of time. Although we did not demonstrate any difference in the EC50 for noradrenaline or endothelin-1, the concentration-response curves are clearly different in the two groups with a significantly greater response to the maximal dose of each agonist in the uraemic patients, and a pattern suggesting a more prolonged contraction with each agent. There was also a trend toward a higher maximal contraction with both agonists that I suspect would be significant if more subjects had been studied.

One could postulate several reasons for an enhanced contraction to noradrenaline and endothelin in uraemic resistance arteries :

- there may be reduced counterbalancing basal or stimulated NO production, as both NA and ET-1 act via α_2 and ET-B receptors respectively to stimulate NO release (in a presumed protective negative feedback role)
- there may be upregulation of endothelin and adrenergic receptors by an unknown mechanism (although unlikely in the face of increased circulating levels of an agonist)
- there may be alterations in the signal transduction pathways

- there may be structural changes in the vessel wall such as increased medial smooth muscle secondary to long-standing hypertension or uraemia itself. We know that essential hypertension is characterised by remodelling of small arteries with an increased media/lumen ratio. The increased pressor response in this present study may reflect the vascular changes of hypertension, and not be influenced by uraemia : previous studies in hypertension have shown an increased pressor response to infused agonists in both humans and spontaneously hypertensive rats (Mulvany 1990)

Despite controlling for smoking habit and serum cholesterol level, one potential confounding factor in our study was the higher systolic blood pressure in the patients compared to controls. It is extremely difficult to control for blood pressure in a study such as this, as obtaining subcutaneous fat biopsies from normal controls is difficult, let alone finding essential hypertensives undergoing abdominal surgery. As several studies in hypertension have demonstrated endothelial dysfunction we cannot conclude that the results of this study are entirely secondary to uraemia.

There were no obvious factors related to the degree of endothelium-dependent vasodilatation (EDV) in either uraemic or control vessels, and in particular, smokers had similar EDV compared with non-smokers. However, the lack of a correlation between any of the background biochemical or BP data may simply reflect the small numbers involved. Interestingly, DBP was associated with the EC_{50} for noradrenaline and creatinine with the EC_{50} for endothelin-1. Thus, uraemia (i.e. a higher creatinine) is related to a lower sensitivity to endothelin : this would fit with the known raised circulating endothelin levels in renal failure and presumed down-regulation of

endothelin receptors. However, this goes against the finding of a trend toward a greater pressor response to endothelin in uraemic patients : the mechanisms involved here are obviously complex and require further study. The relationship between DBP and noradrenaline sensivity is unclear and the opposite of what one would expect.

4.5 CONCLUSION

In conclusion, this study has demonstrated impaired endothelium-dependent vasodilatation with preserved endothelium-independent vasodilatation in uraemic resistance arteries *in vitro*. The results support *in vivo* studies in renal failure, but contrast with those of the only other published myography study in uraemia looking specifically at endothelial function (Thuraisingham 1999). Our results suggest endothelial dysfunction, a defect that may predispose these patients to accelerated atherosclerosis. The underlying patho-physiology remains unclear.

To further investigate the underlying mechanism, one would first have to repeat the above studies in the presence of indomethacin and L-NMMA to ascertain the degree of involvement of NO or prostaglandins. Assuming that these experiments reveal that the defect lies in the L-arginine/NO pathway then the studies could be repeated in the presence of anti-oxidants such as ascorbic acid, to assess the role of increased oxidative stress, or in the presence of L-arginine in an attempt to overcome the effects of circulating inhibitors of NOS.

Chapter 5

A study of endothelial function in CAPD patients

5.1 INTRODUCTION

Continuous ambulatory peritoneal dialysis is an attractive method of renal replacement therapy for many patients with ESRF as it allows a degree of independence from the renal unit. Owing to selection bias, it has not been possible to accurately determine whether patient survival is better or worse on CAPD compared to haemodialysis, although most studies suggest survival is comparable on the two methods. There are no data to suggest that cardiovascular disease is any less common in CAPD patients; the reverse is probably true as patients with heart failure and IHD often tolerate CAPD better than haemodialysis.

CAPD patients are uraemic. They are maintained in a steady state with weekly creatinine clearances of around 60-70 litres, equating roughly to a GFR of 7-10 ml/min. Thus, these patients are exposed to all the cardiovascular risks of patients with advanced CRF, and often over a longer period. Hyperhomocysteinaemia, hyperparathyroidism, dyslipidaemia (different pattern from HD; see Table 1.2), increased oxidative stress and hypertension, are all prevalent. Asymmetric dimethylarginine levels may be lower than those in haemodialysis patients (Kielstein 1999). Nevertheless, one would expect endothelial dysfunction in CAPD patients as a result of uraemia and other cardiovascular risk factors. Our results for a forearm plethysmography study of endothelial function in uraemia detailed in chapter 3 included those from 6 patients on CAPD. While the uraemic group overall exhibited impaired EDV, no differences could be seen between the pre-dialysis patients and the CAPD patients (Figures 3.7 and 3.8). Haemodialysis patients could not be included owing to forearm AV fistulae.

Hypertension is common in CAPD patients, although the prevalence is lower than that in patients treated by haemodialysis (Cheigh 1999). In many patients there is a clear relationship between fluid balance and blood pressure, and CAPD patients are therefore a reasonable model for studying volume-dependent hypertension. The mechanisms underlying volume-dependent hypertension are complex : some studies have shown that the initial rise in cardiac output is transferred to the resistance vasculature with a resulting sustained increased peripheral resistance (Otsuka 1988). What is not clear is the role of the endothelium in this process. Increased intraluminal pressure or stretch of the vessel wall may stimulate contraction either through direct effects on the vascular smooth muscle cells or through endothelial release of contracting factors (or reduced relaxing factors). The role of shear stress is also unclear : shear stress is the result of contact of red blood cells with the endothelium and induces endothelial NO production. If a patient is volume overloaded and haemodilute, the RBCs may flow in the centre of the vessel and shear stress will be reduced with less endothelial NO production.

This small study was designed to examine basal and stimulated endothelial NO production in CAPD patients with varying intravascular volume.

5.2 METHODS

5.2.1 Subjects

The initial plan was to enrol 10 patients. However, recruitment difficulties for this project resulted in only 3 patients being studied. This was related to the requirement for the CAPD regime to be altered and to the need for two separate visits to the plethysmography laboratory. The patients' background characteristics are detailed in table 5.1. The patients were all non-smokers and not diabetic. In addition, none were taking aspirin, statins, ACE inhibitors or other antihypertensive drugs. All suitable patients attending the CAPD clinic were invited to participate (except those prone to pulmonary oedema).

	Patient 1	**Patient 2**	**Patient 3**
Sex	Female	Male	Male
Age	59	43	43
Renal Diagnosis	Membranous GN	Reflux nephropathy	APKD
Months on CAPD	32	14	21
Cholesterol	8.8	7.8	4.4
Triglyceride	2.3	3.5	2.4
Haemoglobin	8.9	10.6	10.2
SBP	176	113	159
DBP	89	63	91

Table 5.1 Background data for 3 CAPD patients.

5.2.2 Materials

Carbachol was obtained from Martindale Pharmaceuticals (England), sodium nitroprusside from Faulding DBL (England) and L-NMMA from ICN (U.K.). All solutions were prepared in 0.9% saline by the sterile pharmacy productions unit at the Western Infirmary, Glasgow.

5.2.3 Protocol

The study was approved by the local ethics committee. Two separate forearm plethysmography studies were performed. The first study was performed when the patients were felt to be at their dry weight. Following this, their CAPD regime was altered in such a way to encourage fluid gain. The aim was for weight to increase by 2kg over a 7-14 day period, with a further forearm plethysmography study being performed at the higher weight. Patients were therefore studied "dry" and "wet" (or relatively fluid-overloaded. (Note : On all occasions, CAPD fluid was left within the abdomen during studies).

Forearm plethysmography was performed as described in Chapters 2 and 3.
All studies were performed in a quiet vascular research laboratory maintained at 24-26°C after an overnight fast. The same protocol was used as in Chapter 3 : 30 mins acclimatisation with 0.9% saline, then incremental doses of carbachol (0.1, 0.3, 1.0 and 3.0 μg/min), then a 30 minute wash-out with saline followed by incremental

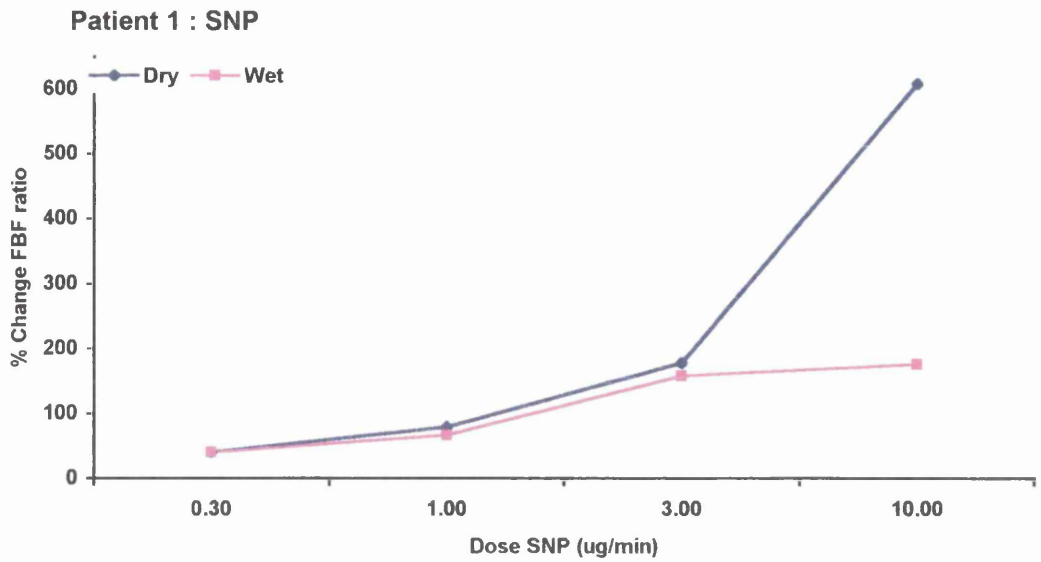
doses of SNP (0.3, 1.0, 3.0, 10.0 μg/min). After a further 30 minutes washout with saline, a single dose of L-NMMA (8μmol/min) was infused (Figure 3.1).

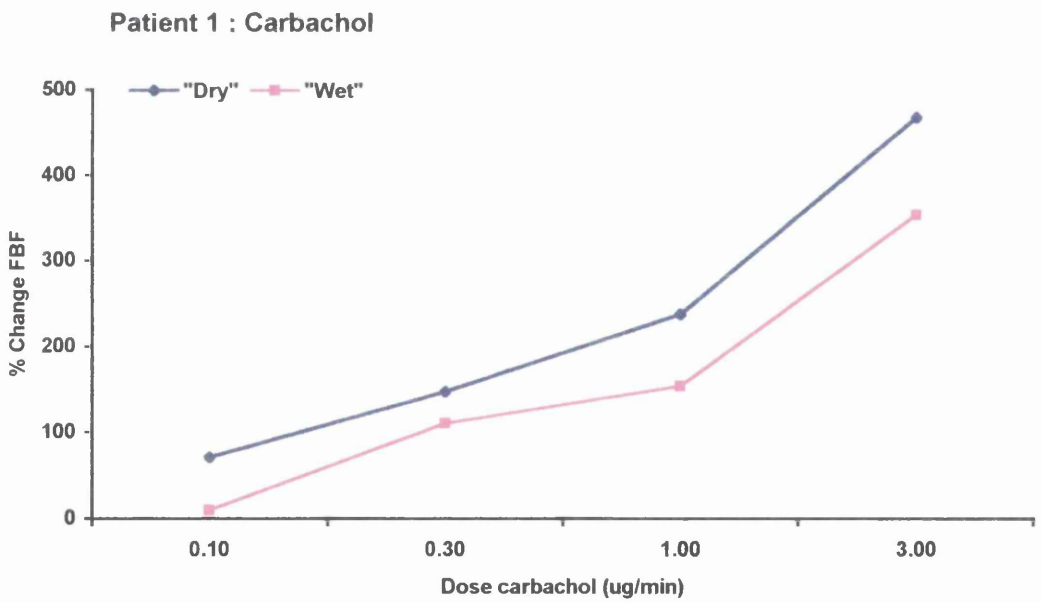
5.2.4 Statistical Analysis

Results are simply expressed in graphical form (Microsoft Excel 97). No formal statistical analysis was performed in view of the small number of patients studied.

