The University of Nottingham

MEASUREMENTS OF VASCULAR FUNCTION IN HAEMODIALYSIS AND OBESE PATIENTS BY MYOGRAPHY

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Abstract

Background: Patients with chronic kidney disease (CKD) face a markedly increased risk of cardiovascular morbidity and mortality. In this setting, aberrant endothelial function is a key initiating event in vascular disease. Haemodialysis (HD) patients characteristically exhibit significant abnormalities in vascular structure and function, which impact cardiovascular morbidity and mortality. Micro- and macro-vascular dysfunctions are the principle factors contributing to the increased risk of morbidity and mortality associated with obesity. Impaired endothelial function represents the earliest abnormality in the development of vascular disease in obesity and exhibits increased risk of cardiovascular disease. We first aimed to investigate the effect of HD and obesity on the vascular reactivity through directly examines the isolated subcutaneous arteries using wire myography. The second goal was to study changes that might underlie altered vascular responses following bariatric surgery and whether reduction in weight improves endothelial function. We also intended to correlate the *ex vivo* myography data with the *in vivo* results of pulse wave velocity (PWV) and blood pressure (BP) in both HD and obese patients. ***Methods:*** Abdominal subcutaneous fat biopsies were obtained from HD patients (n=11) during non-HD visits through small lower abdominal incisions using local anaesthetics; obese patients (n=12) during the time of bariatric surgery (using a laparoscopic port); and non-HD, non-obese healthy controls (n=26) during the time of elective surgery (hernia repair). Additional abdominal subcutaneous fat samples (n=4) were also obtained from obese patients at six months after bariatric surgery through an extra incision in the lower abdominal region using local anaesthetics. Different-sized

arteries (small with internal diameter between 200 μm – 500 μm and large between 600 μm – 900 μm) were dissected, mounted and conducted on a wire myography on the same day. Cumulative concentration-response curves were constructed for the following vasoactive agents: noradrenalin (NA), endothelin-1 (ET-1), U46619, angiotensin II (AngII), vasopressin, bradykinin (BK), acetylecholine (Ach) and sodium nitroprusside (SNP). Carotid-to-femoral arterial PWV was measured using an oscillometric device (Vicorder, Skidmore Medical Ltd., UK) for HD and obese patients in addition to measuring blood pressure (BP). Laboratory data were expressed as mean ± SEM and groups were compared by t-test. ***Results:*** In both HD and obese patients, greater contractile response to different vasoconstrictors was observed in different-sized arteries compared to control group. Although the potency of these drugs was similar between HD patients and controls, large vessels of HD patients were highly potent to U46619 and vasopressin compared to controls. Similarly, in obese patients, large vessels were also significantly more sensitive to U46619 and vasopressin than that of controls, while small vessels were highly potent to vasopressin response. The maximum vasorelaxation response of small and large vessels to Ach and BK (endothelium-dependent vasodilators) was significantly lower in both HD and obese patients than vessels of controls. A similar response to SNP (an endothelium-independent vasodilator) was obtained in all groups. However, the potencies of all vasodilators in all groups were similar. In HD patients, *in vivo* PWV was significantly correlated with the maximum contractile response of large arteries to vasopressin response ($r = 0.829$, $P = 0.042$). PWV was positively correlated with the percentage of maximum contractile response of small arteries to vasopressin ($r = 0.886$, $P = 0.019$). The diastolic but not systolic BP of HD patients was significantly

inversely correlated with the response of large vessels to SNP (r = -0.954, P = 0.012), it was also negatively correlated with the percentage of contractile response of small arteries to vasopressin (r = -0.829 P = 0.042). There was no correlation observed in the responses of isolated small arteries to the other vasoconstrictor substances in terms of PWV or BP. In obese patients, The PWV was significantly correlated with the maximum contractile response of large arteries to U46619 (r = 0.928, P = 0.006), and with the maximum contractile response of small arteries to vasopressin (r = 0.885, P = 0.033). However, positive correlation was obtained between systolic (but not diastolic BP) of obese patients and the response of large vessels to U46619 (r = 0.785, P = 0.048). There was no significant difference in the vasocontractile or vasorelaxation responses of isolated vessels in obese patients before and after surgery; however, a trend of more contractile response to vasoconstrictors was observed in the obese group before surgery compared to those after surgery. ***Conclusion*:** These results suggest that HD and obesity can alter endothelial function via an incremental increase in vasocontractility in response to various stimuli and an impaired vasodilatation response to endothelium-dependent agonists in isolated different-sized vessels. In both groups, ex vivo arterial responses were correlated to *in vivo* assessment of arterial function. The association between these risk groups and endothelial dysfunction in isolated arteries would be expected to accelerate cardiovascular events, which impacts cardiovascular morbidity and mortality among these groups of patients. Therefore, the development of cardiovascular disease is mediated, at least partly, by functional alterations at the level of microcirculation.

Declaration

I hereby declare that this thesis is entirely my own work and effort, and is based upon research carried out in the School of Graduate Entry Medicine and Health, University of Nottingham and Department of Renal Medicine, Derby Hospital.

The work was done under the guidance of my supervisors Professor **Chris W McIntyre** and Associate Professor **Saoirse O'Sullivan.**

All myography experiments on isolated human vessels were undertaken by me (the total experiments conducted by myography were 45). Analysis of all myography data using GraphPad Prism-5 software was also undertaken by me with help of Dr. Saoirse O'Sullivan. Correlation of ex vivo and in vivo data were kindly analysed by our research fellows

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I would like to thank my wife for her support and patience during my study period. I absolutely would like to thank my parents, brothers and sisters for their unending love and support receive my deepest gratitude for their dedication and their support.

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Abbreviations

Ach	Acetylcholine
AngII	Angiotensin II
ADMA	Asymmetric dimethylarginine
BK	Bradykinin
BMI	Body mass index
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DMAs	Dimethylearginines
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
ED	Endothelial dysfunction
ET-1	Endotheline-1
EDHF	Endothelium-derived hyperpolarizing factor
FMD	Flow-mediated dilatation
HD	Haemodialysis
IL-6	Interleukin-6
K/DOQI	kidney disease outcome quality initiative
MDRD	Modification of Diet in Renal Disease
MBF	Myocardial blood flow
NO	Nitric oxide
NOS	NO synthase enzyme
NA	Noradrenalin
PD	Peritoneal dialysis
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SDMA	Symmetric dimethylarginine
SNP	Sodium nitroprusside
U46619	Thromboxane AII

Chapter 1
General Introduction

Chapter 1: General introduction

1.1 Chronic Kidney Disease (CKD)

1.1.1 Background and definition

Each kidney consists of approximately one million nephrons (the functional filtering units of the kidney) that are continuously responsible for filtering blood while removing waste products (urea, creatinine etc.), salts and excess fluid from the body. Kidneys can fail in two ways: rapidly, which occurs over days, weeks or months (acute renal failure), or slowly, which occurs over a period of years (chronic renal failure). The most common diseases associated with chronic renal failure are diabetes mellitus, hypertension and glomerular disease (Sowers and Epstein, 1995). Chronic kidney disease (CKD) is increasingly recognised as a worldwide major health problem that affects the population, resulting in multiple adverse outcomes, including renal failure, cardiovascular disease (CVD) and premature death.

The definition of CKD is based on the impairment of kidney function, damage and an assessment of chronicity. Assessment of kidney function is most usually based on the measurement of serum creatinine and estimation of the glomerular filtration rate (eGFR). According to the kidney disease outcome quality initiative (K/DOQI, 2002) (Eknoyan and Levin, 2002), kidney damage can be defined as structural and functional abnormalities in the kidney with or without decreased GFR for three months or more. This is manifested by either pathologic abnormalities or markers of kidney damage, including abnormalities in the composition of the blood or urine or abnormalities in imaging tests. CKD can also be defined as a reduction in eGFR (< 60 mL/min/1.73m^2)

for three months or more with or without kidney damage, irrespective of cause (Goolsby, 2002). This destruction of renal mass can lead to irreversible fibrosis and loss of nephrons and, in turn, can cause progressive decline in eGFR.

Kidney damage can be ascertained firstly by a reduction in the estimated glomerular filtration rate. This can be estimated from calibrated serum creatinine using the Modification of Diet in Renal Disease (MDRD) study equation, which is an accurate equation that predicts GFR as measured by an accepted method (urinary clearance of ^{125}I-iothalamate) (Levey et al., 2003). Another measurement for clinical assessment of kidney disease is measured creatinine clearance using 24 hr urine collection, which is inconvenient and frequently inaccurate compared to the estimated GFR using MDRD equations (Levey et al., 1999). Also, urinary cystatin C is independently associated with acute kidney injury (Nejat et al., 2010). Haematuria and proteinuria are also predictors of developing CKD stage 3 or higher (Yamagata et al., 2007). The presence of proteinuria (either unselective or albuminuria) is known to be the most important marker of kidney damage, measured as albumin-to-creatinine ratio > 30 mg/g in at least two of three spots of urine specimens.

Other markers of kidney damage include urine sediment abnormalities (epithelial and tubular casts), imaging abnormalities (polycystic kidney disease, hydronephrosis, and small 'echogenic' kidneys) and abnormalities in the urine and blood composition (nephrogenic diabetes insipidus, renal tubular acidosis, Fanconi syndrome, etc.).

1.1.2 Classification of CKD

Following the proposed classification by the National Kidney Foundation K/DOQI 2002, CKD can be divided into five stages (Table1). This simple and initial classification is based on the severity of renal failure and the level of eGFR using eGFR estimated equations.

Table 1 K/DOQI 2002 classification of CKD (Based on the severity)

Stages	renal function	GFR ml/min/1.73 m^2	related terms
Stage 1	normal kidney function	≥ 90	Albuminuria, proteinuria haematuria
Stage 2	mild reductions in RF	60 – 89	Albuminuria, proteinuria haematuria
Stage 3	moderate reduction in RF	30 – 59	Chronic and early renal Insufficiency
Stage 4	sever reduction in RF	15 – 29	Chronic and late renal Insufficiency-pre ESRD
Stage 5	kidney failure and ESRD	< 15	Renal failure, uremia ESRD

Abbreviation: CKD chronic kidney disease, ESRD end-stage renal disease, GFR glomerular filtration rate, RF renal function.

1.2 End stage renal disease

1.2.1 Background

End stage renal disease (ESRD) is the last stage of CKD (CKD stage five), which can be defined as a reduction in eGFR by less than 15 ml/min/1.73 m^2. The reduction in eGFR results from progressive and irreversible loss of renal function that makes the kidneys fail to perform their normal functions. Renal replacement therapy (RRT) must be considered for patients who have developed ESRD or CKD stage five to replace renal function, either by haemodialysis (HD), peritoneal dialysis (PD) or renal transplantation.

1.2.2 Epidemiology of CKD and ESRD

In the twentieth century, the major cause of death and disability were infectious disease; however, in this century, non-communicable, non-infectious chronic diseases have become the major cause of morbidity and mortality (Yach et al., 2004, Beaglehole and Yach, 2003). The number of patients developing ESRD is increasing annually. Diabetes mellitus is the most common chronic disease affecting the worldwide population, and its prevalence is predicted to double in the next decades, particularly in developing countries (Wild et al., 2004). The majority of diabetic nephropathy patients usually die from cardiovascular complications before reaching ESRD (Rossing et al., 1995). Hypertension, together with diabetes, is now the major cause of ESRD worldwide, not only within the developed world, but also increasingly within the developing world.

The incidence of ESRD in the UK, as calculated by the number of new patients undertaking RRT, varies between 80 and 110 patients per million of population (pmp) per year (UK Renal Registry 2002). The prevalence rate of ESRD in the UK increased

from 724 pmp in 2006 to 746 pmp in 2007 according to the UK renal registry (UKRR) in 2008 (Farrington UKRR 2008). This prevalence rate remained lower in England (746 pmp) than in Northern Ireland (791 pmp), Scotland (797 pmp) and Wales (798 pmp), (Farrington et al., 2009) as shown in Table 2.

Table 2 Prevalence of RRT therapy of adults in UK (2007)

	England	Wales	Scotland	N Ireland	UK
All UK centres	37,614	2,377	4,101	1,392	45,484
Total population, mid-2007(millions)	51.1	3.0	5.1	1.8	61.0
Prevalence pmp HD	318	339	346	393	323
Prevalence pmp PD	74	109	77	60	76
Prevalence pmp dialysis	392	448	423	453	399
Prevalence pmp transplant	344	350	374	338	347
Prevalence pmp total	736	798	797	791	746
Confidence intervals Total	729–744	766–830	773–822	750–833	739–753

Abbreviations: RRT, renal replacement therapy; PD, peritoneal dialysis; HD, haemodialysis. *Adapted from Farrington et al 2009.*

Similarly, this elevated trend has also been seen in the United States. The prevalence rate of patients treated by dialysis and transplantation from 1999 to 2004 was higher than in the period from 1988 to 1994 (Palmer et al., 1988b). The study observed

an increase in diagnosed diabetes and hypertension from 10% in 1988-1994 to 13.1% in 1999-2004. It also showed that the percentage of both albuminuria and reduction in eGFR were increased from 1988-1994 to 1999-2004, based on the measurement of persistent microalbuminuria (>30 mg/g) and estimated GFR using the MDRD study equation. The overall increase in the prevalence rate of CKD, particularly among older people and those with diabetes and hypertension suggests that plans for future health services will be focused on the management of CKD.

1.3 Cardiovascular disease in CKD patients

1.3.1 Background

Cardiovascular disease is the major cause of morbidity and mortality in CKD patients, and this is frequently complicated by cardiac and vascular changes. Since the introduction of RRT in the form of dialysis and renal transplantation, patients with ESRD have been successfully treated and no longer die of uraemia; however, the most threatening cause of death among that group is now premature cardiovascular death. Cardiovascular disease was believed to occur only in the late stages of CKD and dialysis patients; however, previous studies have shown evidence of its development even in the early stages of the disease (Go et al., 2004). Go *et al.*, in a community-based study involving one million adults, observed that the risk of both cardiovascular events and death were increased even in the earliest stages of CKD where eGFR decreased below 60ml/min/1.73m^2. The study also showed that the highest risk group were those with CKD stages 4 and 5 (Go et al., 2004, Ritz, 2003) (Figure1). This association between

renal function, death, and cardiovascular events may highlight the importance of determining and controlling modifiable cardiovascular risk factors in the earlier stages of CKD.

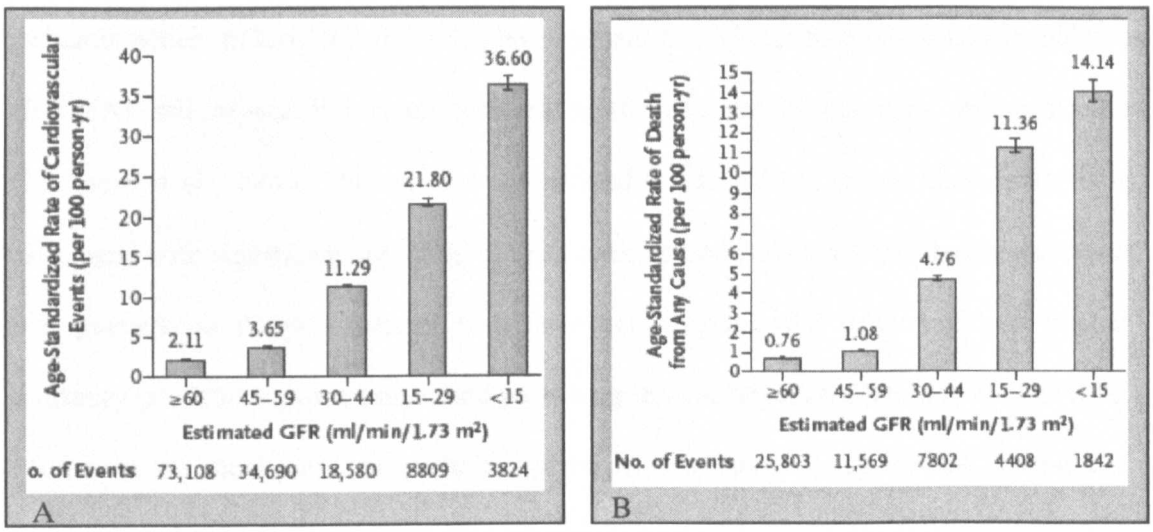

Figure 1 Age standarized rates of cardiovascular events (A) and death from any cause (B) in relation to eGFR
Large population-based longitudinal study (*Adapted from Go et al, 2004*).