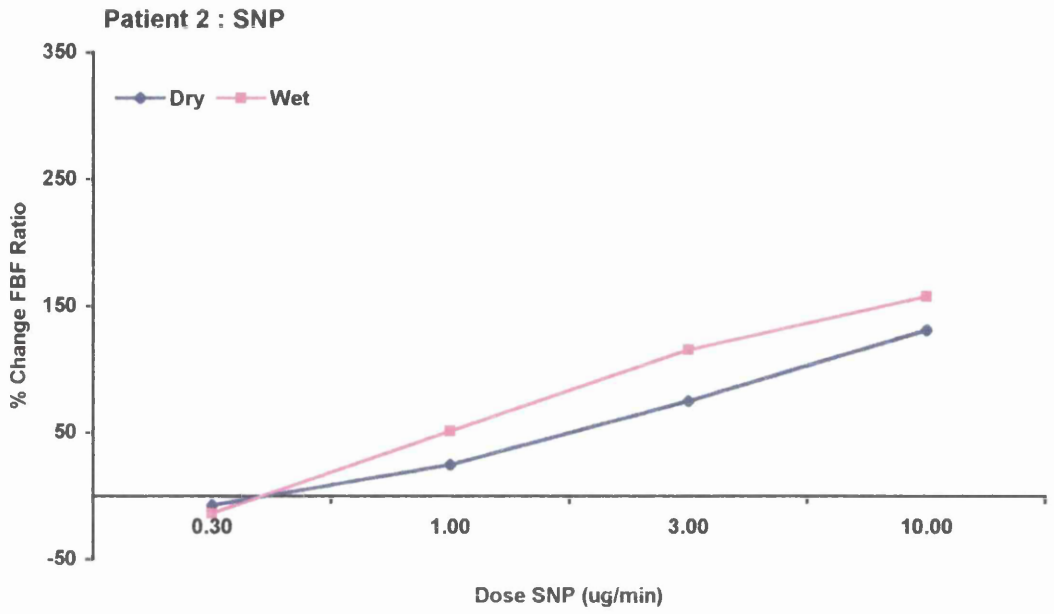
5.3 RESULTS

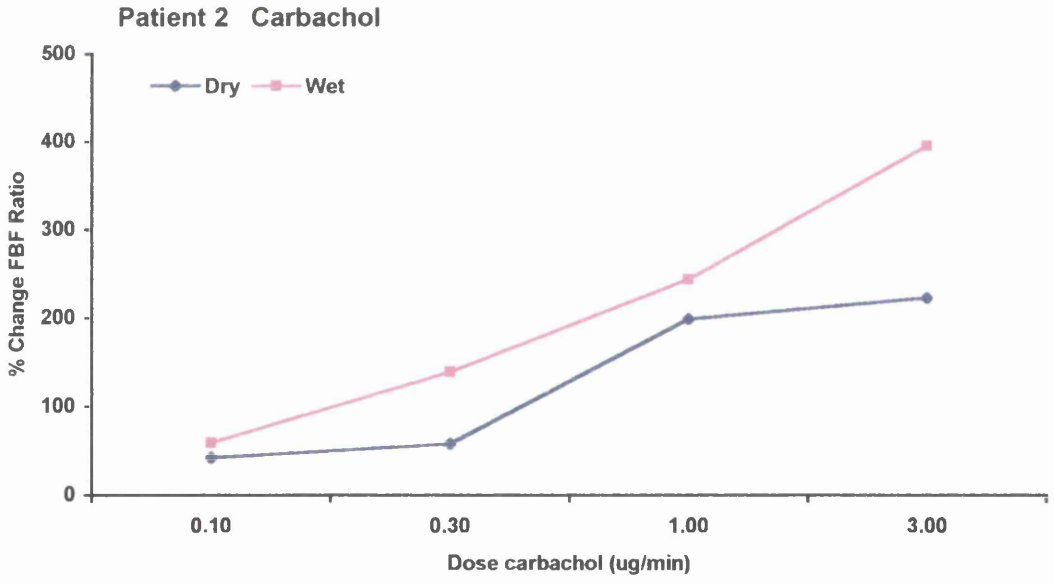
All subjects gained weight between the 2 studies. Subject 1 increased from 76.1kg to 78.5kg, subject 2 from 70.0kg to 71.6kg and subject 3 from 89.0kg to 91.0kg. Results for infusion of carbachol and SNP are illustrated in Figure 5.1-5.3.



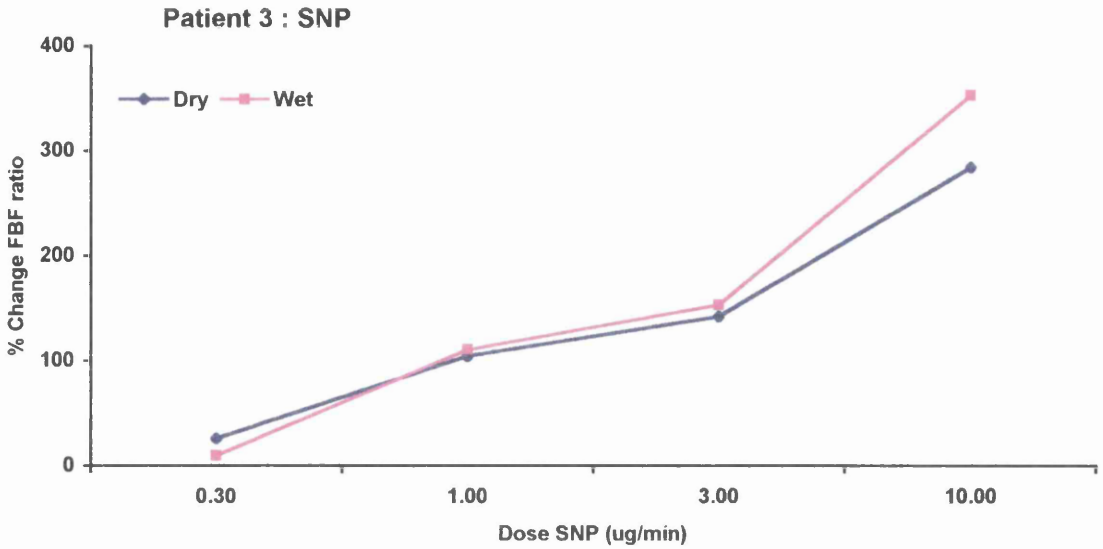


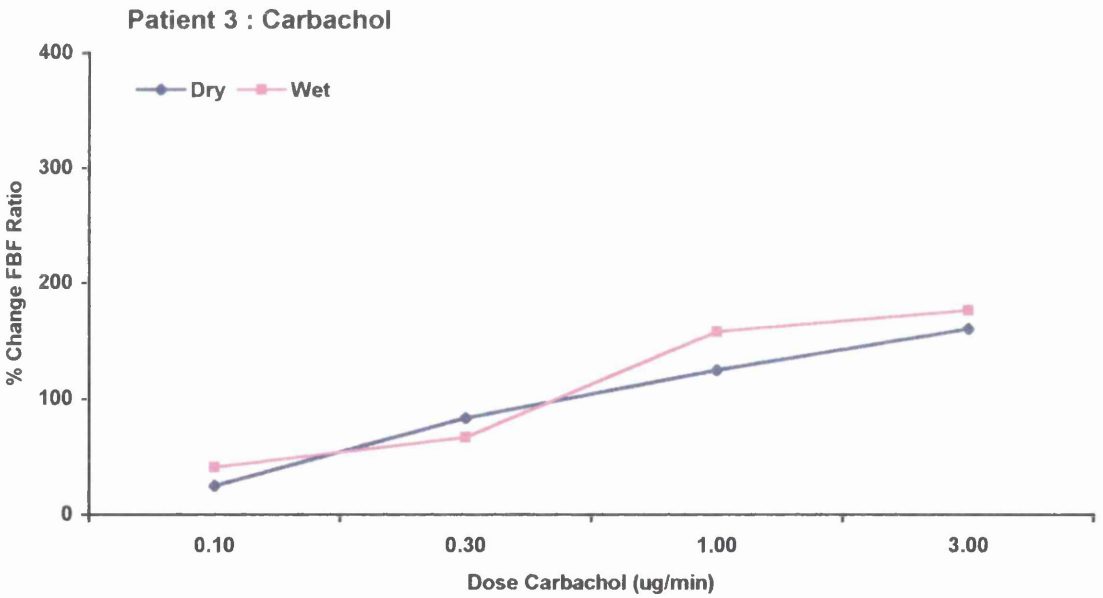
Figures 5.1a and b. Dose response curves for infusion of SNP and carbachol in Patient 1at dry weight and 2.4kg heavier.





Figures 5.2a and b. Dose response curves for infusion of SNP and carbachol in Patient 2 at dry weight and 1.6 kg heavier.





Figures 5.3a and b. Dose response curves for infusion of SNP and carbachol in Patient 3 at dry weight and 2 kg heavier.

L-NMMA results are only available for patients 1 and 3. In patient 1 the % reduction in FBF ratio with L-NMMA was 23.5% dry and 27.3% wet. In patient 3 the % reduction in FBF ratio was 43.6% dry and 27.3% wet. Patient 2 was unable to tolerate the extra time required for the L-NMMA infusion.

5.4 DISCUSSION

This study was clearly limited by small numbers. Recruitment difficulties mainly resulted from the requirement for 2 visits to the vascular lab and patients were also concerned about manipulation of fluid balance. In the 3 patients studied there were no adverse effects of this study.

It is difficult to draw any conclusions from the results. The response to SNP was similar in all 3 patients when "dry" and "wet", although there was a marked reduction in response to the highest dose in patient 1 when "wet". Overall the response curves for SNP were rather flat, the reason for this being unclear. In patient 3 there was no difference in response to carbachol, whether "wet" or "dry". In patient 1, the response to carbachol was greater when "dry" while the opposite was true for patient 2. No obvious pattern is therefore seen in these 3 patients.

L-NMMA was used to assess basal NO production and the effect of changes in fluid balance. Again the changes were different in the 2 patients studied and no firm conclusion can be made. If the numbers had been substantially higher then perhaps a

pattern would appear. Power calculations are almost impossible in a study such as this where there is no precedent.

Thus, the mechanism of volume-dependent hypertension remains unclear. Many theories have been forwarded for this form of hypertension that is common in renal disease. Following an acute volume load, animal studies show that there is initially a rise in cardiac output (Otsuka 1988) followed, in some animals by a sustained rise in blood pressure associated with a raised peripheral resistance and normal circulating noradrenaline and vasopressin levels, and plasma renin activity. It is therefore likely that there are key alterations in resistance vessel function in volume-dependent hypertension.

Postulated changes in resistance vessel function in volume-dependent hypertension include :

- altered basal and stimulated NO production.
- altered endothelin production.
- the Bayliss effect : stretch of the vessel wall by raised intravascular volume leads to vasoconstriction (Bayliss 1902). The mechanism responsible for this is still unknown but is a property of the vascular smooth muscle cells.
- in vitro, raised pressure within small arteries leads to a reduction in lumen diameter that is endothelium-dependent (i.e. pressure induces release of an endothelium-dependent contracting factor) (Mulvany 1990). Increased flow however induces NO release.
- circulating NOS inhibitors.
- circulating Na^+/K^+ ATPase inhibitors.

- changes in shear stress.
- changes in sympathetic tone.
- vessel wall oedema caused by salt and water retention leading to vessel dysfunction.

5.5 CONCLUSION

The mechanisms underlying volume-dependent hypertension are thus unclear. Unfortunately, this small study using the manipulation of CAPD patients' volume status has added little to our understanding of this complicated subject. Further study is warranted.

Chapter 6

An *in vivo* study of endothelial function in renal transplant recipients

6.1 INTRODUCTION

The introduction of cyclosporin to standard immunosuppressive protocols in 1984 has greatly improved graft survival in renal transplantation, but patients continue to die prematurely of cardiovascular disease. Figures from the European Renal Association Registry show around a 10-fold increase in mortality from cardiovascular disease in renal transplant recipients compared to the general population.

Hypertension occurs in almost all patients by the time ESRF is reached, and then often improves significantly with adequate salt and water removal on dialysis. Paradoxically, hypertension again becomes extremely common (up to 90% of patients) following renal transplantation (Schwenger 2001), and is likely to play a major role in the development of cardiovascular disease in this group. In keeping with its role in native renal disease progression, systemic hypertension is also a major independent predictor of renal graft failure (Opelz 1998), with a recent multivariate analysis suggesting that for every 10mmHg increase in M.A.P. the relative risk of graft failure is 1.3 (Mange 2000). Furthermore, post-transplant hypertension is associated with poorer patient survival (Kasiske 1996). The potential causes of post-transplant hypertension are manifold (Schwenger 2001) :

- impaired renal function (secondary to chronic rejection/recurrence of original disease)
- excessive renin production by native kidneys
- calcineurin inhibitors : cyclosporin & tacrolimus
- corticosteroids (at least in the early post-transplant period)
- increased body weight

- transplant renal artery stenosis
- polycythaemia
- genetic predisposition to hypertension in the donor

There is considerable evidence that cyclosporin (and possibly also tacrolimus) is the most important factor in the development of post-transplant hypertension. Prior to cyclosporin's introduction, the incidence of post-transplant hypertension was approximately 10% in bone marrow and cardiac transplantation, and now with its regular use, hypertension occurs in 33-60% of bone marrow and 71-100% of cardiac transplant recipients (Textor 1997). In renal transplantation the incidence of hypertension has increased by around 30% since the introduction of cyclosporin (Textor 1997).

In keeping with most forms of hypertension, cyclosporin-induced hypertension involves an increased systemic vascular resistance (Luke 1991). Early organ bath work showed that cyclosporin vasoconstricted smooth muscle preparations, an effect partially blocked by α-adrenoceptor antagonists (Xue 1987). Since then, several clinical and *in vitro* studies have suggested a range of possible mechanisms for CsA-induced hypertension :

- Sodium retention (Ciresi 1992), possible secondary to afferent arteriolar constriction in the glomerulus.
- Calcium-sensitisation of vascular smooth muscle (Ventura 1997).
- Altered prostaglandin production (Petric 1988).

- Activation of the sympathetic nervous system. The initial report came from animal studies in heart transplantation (Scherrer 1990), however subsequent work in humans has refuted this (Stein 1995, Kaye 1993, Ventura 1997).
- Increased plasma renin activity. This is certainly true in animals, but in humans there is either little change or a reduction in plasma renin activity (Lee 1997), although renin may be inappropriately high for the degree of blood pressure.
- Increased endothelin levels or action. Raised circulating endothelin-1 levels have been documented in both animals and humans with cyclosporin-induced hypertension (Ventura 1997). In rats, ET-A receptor mRNA expression is increased in the aorta and mesenteric arteries while ET-B receptor mRNA expression is reduced in the vascular endothelium (Iwai 1995). In addition, the concomitant administration of bosentan, a non-selective endothelin receptor antagonist, with cyclosporin in rats ameliorates the hypertensive response (Ventrua 1997). In humans, cyclosporin-induced glomerular hypoperfusion is associated with a marked increase in urinary endothelin levels (Perico 1992). Thus, there is a growing body of evidence for a role of endothelin in CsA-induced hypertension.

More recently, attention has focused on another aspect of endothelial function, the L-arginine/nitric oxide system. Over the years, data has accumulated to show that CsA is toxic to endothelial cells in culture, and that this effect is time- and dose-dependent (Zoja 1986). Animal work demonstrates attenuated acetylcholine (NO)-mediated vasodilatation in cyclosporin-treated dogs (Sudhir 1994) and rats (Takenaka 1992), and a reduction in iNOS gene expression in rats (Vaziri 1998). A recent study in

humans failed to show any improvement in endothelium (NO)-dependent vasodilatation after conversion from cyclosporin to azathioprine (van der Dorpel 1998).

Using the *in vivo* technique of forearm strain-gauge plethysmography, the aims of this study were :

1. to establish the presence or absence of endothelial dysfunction in renal transplant recipients with stable graft function
2. to examine both basal and stimulated endothelial nitric oxide production in cyclosporin-treated renal transplant recipients

6.2 METHODS

6.2.1 Subjects

Renal transplant recipients, at least one year post-transplantation, and with stable allograft function, were recruited from the transplant follow-up clinic at the Western Infirmary, Glasgow. The recruitment process was random; one investigator (SM) attended the above clinic whenever possible for 1 year and asked all suitable patients if they were willing to participate. Controls without renal disease were obtained from hospital staff and by advertisement within the hospital and University grounds. Those obtained by advertisement were paid £50 to cover expenses and time away from work. All subjects gave written informed consent, and the protocol was approved by the local ethics committee.

The results detailed below are from 39 subjects who completed the studies. There were around 10 additional patients and subjects who were unable to lie still for the 2-hour studies and their data are not included.

Two separate studies were performed. In study 1, stimulated NO production was assessed in 9 CsA-treated patients, in 7 patients taking an azathioprine-based regime and in 12 controls without renal disease. In study 2, basal NO production was assessed in 9 CsA-treated patients and in 11 controls. Patients in the cyclosporin-treated group were maintained on prednisolone (5-10mg/day), azathioprine (1-2mg/kg/day) and cyclosporin (3-5mg/kg/day). Patients in the azathioprine group were maintained on prednisolone (5-10mg/day) and azathioprine (1-2mg/kg/day). These patients were initially also treated with cyclosporin but this was withdrawn at 6-12 months post-transplantation for a variety of reasons including hirsutism, gingival hyperplasia, gout and hypertension.

The patient and control groups were of a similar age and sex distribution, and had similar smoking habits and serum cholesterol concentrations. Background characteristics of subjects enrolled into study 1 (stimulated NO production) are depicted in Table 6.1, and of those enrolled into study 2 (basal NO production) in Table 6.2. Four of the controls and six of the cyclosporin-treated patients participated in both studies.

Patients were excluded if they were anti-coagulated with warfarin, had forearm AV fistulae, had a history of diabetes mellitus, vasculitis or myocardial infarction, or were

taking anti-anginal medication or statins. Patients were also excluded if they had severe hypertension such that anti-hypertensive drugs could not be withheld prior to the study.

All subjects were studied in the morning after an overnight fast, and were asked to omit aspirin for 7 days and anti-hypertensive medication for at least 48 hours before the study. Immunosuppressive drugs were taken normally on the day of study. Subjects were asked to avoid caffeine and alcohol, and to refrain from smoking, for at least 12 hours prior to the study. As stimulated endothelial NO production was normal in azathioprine-treated patients in Study 1, only cyclosporin-treated patients and controls were examined further in Study 2.

6.2.2 Drugs and solutions

Throughout each plethysmography session, 0.9% saline (Baxter, U.K.) or drug solutions were infused at 1ml/min. The following drugs (supplier) were stored and diluted to the appropriate concentration by the sterile pharmacy production unit at the Western Infirmary, Glasgow : sodium nitroprusside (Faulding DBL, England), carbachol (Martindale Pharmaceuticals, England), L-NMMA (ICN, England) and noradrenaline (Abbott, England). The reasons for the drug choices have been given in chapter 3; noradrenaline was used as a control vasoconstrictor as in other published forearm plethysmography studies (Calver 1992).

	CONTROL	AZA	CsA	p value[1]	p value[2]
male (total)	8 (12)	6 (7)	7 (9)	0.950	-
smoker (no.)	7	4	4	0.950	-
age (years)	40 (25,51)	45 (33,51)	36 (24,55)	0.681	-
urea (mmol/l)	4.4 (2.9,6.6)	6.9 (4.6,10.4)	7.6 (5.7,12.5)	<0.001	0.210
creatinine (μmol/l)	85 (57,108)	133 (80,223)	143 (111,215)	0.001	0.837
glucose (mmol/l)	4.7 (4.3,5.6)	4.4 (3.8,6.4)	4.7 (4.3,6.5)	0.805	-
cholesterol (mmol/l)	5.3 (3.5,6.6)	5.4 (3.6,7.4)	5.6 (3.7,9.3)	0.360	-
triglyceride (mmol/l)	1.1 (0.6,3.1)	1.4 (0.5,2.8)	2.1 (0.6,5.4)	0.242	-
haemoglobin (g/dL)	14.5 (12.4,15.6)	13.6 (11.0,15.6)	12.9 (10.6,14.7)	0.020	0.351
S.B.P. (mmHg)	124 (104,158)	130 (106,155)	158 (127,184)	0.007	0.029
D.B.P. (mmHg)	71 (50,75)	80 (70,91)	89 (70,99)	0.002	0.054
F.B.F. (ml 100ml^{-1})	2.95 (1.68,4.98)	2.40 (1.75,3.88)	2.80 (1.67,7.84)	0.474	-
total R.R.T. (months)	-	232 (112,348)	108 (51,231)	-	0.023
months since Tx	-	129 (83,312)	48 (14, 216)	-	0.142
CCBs (no.)	-	1	6	-	0.200
β blockers (no.)	-	2	3	-	0.900
aspirin (no.)	-	1	1	-	0.900
trough CsA level (nmol/l)	-	-	82 (41,121)	-	-

Table 6.1. Background characteristics for patients and controls in Study 1. Results are expressed as median (range). [1]Represents Kruskall-Wallis or Chi-Square comparison between 3 groups. [2]Represents Mann-Whitney or Chi-Square test between CsA and AZA. (F.B.F.=baseline forearm blood flow in infused arm; R.R.T. =renal replacement therapy; CCBs=calcium channel blockers).

	CONTROL	CsA	p value
male (total)	11 (11)	9 (9)	-
smoker (no.)	5	4	0.980
age (years)	38 (28,42)	36 (24,55)	0.492
urea (mmol/l)	4.5 (2.8,6.7)	8.2 (6.0,11.71)	<0.001
creatinine (μmol/l)	87 (73,100)	157 (90,164)	<0.001
glucose (mmol/l)	5.0 (4.4,5.2)	5.2 (4.1,6.5)	0.182
cholesterol (mmol/l)	5.0 (3.1,6.8)	5.9 (3.6,6.5)	0.604
triglyceride (mmol/l)	1.2 (0.8,1.9)	2.0 (0.8,5.4)	0.211
haemoglobin (g/dL)	13.9 (12.4,15.5)	13.2 (10.5,13.6)	0.019
S.B.P. (mmHg)	125 (101,144)	146 (108,169)	0.056
D.B.P. (mmHg)	72 (56,79)	84 (64,95)	0.002
F.B.F. (ml $100ml^{-1}$)	2.82 (1.70,5.20)	3.06 (1.40,5.00)	0.849
total RRT (months)	-	65 (108,244)	-
months since Tx	-	20 (51,216)	-
CCBs (no.)	-	7	-
β blockers (no.)	-	3	-
aspirin	-	2	-
trough CsA level (nmol/l)	-	96 (37,161)	-

Table 6.2 Background characteristics for patients and controls in study 2. Results are expressed as median (range). Comparison is by Mann-Whitney or Chi-Square test.

6.2.3 Plethysmography protocol

All studies were performed in a quiet, sealed vascular research laboratory maintained at 24-26°C. Details of the equipment and technique have been outlined in chapter 2. In study 1, incremental infusions of carbachol (0.1, 0.3, 1.0, 3.0 μg/min) followed by sodium nitroprusside (0.3, 1.0, 3.0, 10.0 μg/min) were used. In study 2, noradrenaline was infused first (60, 120, 240 pmol/min) followed by L-NMMA (1, 2, 4 μmol/min) in view of its long half-life. There was a 30-minute washout period with 0.9% saline between drug infusions. Each drug was infused for 7 minutes with FBF measurements taken from minutes 3-6, and blood pressure recorded in the seventh minute. During each drug infusion, several FBF measurements were made; the mean of the final five readings was taken as the FBF achieved for each dose of drug. Forearm blood flow was expressed as ml blood flow min^{-1} $100ml^{-1}$ forearm, and bilateral FBF measurements were made with the results expressed as a ratio of infused:non-infused arm.

6.2.4 Blood sampling and laboratory evaluation

Prior to each plethysmography session, a 21gauge cannula was inserted into the dominant arm, and blood drawn after a 20-minute period of supine rest. Thirty ml of blood were drawn into EDTA, lithium heparin or plain tubes, spun and frozen immediately. In addition to standard haematological and biochemical analyses, assays were performed for renin (Millar 1980) and noradrenaline (Goldstein 1981), and for endothelin-1 using a commercially available kit (Cozart Bioscience, England).

6.2.5 Statistical Analysis

All results are expressed as median (range) unless otherwise specified. Comparison between groups is by MANOVA with repeated measures. When significant, post-hoc analyses were performed by Mann-Whitney U to compare individual groups at each drug dose level. Statistical significance was defined at the 5% level.

In keeping with the method used in chapter 3, data for area under the curve are also presented. All statistics were performed using SPSS package for Windows version 7.0.

6.3 RESULTS

6.3.1 Neurohumoral data

The neurohumoral data from studies 1 and 2 are detailed in Tables 6.3 and 6.4 respectively. In study 1, there was a trend toward higher endothelin levels in patients compared to controls (this was significant for azathioprine-treated patients (0.48 (0.10,1.15) fmol/ml) compared to controls (0.19 (0.01,0.49); p=0.022)). Renin levels were similar for all groups studied, however there was a trend toward lower noradrenaline levels in CsA patients compared to AZA patients and controls.