A cardiovascular event was defined as; hospitalization for coronary artery disease, heart failure, ischemic stroke, and peripheral arterial disease.

The risk of morbidity and mortality from cardiovascular disease in CKD patients is significantly higher. Previous studies have indicated that 40% to 50% of deaths from cardiovascular disease were observed in patients with ESRD (Foley et al., 1998). The risk of cardiovascular mortality in patients receiving haemodialysis (HD) or peritoneal dialysis (PD) was shown to be 10 to 20 times higher than that of the general population (Foley et al., 1998). Cardiovascular morbidity was also higher among that group, and

approximately 75% of patients receiving dialysis therapy developed left ventricular hypertrophy as detected by ultrasound. (Foley et al., 1995).

McIntyre *et al.* (2008) has shown that patients on long-term haemodialysis therapy are at high risk of developing vascular ischemia, particularly, myocardial ischemia which, in turn, results in the development of regional wall motion abnormalities (RWMA) and myocardial stunning even in the absence of coronary artery disease (McIntyre et al., 2008). This study demonstrated for the first time that haemodialysis is associated with significant reduction in myocardial blood flow (MBF). It has been shown that patients on dialysis therapy who have acute myocardial infarction have higher mortality rate from cardiac causes and poor long-term survival rate (Herzog et al., 1998). Moreover, the most prevalent pathological forms of cardiovascular disease among that group are: left ventricular hypertrophy, atherosclerosis and arteriosclerosis.

1.3.2 Cardiovascular risk factors in CKD

Cardiovascular diseases (CVD) are quite common in CKD and dialysis patients, due to the high prevalence of conventional risk factors. The increased prevalence of CVD in CKD patients may result from the consequences of higher prevalence of diabetes mellitus, hypertension, hyperlipidaemia, and aging (Fuster et al., 2011). There are some concerns about intervention management to decrease the risk of incidence and prevalence of CVD. These include angiotensin converting enzyme blockers, angiotensin receptor blockers, platelet inhibitors, thrombolytic, and aspirin usage, which may be unexploited among CKD patients, though their utility among this group of patients (Saran and

DuBose, 2008). Additional non-traditional risk factors unique to these patients have been suggested to play a part in the progression of CVD (Sarnak and Levey, 2000). These factors have been identified to be involved in a number of mechanisms of cardiac damage and are predictive of adverse outcomes in individuals with CKD stages 3 and 4 (Sarnak and Levey, 2000, Weiner et al., 2008). However, vascular abnormalities, especially arterial calcification and stiffness are important risk factors contributing to cardiovascular mortality and morbidity in late CKD patients (stages 4 and 5) (London et al., 2003).

Traditional and non-traditional risk factors have been implicated in the elevated CVD development in CKD patients (Menon et al., 2005). The prevalence of hypertension and diabetes mellitus is higher in CKD and play an important role in increased mortality and morbidity among this group. However, these traditional risk factors play major roles in vascular damage and alteration in left ventricular function in CKD patients (Zoccali et al., 2003). One of the most important non-traditional cardiovascular risks in CKD patients is albuminuria, which is known as a continuous cardiovascular risk factor in CKD and diabetic patients. Normal albuminuria is defined as the urinary albumin / creatinine ratio (ACR) < 30 mg/g. The role of albuminuria as cardiovascular risk was first recognised to macroalbuminuria, in which the ACR > 300 mg/g (Grimm et al., 1997), but this level was extend to microalbumiuria (ACR, 30 to 300 mg/g) (Keane and Eknoyan, 1999). The reason why microalbuminuria is a powerful predictor of CVD is yet unknown; however, one of the principle pathophysiological mechanisms has been proposed to the consequences of endothelial damage or to the pathogenicity of

miroalbuminuria itself (Schiffrin et al., 2007). The other important non-traditional risk factors are discussed in section 1.4.3.

Table 3 Traditional and non-traditional risk factors

Pathology	Traditional risk factors	Non-traditional risk factors
Cardiomyopathy	Older age Hypertension Valvular disease Dyslipidemia Smoking Diabetes	Albuminuria Reduced glomerular filtration rate Anemia Inflammation Arteriosclerosis Extracellular fluid volume overload
Atherosclerosis	Older age Male gender Hypertension Diabetes Dyslipidemia Smoking Physical inactivity Left ventricular hypertrophy	Albuminuria Reduced glomerular filtration rate Anemia Inflammation Oxidative stress Endothelial dysfunction Homocysteine Lipoprotein Malnutrition Thrombogenic factors Sympathetic activity Insulin resistance/metabolic syndrome
Arteriosclerosis	Older age Male gender Smoking Hypertension Diabetes Dyslipidemia	Albuminuria Reduced glomerular filtration rate Endothelial dysfunction Abnormalcalcium/phosphate metabolism Metabolic syndrome

Adapted from (Menon et al., 2005)

1.4 Vascular endothelium and CKD

1.4.1 Vascular endothelium

The interior surface of blood vessels of the circulatory system from heart to capillaries is lined with a single layer of endothelial cells, which is a specialized type of simple squamous epithelium separated from the surrounding outer layers by a basal lamina (Gunthner et al., 2009) see Figure 2. These cells control the passage of materials—and the transit of white blood cells—into and out of the bloodstream. Endothelial cells have mechanoreceptors that allow them to sense shear stress due to flow of blood over their surface. By signalling this information to the surrounding cells (smooth muscle cells), it enables blood vessels to adapt their diameter and wall thickness to suit the blood flow (Simionescu et al., 1984), a maintain end organ perfusion in the setting of altering systemic blood flow.

Endothelium normally provides a non-thrombogenic surface and reduces turbulence of blood flow. This layer acts as a selective barrier between the vessel lumen and surrounding tissue, and regulates angiogenesis, vessel tone and function, and mediates inflammatory processes (Cines et al., 1998). It additionally plays an important role in the biology of arterial wall via release of vasoactive and trophic factor. The most important cellular component in the tunica media is the vascular smooth muscle layer that responsible for vasodilatation and vasoconstriction through secretion of various hormones and growth factors that regulate proliferation, migration and extracellular matrix formation in this layer (Betsholtz and Armulik, 2006).

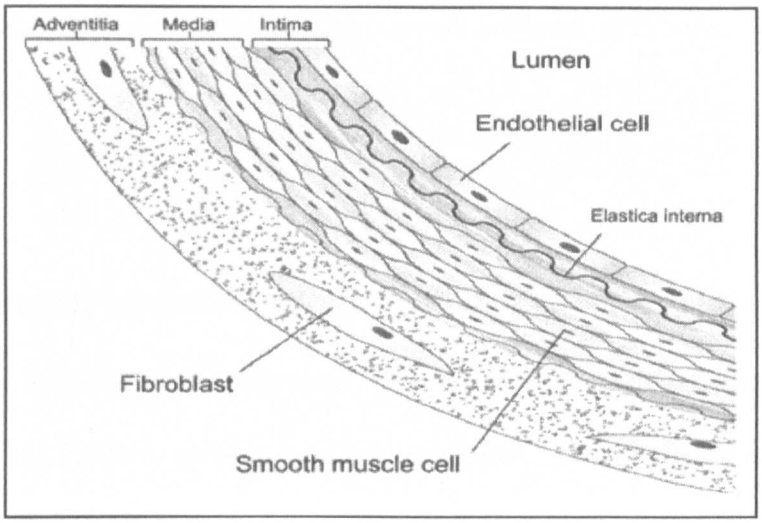

Figure 2 Cross-section of a vessel wall

Adapted from (Gunthner et al., 2009)

Vascular endothelium plays an important role in the regulation of vascular function by providing a protective action through modulation of vascular structure, tone and interaction of blood components with the vascular wall (Annuk et al., 2001, Baragetti et al., 2007). It has an atheroprotective effects through production and release of a wide range of vasoactive substances that include vasoconstrictors such as endothelins, thromboxane A2, and vasodilators nitric oxide (NO), prostacyclin, arachidonic acid derivatives and endothelium-derived hyperpolarising factors (EDHF) (Cross, 2002). Different chemical substances such as acetylcholine (Ach), bradykinin (BK), serotonin, and substance P stimulate the endothelium to initiate the vasodilatation mechanism (Vallance and Chan, 2001). This occur either through endothelium-deriving relaxing factor which was first demonstrated as NO (Furchgott and Zawadzki, 1980) or through

mediated hyperpolarization of vascular smooth muscle cells via NO-independent pathway (Busse et al., 2002).

1.4.2 Measurement of endothelial function

Several techniques are now available for the assessment of endothelial function in humans including invasive and non-invasive techniques. These methods are based on the assessment of endothelium-mediated responses of peripheral arteries by measuring the effects of their receptor agonists on the vascular resistance.

The initial *in vivo* studies on the endothelial function was on investigating coronary circulation, involved local infusion of Ach with measurement of the vascular diameter using coronary angiography (Cox et al., 1989). Subsequently, these methods have been repeated using Doppler flow wires to measure resistance of the vascular function (Drexler and Zeiher, 1991). The physiological responses of vascular endothelium to various stimuli including Ach, BK, and substance P have also been investigated in vivo using flow-mediated dilatation (FMD) of conduit arteries (Nabel et al., 1990). A number of alternative non-invasive methods have also developed to investigate endothelial function in peripheral circulation, particularly, forearm resistance vessels. These include venous occlusion plethysmography, which is one of the widely used techniques in cardiovascular research (Benjamin et al., 1995). This method is based on the measurement of increase in forearm volume after suppression of the venous return by using specific pneumatic cuff placed at the level of the arm. Another non-invasive assessment method of endothelium function in peripheral arteries is studying the effects

of their receptor agonists on blood flow using non-invasive Doppler and intravascular ultrasound imaging (Donald et al., 2006). Laser Doppler is also one of the widely used method to assess the endothelium function of cutaneous microcirculation (Johnson et al., 1984).

A number of *ex vivo* technique have been also introduced to investigate the endothelial function through measurement of vascular contractility and various aspects of excitation-contraction coupling process. These include wire myograph (Mulvany and Aalkjaer, 1990), that described in details in methodology chapter section 2.4. The other technique is pressure myograph, which is used to study the physiological function of small isolated vessels, it allows to investigate the pharmacological effects of different vasoactive stimuli on the isolated vessels under near physiological conditions (Halpern and Kelley, 1991). Both techniques are widely used in the *ex vivo* research which enabling the researchers to directly investigate the vascular function in isolated vessels from different tissues. However, in these methods, vessels possess many of their *in vivo* properties.

1.4.3 Endothelial dysfunction in CKD

Endothelial dysfunction can be defined as an alteration in the normal function of the endothelium. It can also be defined as partial or complete loss of balance between vasoconstrictors, growth and inhibiting factors, pro-coagulant and anti-coagulant factors (Caballero, 2003). During endothelial dysfunction, endothelial cells release paracrine factors such as vascular endothelial growth factor (VEGF), endothelin-1 and interleukin-

6 that act either as growth factors to induce smooth muscle cell proliferation, or as chemokines to stimulate circulating inflammatory cells (Bolton et al., 2001). Endothelial damage is recognised as a common risk of vascular damage in many conditions associated with increased cardiovascular risk including CKD.

Endothelial dysfunction has been implicated in different pathological diseases including CVD, CKD, obesity, hypertension, diabetes mellitus, and peripheral vascular disease (Schiffrin, 2004). Endothelial dysfunction has been reported to occur in various stages of CKD even in the early stages (Go et al., 2004). However, the occurrence of endothelial dysfunction in CKD can be independently to traditional factors, this may highlight the presence of other 'kidney-specific' mechanisms that contribute to CKD-related endothelial dysfunction. For example, Kari et *al.* observed the presence of endothelial dysfunction in uremic children without hypertension and dyslipidaemia (Kari et al., 1997). In 1990, the endothelial dysfunction was first demonstrated in forearm vessels of hypertensive patients (Panza et al., 1990). Impaired endothelium-dependent vasodilatory function was demonstrated in ESRD patients in *ex vivo* (Morris et al., 2001, Luksha et al., 2011) and *in vivo* studies (Morris et al., 2000, Bolton et al., 2001, Yildiz et al., 2003). Moreover, such impairment has also been observed in obese patients in *ex vivo* (Georgescu et al., 2011, De Ciuceis et al., 2011) and *in vivo* studies (Nielsen et al., 2004). endothelial dysfunction can also occurs in other conditions including assessment of the vascular function in peripheral vascular beds of hypertensive (Park et al., 2001) (Schiffrin et al., 2000) and diabetic patients (Rizzoni et al., 2001a, Schofield et al., 2002). The pathophysiological mechanisms of endothelial dysfunction in CKD patients is

complex and incompletely understood, however many proposed mechanisms have been suggested.

Several studies investigating the mechanisms of endothelial dysfunction in uremic patients have focused mainly on the impairment of NO bioavailability resulting to impaired endothelium-dependent vasodilation as the principal event that leading to endothelial dysfunction. The exact mechanisms for altered NO activity in uremic patients is yet unclear, however some non-traditional uraemia-specific risk factors are being widely discussed, including excessive oxidative stress and reactive oxygen species (Ferraro et al., 2003, Hasdan et al., 2002, Miyazaki et al., 2000), hyperhomocysteinemia that found in the majority of renal patients (Bostom and Culleton, 1999) and elevated plasma levels of endogenous competitive inhibitors of nitric oxide synthase (eNOS), such as asymmetric dimethylarginine (ADMA) in dialysis patients (Vallance et al., 1992), which has been discussed in details in this chapter.

1.4.3.1 L-argentine- NO pathway and endothelial dysfunction

NO is a soluble gas that is synthesized from the amino acid l-arginine in endothelial cells by a calcium-calmodulin-dependent enzyme (endothelium nitric oxide synthase eNOS (Palmer et al., 1988b). L-arginine is an amino acid known to be a physiological precursor for NO synthesis in the culture of vascular endothelial cells (Palmer et al., 1988a). The synthesis of NO occurs after oxidation of guanidine nitrogens terminal of L-arginine by NO synthase enzyme, activated by an increase in endothelial intracellular calcium concentration, to produce NO and L-citruline (Bredt, 1999). The

generator cell (endothelial cell) releases NO as a gas or attached to other molecules to the target cell (smooth muscle cell) (figures 3 and 3.1). In the target cell, NO stimulates soluble guanylayl cyclase and subsequently produce an increased concentration of cyclic guanosine monophosphate (cGMP) (MacAllister and Vallance, 1994). This in turn leads to different activities, such as decrease intracellular calcium levels and smooth muscle relaxation.

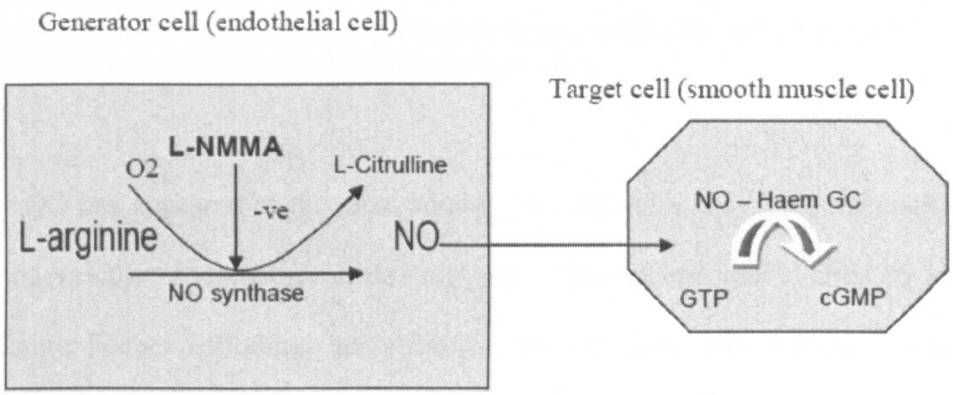

Figure 3 L-arginine- NO pathway.

Abbreviation, L-NMMA (N^G-monomethyl-*L*-arginine), GC (guanylayl cyclise), GTP (guanosine triphosphate), cGMP(cyclic guanosine monophosphate).