	CONTROL	AZA	CsA	p value[1]	p value[2]	p value[3]
endothelin-1 (fmol/ml)	0.19 (0.01,0.49)	0.48 (0.1,1.15)	0.36 (0.18,2.1)	0.019	0.068	0.397
renin (uU/ml)	20 (2,44)	27 (16,74)	20 (6,57)	0.104	0.840	0.281
noradrenaline (nmol/l)	1.15 (0.01,4.20)	0.30 (0.01,5.30)	0.05 (0.01,4.30)	0.596	0.238	0.463

Table 6.3. Neurohumoral data for study 1. Results expressed as median (range). Statistical comparison is by Mann-Whitney test : [1]between control & AZA; [2]between control & CsA; [3]between AZA &CsA.

	CONTROL	CsA	p value
endothelin-1 (fmol/ml)	0.12 (0.01,0.30)	0.13 (0.01,0.30)	0.439
renin (uU/ml)	12.0 (6.0,28.0)	14.0 (7.0,56.0)	0.385
noradrenaline (nmol/l)	0.70 (0.01,1.90)	0.30 (0.01,1.00)	0.558

Table 6.4. Neurohumoral data from study 2. Results expressed as median (range), and comparison is by Mann-Whitney test

6.3.2 Effect of infusion of carbachol and SNP in transplant recipients and controls

Results for study 1 are illustrated in dose-response curve form, with results for infusion of S.N.P. in Fig. 6.1 and 6.2 and for carbachol in Fig. 6.3 and 6.4.

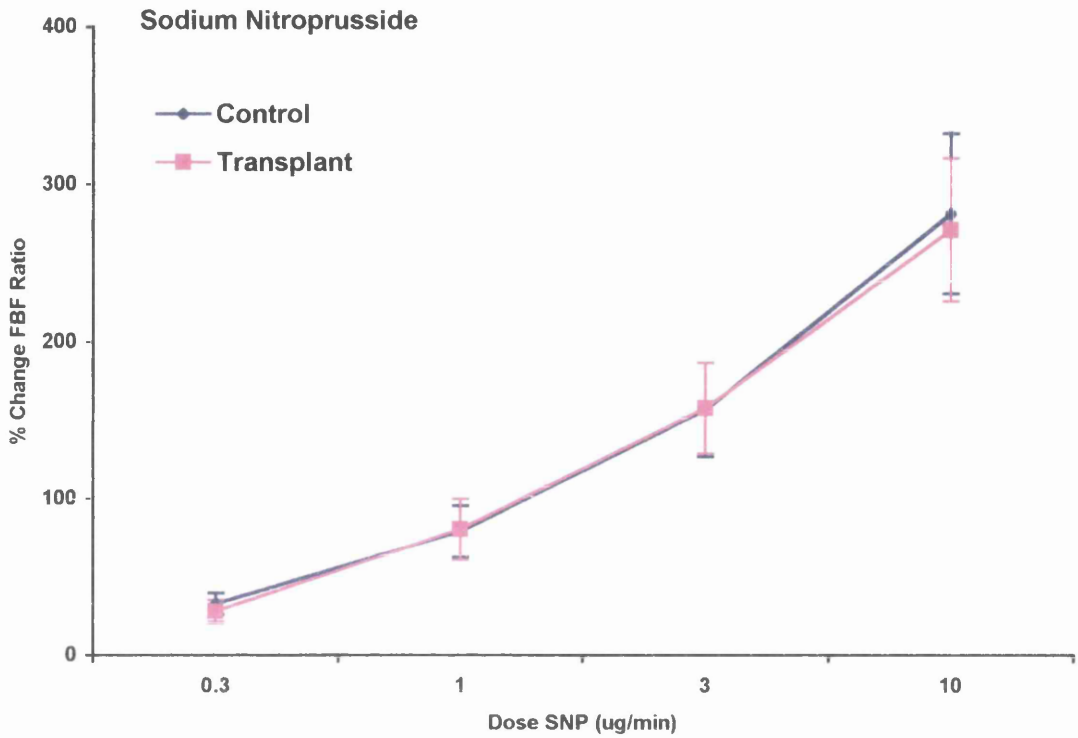


Figure 6.1 Dose-response curves for infusion of SNP in 12 controls and 16 renal transplant recipients. Data are mean ± sem.

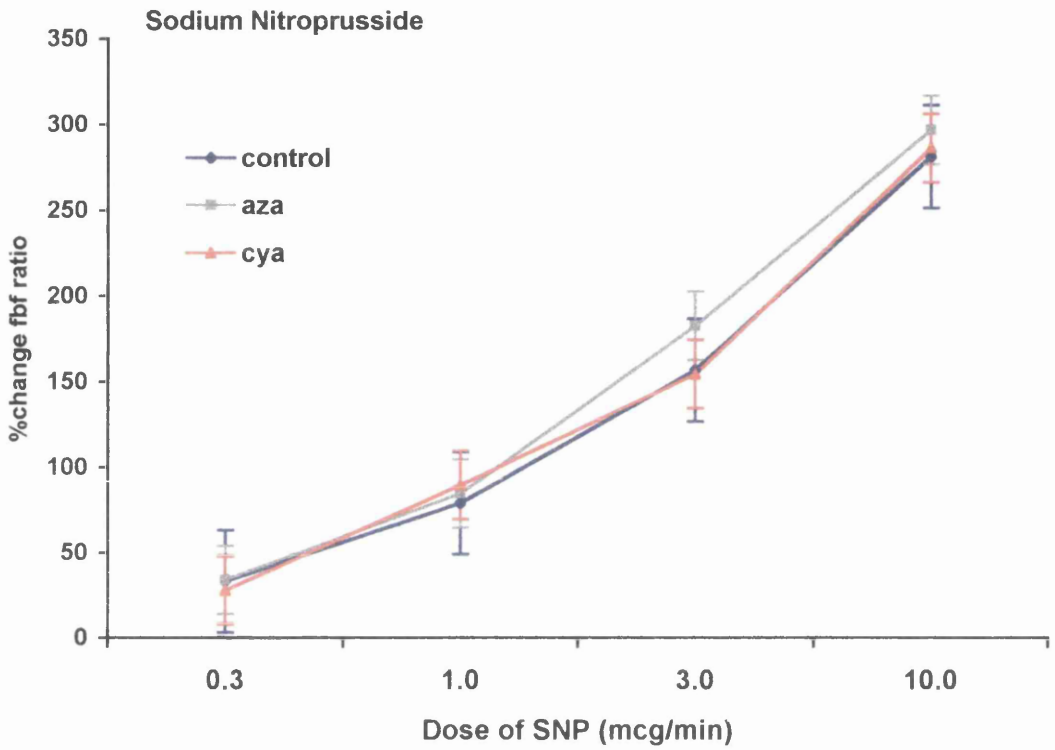


Figure 6.2 Dose response curves for infusion of sodium nitroprusside in renal transplant recipients maintained on CsA (n=9) or not (aza; n=7), and 12 normal controls. Results are mean ± sem. Comparison by MANOVA showed no significant difference between groups.

All three groups vasodilated similarly to S.N.P. (p=0.985 by MANOVA) demonstrating that endothelium-independent vasodilatation was comparable between groups. However, vasodilatation to carbachol (Fig. 6.3) was attenuated in the renal transplant recipients although did not reach statistical significance by MANOVA (p=0.10). Figure 6.4 shows the results for carbachol when the transplant recipients are divided into those taking a CsA-based regime and those taking an azathioprine-based regime. Vasodilatation to carbachol was attenuated in cyclosporin-treated patients (p=0.008 by MANOVA) with a significant reduction in response to 3μg/min carbachol in the cyclosporin group (188.8 (72.5,385.0) %change FBF ratio) compared to controls (303.8 (124.8, 813.3) p=0.028) and to the azathioprine group (378.1 (124.0,548.9); p=0.042).

For study 1 the corresponding area under the curve data are as follows. The AUC for carbachol was 660.9 (259.6,1576.6) for controls, 471.6 (150.4,939.4) for CsA group and 698.4 (241.9,959.4) for AZA group; p=0.09 by Kruskall-Wallis test. The AUC for SNP was 1417.0 (857.8,4717.1) for controls, 1010.5 (736.7,4311.2) for CsA group and 1504.8 (1192.6,4939.2) for Aza group; p=0.26 by Kruskall-Wallis.

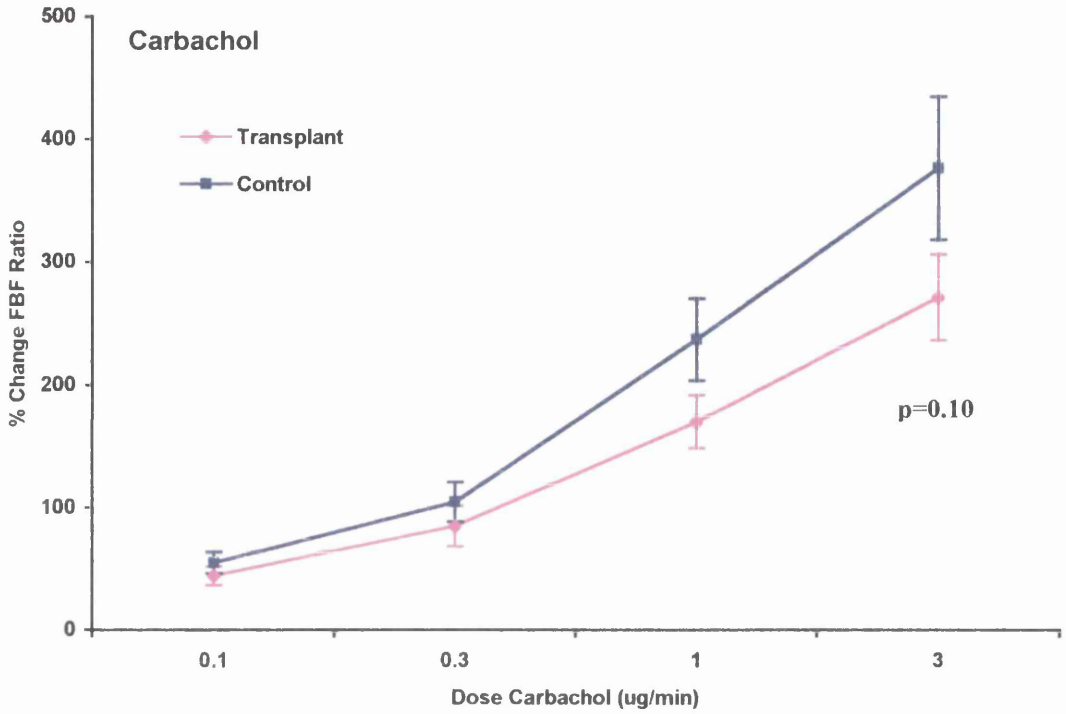


Figure 6.3. Dose response curves for infusion of carbachol in 12 controls and 16 renal transplant recipients. Results are mean ± sem, and comparison is by MANOVA.

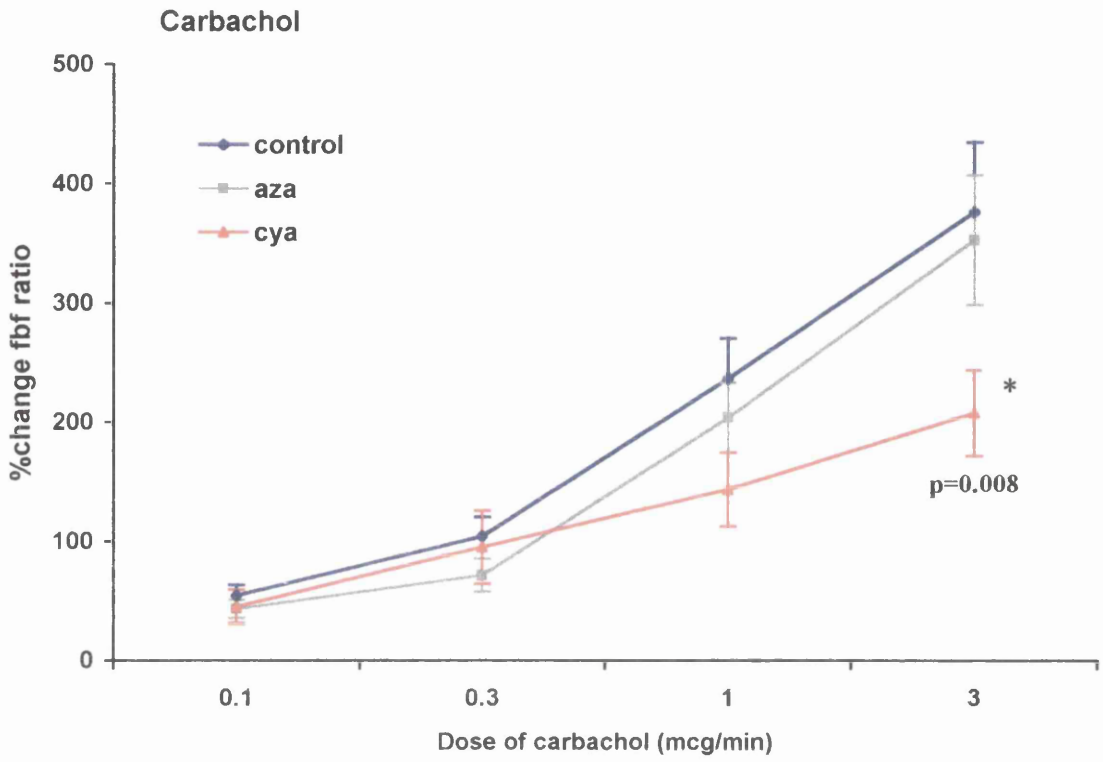


Figure 6.4. Dose response curves for infusion of carbachol in renal transplant recipients maintained on CsA (n=9) or not (n=7; aza), and 12 normal controls. Results are mean ± sem. Comparison is by MANOVA.