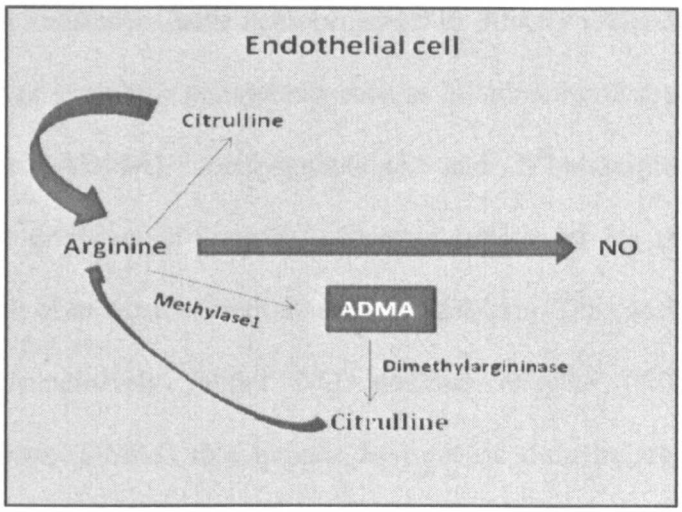

Figure 3.1 Proposed metabolisms of methylearginines within the endothelial cell
Figures 3 and 3.1 are *Adapted from* MacAllister 1994.

NO has appeared as the most notable endothelium-derived vasorelaxant released from the vascular endothelium following stimulation of endothelial cells by numerous stimulating factors including; acetylcholine, thromboxane AII, thrombin, bradykinin, histamine (Cannon, 1998). It can promotes vascular homeostasis such as maintaining vascular function, regulates local cell growth and protects the vessel from blood circulating cells and platelet aggregation (Michel and Vanhoutte, 2010). In addition to its vasodilator effect, NO can inhibit the following; Platelet aggregation and adhesion, vascular smooth muscle proliferation, vascular smooth muscle contraction, endothelin production, monocyte adhesion and migration, and expression of adhesion molecules (Radomski et al., 1987).

1.4.3.2 NO inhibitors and the effect of renal failure on NO synthesis.

Different substances have been proposed to directly affect NO synthesis. These include a group of guanidine compounds such as N^G-dimethyl-*L*-arginine (asymmetrical dimethylarginine, ADMA), methylguanidine, and N^G-monomethyl-*L*-arginine (L-NMMA). These endogenous naturally occurring substances are accumulated in renal failure as a result of reduced excretion and/or metabolism. They accumulate in sufficient amounts to competitively inhibit NO synthase enzyme (NOS) (Cross, 2002). Dimethylearginines (DMAs) that include asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) have been shown to be accumulated in plasma of patients suffering from renal failure (Cross, 2002).

Plasma ADMA is water soluble substance derived from the catabolism of proteins containing methylated arginine residues (Chan and Chan, 2002). It has been shown that more than 10 mg of plasma ADMA is normally excreted in urine over 24 hr (Vallance et al., 1992), however patients with ESRD who are oligouric or anuric, excretion of plasma ADMA is blocked (Matsuguma et al., 2006). ADMA directly inhibits eNOS and reduces NO synthesis (Vallance et al., 1992), while SDMA does not inhibits eNOS, but may competes with L-arginine for transport into endothelial cells (Teerlink et al., 2002). These circulating inhibitors are accumulated in a sufficient amount to inhibit NO synthesis in patients with renal failure, though the precise mechanism underlying accumulation of ADMA and SDMA in uremic CKD patients is not fully understood. DMAs are freely filtered and removed from the plasma of dialysis patients along with various uremic toxins during dialysis procedure (Anderstam et al., 1997). However, accumulation of these compounds occurs as a result of poor clearance

during dialysis procedure and substantial rebound that occurs at the end of dialysis session (Eloot et al., 2005).

Many studies have demonstrated marked elevation of plasma ADMA in patients with ESRD. In 1992, Vallance *et al.* reported for the first time that concentration of plasma ADMA and SDMA are up-to 4-fold increase in uremic patients compared to control groups in a study recruited 9 HD patients and 6 normal controls (Vallance et al., 1992). Kielestein *et al.* highlighted markedly increased plasma ADMA in early stages of non-smoking, non-diabetic CKD patients (even those with normal eGFR), with highly significant differences between patients and control groups (Kielstein et al., 2002). Moreover, Fleck *et al.* in his large study that include 221 patients with different stages of renal diseases, observed that plasma ADMA concentration were raised by 38% in CKD group, whereas plasma SDMA was elevated by 250% in CKD compared to healthy subjects (Fleck et al., 2003) (see figure 4). The study also showed that both ADMA and SDMA are elevated further by about 5.5-fold in haemodialysis patients (HD) compared to normal controls. However, following renal transplantation, only the concentrations of SDMA were decreased compared to non-transplant HD group, whereas ADMA remains nearly unchanged. Furthermore, the study suggested that SDMA is another risk marker for endothelial dysfunction and both DMAs may contribute to increased risk of CV mortality and enhancement of hypertension in CKD patients.

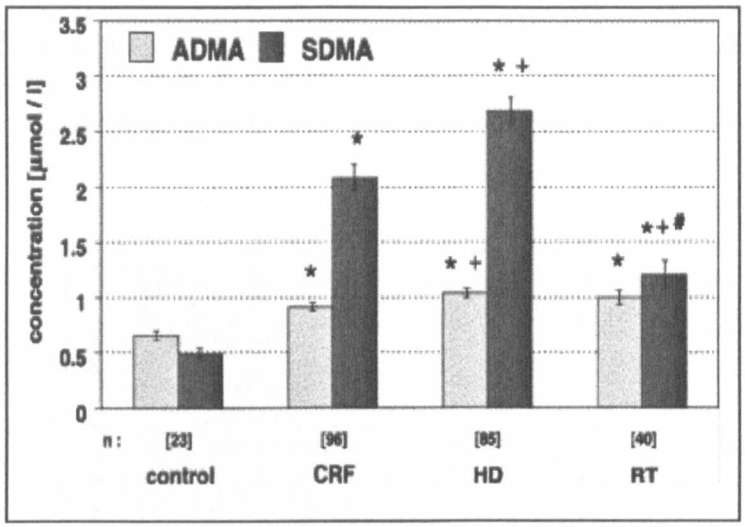

Figure 4 ADMA and SDMA concentrations in patients with different RRT Renal failure (CRF), haemodialysis (HD), renal transplant (RT), and Control group

n= patients numbers, *significantly different from control; +significant difference to CRF; #significant difference between RT and HD ($p < 0.05$). ***Adapted from Fleck et al 2003.***

1.4.3.3 NO inhibitor (ADMA) and cardiovascular dysfunction

It has been shown that NO inhibitors may contribute to cardiovascular dysfunction. Kielstein *et al.* demonstrated the effect of plasma ADMA on cardiac output, systemic vascular resistance and blood pressure. The study involved healthy subjects infused with different concentrations of ADMA intravenously to assess the effect of ADMA on NO production and renal haemodynamic (Kielstein et al., 2004). It observed that an acute elevation in plasma ADMA concentrations within the physiological relevant range (2-10µmol/L) can cause a significant decrease in plasma cyclic guanosine monophosphate concentration (cGMP), the main second messenger of NO in the

cardiovascular system. It also showed that infusion of healthy subjects with 0.10 mg.kg^{-1}.min^{-1} concentration of ADMA can result to a significant sustained reduction in cardiac output and increased systemic vascular resistance (figure 5 and 5.1 respectively).

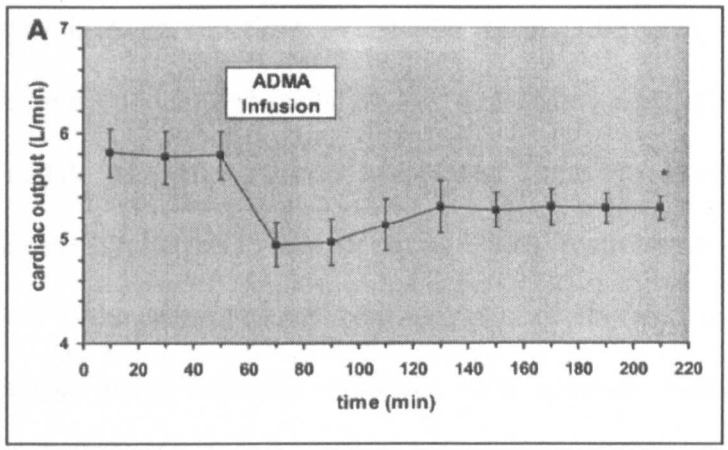

Figure 5 Effects of ADMA on cardiac output on healthy volunteers. Data adapted from (Kielstein et al., 2004).

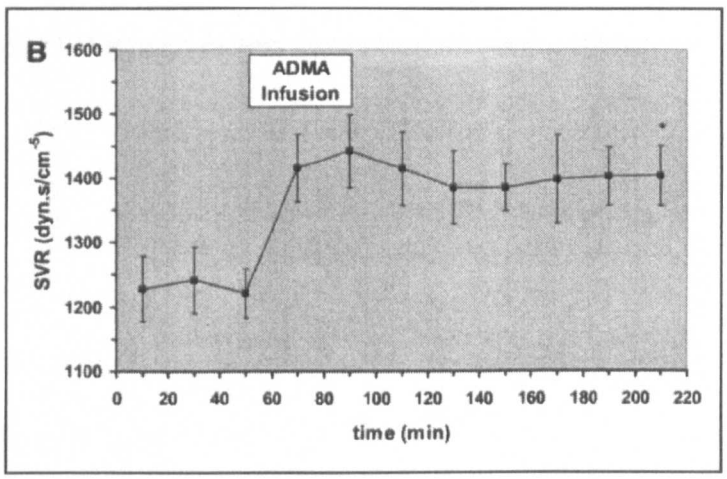

Figure 5.1 Effects of ADMA on systemic vascular resistance on healthy volunteers.

1.4.3.4 Uremia and endothelial dysfunction

Uremic toxins are attributed to the progressive retention of a large number of compounds, which under normal conditions are excreted by the healthy kidneys. These compounds are called uremic retention solutes, or uremic toxins when they interact negatively with biologic functions (Vanholder et al., 2001). A number of uremic compounds have been identified including low molecular weight solutes, protein-bound solutes and middle molecules such as guanidines. These physiological molecules are difficult to remove by dialysis (Vanholder et al., 2001, Vanholder et al., 2008). Table 4, illustrates some of the currently known uremic retention solutes, classified according to the characteristics that potentially influence their removal pattern during dialysis (Vanholder et al., 2003, Vanholder et al., 2008). The pathophysiological mechanisms by which uremic toxins can cause endothelial damage is still understood, however some of these toxins can induce release of endothelins and enhance angiogenesis by stimulating vascular endothelial growth factors (Anagnostoulis et al., 2008).

Table 4 Compounds with the potential to provoke vascular damage.

Small water-soluble compounds	Guanidines (ADMA, SDMA, Methylguanidine, Argininic acid, guanidinopropionic acid, Guanidinoacetic acid, Guanidonosuccinic acid, Taurocyamine).
Protein-bound molecules	AGE (3-deoxyglucosone, Fructoselysine, Glyoxal, Methylglyoxal, Pentosidine, dinucleotide polyphosphates, indoxyl sulphate), indoles (Indole-3-acetic acid, Indoxyl sulphate, Kinurenine). Homocysteine, leptin, Phenylacetic acid, TNF- α.
Middle molecules	AGE, dinucleotide polyphosphates, leptin, TNF- α, Adrenomedullin , Atrial natriuretic peptide, -endorphin B2-microglobulin, Cystatin, Endothelin, Interleukin-1B, Interleukin-6 Methionine-enkephalin, Neuropeptide, Parathyroid hormone.

Abbreviation: AGE, advanced glycation end products; TNF tumour necrosis factor alpha,ADMA, asymmetric dimethyl argentine; SDMA, symmetric dimethyl argentine. Data *adapted from Vanholder et al. 2008.*

Uremia is associated with other conditions that probably accelerate the development of atherosclerosis, such as increased production of reactive oxygen species (ROS), increased homocysteine levels, acidosis, recurrent infections, and complementing activation, which may contribute to impaired endothelial function (Gunthner et al., 2009). Similarly, other conditions including diabetes, hypertension, hyper-cholesterolaemia, congestive heart failure, and hyper-homocystinaemia have been

associated with endothelial dysfunction (Cannon, 1998). In these conditions, alteration in endothelial cells can promote inflammation, oxidation of lipoproteins, smooth muscle proliferation, platelet activation, thrombus formation, extracellular matrix deposition and accumulation of lipid-rich materials. All these consequences may contribute to the pathogenesis of atherosclerosis. However, these inflammatory conditions may predispose to endothelial damage.

1.4.3.5 Oxidative stress and endothelial dysfunction

Oxidative stress plays a pivotal role in the pathogenesis of vascular injury and in the progression of atherosclerosis. This can occurs through several mechanisms, some of which are associated with inhibition of NO synthase activity and inactivation of NO by reactive oxygen species (Harrison, 1997). Oxidative stress has been found to be elevated in different stages of CKD patients (Pawlak et al., 2004). Release of reactive oxygen species (ROS) in uremic patients can occur as a result of an imbalance between pro-inflammatory and anti-inflammatory mechanisms which can be manifested by decreased NO levels (Morena et al., 2005). Impaired NO bioavailability as a result of excessive production of ROS may contribute to endothelial cell damage and vascular dysfunction (Morena et al., 2005). Although endothelial cells have an antioxidant defence mechanism against these products, over production of ROS gradually over time can damage these cells. Many experimental and clinical studies have demonstrated that uraemia is associated with an increased state of oxidative stress (Ferraro et al., 2003, Hasdan et al., 2002). This condition is characterized by an increase of lipid peroxidation products and

retention of oxidized solutes (Cruz et al., 2008). Moreover, Annuk *et al.* observed the direct relation between increase plasma markers of uremic oxidative stress and endothelial dysfunction (Annuk et al., 2001).

1.4.3.6 Homocysteine and endothelial dysfunction

Homocysteine is a sulphur-containing amino acid, known to be an independent cardiovascular factor that is associated with endothelial dysfunction (Clarke et al., 1991). Homocysteine has been shown to be associated with increased risk of atherosclerosis and venous thromboembolism. Approximately 50% of patients with severe hyper-homocysteinaemia develop a clinically significant vascular event even prior to the age of 30 (Mudd et al., 1985). Homocysteine is frequently seen in patients with CKD and it has been found in more than 90% of patients on dialysis treatment (Francis et al., 2004). It is one of the uremic factors that accumulates in renal failure and contributes to endothelial and cardiovascular dysfunctions (Mallamaci et al., 2002). Although, the precise mechanism underlying the effect of hyper-homocysteinaemia on endothelial dysfunction remains unclear, several studies have suggested that hyper-homocysteinaemia is indirectly associated with endothelial dysfunction. One mechanism proposed that the defect can occurs via impaired NO bioavailability (Sydow et al., 2003), either through its oxidation to hydrogen peroxide and other reactive oxygen species (Lang et al., 2000) or secondary to accumulation of endogenous NO synthase inhibitor (ADMA) (Boger et al., 2000). It has also been shown that increased plasma homocysteine but not cysteine

concentration between 10-50 μmol/L might inhibit endothelial cell proliferation (Wang et al., 1997).

Various studies have determined that hyper-homocysteinaemia is a risk marker of cardiovascular mortality and morbidity in haemodialysis patients (Mallamaci et al., 2002, Buccianti et al., 2004). It has been shown, in dialysis patients, that hyper-homocysteinaemia can be successfully treated by folic acid therapy via administration of folate and B-vitamins. Baragetti *et al.* compared 19 peritoneal dialysis patients receiving oral folic acid (5-methyltetrahydrofolate therapy) with other dialysis group without treatment for the same period of time (12 week follow-up) (Baragetti et al., 2007). The study showed that treatment with folic acid can lowers, but does not normalize, plasma homocysteine level with 30% reduction in plasma homocysteine levels among the treatment group (see figure 6). It also observed significant improvement in the endothelial function measured using B-mode ultrasonography in the brachial artery by daily administration of the drug among treatment group, independently to the reduction in homocysteine plasma levels. However, recent randomised clinical trials assessing clinical effectiveness of homocystein-lowering interventions (HLI) in non-CKD people with or without pre-existing cardiovascular disease did not observe the evidence to support the use of HLI to prevent cardiovascular events (Marti-Carvajal et al., 2009).

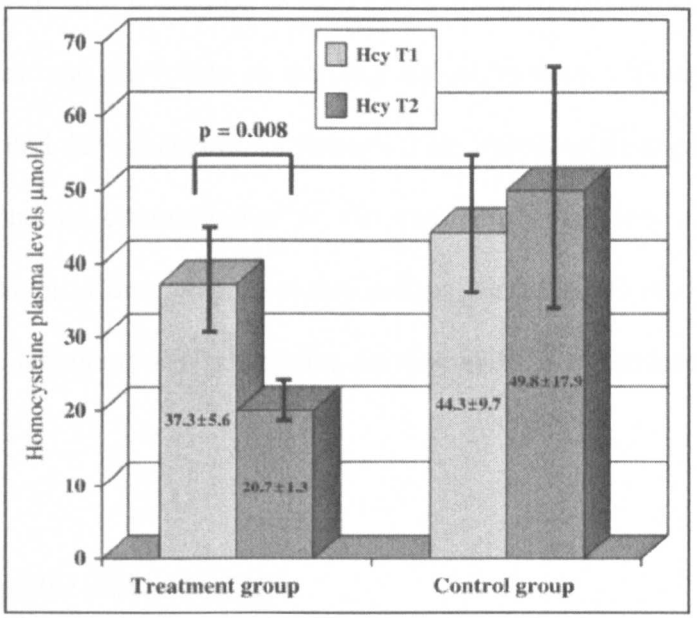

Figure 6 Effects of 5-MTHF treatment on plasma homocysteine level
Assessment of homocysteine level was at (T1 and T2) in dialysis (treatment and control group). Endothelial function measured by B-mode ultrasonography in the brachial artery (Baragetti et al., 2007).

1.5 Wire myograph

Wire myograph is an *ex vivo* technique that used to investigate the vascular function of isolated small resistance vessels and tubular tissues including ureters and bronchi. Bevan and Osher firstly described the technique in 1972. They investigated small vessels with internal diameters around 100 µm to assess the contractility of small isolated arteries to agonist stimulation (Bevan and Osher, 1972). This technique, thereafter, was developed by Mulvany and Halpern to allow an *in vitro* measurement of both active and passive properties of small arteries (with internal diameter 60 µm to 300 µm) under isometric conditions using wire myograph (Mulvany and Halpern, 1977). In this system, vessel segments are threaded on two small wires (40 µm) as a ring

preparation. Each wire is secured to each of the jaws, one of the wires is fastened to a force transducer and the other is attached to micrometer for adjustment of vessel circumference and the application of tension. This arrangement helps the researchers to measure the internal circumference of the vessel, wall tension, and isometric force responses to chemical drugs (agonists and antagonists).(Rizzoni et al., 2003). Details of the myography technique and procedures are described in methodology chapter, section 2.4.

1.6 In vivo vascular studies in CKD

Structural remodelling in vascular resistance has been observed in uremic and hypertensive humans and animal models. In experimental uremic hypertensive animals, structural changes characterised by an increased wall/lumen ratio with decreased lumen diameter have been observed in the mesenteric arteries of Wistar-Kyoto rats, suggesting that these vessels undergo remodelling in uremic hypertension (New et al., 2004). Impaired peripheral vascular perfusion, measured *in vivo* using laser Doppler perfusion imaging, has been observed in uremic rats induced by subtotal nephrectomy (Jacobi et al., 2006).

In humans, several *in vivo* studies have confirmed impaired endothelial-dependent vasodilation in peripheral vascular beds of uremic patients. Morris *et al.* examined endothelial function in uremic peripheral forearm vessels by measuring changes in the forearm blood flow induced by carbachol (endothelium-dependent vasodilator) and SNP (endothelium-independent vasodilator) using forearm plethysmography (Morris et al., 2000). The study observed reduced vasodilatation

responses to carbacol in uremic patients, with preserved vasorelaxation function in response to SNP. Hand *et al.* identified impaired endothelium-dependent vasodilatation in adult haemodialysis patients using non-invasive vascular measurements of dorsal hand veins (Hand et al., 1998). The study examined 12 haemodialysis patients and 8 healthy controls (non-HD) comparing responses of Ach (activator of NO synthase) and glyceryl trinitrate (GTN, a NO donor) to preconstricted dorsal hand vessels before and after dialysis using dorsal hand vein measurement. This study observed an impaired venodilatation response to Ach before dialysis, which corrected after each dialysis session, whereas venodilatation in response to GTN was similar before and after dialysis. The study also showed that Ach-dependent venodilatation was corrected before dialysis by co-infusion of L- but not D-arginine. From these findings, the authors suggested that patients on haemodialysis have impaired Ach-mediated venodilation as a result of accumulated NOs inhibitors, which are cleared by dialysis therapy. Baragetti *et al.* also examined the endothelial function in the forearm vessels of uremic dialysis patients (Baragetti et al., 2007) using B-mode (Biosound) ultrasonography on the brachial artery as discussed in detail in section 1.4.3.6.

1.7 Ex vivo vascular studies in CKD

An *ex vivo* study of small arteries is an important and helpful tool for assessing vascular abnormality in uremic patients who are at high risk of developing the structural and functional cardiovascular abnormalities that may occur as a result of microvascular dysregulation.

In *ex vivo* animal studies, altered myogenic constriction and impaired EDHF-mediated relaxation has been observed in isolated small vessels of uremic hypertensive rats (Vettoretti et al., 2006). Decreases in Ach-mediated vasorelaxation but not in SNP-mediated response have been demonstrated in isolated small vessels of severely hypertensive uremic rats induced by renal mass reduction (Benchetrit et al., 2003). Moreover, endothelial function in isolated uremic arteries has been investigated using arterial responses to different concentrations of acetylcholine (Ach) and sodium nitoprusside (SNP) in controls (non-uremic) and uremic rats induced by nephrectomy (Thuraisingham and Raine, 1999). The study observed that normal agonist-induced endothelium-dependent relaxation was maintained in experimental uraemia.

Despite the high prevalence of cardiovascular events in CKD, few studies have examined vascular and endothelial function in isolated subcutaneous vessels of uremic patients. For example, Morris *et al.* examined the effects of uraemia on the vascular function through measurement of isolated human subcutaneous resistant arteries using wire myography. In this study, subcutaneous fat biopsies were obtained from the anterior abdominal wall from twelve uremic patients with different stages of CKD at the time of peritoneal dialysis catheter insertion or renal transplantation, and eight control samples without kidney disease at the time of abdominal elective surgery. Small resistant arteries were dissected and conducted by wire myography, and cumulative concentration-response curves for norepinephrine (NA), endotheline-1 (ET-1), acetylcholine (Ach), and sodium nitroprusside (SNP) were constructed (Morris et al., 2001). This study showed no significant differences between both groups in response to NA and ET-1 (see Figure 7), however more tendency of increased maximum contraction to the highest doses of these

compounds were observed in uremic patients than control group. The potency and maximum relaxation to SNP (endothelium-independent vasodilator) were similar in the two groups, while the maximum % of relaxation to Ach (endothelium-dependent vasodilator) was significantly lower in uremic patients compared to control group (Figure 8).

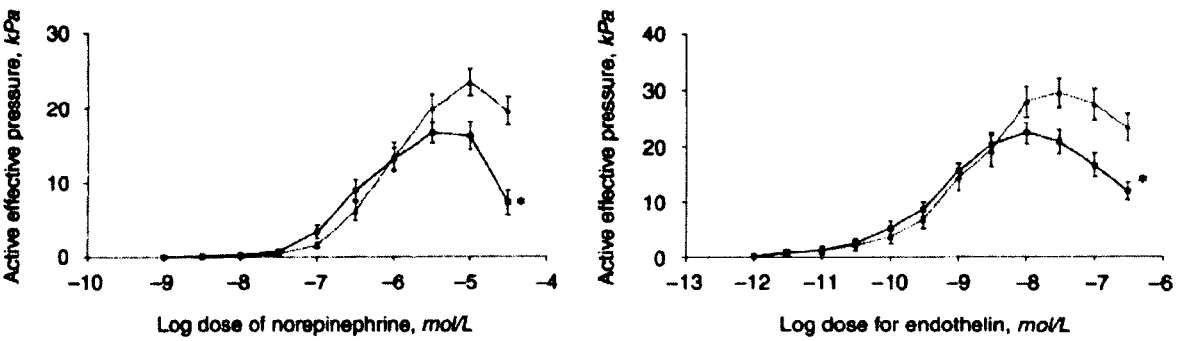

Figure 7 Concentration response curves for vasoconstrictors in uremic and controls Data are mean ± SEM. Norepinephrine (NA), endotheline-1 (ET-1) in uremic (▲) and control (■) groups.*P = 0.001 and *P = 0.01 respectively (Morris et al., 2001).

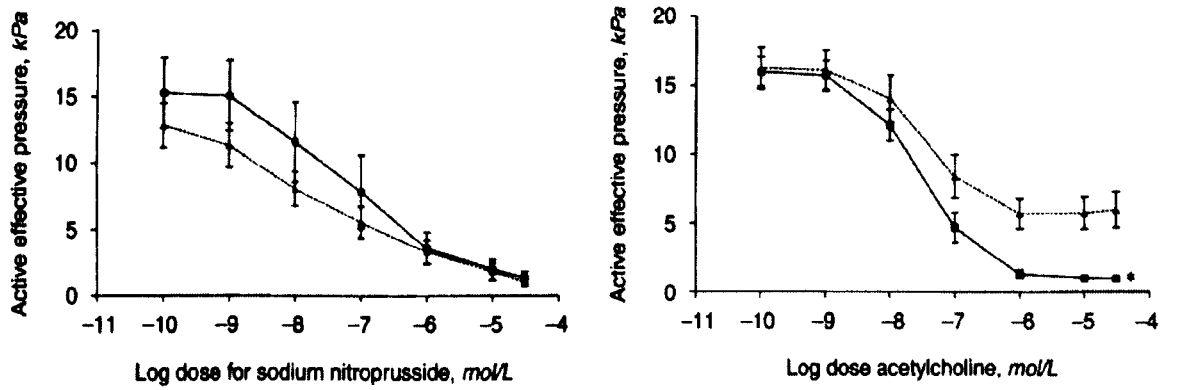

Figure 8 Concentration-response curves for vasodilators in uremic and controls Data are mean ± SEM, (*P = 0.001, Sodium nitroprusside (C) and acetylcholine (D) in uremic (▲) and control (■) groups. Figures Adapted from Morris *et al., 2001*.

Effects of uremia on the structure and function of isolated subcutaneous small arteries has also been studied recently in ESRD patients using both wire and pressure myography (Luksha et al., 2011). In this study, 35 patients starting peritoneal dialysis were matched with 30 healthy controls. It observed impaired endothelium-dependent vasodilatation in response to Ach, while preserved endothelium-independent function in response to SNP. However, the study found that the vasocontractile function of isolated vessels in response to NA, ET-1, and angiotenisn II was similar between both groups.

On the other hand, vascular dysfunction due to other disorders such as hypertension has been well investigated in human subcutaneous resistance arteries by myography. Such studies have examined vascular dysfunction and remodelling (James et al., 2006, Rizzoni et al., 2006), as well as, evaluate the use of some pharmacological interventions (Buus et al., 2007, Schiffrin et al., 2002). Early morphological studies on the conduit arteries have shown that vascular alterations are characterized by atherosclerosis in HD patients (Pascazio et al., 1996, London and Drueke, 1997). In experimental uraemia, increased wall-to-lumen ratio in intra-myocardial small vessels with significant architectural abnormalities in the aorta has been shown in uremic rats (Amann et al., 1995a).

1.8 Obesity and cardiovascular dysfunction

1.8.1 Background and Epidemiology

Obesity is a major worldwide health problem, and well known as one of the most common risk factors for cardiovascular disease. The Framingham Heart study data has

indicated that obese and overweight people are associated with an increased relative risk for development of cardiovascular events (Wilson et al., 2002). It has been recognised as a major modifiable cardiovascular risk factor. Obesity and excessive weight gain significantly increase the risk for coronary artery disease, hypertension, and diabetes in both men and women (Sharma, 2003). Increased risk of developing congestive heart failure has been observed in obese people compared to controls with normal BMI (Kenchaiah et al., 2002).

The risk of cardiovascular morbidity and mortality is increased in obese people as a result of vascular dysfunction, particularly coronary complications (Ninomiya et al., 2004). Obesity is associated with increased risk of premature death, particularly from cardiovascular events. It has been recognised that the life expectancy of obese people in the current and future generations is expected to be reduced as a result of cardiovascular events and other associated chronic diseases (Karuparthi et al., 2008). Several central and peripheral abnormalities have been identified in obesity that contributes to the development of high blood pressure (Rahmouni et al., 2005). These include activation of the rennin-angiotensin-aldosteron system, and activation of the sympathetic nervous system (Figure 9).

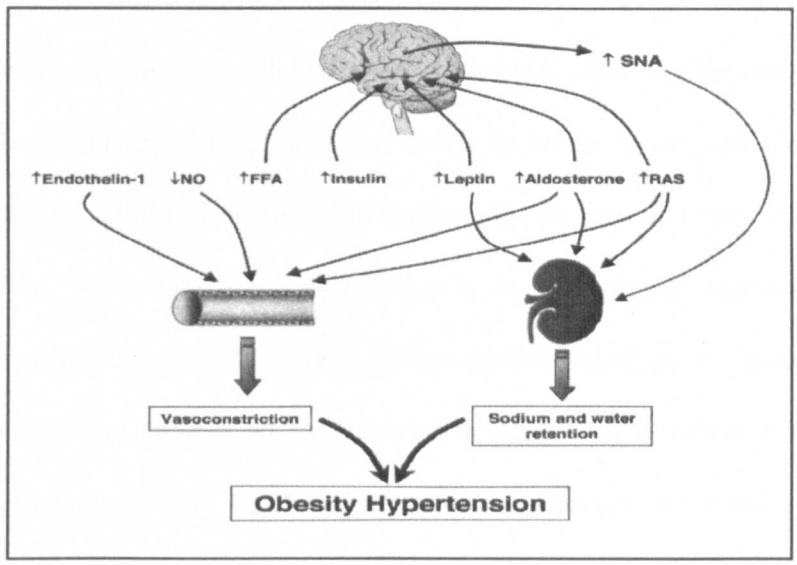

Figure 9 Mechanisms and hormonal system involved in obesity
Abbreviations: SNA, sympathetic nerve activity; FFA, free fatty acid; RAS, rennin angiotensin system. Data adapted from (Rahmouni et al., 2005).

1.8.2 Obesity and endothelial dysfunction

Obesity, insulin resistance, and metabolic syndrome have been shown contribute to vascular endothelial dysfunction and decreased arterial compliance (Fornoni and Raij, 2005, Ritchie et al., 2004). The association between obesity and endothelial dysfunction is decisive because the crucial role of endothelial dysfunction in the pathogenesis of atherosclerosis and development of cardiovascular events (Widlansky et al., 2003).

It has been shown that obesity-related conditions such as diabetes mellitus, hypertension, and hyperlipidaemia may cumulatively damage vascular endothelium (Meyers and Gokce, 2007). The mechanisms responsible for endothelial dysfunction in obese patients are yet unclear; however it is likely that various mechanisms may be involved. An endothelial cell adhesion molecule that facilitates cellular adhesion and

endothelial cell migration has been reported in obese patients (Escobar-Morreale et al., 2003). Increased vascular endothelial expressions of some oxidase enzymes and elevated endothelial oxidative stress have also been found in obese and overweight subjects (Silver et al., 2007). Impaired NO bioavailability has a major role in endothelial dysfunction. Since NO plays a crucial role in maintaining vascular tone, monocyte adhesion, cellular proliferation and inhibits platelet aggregation, therefore reduction in NO function may contributes to the development of macrovascular disease in obesity. The exact mechanism by which impaired NO function can cause endothelial dysfunction in obesity is not completely understood. However, a number of contributory factors including insulin resistance (Steinberg et al., 1996), increased concentration of pro-inflammatory cytokines (Aldhahi and Hamdy, 2003), increased free fatty acid levels (Steinberg et al., 2000), and increased endothelin-dependent vascular tone (Mather et al., 2002), have all been observed in obesity. Therefore these factors may responsible for the underlying mechanisms that mediate vascular endothelial dysfunction (Poirier et al., 2006).