6.3 Effect of infusion of L-NMMA and noradrenaline in CsA patients and controls

In study 2, the response to infusion of L-NMMA is illustrated in Fig. 6.5. There was a graphical trend toward reduced vasoconstriction in the cyclosporin group, (overall MANOVA p=0.378), with the difference bordering on statistical significance at 4μmol/min L-NMMA (-39.4 (-15.7,-52.8) controls v -19.5 (-4.7,-63.1) for CsA; p=0.056 by Mann-Whitney test).

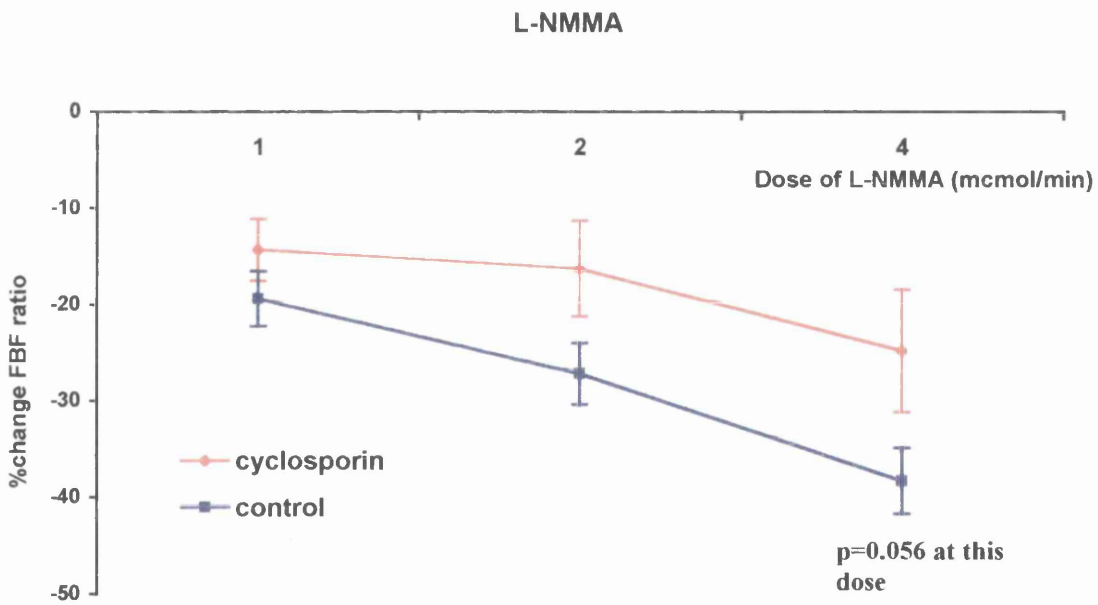


Figure 6.5. Dose-response curves for infusion of the NOS inhibitor L-NMMA in CsA-treated renal transplant recipients (n=9) and normal controls (n=11). Comparison by MANOVA was not statistically significant, but at 4μmol/min comparison by Mann-Whitney approached significance (p=0.056). Results are mean ± sem.

Results for infusion of noradrenaline are illustrated in Fig. 6.6. Cyclosporin-treated patients as a group tended to vasodilate slightly while controls vasoconstricted as expected (p=0.016 by MANOVA). The maximum %change FBFratio for 240pmol/min noradrenaline was -27.0 (-1.4, -38.6) for controls compared to +7.9 (-36.8, +92.6) for CsA-treated patients (p=0.02 by Mann-Whitney test).

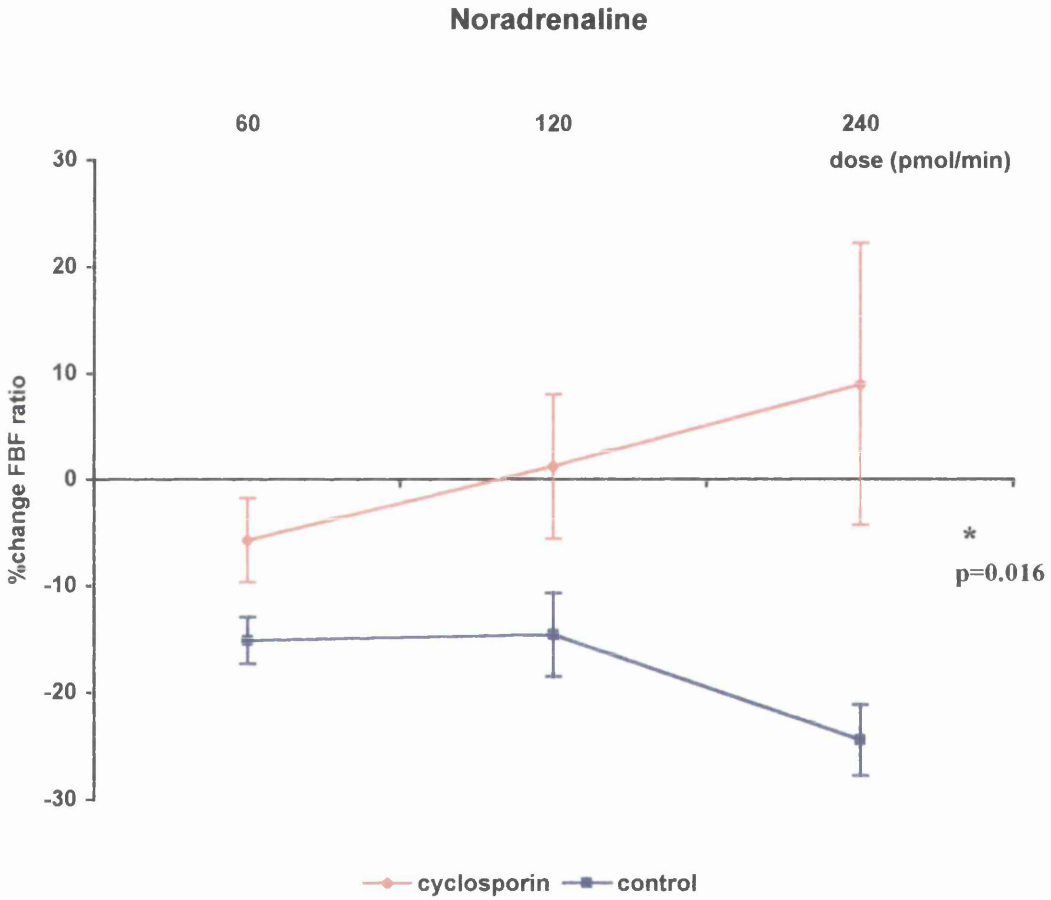


Figure 6.6. Dose response curves for infusion of noradrenaline in CsA-treated renal transplant recipients and normal controls. Results are mean ± sem. Comparison by MANOVA showed a significant difference between the 2 groups (p=0.016).

The error bars indicate a considerable degree of variation in the response to noradrenaline amongst the CsA-treated patients. When these results are examined more closely, it is clear that the patients are behaving differently : 3 vasoconstrict normally (to a similar degree as the control group). One patient had a minimal vasoconstrictor response, while 5 vasodilated. These patients were therefore divided into those who vasoconstricted (n=4) and those who did not (n=5) to see if any factors could be identified from the background history and results that would explain the discrepancy. These data are presented in Table 6.5

Of the patients who vasodilated, 2 were taking aspirin, 4 were taking CCBs and 1 was taking a beta-blocker. Of the (normal) vasoconstrictors, none were taking aspirin, 3 were taking CCBs and 2 were taking beta-blockers. No patients were taking alpha-blockers. As the numbers were so small in each group, there was no statistically significant difference in drug use between groups. In addition, there were no significant differences between the neurohumoral data in vasodilators compared to vasoconstrictors. Spearmann correlation coefficients were performed for each factor in Table 6.5 and the response to 240pmol/min noradrenaline. None were significantly correlated, except for age. The plot for this correlation is demonstrated in Figure 6.7

Pulse and blood pressure did not change significantly throughout the studies, as the doses of drugs infused were chosen to exert only local effects in the forearm.

	NA Vasodilators	NA Vasoconstrictors	p value
Age	46.4 (9.3)	28.7 (5.1)	0.016 *
Creatinine (μmol/l)	137.8 (16.9)	144.0 (36.2)	0.286
Glucose (mmol/l)	5.2 (0.7)	5.2 (0.7)	0.730
Hb (g/dL)	12.8 (0.7)	12.8 (1.6)	0.556
Cholesterol (mmol/l)	5.3 (1.1)	5.6 (0.9)	0.730
Triglyceride (mmol/l)	1.74 (0.87)	2.38 (2.04)	0.556
SBP (mmHg)	144.6 (25.7)	136.5 (16.1)	0.730
DBP (mmHg)	86.0 (7.3)	79.2 (10.2)	0.413
CsA level#	108.5 (44.7)	80.4 (42.3)	0.556
Tx time	88.6 (77.4)	49.7 (28.5)	0.413

Table 6.5 Background data for CsA-treated patients split into those who vasoconstricted normally to NA (n=4) and those who vasodilated (n=5). In view of small numbers data are mean ± sd, comparison by Mann-Whitney U test. # CsA level is mean of last 3 trough levels prior to the study.

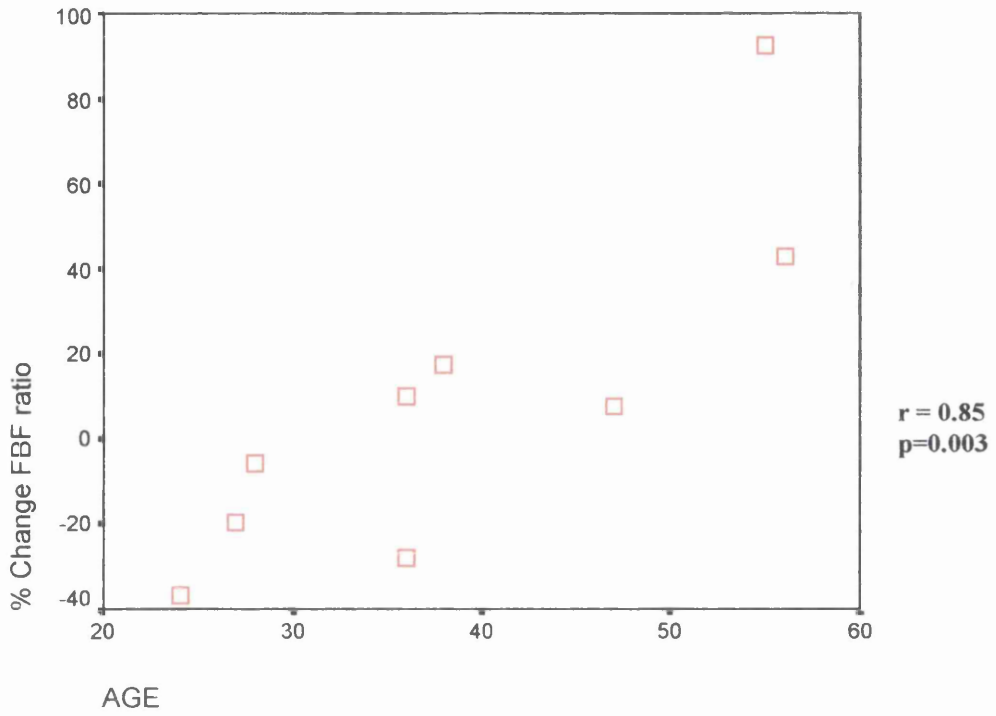


Figure 6.7 Scatter plot to illustrate the correlation between age and response to infusion of 240 pmol/min noradrenaline in CsA treated renal transplant recipients.

6.4 DISCUSSION

The first aim of this study was to examine endothelial function in renal transplant recipients compared to controls. The results from Study 1 show that, among 12 controls and 16 renal transplant recipients, there was a trend toward attenuated endothelium-dependent vasodilatation in the transplant group compared to controls which may have reached statistical significance if more subjects had been recruited. Responses to the endothelium-independent vasodilator, SNP, were preserved. Thus

the pattern suggests endothelial dysfunction in the transplant group, and in particular, a reduced ability of the vascular endothelium to produce and release NO upon stimulation, or indeed, a reduced effect of NO through increased inactivation. It is also possible, that there is impairment of production and release of other endothelium-dependent vasodilators such as EDHF. To test for the relative contribution of NO and other vasodilators, one would have to repeat the studies in the presence of a NOS inhibitor such as L-NMMA. This was not done in these studies as it proved difficult for subjects to lie still for over 2 hours, and a second study would have been required in all cases.

With knowledge of the association between cyclosporin and hypertension, the second aim of this study was to examine the effects of cyclosporin treatment on basal and stimulated endothelial NO production. The results for Study 1 were therefore examined with the transplant group split into those patients taking cyclosporin and those who were not. This time, there was a statistically significant reduction in the response to carbachol in the CsA-treated patients compared to controls and the azathioprine-treated group. In study 2, the CsA-treated group vasoconstricted less well to the NO synthase inhibitor, L-NMMA, suggesting reduced basal endothelial NO production. The results of both studies indicate endothelial dysfunction in cyclosporin-treated renal transplant recipients.

This is unlikely to represent the effects of renal transplantation itself, as azathioprine-treated patients had preserved stimulated NO production. Neither is it likely to reflect years of uraemia prior to transplantation, as the azathioprine group had a longer total time on renal replacement therapy (Table 6.1). Endothelial dysfunction has been

demonstrated in several conditions including hypercholesterolaemia, cigarette smoking, diabetes mellitus, hypertension and cardiac failure. However, diabetics were excluded from this study and both groups were matched for smoking habit and total cholesterol, although blood pressure was higher in the cyclosporin group (both compared to controls and compared to azathioprine-treated patients). As stated before, it is difficult to control for blood pressure in studies of this nature and the results are therefore limited by the possibility that the endothelial dysfunction is related to hypertension and not to treatment with cyclosporin. Another "unmatched" variable was triglyceride level, although studies in severe hypertriglyceridaemia have shown preserved endothelial function (Chowienczyk 1997).

Thus it remains possible that endothelial dysfunction in the CsA-treated patients is a direct effect of cyclosporin therapy, a theory supported by evidence from other studies. Bossaller et al demonstrated in 1988 that aortic rings from rats treated with cyclosporin showed attenuated relaxation to acetylcholine with preserved responses to GTN (Bossaller 1988). A similar picture was observed by adding cyclosporin to human subcutaneous resistance arteries in a wire myograph (Richards 1990) although this group felt the main inhibitory action was on the production of endothelium-derived prostanoids.

A more recent and well-designed study examined responses of thoracic aortic rings from rats treated with either cyclosporin or control (olive oil) (Oriji 1998). Rings from the CsA-treated animals demonstrated a 35% increase in tension when exposed to endothelin-1 compared to rings from control animals. Furthermore, the subsequent vasorelaxation to acetylcholine was reduced by 65% in the CsA rings, and this

inhibitory action was prevented by pre-treatment of the rats with L-arginine. In whole animals, mean arterial pressure rose by 42% after administration of CsA and this was again prevented by pre-treatment with L-arginine. The rise in MAP was accompanied by a significant fall in the urinary nitrite, nitrate and cGMP levels, suggesting that cyclosporin-induced hypertension, at least in rats, is associated with inhibition of NO production. Similarly, Gonzalez-Santiago et al found that treatment of rats with CsA reduced endothelial NOS mRNA expression and increased ECE mRNA expression (Gonzalez-Santiago 2000). In humans, Schrama found evidence of endothelial dysfunction in CsA-treated renal transplant recipients (Schrama 2001). This group measured von Willebrand factor and sP-selectin (both markers of endothelial injury) during stepwise withdrawal of cyclosporin and found elevated levels during treatment with full dose CsA that subsequently fell after withdrawal of the drug.

Thus there is a growing body of evidence that cyclosporin-induced hypertension is related to endothelial dysfunction, and in particular reduced production &/or effect of NO. Cyclosporin-related haemolytic uraemic syndrome may simply be a more extreme manifestation of the endothelial damage produced by this drug.

A surprising finding of this study was a trend toward reduced vasoconstriction or a slight vasodilatation to noradrenaline in cyclosporin-treated patients. In a study using isolated rat aortic strips, cyclosporin added to the organ bath induced a slowly-developing contraction that could be blocked by either the calcium-channel blocker verapamil, or by the α-antagonist phenoxybenzamine (Xue 1987). It is therefore possible that cyclosporin interferes with α-mediated vasoconstriction, such that infusion of noradrenaline at plethysmography has little effect or vasodilates through

the β_2 adrenoceptor. The reason for the age effect is unclear. The strong correlation would suggest that this is a true effect and not a type II error, but a possible mechanism is hard to explain as there was no correlation between the time since transplantation or total RRT duration. No previous studies have documented a change in adrenoceptor function with aging and this finding merits further investigation.

A limitation of study 2 was the absence of a non-CsA treated group, thus it is plausible that the results could reflect years of uraemia rather than the effects of cyclosporin. A larger study is warranted to examine the effects of noradrenaline further.

The neurohumoral studies failed to demonstrate significantly raised circulating endothelin levels in the cyclosporin-treated patients as reported previously (Textor 1992), perhaps reflecting the small numbers studied. Interestingly, the azathioprine-treated group tended to have higher endothelin levels, although this failed to alter the response of this group to vasodilators. There was a trend toward lower noradrenaline levels in CsA patients compared to other groups in both studies, in contrast to a previous study where the noradrenaline level was elevated along with increased sympathetic nerve activity in CsA treated patients (Scherrer 1990). The reason for the lower noradrenaline levels and any possible link to the paradoxical vasodilatation is unclear and requires further study. Renin levels were lower in study 2, again perhaps reflecting the small numbers studied. There was no difference in diuretic use between studies.

Forearm plethysmography studies are often small as recruitment of patients for brachial artery cannulation can be difficult. We therefore accept that a limitation of this study is the relatively small study group, and that a larger study is warranted to examine the effect of noradrenaline infusion in more detail.

6.5 CONCLUSION

In conclusion, this study demonstrates attenuated endothelium-dependent vasodilatation in cyclosporin-treated renal transplant recipients compared to controls and azathioprine-treated patients. As endothelium-independent vasodilatation remained intact, this pattern implies altered endothelial function in CsA-treated patients (rather than altered smooth muscle action). Basal NO production was also reduced in CsA-treated patients compared to controls. Cyclosporin-induced endothelial dysfunction may therefore explain at least some of the increased cardiovascular risk in this group, and provide a potential mechanism to explain cyclosporin-induced hypertension. The most surprising finding of this study, that some (predominantly older) CsA-treated patients reacted abnormally to infusion of noradrenaline, requires further study.

Chapter 7

General Discussion & Conclusions

7.1 Endothelial function in uraemia

The principal aim of this project was to examine endothelial function in patients with chronic renal failure. The work contained in this thesis concentrates on endothelium-dependent vasodilatation, as this is the most amenable aspect of endothelial cell function to study in humans. Two methods were employed, one *in vivo*, the other *in vitro*.

Using forearm venous occlusion plethysmography it was demonstrated that endothelium-independent vasodilatation to SNP was similar in 10 uraemic patients (pre-dialysis and on CAPD) and 10 controls. In contrast, endothelium-dependent vasodilatation to carbachol was impaired in the uraemic patients compared to controls, and the pattern therefore suggests endothelial dysfunction in uraemia. As many authors have previously suggested that there may be a circulating "uraemic factor" that promotes atherosclerosis (presumably through endothelial damage) a similar study was performed using the *in vitro* technique of wire myography. This confirmed the findings of the plethysmography study and would suggest that a short-lived, readily-reversible circulating factor is not entirely responsible. However, the effects of years of exposure of the endothelium to one or many "circulating factors" (including traditional cardiovascular risk factors such as oxLDL) may not reverse easily on short-term removal of the offending agents, and thus it remains unproved that circulating factors are not involved. One particular lesson that was learned from the study described in Chapter 3 is that the order of drugs infused in plethysmography should probably not be randomised, especially with SNP as this has a long-lasting

influence on basal vascular tone. Randomising drug order also increases variability and necessitates an increased sample size to show a difference between two groups.

What is likely to be the mechanism underlying endothelial dysfunction in chronic renal failure? The studies performed in this thesis do not allow an adequate explanation, and further work is required. Repeating the experiments in the presence of a NOS inhibitor, such as L-NMMA, would allow a more accurate assessment of the degree of involvement of NO. However, work performed by Newby et al (Newby 1997) has previously demonstrated that endothelium-dependent vasodilatation in the resistance vasculature is predominantly through NO, thus one could speculate that the results from the above experiments suggest dysfunction of the L-arginine/NO pathway in uraemia. This may occur at various levels :

1. altered expression of endothelial receptors
2. impaired signal transduction mechanisms
3. decreased activity of NO synthase
4. reduced intracellular availability of L-arginine
5. increased breakdown of NO
6. reduced responsiveness of the VSMC to NO
7. increased production of an endogenous inhibitor of NOS

Further studies are required in uraemia to elucidate the underlying defect(s).

7.2 Risk factors linked to endothelial dysfunction

The mechanism whereby uraemia leads to endothelial dysfunction has not been elucidated. Patients with CRF often have several traditional cardiovascular risk factors and we may simply be seeing the cumulative effect of these factors rather than an effect specific to uraemia itself. The following risk factors have been linked to endothelial dysfunction :

7.2.1 Essential hypertension

Despite Cockcroft's initial report (Cockcroft 1994), there are now considerable data supporting an association between essential hypertension and impaired endothelium-dependent vasodilatation (Panza 1990; Taddei 1998; Ferro 1997; Iiyama 1996). In animals, chronic inhibition of NOS leads to hypertension, and mice that overproduce NO are chronically hypotensive. However, in humans it remains to be seen whether endothelial dysfunction is the cause or effect of hypertension, and genetic studies have thus far failed to show a link between hypertension and a defect in the eNOS gene (Bonnardeaux 1995). As hypertension is an almost invariable accompaniment of CRF it is likely that if essential hypertension is associated with endothelial dysfunction then uraemia will also be associated with endothelial dysfunction. Designing studies to control for the effect of hypertension is difficult as most uraemic adults will be hypertensive, and thus a second hypertensive control arm would be necessary. Kari et al (Kari 1997) bypassed this problem by examining endothelial function in normotensive children with CRF, and found that endothelial dysfunction

was present despite the absence of hypertension. This group concluded that uraemia per se was associated with endothelial dysfunction.

7.2.2 Aging

In a study of 119 healthy subjects aged from 19 to 69 years it was shown by venous occlusion plethysmography that endothelium-dependent vasodilatation declined with increasing age, while endothelium-independent vasodilatation remained constant (Gerhard 1996). Endothelial dysfunction was apparent from age 30-39 onwards. This group proposed that the reduction in NO production &/or release with advancing age may explain the increased risk of atherosclerosis with aging. Their results were supported by a subsequent study demonstrating reduced vasoconstriction to L-NMMA in elderly subjects compared to younger controls (with preserved constriction to noradrenaline) inferring reduced basal NO production in old age (Lyons 1997). In the studies performed in this thesis age is not likely to be relevant as the study and control groups were matched for age.

7.2.3 Tobacco smoking

Smoking is a major risk factor for the development of atherosclerosis in the general population and has been linked to adverse cardiovascular outcome in CRF. The first study to examine the effects of cigarette smoking on endothelial function was published in 1993 (Jacobs 1993) and had surprising results - in this small forearm plethysmography study stimulated endothelium-dependent vasodilatation appeared to be preserved in young cigarette smokers. A subsequent larger study by Kiowski et al

looked at basal NO production using L-NMMA and showed that long-term smokers had attenuated vasoconstriction to this agent implying reduced basal NO production in smokers (Kiowski 1994). Several studies have confirmed this finding and it is now well established that chronic, acute and passive cigarette smoking are all associated with endothelial dysfunction (Schoenberger 2001). The mechanisms underlying the adverse effects of smoking on endothelial function are complex but in part are related to the generation of oxLDL by free radicals contained in cigarette smoke (Miller 1997). Again, the results from the studies contained within this thesis are unlikely to be influenced by smoking, as there were a similar number of smokers in both the study and control groups.

7.2.4 Diabetes Mellitus

Patients with diabetes mellitus were excluded from the studies performed in this thesis as diabetes is an established risk factor for endothelial dysfunction. The most obvious manifestation of endothelial dysfunction in diabetes mellitus is the characteristic increased permeability of the microcirculation with resultant microalbuminuria. One of the earliest human studies to examine endothelial responses demonstrated impaired endothelium-dependent vasodilatation of isolated vessels from the corpus cavernosum of impotent diabetic males, with preserved responses to SNP and normal responses in non-diabetic impotent males (De Tejada 1989). Subsequent studies, many utilising forearm plethysmography, have confirmed these findings in type I and type II diabetic patients (Williams 1996; O'Driscoll 1997; Watts 1996) and also with acute hyperglycaemia induced by glucose loading in normal subjects and in people with impaired glucose tolerance (Kawano 1999).