1.8.2.1 Increased concentrations of pro-inflammatory cytokines and lipotoxicity

It is now clear that adipose tissue secretes different bioactive proinflammatory substances and hormones, such as adiponectin and leptin, which have been shown elevated in obese people. Dysregulation of these substances can lead to the incidence of obesity-related diseases including glomerulopathy and CKD (Trayhurn et al., 2008). Leptin is a hormone produced from adipocytes that stimulates the hypothalamus gland

resulting in appetite suppression (Trayhurn et al., 2008). It has been found that the concentration of circulating leptin is directly proportionate to the amount of adipose tissue present (Bagby, 2004, Sharma and Considine, 1998). Reduction in the concentration of circulating leptin has been observed in obese people who undergo weight loss (Leichman et al., 2008). In *ex vivo* animal studies, it has been shown that leptin stimulates the proliferation of cultured glomerular endothelial cells through the generation of reactive oxygen species (Bouloumie et al., 1999). In rats, administration of high doses of leptin can increase glomerulosclerosis and proteinuria (Wolf et al., 1999). Lipotoxicity is a process of lipid overload seen in obesity that promotes the cellular deposition of free fatty acids (FFAs) and triglycerides (TGs) which contributes to organ dysfunction (Wahba and Mak, 2007). In lipotoxicity, the continuous production of mediators, such as FFAs, other adipokines like interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- α), can increase platelet activity, activate renin-angiotensin system, impair insulin action and mediate insulin resistance. All of which contribute to glomerulonephritis, acute renal failure and tubulo-interstitial nephritis (Wahba and Mak, 2007). It has also been demonstrated that different mediators in obesity, such as TNF- α, interleukin (IL)-6, resistin and leptin, have some direct and / or indirect effects on the vascular endothelium (Aldhahi and Hamdy, 2003). The chronic inflammatory cascade that occurs in obesity contributes to the development of atherosclerotic diseases which predict increased risk of cardiovascular mortality and morbidity in this group.

1.8.2.2 Increased insulin resistance

It has been shown that obese patients with hyperfiltration have extremely high levels of insulin resistance (Chagnac et al., 2000), however, the exact underlying mechanism behind this remains unclear. One potential underlying mechanism is that insulin increases sodium reabsorption in the proximal tubules, thick ascending limb and distal tubule. This leads to associated fluid retention and the development of systemic hypertension (Tiwari, 2007, El-Atat, 2004). There is also increased extracellular proliferation, increased lipid production, and hyaluronate deposition, which act to increase intrarenal pressure. This causes compensatory lowered renal vascular resistance, raised kidney plasma flow, glomerular hyperfiltration and activation of the renin-angiotensin system (El-Atat, 2004).

It remains to be determined whether changing these potential factors contribute to improvement in the vascular function in obesity. Therefore, decreased obesity may improve vascular function and reduce cardiovascular disease in these high-risk subjects.

1.8.3 Bariatric surgery

Different bariatric surgical procedures are available to reduce the weight in obese people. The principle target in this procedure is to achieve weight loss by reducing the size of stomach. This can be achieved either by gastric banding which is performed by implanting a medical device, or gastric bypass surgery, which resections and reroutes the small intestines to a small stomach pouch. A third approach is billiopancreatic diversion, which is performed through the removal of a small portion of the stomach with a

duodenal switch. Long term studies on obese people have observed that a significant reduction in the body weight is associated with significant improvement in the cardiovascular risk factors (Robinson, 2009).

1.8.4 Bariatric surgery and renal function

Few studies have established the relation between bariatric surgery and renal function. In a study of eight obese patients without kidney disease, the glomerular filtration rate (GFR), renal plasma flow (RPF), BMI, and albuminuria were studied before and after bariatric surgery and compared with controls (Chagnac et al., 2003). In this study, obese patients had a higher baseline GFR and RPF than controls by 61% and 32% respectively. Over a period of 12 to 17 months, BMI decreased from 48 +/- 2.4kg/m2 to 32.1+/-1.5 kg/m2 and GFR and RPF both decreased by 24% and 13% respectively. Albumin excretion also reduced with weight loss from 16μg/ml (range, 4 to 152μg/ml) to 5μg/ml (range, 3 to 37μg/ml). Another study followed-up 61 extremely obese patients over a period of 24 months after bariatric surgery and compared with normal weight controls (Navarro-Diaz et al., 2006). At baseline the weight, BMI, GFR (calculated by 24 hour creatinine clearance), and 24-hour proteinuria were higher in the obese patients than in the controls. After one year, all the above parameters improved in obese patients with only albuminuria continuing to decrease at the end of the second year. This was attributed to the slowing of weight loss after the first year. In obese patients with established CKD and ESRD, one study showed stabilisation or improvement of the initial renal disease in nine patients out of 31 after bariatric surgery,

but each was presented as a case study, and measurements were not uniform (Alexander et al., 2009).

Decrease in weight following bariatric surgery is associated with an improvement in a number of cardiovascular risk factors. At two and ten-year follow ups it was shown that surgery patients had a lower incidence rate of diabetes, hypertriglyceridemia, and hyperuricemia than controls (van Etten et al., 2002, Dixon et al., 2008). Improvement in left ventricular relaxation has been shown in adult obese patients after nine months post-surgery (Ouchi et al., 2003, Leichman et al., 2008). Reduction in left ventricular hypertrophy has also been shown in adults with weight reduction post-surgery (Ikonomidis et al., 2007).

1.8.5 In vivo vascular studies in obesity

Endothelial dysfunction that manifests either as abnormal circulating products of endothelial cells or impaired physiologic responses to endothelium-dependent vasodilator stimuli has been demonstrated in both human and animal obesity (Laight et al., 2000). In the animal model, impaired NO-dependent dilatation in muscular arterioles has been observed in hypertensive diabetic obese rats using television microscopy, and vessel dilatation was measured with a video micrometre (Frisbee and Stepp, 2001). The study found that impaired vascular perfusion in obese rats was due to elevated oxidative stress.

In humans, several *in vivo* studies using non-invasive techniques have suggested impaired endothelial function in the peripheral vessels of obese patients. Perticone *et al.* assessed endothelial function through measurement of forearm blood flow in response to acetylcholine (Perticone et al., 2001). The authors observed that the presence of

endothelial dysfunction in obese humans is due to reduce NO bioavailability secondary to the increased production of reactive oxygen species. Another *in vivo* study assessed endothelial function in obese patients underwent a dietary restriction regimen through the measurement of forearm blood vessels (Sciacqua et al., 2003). The study demonstrated significant improvement in the maximal vasodilator responses of forearm vessels to the high dose of acetylcholine in healthy obese subjects following an energy-restricted diet. Moreover, endothelium-dependent and independent vasodilatation has also been assessed in the forearm vessels of patients with visceral obesity using non-invasive plethysmography (Nielsen et al., 2004). Arkin *et al.* examined endothelial function in two groups of obese patients including super-obese group (BMI≥50) and morbidly obese patients (BMI≥40), investigating the vascular response to endothelium-dependent and independent vasodilatation using flow-mediated dilatation on brachial artery (Arkin et al., 2008). The study observed significant impairment in endothelium-dependent dilatation in the super-obese group compared with the other obese group, with similar vasorelaxation response to nitroglycerin-mediated dilatation has observed between both groups.

1.8.6 Ex vivo vascular studies in obesity

Several *ex vivo* studies have been conducted in animal models. Vascular and cardiac functions have been examined in rats with diet-induced obesity (Boustany-Kari et al., 2007). The study observed increased vascular contractility and decreased coronary vascular relaxation of isolated vessels to various stimuli. Chinen *et al.* demonstrated

impaired endothelium-dependent vasodilatation in isolated aortic vessels of obese rats using wire myography (Chinen et al., 2007). The author found that reduction in the vasorelaxation response to Ach in obese Zucker rats was induced by FFAs and the overproduction of ROS. It also observed that the vasorelaxation response to SNP was identical in the two groups.

In humans, some studies have identified impaired endothelium-dependent vasodilatation in isolated resistance arteries of obese patients. De Ciuceis *et al.* examined endothelium-dependent vasodilatation in the isolated subcutaneous arteries of normotensive and hypertensive obese patients compared to non-obese controls (De Ciuceis et al., 2011). The study showed significant impairment in acetylcholine-dependent relaxation in both normotensive obese and hypertensive obese patients compared to controls. The authors also observed improvement in endothelial relaxation in response to acetylcholine in a small number of obese patients one year after bariatric surgery. Significant impairment in acetylcholine-induced relaxation has been observed in isolated subcutaneous arteries from severely obese patients compared to lean subjects ($P < 0.01$), with preserved endothelium-independent vasodilatation in response to SNP (Grassi et al., 2010b). Impaired endothelial-dependent vasodilatation response has been reported in other conditions such as diabetes mellitus. Rizzoni *et al.* observed impaired vasorelaxation response of small isolated vessels to Ach and BK in normotensive and hypertensive diabetic patients (Rizzoni et al., 2001b).

In addition to microvascular disturbances, structural remodelling in small vessels characterised by an increased media-to-lumen ratio has been observed in the obesity milieu (Rizzoni et al., 2012). Vascular alterations such as thickening of the intima and

media of the vessel wall are frequently found in obesity, promoting endothelial damage and initiating atherosclerosis and cardiovascular disease (Georgescu et al., 2011). Grassi *et al.* also found that media thickness and media-to-lumen ratio were significantly greater in the subcutaneous resistance arteries of obese patients compared with non-obese controls (Grassi et al., 2010b). The authors also observed that these structural alterations were accompanied by changes in endothelial function. Moreover, in a study compared 14 lean healthy controls, 13 obese patients, and 12 participants with metabolic syndrome (Grassi et al., 2010a). Small arteries isolated from abdominal subcutaneous fats were investigated for structural and functional properties. The study found that media thickness and media-to-lumen ratio of the resistance arteries were significantly greater in the metabolic syndrome and obese groups compared with controls. Impaired Ach-induced endothelium-dependent relaxation was also observed in the obese and metabolic syndrome groups but not in the control group.

1.9 Vasoconstrictors

Vasoconstriction can be defined as a narrowing of the internal diameter of blood vessels as a result of contraction of their muscular layer, particularly small arterioles, large arteries and veins. This process is important for controlling haemorrhage and acute blood loss (Hynynen and Khalil, 2006). The muscular layer of all blood vessels from heart to capillaries are involuntary controlled in response to various substances, chemicals and hormones resulting in either vasoconstriction or vasodilatation. During constriction of blood vessels, blood flow is restricted or decreased resulting to an increase in vascular resistance (Groeneveld et al., 1988). Generalised vasoconstriction

usually causes an increase in systemic blood pressure; however, vasoconstriction may also occur in particular tissues leading to localised reduction in the blood flow. This process can initiate tissue ischemia and necrosis (Black et al., 2003). One proposed mechanism of vasoconstriction is that increased concentration of calcium (Ca^{2+} ions) within vascular smooth muscle cells (Brayden and Nelson, 1992).

1.9.1 Noradrenaline (Norepinephrine)

Noradrenaline (NA) is an endogenous potent vasoconstrictor catecholamine that acts as α-adrenergic receptor agonist. It is the neurotransmitter of the sympathetic nervous system. It stimulates alpha adrenoceptors that are found throughout the vascular system resulting in contraction of the muscular layer within the vascular system, therefore causing restriction of blood vessels and elevation of blood pressure (Teerlink et al., 1994). NA is chemically similar to adrenaline; both are synthesized and secreted from adrenal glands. It is well-recognised that NA promotes high K^{+}- depolarisation which activates Ca^{2+} influx and induces maintained contraction whereas epinephrine induces an initial transient contraction through releases of Ca^{2+} from the vascular cells, followed by activation of Ca^{2+} influx to induce sustained contractions (Bolton, 1979). In humans and animals, it has been shown that infusions of NA can elevates arterial pressure and peripheral resistance without any changes on the cardiac output (Lansing and Stevenson, 1958). The vascular endothelium when exposed to vasoconstrictor stimuli such as NA, it can modulates and maintains the normal vascular tones and homeostasis through the secretion of endothelium-derived relaxing factors such as NO. NA has been tested in

several *ex vivo* studies to assess the vascular function in uremic CKD patients (Morris et al., 2001), hypertensive (Rizzoni et al., 2001a, Amann et al., 2001, Black et al., 2003), and obese patients (Georgescu et al., 2011). Moreover, it has been tested *in vivo* to examine the vascular reactivity in haemodialysis patients (HD)(Morris et al., 2000). Intravenous infusion of NA in haemodialysis patients during haemodialysis session can induce vasoconstriction and increased in the total peripheral resistance that improve haemodynamic instability (Nette et al., 2006).

1.9.2 Endothelins

Endothelin is an endogenous potent 21-amino-acid vasoconstrictor amino acid peptide produced primarily in the endothelium, discovered in 1980s (Yanagisawa et al., 1988). There are four subtypes of endothelins including; ET-1, ET-2, ET-3, and ET-4. The main isoform is endothelin-1 (ET-1) which released from the endothelial cells and acts by stimulating endothelin receptors in the endothelial cells and vascular smooth muscles (VSM) resulting to control of cellular growth, proliferation and regulation of vascular function (Luscher and Barton, 2000). The vascular endothelin system can control and modulate vascular tone, growth, and function. ET-1 may contribute to the elevation of blood pressure that observed in both human and experimental models, seems to be a risk factor in many cardiovascular diseases (Hynynen and Khalil, 2006). However, the exact roles of endothelins and its receptors in the regulation and pathogenesis of hypertension is still unclear. The proinflammatory effects of ET-1 induces vasoconstriction may promotes fibrosis and it has an effects on the VSM through

stimulation of specific receptors on the VSM cells resulting in VSM proliferation. This may suggest the involvement of ET-1 in the process of thickening of vascular intima and media in uremic patients and therefore development of cardiovascular disease (Amann et al., 2001).

1.9.3 Thromboxane AII (U46619)

Thromboxane A2 (TXA2) is a vasoconstrictor and potent hypertensive agent that potentiates platelets aggregation. Firstly discovered by Hamberg *et al.* (1975) as an eicosanoid that synthesized in platelets from prostaglandin H2 (archidonic acid derivatives) through thromboxane-A synthase enzyme (Hamberg et al., 1975). The extent of TXA2 induced alteration in the microcirculation is basically dependent on the endothelial function. Endothelial damage such as endothelial calcification or atherosclerosis may associated with a reduction in TXA2 inhibitors and thus promotes platelet activation and adhesion at the site of endothelial injury, which ends by thrombus formation (Schror, 1990). For that reason, in myocardial ischemia, the major source of TXA2 seems to be due to platelet activation. TXA2 was shown to cause contraction in human coronary arteries by thrombin-stimulated platelets, this kind of response was resulting from platelet-derived TX2 (Ellis et al., 1976). It has been tested *in vivo* to induce coronary vasoconstriction which in turn resulting to sever myocardial ischemia and sudden death in rabbits (Lefer et al., 1980).

Similarly, Terashita *et al. 1978* confirmed these vasocontractile effects in isolated small coronary microcirculation in *ex vivo* animal model (Terashita et al., 1978). The

study observed that TXA2 can cause strong contraction in renal vascular beds in isolated glomerulous of rats (Cavarape et al., 2003). TXA2 was believed as predisposing factor that lead to coronary and cerebral vascular ischemia as a result of its potent vasocontractile effects associated with platelets aggregation (Smith et al., 1980).

1.9.4 Angiotensin II

Angiotensin is an oligopeptide hormone that acts as an endocrine hormone constricting blood vessels and involved in the regulation of rennin-angiotensin system. Angiotensin II (AngII) when given in low doses, may raise blood pressure slowly and progressively (Griffin et al., 1991). It is derived from the precursor angiotensinogen, a globulin that synthesized in the liver. Angiotensin I converted to AngII through removal of two C-terminal residues via the enzyme called angiotensin converting enzyme (ACE) (Skurk et al., 2001). AngII has a prothrombotic effect through aggregation and adhesion of platelets (Skurk et al., 2001, Gesualdo et al., 1999). Renin-angiotensin system can be activated during cardiac and vascular damage such as atherosclerosis and endothelial alteration (Gesualdo et al., 1999). By stimulation of angiotensin-1 receptor, AngII can initiates different actions including vasoconstriction, sodium and water retention, facilitates adrenergic nerve activity and production of reactive oxygen species.