Diabetes mellitus is likely to cause endothelial dysfunction in several ways including decreased availability of NADPH (a co-factor for eNOS), increased production of oxygen free-radicals, increased shear stress through insulin-mediated increased flow and hyperinsulinaemia-induced alterations in post-receptor pathways such as PI3 kinase (Storey 2001). In addition, diabetes is associated with inflammation, dyslipidaemia and hypertension, all of which are linked to endothelial dysfunction.

7.2.5 Dyslipidaemia

Dyslipidaemia is common in chronic renal failure and is likely to contribute to the pathogenesis of endothelial dysfunction. While total cholesterol may be normal, uraemia is characterised by hypertriglyceridaemia, increased total:HDL cholesterol ratio and increased small dense LDL concentrations. Most of the human studies examining endothelial function in dyslipidaemia have demonstrated impaired endothelium-dependent vasodilatation in patients with raised total cholesterol levels (Creager 1992; Stroes 1995), although recent studies have shown a link between low HDL levels and endothelial dysfunction in healthy young men (Toikka 1999), and LDL particle size and endothelial responses in diabetics (Skyrme-Jones 2000). Studies in hypertriglyceridaemia have given conflicting results with Chowienczyk (1997) finding preserved endothelial function and Lewis et al (1999) finding impaired endothelium-dependent vasodilatation in patients with high fasting triglyceride levels and normal LDL cholesterols. In chapters 3 and 4, patients had higher triglyceride levels than controls and this may be a confounding issue, and again an issue for which it is extremely difficult to control.

There is clear evidence that dyslipidaemia is intimately related to endothelial dysfunction, even in patients without clinically apparent atherosclerosis. The pathogenesis of the endothelial dysfunction is again likely to be multifactorial, but several pieces of information are known :

- Oxidised LDL more potently impairs endothelial function than does native LDL (Vogel 1999). Oxidised lipoprotein(a) is of even greater potency.
- HDL counteracts the inhibitory effect of oxLDL on endothelium-dependent vaso-dilatation (Matsuda 1993).
- Oxidised LDL inhibits NO synthase in cultured endothelial cells (Vogel 1999).
- Hypercholesterolaemia increases platelet aggregability and endothelial cell adhesion molecule expression, thereby generating a pro-thrombotic state (Vogel 1999).
- Hypercholesterolaemia may increase ADMA levels (Luscher 1997).
- Oxidised LDL may stimulate endothelin-1 production (Luscher 1997), and enhance the sensitivity of the VSMC to endothelin-1.
- Hypercholesterolaemia is associated with reduced bioavailability of NO, through increased inactivation of NO by superoxide anion generation (Vogel 1999).

Thus, many traditional cardiovascular risk factors are prevalent in patients with chronic renal failure and are likely to be involved in the pathogenesis of endothelial dysfunction. Several less traditional risk factors may also be involved including ADMA, homocysteine, inflammation, oxidative stress and hyperparathyroidism, and these have been discussed in more detail in chapter 1. The degree of interplay

between these various risk factors in the pathogenesis of endothelial dysfunction in uraemia remains unknown. Nor do we know if other, unidentified, uraemic factors are involved.

The clinical relevance of endothelial dysfunction in uraemia is visible to all clinical nephrologists : accelerated atherosclerosis, manifest as angina and myocardial infarction, stroke, renovascular disease, mesenteric ischaemia and peripheral vascular disease. While many deaths in our patients occur suddenly, often out-of-hospital, and may be related to left ventricular hypertrophy and arrhythmias, we are increasingly seeing patients with atherosclerotic complications leading to significant morbidity and mortality. With an aging and increasingly diabetic population on RRT, then perhaps the biggest problem facing nephrologists in the near future will be the morbidity and use of resources associated with peripheral vascular disease and cerebrovascular disease.

7.3 Endothelial function following renal transplantation

While renal transplantation undoubtedly improves the quality of life of patients on dialysis, there remains a debate as to whether survival is prolonged, as studies are confounded by selection bias. Cardiovascular disease, common in dialysis patients, remains the commonest cause of death following renal transplantation and death with a functioning allograft (most often from cardiovascular causes) is now the main reason for graft loss.

The most likely explanation for this is that by the time a patient reaches renal transplantation the damage is already done. It is widely believed that increased cardiovascular risk begins at a very early stage in a patient's "renal life", with hypertension, dyslipidaemia, hyperhomocysteinaemia and other factors increasing in prevalence and severity as GFR falls. Not surprisingly, endothelial dysfunction also begins at an early stage and in this thesis has been demonstrated in pre-dialysis uraemic patients, as well as in uraemic children (Kari 1997). Thus by the time a patient is commenced on dialysis they already have established endothelial dysfunction, early (or possibly advanced) atherosclerosis and left ventricular hypertrophy. It is not surprising that endothelial dysfunction is also found in patients following renal transplantation.

Using forearm plethysmography it was demonstrated that endothelium-independent vasodilatation to SNP was similar in 16 renal transplant recipients and 12 controls, while there was a trend toward impaired endothelium-dependent vasodilatation in the transplant recipients. When this group was divided into those taking cyclosporin and those not, it was clear that there was endothelial dysfunction in the CsA group. One would have expected some impairment of endothelium-dependent vasodilatation in the azathioprine and prednisolone group, but this was not seen perhaps reflecting small numbers. The CsA group also had less vasoconstriction to L-NMMA implying that basal NO production was impaired. Thus, CsA-treated patients had impaired stimulated and basal NO production and this may partly explain the pathogenesis of CsA-induced hypertension and the increased cardiovascular risk in renal transplant recipients.

A most surprising finding was of paradoxical vasodilatation to noradrenaline in some of the CsA-treated patients – this was difficult to explain but seemed to be related to age. The clinical relevance of this is unclear but may suggest that CsA, at least in some patients, interferes with α_1-adrenoceptors such that infusion of noradrenaline vasodilates through the β_2-adrenoceptor. Whether this mechanism is involved in CsA-induced hypertension is not known.

7.4 Therapies that may improve endothelial function

Given that patients with CRF, both pre-dialysis, on dialysis and following renal transplantation have endothelial dysfunction and unacceptably high death rates from cardiovascular disease, can we reverse endothelial dysfunction and use this as a surrogate marker for reducing cardiovascular events?

There have been several published reports of improvements in endothelial function following drug therapy and lifestyle changes :

7.4.1 ACE-Inhibitors

Theoretically, angiotensin II may induce endothelial dysfunction through increased ET-1 and prostaglandin H2 production, inhibition of NOS activity by activation of protein kinase C, and through increased free radical production (Virdis 1998). In cigarette smokers (Butler 2001), type I diabetics (O'Driscoll 1997a) and patients with IHD (Prasad 1999), ACE inhibitor treatment has been shown to improve endothelium-

dependent vasodilatation. In essential hypertension the situation is less clear, with some studies showing improved endothelium-dependent vasodilatation in the subcutaneous microcirculation but others demonstrating no benefit in the forearm circulation (Virdis 1998). The reason for the discrepancy is poorly understood. To date, no studies have looked at the effect of ACE inhibitors on vascular endothelial function in uraemia.

7.4.2 Calcium Antagonists

In patients with essential hypertension, data are available showing that dihydropyridine calcium channel blockers improve endothelial dysfunction in various vascular beds : subcutaneous resistance vessels (Schiffrin 1996), the coronary circulation (Frielingsdorf 1996) and the forearm vasculature (Taddei 1997). Interestingly, in the forearm study there was no immediate benefit of intra-arterial infusion of lacidipine while an improvement in endothelial function was apparent after 2 months of oral treatment. Moreover, this improvement persisted for 2 weeks after discontinuation of the drug suggesting that the drug acted independently of blood pressure reduction. Similar improvements in endothelial function have also been demonstrated in patients with hyperlipidaemia (Bracht 2001) but not yet in CRF.

The reason for the beneficial effect of calcium antagonists is uncertain as these drugs act on voltage-dependent calcium channels that are not expressed on endothelial cells, but rather on vascular smooth muscle cells. Some authors have postulated that the inhibition of calcium influx into the smooth muscle cell simply potentiates the vasodilatory effect of NO. However, if this were the case then the responses to

endothelium-independent vasodilators would also be enhanced, and this was not the case in the forearm plethysmography study detailed above. More likely is that calcium antagonists act via an antioxidant mechanism that involves endothelial protection through free radical scavenging (Virdis 1998; Cominacini 1998). In addition, there may be a direct effect of these drugs on the endothelium with some evidence available to suggest that they alter the redox potential of endothelial cells and that there may be an effect on endothelial stretch-operated calcium channels (Born 1998).

7.4.3 Beta Blockers

"Standard" beta-blockers such as atenolol have no specific effect on endothelial function. However, newer drugs such as nebivolol and carvedilol may be different. In two separate studies nebivolol, infused into normotensive volunteers, vasodilated preconstricted dorsal hand veins and increased forearm blood flow, with the effects being blocked by L-NMMA (Bowman 1994; Cockcroft 1995). These data suggest that nebivolol acts partly through increased NO release. Carvedilol is another newer beta-blocker with vasodilating activity *in vivo* and anti-oxidant effects *in vitro*. A recent study has demonstrated that this drug also improves endothelial function in patients with coronary artery disease (Matsuda 2000). No studies are available in renal failure.

7.4.4 Cholesterol reduction

LDL plays a pivotal role in the pathogenesis of atherosclerosis as discussed in Chapter 1, and there are considerable data linking hypercholesterolaemia to

endothelial dysfunction in patients without renal impairment. In this "non-CRF" population there are now over 20 published studies demonstrating improved endothelial function with lowering of serum cholesterol. While the majority of these studies have examined the effects of HMG CoA reductase inhibitors or "statins" on endothelial function, there is reasonable evidence that endothelial function can be improved by any method of cholesterol lowering, including apheresis (Tamai 1997), cholestyramine (Leung 1993) and bezafibrate (Seiler 1995).

Most interest, however, has focused on statin therapy as these drugs offer the most potent therapeutic means of lowering total, and more specifically, LDL cholesterol. Some of these agents are also associated with a modest increase in beneficial HDL cholesterol levels. The first study to demonstrate an improvement in endothelial function with statins was published in 1989 and looked at the effect of lovastatin on rabbits fed a cholesterol-rich diet (Osborne 1989). Subsequent publications have confirmed that statins improve endothelial function in humans with (O'Driscoll 1997b; Drury 1996) and without coronary heart disease (Stroes 1995; Laufs 2001), as well as in a range of other conditions including diabetes (Mullen 2000) and hypertension (Wassmann 2001) . In addition, the benefits may be seen in patients who would traditionally have been classed as normocholesterolaemic (Laufs 2001).

The mechanism of action of statins in restoring endothelial function may be more complex than simple cholesterol reduction. Initial studies suggested that the beneficial effects of these drugs on endothelial function could be observed after 4 weeks of treatment (O'Driscoll 1997). Recent work, however, suggests that these drugs may work over a much shorter timescale with one group demonstrating an improvement in

endothelial function after 3 days treatment of elderly diabetics with cerivastatin (Tsunekawa 2001) and quite remarkably, another group within 24 hours of treatment of normocholesterolaemic, healthy men with high dose (80mg/day) atorvastation (Laufs 2001). In both of these studies the improvements in endothelial function occurred before there was a significant fall in serum cholesterol, implying that the statin improved endothelial function via a cholesterol-independent mechanism. These findings are supported by work on cultured endothelial cells that demonstrates upregulation of eNOS by statins (Laufs 1998) and activation of eNOS by atorvastation via a reduction in the concentration of the eNOS inhibitory substance caveolin-1 (Feron 2001). An antioxidant mechanism may also be involved (Wassmann 2001).

Thus, there is a large body of evidence to support the lowering of LDL-cholesterol with statin therapy in high-risk groups (and possibly lower risk groups) without CRF to improve endothelial function and reduce cardiovascular events. Unfortunately there are currently no published outcome studies looking at cholesterol reduction in patients with CRF. Nevertheless, in renal transplant recipients we now have evidence that statin therapy improves endothelium-dependent vasodilatation (Asberg 2001; Hausberg 2001) and on-going studies will soon report on the effect of statins on cardiovascular outcomes in this group.

7.4.5 L-Arginine

The rationale behind the use of L-arginine to improve endothelial function is simple : either restore availability of the substrate for NO synthesis to normal (if deficient) or

increase the substrate concentration above normal levels in order to compete with antagonists for the enzyme such as ADMA. Under normal conditions however, L-arginine is neither deficient nor rate-limiting with intracellular concentrations being in the millimolar range and the Michaelis constant for NOS being in the micromolar range (Maxwell 1998). The observation that L-arginine improves NO-dependent vasodilatation in a number of clinical scenarios is therefore difficult to explain, although intracellular concentrations of ADMA may be many times greater than circulating levels in these conditions.

Early animal studies demonstrated that supplemental dietary L-arginine reduced atherosclerotic plaque formation in hypercholesterolaemic rabbits (Cooke 1992) while intravenous L-arginine improved endothelium-dependent vasodilatation in a similar rabbit model (Girerd 1990). Subsequent studies in humans have confirmed these findings with the demonstration of improved endothelium-dependent vasodilatation with L-arginine in healthy young adults (Imaizumi 1992), in hypercholesterolaemia (Creager 1992), heart failure (Hirooka 1994) and ischaemic heart disease (Maxwell 1998). In contrast, L-arginine infusion had no effect on endothelial responses in patients with essential hypertension (Panza 1993).

In uraemia, the effects of L-arginine are controversial. Using a dorsal hand vein technique, Hand et al (1998) showed that endothelium-dependent responses were impaired in patients undergoing haemodialysis and that these responses were improved with dialysis, with infusion of L-arginine but not with D-arginine. This group hypothesised that their results supported the belief that accumulation of endogenous NOS inhibitors in uraemia is functionally important. However, a

subsequent study using forearm plethysmography failed to demonstrate any effect of local or systemic infusion of L-arginine in pre-dialysis and haemodialysis patients (Cross 2001), and therefore, without outcome studies, the potential therapeutic benefit of L-arginine supplementation in CRF remains unclear. A further problem may be tolerability as many studies have used over 20g of arginine powder. In patients who are already subject to polypharmacy, this may prove unpalatable.

7.4.6 Antioxidants

Oxidative stress, particularly in CRF, is thought to play an important role in the development of atherosclerosis. On this premise, it has been hoped that antioxidant therapy with ascorbic acid (vitamin C) &/or alpha-tocopherol (vitamin E) would improve endothelial function and slow or prevent the development of atherosclerosis in general and CRF populations. The mechanisms underlying the potentially beneficial effects of antioxidants include (Carr 2000):

- Scavenging of free radicals by ascorbate thereby preventing oxidation of LDL
- Prevention of LDL lipid peroxidation by alpha-tocopherol (contained within the LDL particle)
- Prevention of the pro-oxidant activity of vitamin E by ascorbate
- Inhibition of leucocyte-endothelial cell interaction
- Reduction in oxLDL and superoxide levels to enhance bio-availability of endothelium-derived NO
- Ascorbate may also increase endothelial cell NO production directly by assisting in the regeneration of tetrahydrobiopterin

In keeping with these theoretical benefits, there have been numerous studies demonstrating improved endothelial function with vitamin C and vitamin E in a range of condition including diabetes (Ting 1996), hyperhomocysteinaemia (Chambers 1999), heart failure (Hornig 1998), hypertension (Solzbach 1997) and hypercholesterolaemia (Green 1998). Unfortunately, in the general population these effects on endothelial function have not been translated into clinical benefits with the HOPE and GISSI studies failing to show any effect of antioxidant therapy on cardiovascular endpoints.

In CRF, the coating of haemodialysis membranes with vitamin E has been shown to reduce oxidised LDL formation and endothelial dysfunction associated with the haemodialysis process (Miyazaki 2000). In renal transplant recipients, oral administration of 2g ascorbic acid 2 hours prior to plethysmography significantly increased endothelium-dependent vasodilatation (Williams 2001). Furthermore, a recent study by Fellstrom's group has shown a significant relationship between several markers of oxidative stress and endothelial dysfunction in patients with CRF (Annuk 2001). This suggests that increased oxidative stress, perhaps mediated through hyperhomocysteinaemia, may be the principal mechanism underlying endothelial dysfunction in uraemia. Supporting evidence for this theory comes from the SPACE study, a prospective, randomised, double-blind study of high dose vitamin E or placebo in 196 haemodialysis patients with pre-existing cardiovascular disease (Boaz 2000). Remarkably, after a median follow-up period of only 1.5 years there was a 54% reduction in cardiovascular endpoints in the vitamin E treated group.

7.4.7 Folic Acid

Hyperhomocysteinaemia is extremely prevalent in patients with CRF and is now established as a major risk factor for atherosclerotic cardiovascular disease in this group and in the general population. Folic acid, vitamin B6 and vitamin B12 are required for the metabolism of homocysteine to methionine and cysteine, with deficiencies of these vitamins being associated with hyperhomocysteinaemia and atherosclerosis (Robinson 1998).

In subjects without renal failure, dietary supplementation with folic acid (0.5-5mg/day) reduces blood homocysteine concentration by around 25% (Anonymous, 1998), with an additional 7% reduction with vitamin B12 0.5mg/day. Vitamin B6 had no effect in this meta-analysis. To date, there are no published studies examining the effect of homocysteine-lowering on cardiovascular outcomes although one small unblinded study reported a reduction in the progression of carotid artery plaque thickness with a combination of folic acid, vitamin B6 and vitamin B12 (Peterson 1998). In contrast, many studies have examined the effect of homocysteine-lowering on endothelial function in patients without CRF. Several have shown improved endothelium-dependent vasodilatation (Chambers 2000; Title 2000; Bellamy 1999) while others have not (van Dijk 2001, Pullin 2001), and the reason for the discrepancy is unclear.

In CRF, folic acid supplementation (5-15mg/day) lowers fasting tHcy by around 30-50% although only a third of patients reach "normal" tHcy levels (Bostom 1996). In keeping with the general population, there is a paucity of information on the long-

term effects of folic acid supplementation on cardiovascular events. Additionally, three published studies have failed to show any benefit of homocysteine-lowering (with 5mg folic acid daily) on endothelial function in pre-dialysis patients (Thambyrajah 2000b), CAPD patients (van Guldener 1998a) and haemodialysis patients (van Guldener 1998b). Thus the role of folic acid and B vitamin supplementation in this group is unclear, and it is possible that severe, irreversible endothelial dysfunction occurs at an early stage in CRF and that interventions will be required before GFR has significantly declined. Alternatively, significantly higher doses of folic acid may be required in CRF with or without B vitamins and anti-oxidants, and the studies have not yet been performed to identify the best dose and regime.

7.4.8 Lifestyle Changes/Miscellaneous

In addition to the drug therapies described above, there are many other potential means of improving endothelial function in populations without CRF including hormone replacement therapy (de Kleijn 2001), weight reduction (Ziccardi 2002), smoking cessation (Moreno 1998) and physical exercise (DeSouza 2000). It remains to be seen if these methods will also improve endothelial function in CRF.

7.5 CONCLUSIONS

- Cardiovascular disease is the commonest cause of death in patients with chronic renal failure, both pre-dialysis and on renal replacement therapy. The incidence is several folds greater than that of the general population and indeed of diabetics.
- The increased cardiovascular risk is related to changes in both the heart and the vessels. Left ventricular hypertrophy with resultant arrhythmias and cardiac failure is an extremely common problem in ESRF.
- There are also changes in the blood vessels in uraemia with several reports suggesting accelerated atherosclerosis. As the vascular endothelium protects against the development of atherosclerosis, it is likely that there are fundamental changes in endothelial function in uraemia.
- Studies contained within this thesis demonstrate endothelial dysfunction in adult patients with chronic renal failure, both pre-dialysis and on CAPD. In addition, post-transplant patients receiving cyclosporin therapy also exhibited endothelial dysfunction.
- Endothelial dysfunction in uraemia was demonstrated both *in vivo* using forearm plethysmography, and *in vitro* using wire myography. This would suggest (but not prove) that a short-lived, readily-reversible circulating factor was not entirely responsible. The studies do not allow a more detailed analysis of the aetiology of endothelial dysfunction in uraemia.
- Endothelial dysfunction was demonstrated in CsA-treated renal transplant recipients and this may partly explain the mechanism underlying CsA-related hypertension. Some of these patients had abnormal responses to infusion of noradrenaline, suggesting an abnormality of adrenergic receptors or post-receptor

signalling. The relevance of this to the pathogenesis of CsA-related hypertension is unclear, and the underlying mechanism remains to be elucidated.

- The clinical relevance of endothelial dysfunction in uraemia is a predisposition to atherosclerosis. Traditional cardiovascular risk factors are highly prevalent in CRF patients and may simply act synergistically to accelerate atherosclerosis. However, there may also be more unusual risk factors at play including homocysteine, ADMA, increased oxidative stress, low-grade inflammation and other, as yet unidentified, uraemic factors.
- In the general population, reversal of endothelial dysfunction has been successfully demonstrated using anti-hypertensive agents, cholesterol-lowering, L-arginine, lifestyle changes, folic acid and some antioxidants. It remains to be seen if these methods will also be beneficial in uraemic patients. If so, then reversal of endothelial dysfunction may be viewed as a surrogate marker for treatments to reduce cardiovascular endpoints in this high-risk group.

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Publications Containing Work Undertaken for this Thesis

1. Impaired vascular responsiveness to nitric oxide in renal transplant recipients. Morris STW et al. Transplant Proc 1999; 31, 304-305.
2. Endothelial dysfunction in renal transplant recipients maintained on cyclosporin. Morris STW et al. Kidney International 2000; 57: 1100-1106.
3. Impaired endothelium-dependent vasodilatation in uraemia. Morris STW et al Nephrol Dial Transplant 2000; 15, 1194-1200.
4. The vascular endothelium in chronic renal failure. Morris STW, Jardine AG. J Nephrol 2000; 13, 96-105.
5. Impaired endothelium-dependent vasodilatation in isolated uraemic resistance arteries. Morris ST, McMurray JJV and Jardine AG. Kidney International 2001; 60: 1077-1082.

Presentations to Learned Societies Containing Work Undertaken for this Thesis

1. Impaired vascular responsiveness to nitric oxide in renal transplant recipients. April 1998. Transplant 98, Montreal.
2. Impaired endothelial function in cyclosporin-treated renal transplant recipients. November 1998. Scottish Renal Association, Dumfries.
3. Vascular endothelial dysfunction in renal transplant recipients maintained on cyclosporin. April 1999, Renal Association, Dublin.

4. Impaired endothelial nitric oxide production in renal transplant recipients maintained on cyclosporin. May 1999, ASTP, Chicago. (Winner of an International Young Investigator award).

5. Impaired endothelium-dependent vasodilatation in uraemia. November 1999 A.S.N. Miami.

6. Impaired endothelium-dependent vasodilatation in uraemia. March 2000, Scottish Renal Association.

7. Impaired endothelium-dependent vasodilatation in isolated uraemic resistance arteries. A.S.N. Toronto, October 2000.

8. Endothelial dysfunction in isolated uraemic resistance arteries. European Cardionephrology Meeting, Assisi, Italy, Nov 2000Endothelial dysfunction in isolated uraemic resistance arteries. Nov 2000, Scottish Renal Association.

List of Abbreviations

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
AGE	Advanced glycosylation end product
ANOVA	Analysis of variance
APKD	Adult polycystic kidney disease
AUC	Area under the curve
AV	Arteriovenous
AZA	Azathioprine
BMI	Body mass index
CAPD	Continuous ambulatory peritoneal dialysis
CCB	Calcium channel blocker
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CNS	Central nervous system
CRF	Chronic renal failure
CRP	C-reactive protein
CsA	Cyclosporine A
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ECE	Endothelin converting enzyme
EDV	Endothelium-dependent vasodilatation
EDRF	Endothelium-derived relaxing factor
EDHF	Endothelium-derived hyperpolarising factor
EIDV	Endothelium-independent vasodilatation
EPO	Erythropoietin
ESRF	End-stage renal failure
ET-1	Endothelin-1
FBF	Forearm blood flow
GFR	Glomerular filtration rate

GTP	Glutamyl 5'triphosphate
HD	Haemodialysis
HDL	High density lipoprotein
HUVEC	Human umbilical vein endothelial cell
IHD	Ischaemic heart disease
IL	Interleukin
JAF	Junction-associated actin filament
LDL	Low density lipoprotein
L-NMMA	N_g-monomethyl-L-arginine
LVH	Left ventricular hypertrophy
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthase
PGI2	Prostacyclin
PSS	Physiological saline solution
PTH	Parathyroid hormone
PVD	Peripheral vascular disease
RRT	Renal replacement therapy
SBP	Systolic blood pressure
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TGFβ	Transforming growth factor-beta
tHcy	Total homocysteine
TNFα	Tumour necrosis factor-alpha
USRDS	United States Renal Data System
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
vWf	von Willebrand factor