AngII also acts as a vascular growth factor promoting vascular smooth muscle proliferation and atherogenesis (Kanaide et al., 2003). An *in vitro* testing of AngII on the vascular smooth muscle has been shown to have a mitogenic (Lyall et al., 1988) and trophic effects (Berk et al., 1989). It has not been demonstrated to have these effects *in*

vivo, though it's capability to promote a new vessel formation (Fernandez et al., 1985). In the animal model, it has been observed that infusion of AngII in low doses for 10 days raises arterial pressure slowly and progressively, causing structural change in resistance vessels and increased cardiac weight (Griffin et al., 1991). AngII has been shown to induce other vascular changes such as vascular smooth muscle growth, vascular cell adhesion, deposition of extracellular matrix proteins, cell migration and inflammation (Intengan and Schiffrin, 2001). AngII has a role in the vascular hypertrophy through significantly increase vascular media thickness and media / lumen ratio (Griffin et al., 1991). The contractile effect of AngII has been investigated *in vivo* in rats by intravenous perfusion of 100 nM (10^{-7} M) of the drug which potentiates the contraction of resistant arteries through endothelial production of endothelin (Dohi et al., 1992).

1.9.5 Vasopressin

Vasopressin is a vasoconstrictor peptide hormone (also known as anti-diuretic hormone), synthesized in the hypothalamus and secreted in response to hypovolemia, hypotension, hyperosmolarity and sympathetic stimulation (see Figure 10). The vasopressin prohormone with its glycopeptides is transported through nerve axons to pars nervosa of the posterior pituitary gland where it is stored as granules (Giovannucci and Stuenkel, 1997). It has the following two main principle sites of action: the first place is in the kidney, it stimulates vasopressin 2 receptor (V2 receptor) located on the renal collecting tubules and regulates extracellular fluid volume by increase water permeability and decrease urine formation, leading to increase blood volume, cardiac output and blood

pressure (Maturi et al., 1991). The second action is on blood vessels, vasopressin can initiate the process of vasoconstriction and elevation of arterial pressure through stimulation of V1 receptor on the vascular smooth muscle.

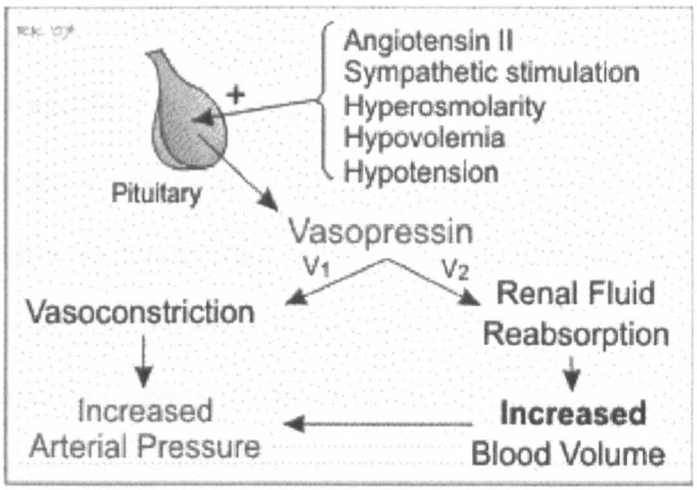

Figure 10 Vasopressin physiology
Adapted from (den Ouden and Meinders, 2005)

The normal physiological concentrations of vasopressin are less than its vasocontractile range. However, in severe hypovolemic shock or hypotension, the production of vasopressin is increased resulting to compensatory increase in systemic vascular resistance (den Ouden and Meinders, 2005). Vasopressin is a contractile agent that increases systemic vascular resistance and causes an elevation in arterial blood pressure. It has also been shown that infusion of low-dose vasopressin can promote renal, cerebral and pulmonary vasodilatation mediated by endothelial release of nitric oxide (Vanhoutte et al., 1984). Vasopressin has been tested to study vascular contraction in isolated human cerebral arteries (de Aguilera et al., 1990). In this study human cerebral medium size arteries (500 – 700 μm diameter) were isolated during autopsy of 15

patients and cumulative concentration response curves for vasopressin were constructed in arteries with endothelium and without endothelium (mechanically removed), the study observed powerful constrictor effects of vasopressin on both groups through direct stimulation of vasopressin-1 receptor that located in the vascular smooth muscle.

1.10 Vasodilator agents

Vasodilatation is a mechanism that leads to relaxation of the smooth muscle of blood vessels, either in the arteries, which results in decrease in systemic vascular resistance or in veins, which leads to reduction in venous blood pressure (Shapira et al., 1999). Some vasodilator agents, including arteriolar vasodilators, can be used in the treatment of hypertension and heart failure, while venous vasodilators are effective in the treatment of angina (Daly et al., 1984). Some vasodilator agents have functional ability to dilate both arteries and veins. The vascular endothelium plays an important role in the regulation of blood flow through mechanical control of vascular smooth muscle. Common substances that mediate endothelium relaxation are nitric oxide (NO) and prostaglandin I2 (prostacyclin, PGI2), both of which are known as endothelium-derived chemicals that exhibit a potent vasorelaxation (Tormakangas et al., 2006). The third substance is endothelium-derived hyperpolarizing factor (EDHF) that mediates hyperpolarization of smooth muscle cells through activation of potassium channels in both smooth muscle and endothelial cells (Feletou and Vanhoutte, 1999). Arterial pressure is also regulated by vasodilator substances such as bradykinin, acetylcholine, mineral ions (potassium and magnesium), endogenous nitric oxide, carbon dioxide and hydrogen gases.

1.10.1 Bradykinin

Bradykinin (BK) is one of the most potent endogenous endothelium-dependent vasodilator amino acid peptide that formed of nine amino acids. It causes vasodilatation, increase vascular permeability and natriuresis (increase urinary sodium excretion) causing reduction in blood pressure (Han et al., 2002). BK stimulates two types of BK receptors including B1 receptor which expressed during tissue injury and inflammation (McLean et al., 2000) and has a role in endothelial cells receptor pathway (Duchene et al., 2007). The action of BK on B2 receptor is mainly associated with vasodilatation. It is well recognized that BK stimulates endothelial cells resulting to release of different vasorelaxant agents such as NO and EDHF (O'Kane et al., 1994). BK has been widely investigated in many *ex vivo* studies of the vascular function (Rizzoni et al., 2001b, Hadoke et al., 2000, Lang et al., 2007). Moreover, it has been demonstrated that BK-induced relaxation of isolated human coronary artery was principally endothelium dependent (Forstermann et al., 1988, Okamura et al., 1989). Some studies showed that BK stimulates the release of endothelium-derived nitric oxide (Forstermann et al., 1988) and hyperpolarizing factor (Nakashima et al., 1993). BK has also been tested *in vivo* as a potent vasodilator in human forearm resistance vessels, resulting to vasorelaxation via hyperpolarization of the vascular wall independent of NO (Honing et al., 2000).

1.10.2 Acetylcholine (Ach)

Ach is a neurotransmitter chemical compound that stimulates both peripheral and central nervous system. Ach is primarily synthesized from their precursor compounds

choline and acetate in neurons through the enzyme choline acetyltransferase (Blusztajn et al., 1987). Ach acts to stimulate two main types of receptors including nicotinic receptors which located in the muscles, central nervous system and autonomic ganglia. This type of receptor is stimulated by both nicotine and Ach to promote sodium, potassium and chloride permeability (Jones, 2005). The second type is muscarinic receptors, these located in different tissue and organs in the body resulting to various biological functions. Stimulation of Ach to muascarinic receptors in blood vessels and heart can results to decrease cardiac contraction and cardiac output. Ach stimulates endothelium-dependent vasodilation in most of isolated mammalian arteries (Vanhoutte and Miller, 1985). This relaxation is mediated by activation of muscarinic receptors on the endothelial cells (Furchgott and Zawadzki, 1980), resulting to increase production of cyclic guanosine monophosphate (cGMP). Endothelial cells that exposed to Ach stimulation can produce various diffusible substances known as endothelium-derived relaxants. Early studies have proposed that adenosine and adenosine monophosphate are endothelium-dependent relaxant factors, however many substances released from the endothelium in response to Ach promotes the vasorelaxation mechanism, these factors are known as endothelium-derived hyperpolarizing factors (EDHF) (Furchgott and Zawadzki, 1980).

1.10.3 Sodium nitroprusside (SNP)

SNP is a complex chemical compound that has a potent endothelium-independent vasorelaxation effect. It was first prepared and investigated in the middle of nineteenth

century (Lefebvre, 1995). The hypotensive action of SNP was established first in 1929. It has been given intravenously in severe and acute hypertensive emergencies and during surgical procedures to control blood pressure. It is also an effective vasodilator agent in congestive heart failure (Subramanyam et al., 1982). SNP breaks down in the blood and releases NO which activates the guanylate cyclase in the vascular smooth muscle and enhances the production of intracellular (cGMP) which in turn promotes the process of smooth muscle relaxation and therefore vasodilation (Grossi and D'Angelo, 2005). It has been frequently used to test endothelium-independent vasodilation in many *ex vivo* (Morris et al., 2001, James et al., 2006, Rizzoni et al., 2006, Thuraisingham and Raine, 1999) and *in vivo* studies (Morris et al., 2000, Hand et al., 1998, Annuk et al., 2001) in humans and animals.

1.11 Aims of the thesis

Alterations in the microcirculatory structure and function may be considered an important mechanism of organ damage. This project has been planned to test the following hypothesis:

Uremia and obesity induces alteration in the vascular function through variable enhancement in the contractile responses of isolated different-sized arteries of HD and obese patients respectively in response to various vasoconstrictors, and impaired endothelium-dependent vasodilatation with preserved endothelium-independent vasodilatation. This alteration may progress to development of major cardiovascular consequences in both risk groups.

Ex vivo assessment of vascular function in uremic and obese patients is not a new technique and has been carried-out in few studies. However, most of previous studies were focused on investigating small-sized arteries in response to a limited suit of vasoactive agents. This study is novel in that it is designed to investigate the vascular function in different-sized arteries isolated from subcutaneous fats of a homogenous uremic group of patients who purely on HD and obese patients underwent bariatric surgery with comparison to non-uremic non obese control groups. Since pulse wave velocity (PWV) as measures of arterial stiffness is correlated with increased risk of cardiovascular disease in both risk groups, we hypothesised presence of a relationship between elevated PWV and changes in the ex vivo vascular function. Therefore to test this hypothesis the following aims will be addressed:

Firstly, to investigate the vascular function in isolated different-sized arteries obtained from subcutaneous fats of HD patients using wire myography, to establish whether uremic HD patients show enhanced vasocontractility and impaired endothelial-dependent vasodilation. In addition, to assess how that *ex vivo* intrinsic function correlates to *in vivo* assessments of cardiovascular status. The results of these aims are presented in chapter 3.

Secondly, this study also aimed to assess an arterial function in isolated different-sized arterial segments obtained from subcutaneous fat of obese patients undergoing bariatric surgery. This was to investigate the vascular reactivity in obese patients through measurement of their responses to different vasoconstrictors and vasodilators. The study also addressed changes that might underlie altered vascular responses associated with obesity and following surgery (decrease in weight). This work is presented in chapter 4.

Chapter 2
General Methodology

Chapter 2: Methodology

2.1 Ethical approval

The study recruited haemodialysis (HD) patients for subcutaneous fat samples, entering them in multi-centre randomised controlled cross-sectional trials. The recruited patients were all males and females willing and able to provide consent, age ≥ 16 years old, all within the 90 days of having started dialysis at least 03 times a week. They will be recruited from both main hospital and satellite dialysis units of five centres (Derby Hospitals NHS Foundation Trust, University Hospitals of North Staffordshire NHS Trust at Stoke, University Hospitals of Leicester NHS Trust, Heart of England NHS Foundation Trust and University Hospital Birmingham NHS Foundation Trust in Birmingham. Obese patients involved in the observational study "Improvement of Renal Disease and Cardiovascular Function in Patients Undergoing Bariatric Surgery" were recruited for subcutaneous fat samples. This study was conducted according to the standards of Good Clinical Practice Guideline, Research Ethics Committee regulations, Trust and Research Office policies and procedures. All protocols were approved and granted by the Derbyshire Research Ethics Committee and the local NHS Research & Development department to allow for the collection of subcutaneous fat biopsy samples from HD patients, obese patients, and normal controls (non-HD, non-obese patients).

All participants were provided with an information sheet describing their biopsy procedure and with sufficient information for subjects to make an informed decision about their participation in the study. Subjects signed a consent form to indicate that they

were giving their valid consent to participate. Few and minimal risks to the subjects were associated with the biopsy technique.

2.2 Human subcutaneous fat samples

2.2.1 Background

The majority of subcutaneous fat biopsies that have been previously studied to examine vascular function by myography were obtained from either a patient's gluteal region or an anterior abdominal wall, and they were obtained under local anaesthesia using 3-5 ml of 1% or 2% lignocain hydrochloride (Rizzoni et al., 2003, Aalkjaer et al., 1987, De Ciuceis et al., 2007). In these studies, the biopsy size varied from 1-3 cm long, 0.5-1 cm wide and 0.5-1.5 cm deep. These biopsies were taken through a standard skin biopsy technique using a horizontal (1-2 cm-long) incision of the skin in the upper external gluteal quadrant or lateral abdominal wall. Many *ex vivo* studies have used this technique to obtain fat samples of 1 cm long, 0.5 cm wide and 0.5 cm deep from superficial gluteal tissue (Joannides et al., 2006, Wang et al., 2000, Wang et al., 2003, Intengan et al., 1999). In the present study, human subcutaneous fat biopsies were obtained from the following sources: haemodialysis patients (to measure *ex vivo* vascular function in haemodialysis patients, and to establish the effects of uraemia on vascular function); obese patients (to investigate the vascular reactivity in obese patients, and to study changes in the vascular response that might occur following bariatric surgery); and from non-haemodialysis, non-obese control patients (to compare them with the haemodialysis and obese patient samples).

2.2.2 Human haemodialysis fat samples

The present study involved HD patients in a multi-centre, randomised controlled cross-sectional trials "The effects of cooling a dialysate on systolic dysfunction in Haemodialysis patients". This study is conducted by our research team to investigate whether or not cooling the dialysate fluid will reduce the degree of cardiac systolic dysfunction and the incidence of cerebral ischemia. In this study, patients were randomly given either standard temperature dialysis or individualised cooled dialysis within 90 days of starting HD. The recruited patients were included males and females aged ≥16 years old having HD treatment at least three times per week. Patients with heart failure grad IV and cardiac transplant recipients were excluded. Consenting patients underwent two visits: a baseline assessment visit and a follow-up visit after one year. Investigations that were carried out in each visit are illustrated in (Figure 11). In each visit (that taken on the non-HD session day), all patients had cardiac and brain MRI at Nuffield Hospital / Derby. Thereafter, each patient was transferred to the study area at Graduate Entry Medical School, University of Nottingham, Royal Derby Hospital / Department of Renal Unit for fat sampling.

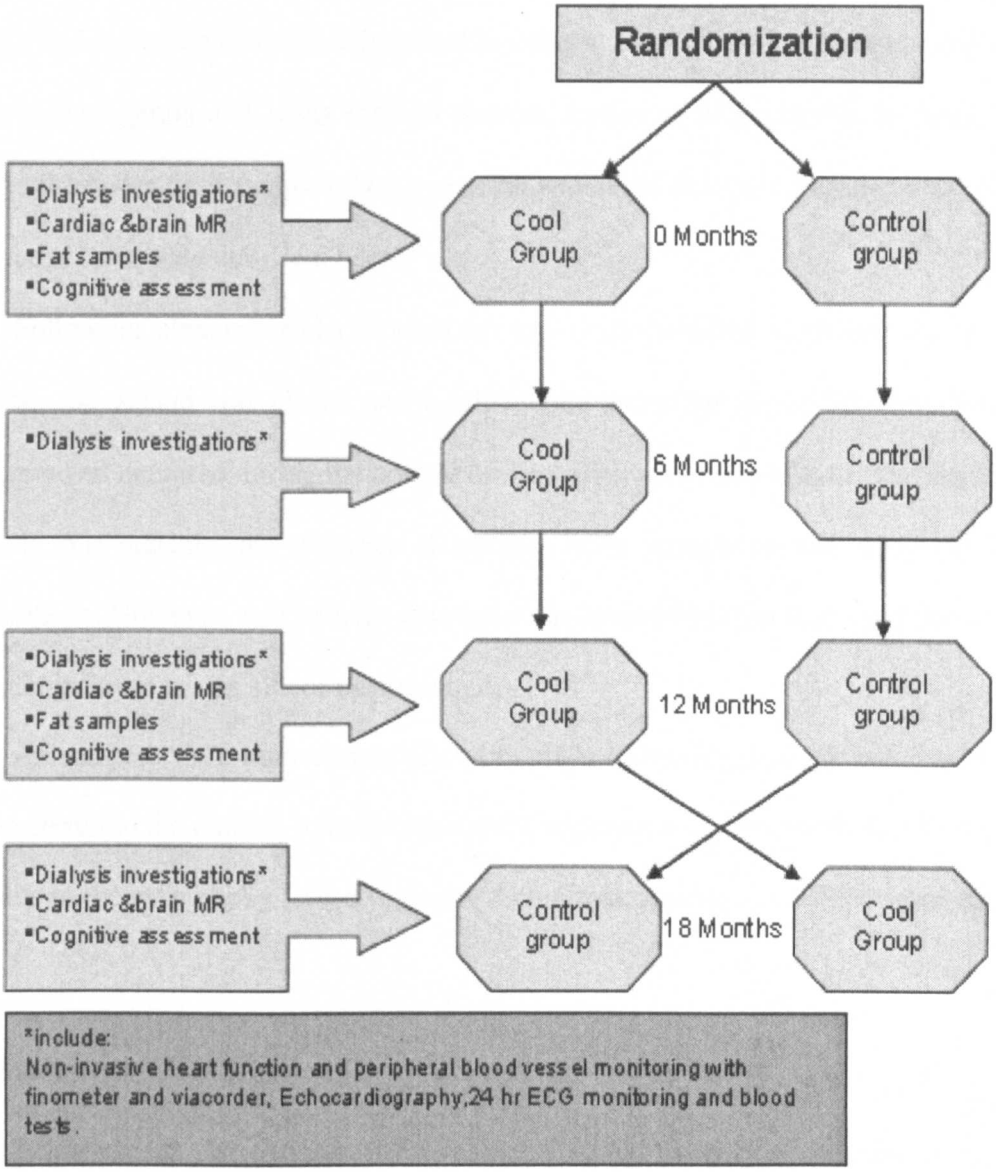

Figure 11 Summary of cooling study including the time of fat sampling.

All subcutaneous fat biopsies were performed by trained renal researchers who conducted the cooling study using local anaesthetic (1% lignocaine, 2-3 ml). A small (1-2 cm long) right or left lower lateral abdominal single transverse incision was made

under full aseptic technique. The subcutaneous adipose tissue was then dissected carefully and separated from the adherent skin using a scalpel. Using a forceps, a piece of fat was held up gently and, using surgical scissors, a piece of subcutaneous fat tissue was taken (in the majority of samples the piece of fat was small diamond in shape with nearly 1.5cm long, 1 cm wide and 1 cm deep).

Following cleaning and cessation of any small bleeding from the site of the operation, the wound was closed under full aseptic technique using 2-3 wire sutures. Suture removal occurred during the normal healing time, which was usually less than two weeks. In our patients, the majority of samples were straightforward, taken without complications. However, two patients developed prolonged bleeding that was relieved by prolonged pressure on the site of operation.

All samples were immediately placed in chilled physiological salt solution (PSS) and transferred to the clinical sciences laboratory department for dissection and mounting of vessels to the myography (see myography techniques, section 2.4) on the same day of biopsy.

2.2.3 Human obese fat samples

Subcutaneous fat samples were obtained from patients undergoing bariatric surgery. This was part of a prospective observational study "Improvement of Renal Disease and Cardiovascular Function in Patients Undergoing Bariatric Surgery, observational single centre pilot study". The study was designed to investigate the effects of obesity on vascular function, renal function and cardiovascular function before and after surgery. All non-diabetic, non-cirrhotic obese patients with age of 18 or over were

invited to participate in the study. Each patient was assessed at four weeks before surgery and then again at three and six months post-surgery (details of patient's assessment in this study are described in Figure 12). Samples of subcutaneous fat tissue were obtained at zero months (baseline samples) and at six months post-surgery. The baseline subcutaneous fat samples were approximately 2cm long, 1.5 cm wide and 1.5 cm deep was taken at the time of operation using laparoscopic port. The second subcutaneous fat samples were obtained at six months following bariatric surgery using extra lower abdominal incision (as described in HD fat samples). Once harvested, the biopsy was immediately transferred in a container with chilled PSS to the laboratory sciences department for dissection and mounting on the same day of the biopsy (as described in section 2.4.3).

2.2.4 Control fat samples

Normal control samples were provided from appropriately consented patients (non-obese non-haemodialysis patients) who underwent either elective laparoscopic or abdominal surgery (elective hernia repair). All information about the size of fat biopsy that must be obtained to get enough sample size was provided to the surgeon who responsible to take the biopsy. Most of the samples that harvested from these patients were approximately 2cm long, 1.5 cm wide and 1.5 cm deep). Following harvesting, samples were immediately transferred in cold PSS to the laboratory sciences to be dissected and experimented on the day of biopsy as described before.

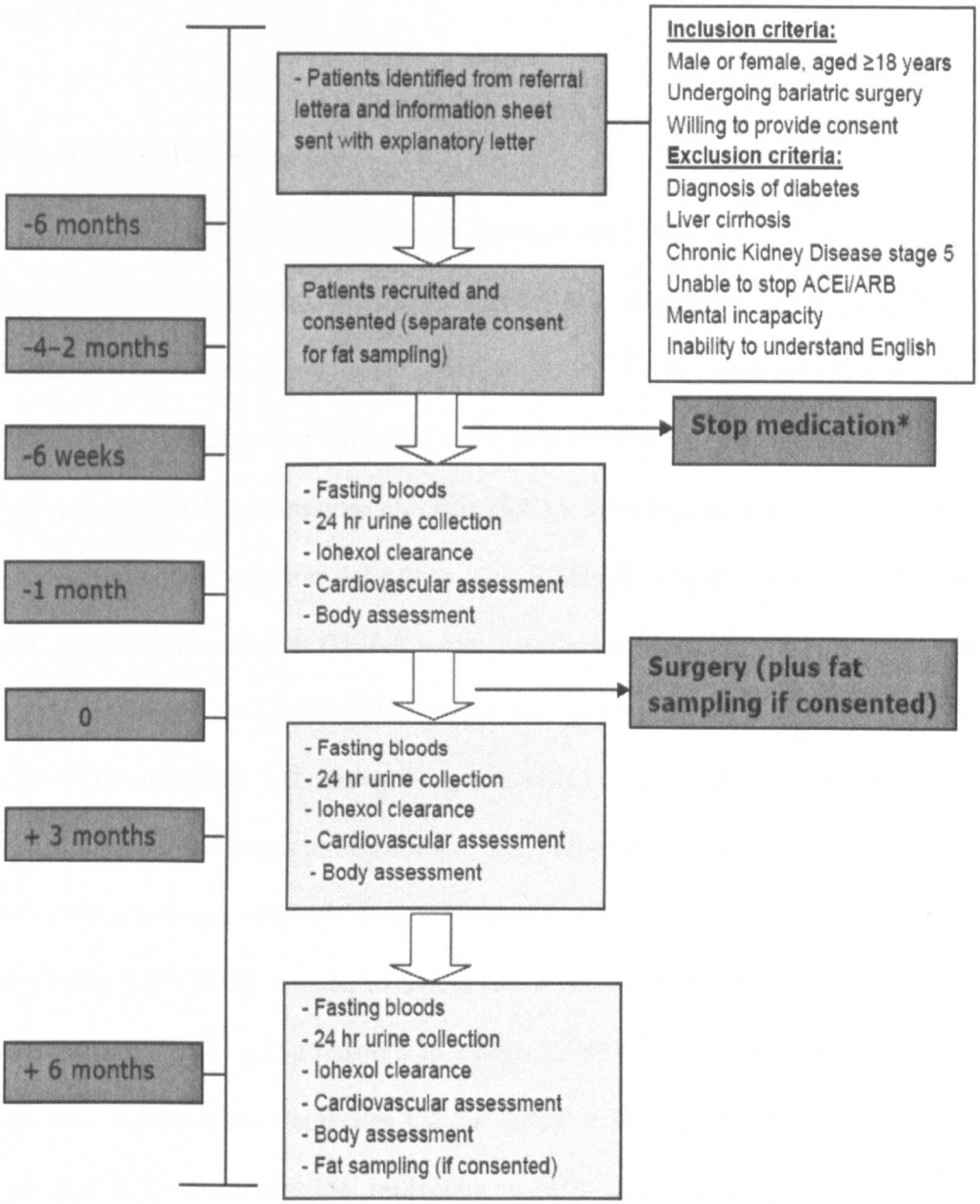

Figure 12 Bariatric study design and timeline.
*** Angiotensin Converting Enzyme inhibitors (ACE) and Angiotensin Receptor Blockers (ARB).**
(The figure *Adapted* from bariatric study protocol).

2.3 Materials

2.3.1 Physiologic salt solutions

Two types of solution were made to conduct each experiment. These including; Physiologic Salt Solution (PSS), which is a solution that intended to act as a medium for maintaining live tissue and blood vessels providing some buffering to maintain the pH of the solution. It is composed of different salts with different concentrations (Table 5). Sodium chloride (NaCL), potassium chloride (KCL), calcium chloride (CaCL), sodium bicarbonate (NaHCO3), calcium dehydrate ($CaCl_2.2H_2O$), D-glucose, and EDTA were purchased from Sigma-Aldrich (USA), while magnesium sulphate (MgSO4.7h2o) and potassium dihydrogen sulphate (KH_2PO_4) were purchased from Fisher Scientific Chemicals (UK) and BDH Laboratory Suppliers (UK) respectively. The second type of physiologic solution is Potassium Physiologic Salt Solution (KPSS), which is the same as PSS, but with potassium chloride concentration (124 mmol/L) substituted for sodium chloride (Table 5.1). KPSS is used to assess the integrity of arteries by their ability to contract by at least 5 mN (0.5g tension) to a high KPSS buffer. It has been determinant that high K+- depolarization activates Ca 2+ influx to induce maintained contraction. Table (5 and 5.1) illustrates the molecular weight and concentration of different chemicals that make both PSS and KPSS that have been used in all experiments. Salts were dissolved completely in double distilled water before adding the calcium chloride. The mixture was bubbled in 5% CO_2 in O_2 for 20 minutes and then the calcium chloride was added to the mixture. The solution was bubbled again for a further 10 minutes until

the pH of the solution reached 7.4. Both solutions were made on the day before the biopsy conduction and stored in cold room (-4c).

Table 5 The composition of PSS.

Chemical	Molecular weight	Concentration (mmol/L)	Amount g/L
Sodium chloride (NaCL)	58.44	119	6.954
Potassium chloride (KCL)	74.56	4.7	0.35
Calcium dehydrate (CaCL2.2H2O)	147.02	2.5	0.368
Mesium sulphate ($MgSO_4$. 7H2O)	246.68	5.5	1.091
Sodium bicarbonate ($NaHCO_3$)	84.01	25	2.1
Potassium phosphate ($KH2PO_4$)	136.1	1.18	0.161
EDTA	372.24	0.027	0.010
D-Glucose	198.77	1.17	0.289

Table 5.1 The composition of KPSS.

Chemical	Molecular weight	Concentration (mmol/L)	Amount g/L
Sodium chloride (NaCL)	58.44	0	0
Potassium chloride (KCL)	74.56	123.7	9.223
Calcium dehydrate (CaCL2.2H2O)	147.02	2.5	0.368
Mesium sulphate ($MgSO_4$. 7H2O)	246.68	1.17	0.289
Sodium bicarbonate ($NaHCO_3$)	84.01	25	2.1
Potassium phosphate ($KH2PO_4$)	136.1	1.18	0.161
EDTA	372.24	0.027	0.010
D-Glucose	198.77	5.5	1.091

2.3.2 Vasocontractile agents

Vasoconstrictor agents that have been investigated in this project included; L-Noradrenalin hydrochloride (NA), endothelin-1 (ET-1), a thromboxane AII mimetic (U46619), angiotensin II (AngII) and vasopressin. L-Noradrenalin hydrochloride (100 mg) and vasopressin acetate salt (1mg) were purchased from Sigma-Aldrich (UK), while ET-1, a human porcine (100 µg), U46619, (1mg) and AngII (5mg) were purchased from Tocris Bioscience (USA). These drugs have been tested to examine vascular function in resistant arteries in *ex vivo* and *in vivo* studies as described in introduction chapter. 1 mg of L-noradrenalin hydrochloride powder (M. Wt = 205.64) was dissolved in 486.3 µL of distilled

water and stock concentration of 10^{-2} M (10 mM) of the solution was prepared. ET-1 10^{-4} M (100 µM) was made and stored as stock concentration by adding ET-1 human porcine 100 µg (M. Wt = 2492) to 401.3 µL distilled water. Stock concentration of U46619 10^{-2} M (10 mM) was made by dissolve 1 mg of U46619 (M. Wt = 350.5) in 285.3 µL of methyl acetate. AngII (M. Wt = 1046.2) was purchased as 5 mg powder and the drug was dissolved in 477.9 µL of distilled water to make 10^{-2} M (10 mM) stock concentration. Vasopressin 10^{-3} M (1 mM) stock concentration was prepared by dissolve 1 mg of vasopressin acetate salt (M. Wt = 1084.2) in 922.3 µL distilled water. Fresh serial dilutions from stock concentration of these vasocontractile agents were prepared on the same day of experiment.

2.3.3 Vasodilator agents

Vasodilators that have been examined in this project were include; acetylcholine (Ach), sodium nitroprusside (SNP) and bradykinin (BK). Ach and SNP (25 g bottles) were purchased from Sigma-Aldrich (UK), while BK (5 mg) was purchased from Tocris Bioscience (USA). These agents have been used to investigate vascular function in *ex vivo* and *in vivo* studies (see introduction chapter). Ach 10^{-2} M (10 mM) stock concentration was made by dissolve 1 mg of Ach powder (M. Wt = 181.66) in 550.4 µL distilled water. SNP 10^{-2} M (10 mM) stock concentration was made by dissolve 1 mg of SNP dehydrate solid powder (M. Wt = 298.0) in 335.5 µL distilled water. The Ach and SNP stock concentrations and fresh serial dilutions were prepared on the same day of experiment. BK 10^{-2} M (10 mM) was made and stored as stock concentration by

dissolve 5 mg of BK white solid powder (M. Wt = 1060.2) in 471.6 µL distilled water. Fresh serial dilutions of BK were prepared on the same day of biopsy. Details of different vasocontractile agents and their stock concentration, dissolves and concentration curves are described in Table 6.

Table 6 Vasoactive drug concentrations.

Drug	Stock Concentration	Dissolved in	Cumulative Concentration
Noradrenaline	10^{-2} M (10 mM)	Distilled water	100 pM - 100 µM
Endothelin-1	10^{-4} M (100 µM)	Distilled water	1 pM - 1 µM
U46619	10^{-2} M (10 mM)	Methyl acetate	1 pM - 1 µM
Angiotensin II	10^{-2} M (10 mM)	Distilled water	1 pM - 1 µM
Vasopressin	10^{-3} M (1 mM)	Distilled water	1 pM - 1 µM
Bradykinin (BK)	10^{-2} M (10 mM)	Distilled water	100 pM - 100 µM
Acetylcholine (Ach)	10^{-2} M (10 mM)	Distilled water	100 pM - 100 µM
Sodium nitroprusside (SNP)	10^{-2} M (10 mM)	Distilled water	100 pM - 100 µM

Abbreviations are: M, molar; mM, millimolar; µM, micromolar; pM, picomolar

2.4 Wire myography

2.4.1 Background

Wire myography is an in vitro technique that is used to investigate the functional responses and vascular reactivity of isolated low-resistance vessels. This technique allows an in vitro measurement of both active and passive properties of small arteries (with internal diameter 60 to 300 µm) under isometric conditions as described before

(Mulvany and Halpern, 1977). In the present study we used multi-chamber wire myography. This model of myography was intended for the study of four isolated vessel ring preparations with diameters ranging from 60 to 1000 μm, which were assessed simultaneously, arteries with diameter larger than 1000 μm were mounted using hocks. Each round chamber is stainless steel (for easy cleaning) and it has separate controlled gas inflow and suction conduits (Figure 13). The sensitive force transducer permits measurements of isometric muscle tension, while the micrometer site allows the setting of vessel diameter. This system is automatically heated to a user-defined set temperature.

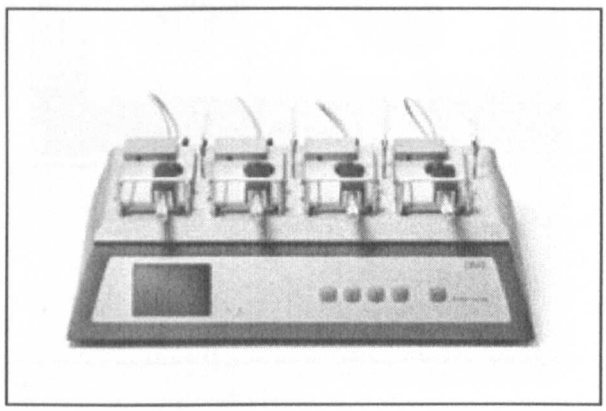

Figure 13 Multi chamber wire myograph system-620 M.

The apparatus composed of; two stainless steel jaws, micrometer, force transducer, ports for oxygenation and solution removal and two mounting wires (30 μm to 40 μm diameters) (Figure 14). In this system, vessel segments are threaded on two small wires (40 μm) as a ring preparation. Each wire is secured to each of the jaws, one of the wires is fastened to a force transducer and the other is attached to micrometer for adjustment of vessel circumference and the application of tension (Figure 15).

This arrangement can helps to measure the following parameters; internal circumference of the vessel, wall tension, isometric force responses to chemical drugs (agonists and antagonists), effective pressure, and media to lumen ratio (M/L) of blood vessels in normalized condition.(Rizzoni et al., 2003).

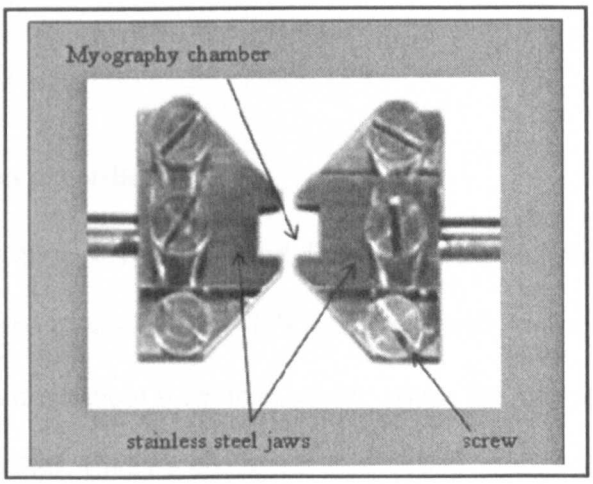

Figure 14 Myography jaws.

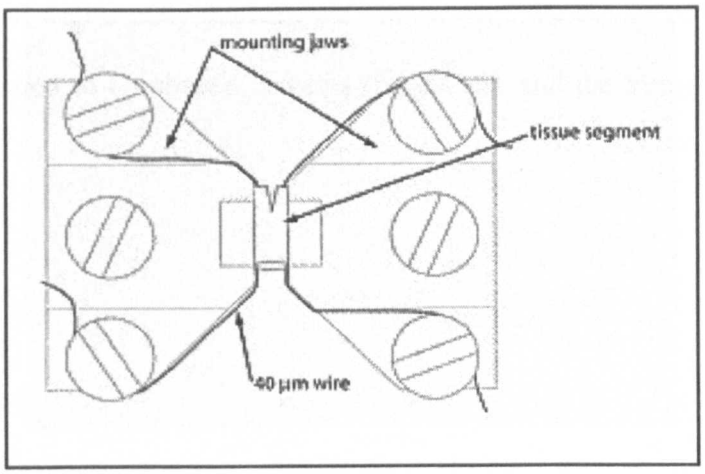

Figure 15 Vessel segments are threaded on 2 stainless steel wires in the myography.

2.4.2 Myography calibration

The myography was calibrated before each experiment. Standard operating procedures, settings and the protocol for myography calibration are attached in the appendix 1 (chapter 6).

2.4.3 Myography

2.4.3.1 Dissection procedure

After the biopsy was obtained following surgical procedure, the sample was immediately placed in cold PSS (4 C°) and transferred to the laboratory. It was placed onto a dissection tray (about 9 cm in diameter) under the microscope. The dissection tray contains ice-cold PSS. Under a dissecting microscope, manipulation and dissection of the vessels was carefully and gently carried out using dissection scissors and Dumont forceps to search for arteries. Some features can helps to differentiate between arteries and vein such as arterial wall usually thicker than venous wall, the branch point of arteries are V-shaped compared to U- shaped in veins (figure 16), and the lumen of the vein usually collapsed.

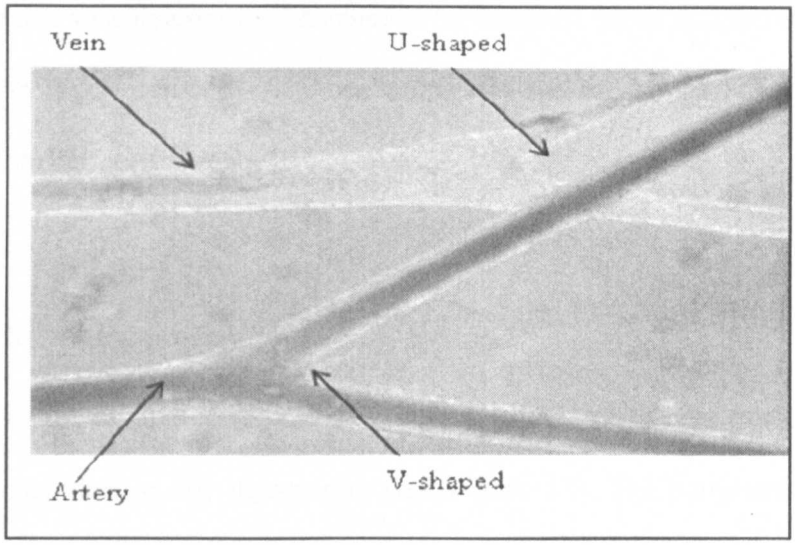

Figure 16 Differences between the artery and the vein

During the dissection procedure, adipose tissue was dissected away to expose the artery and vein to identify them, then the vein was dissected away from the artery using scissors to cut the connective and adipose tissue between them. The fine membrane between the artery and adipose tissue was carefully cut to avoid any damage that might happen to the artery, and then the artery was cut away from the tissue and placed in a fresh beaker of iced PSS. The excess sample was disposed in a container tube to be wasted in disposable bin and not be used for further investigation. The arteries were taken from the beaker and placed back onto a clean dissecting petri dish with fresh PSS. The arteries were further cleaned of all excess tissue using scissors and forceps. Once the artery was cleaned, it was cut into small segments (2-3 mm). Arterial segments were placed into a clean beaker with fresh PSS to be ready for mounting. The duration of vessel dissection, cleaning and cutting into segments were ranged from 1-3 hr depends on

the size and vasculature of subcutaneous fat samples. Some samples especially from HD and controls patients were small containing few and small superficial branches that been difficult to dissect and mount.

2.4.3.2 Mounting procedure

Following dissection and cleaning procedure, vessels are ready for mounting as a ring preparation on the myography (see Figure 17). The equipment that required for mounting procedure includes: a stereomicroscope, fibre light source, two pairs of fine forceps, and pair of fine scissors, small screwdriver and wires.

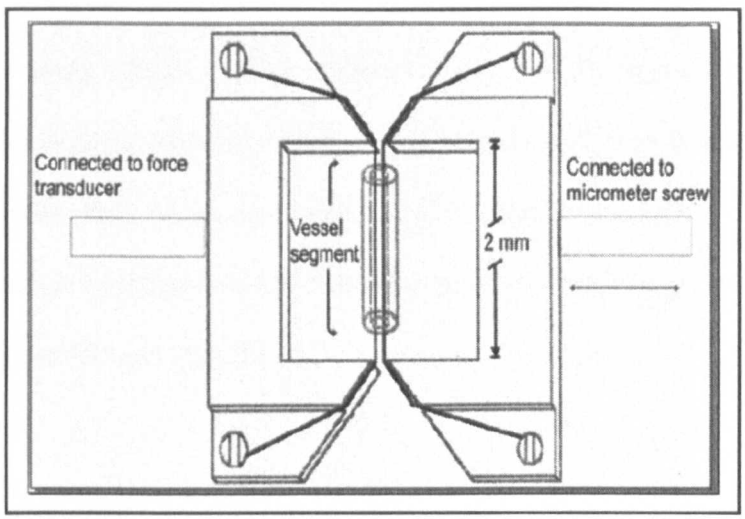

Figure 17 Diagram of small arterial segments mounted in wire myography The figure is adapted from (Wang et al., 2000).

Myography chambers were filled with cold PSS (about 3 ml), and arteries were mounted one by one under the microscope with the micrometer support to my left, and partly both jaws were closes. The first wire was hold with forceps and placed between the two heads of the jaws, when the wire was in position, both jaws were tightening together. The bottom end of the first wire was fixed to the bottom left screw. The vessel segment to be investigated was gently hold and placed close to the proximal end of the wire (I had hold the arteries using the adherent tissue of the vessel by the tips of forceps, then the artery was mounted onto the wire. Both jaws now were released (jaws were opened) and the segment was pulled gently towards the space between both jaws. The top end of the wire was fixed to the top left screw. The second wire was carefully guided into the lumen of the vessel along the first wire. Both jaws were adjusted closely together, and carefully the top end of the second wire was fixed under the top right screw head (at this stage I have looked at the vessel to be not stretched or moved in the longitudinal direction). The lower end of the second wire was fixed under the bottom right screw head. Both wires now were parallel, straight and tight. Once mounted, the myography tail is connected to the interface, with all vessels kept at 37°C in PSS and aerated with 5%CO2 and 95%O2.

2.4.3.3 Normalization

The normalization of microvessels has been investigated by Professor Michael Mulvany who determined that the optimal pre-tension conditions (internal circumference) for microvascular studies is best defined as the size when the vessel is

fully relaxed and under a trans-mural pressure of 100 mmHg (Mulvany and Halpern, 1977). It is a process during which the artery will be measured and stretched to an optimum tension (0.9 L100). The aim of the normalization procedure is to determine the internal circumference at which the vessel would be relaxed and under a transmural pressure of 100 mmHg. A standard normalization procedure can be achieved after three to four stretches of arteries by applying effective transmural pressure exceeding 13.3kpa (100 mmHg) (Hadoke et al., 2000, Schiffrin et al., 2002), then the internal circumference of the vessel will be calculated (Mulvany and Halpern, 1977). In the present study, we aimed to dissect different-sized subcutaneous arteries which categorized into small subcutaneous arteries with diameter < 600 μm and large subcutaneous-sized arteries with diameter > 600 μm. This classification based on the literature, where some studies have classify small arteries with diameters < 600 μm (Paisley et al., 2009, Rizzoni et al., 2012) and the others categorized large arteries with ID > 600 μm (Blacher et al., 2002, Hadoke et al., 2000, Lu and Kassab, 2011). Before the experiment has been conducted, the myography was calibrated one day before the experiment or being calibrated recently during two weeks before the experiment. During the normalization of vessels, all myography jaws were closed together and the normalization process was started by applying the tension on the vessel (stretching of vessels) gradually using the adjusting micrometer until the pressure of 13.3 KPa or more has obtained and the internal circumference (IC) of the vessel was recorded by Lab Chart Program. The internal diameter of the arteries (ID) was then calculated by dividing the internal circumference by π as following:

$$ID\ (\mu m) = IC / \pi \quad (3.12)$$

2.5 General protocol of experiment

Following mounting, and warming process, resistance vessels were continuously bubbled with 5% CO2 and 95% O2 for about 30 minutes, and then arteries were subjected to standard normalization process. The integrity of arteries was tested by their ability to contract by at least 5 mN (0.5g) tensions either to a high potassium physiologic buffer KPSS or contract to a thromboxane A_2 mimetic (U46619). Following normalization procedure, arteries were first exposed twice to a high KPSS solution. After KPSS stimulation in each conducted segments, arteries were washed-out three times (every 2-3 minutes) with PSS solution to allow arteries relaxed back to the baseline. All arteries were then contracted by stimulating them using 50 nmol/L (5×10^{-8} molar) thromboxan A_2 (U46619) with addition of 10 nmol/L U46619 every 3-5 minutes for each artery until the plateau contraction phase had been reached. Once the fixed contraction has reached, then 10 μM of bradykinin (endogenous vasorelaxant) was added to assess whether the endothelium function / intact. Then all arteries were washed-out again three times (every 2-3 minutes) with PSS until the base line have been reached. Vessels that respond to contraction by at least (5 mN) to either KPSS or U46619 were completed the cumulative concentration response curves for different vasoconstrictor agents. Whereas those did not respond to both KPSS and U46619 were discarded.

2.5.1 Vasoconstrictor protocol

Once all arteries have been relaxed back following a wash out of vessels and re-establish the base line (as described in the general protocol), cumulative-concentration

response curves were constructed for the following vasoconstrictors; NA (10^{-10} mol/L - 10^{-4} mol/L), ET-1 (10^{-12} mol/L - 10^{-6} mol/L), U46619 (10^{-12} mol/L - 10^{-6} mol/L), AngII (10^{-12} mol/L - 10^{-6} mol/L), and vasopressin (10^{-12} mol/L - 10^{-6} mol/L). In each myography chamber, stimulation of each arterial segment with specific drug was starting with the lowest concentration of the drug allowing 3-5 minutes per stimulation until the highest concentration. The cumulative concentration of each drug is detailed in Table 6. In each experiment, we dissected different-sized arteries (ranged from 6-12 segments depends on the arteries available in each fat sample). Therefore, we could not test the arterial segments that dissected from each patient to all vasoconstrictors. We planned that the target number of arterial segments in each sized-arteries of HD and obese groups is 6, while in control group is double of those of HD which is 12. However, we did not dissect different-sized arteries from each patient (in all groups), the reason for that because few numbers of samples were small in size and consists only of small arteries and capillaries.

2.5.2 Vasodilators protocol

Separate protocol was carried out for vasodilator drugs. Following a washout period and re-establishment of the baseline (as described in the general protocol), arteries was first contracted using a combination of (100 nmol/L U46619 and 1 nmol/L ET-1). Once the peak steady contraction of arteries had reached, cumulative-concentration response curves were constructed for different vasodilator drugs including BK, Ach, and SNP. Stimulation of each artery with a specific drug was starting from the lowest concentration of the drug to the highest concentration, allowing 3-5 minutes per

stimulation (Table 6). The drug concentration that used for these vasodilators was started from 100 pM (10^{-10} molar) to 100 μM (10^{-4} molar).

Similarly, we could not test the arterial segments of each patient (in all groups) to all vasodilators. For example, some patients were tested only to BK and SNP but not Ach, since in some samples; there were no enough segments to test them to each of vasoactive agent.

2.5.3 In vivo haemodynamic measurements

PWV and blood pressure were measured in a supine position after 10 minutes rest. Brachial artery blood pressure was measured in the non-fistula arm using an oscillometric device (Datex-Ohmeda, GE Healthcare, USA). Carotid-to-femoral arterial PWV was measured using an oscillometric device (Vicorder, Skidmore Medical Ltd, Bristol, UK) as previously described (Pucci et al., 2013). A carotid pressure cuff is applied over the neck to detect the right carotid artery and a pressure cuff is placed around the proximal right upper thigh to detect the femoral artery. The distance from the suprasternal notch to the middle of the thigh cuff was measured and entered into a PC running the Vicorder software. The cuffs are simultaneously inflated to a low pressure 40-60 mm Hg and signal from each cuff is analysed to derive arterial transit time. PWV is calculated by the software by dividing arterial transit time by measured distance. The mean of 2 measurements was recorded.

2.6 Statistical methods

The statistical methodology varied depending on the individual analysis used. All statistical analysis was undertook by the author using software package prism 5 for windows (GraphPad Prism-5 software Inc, San Diego, USA) and SPSS V12 (SPSS Inc, Chicago, USA). Some complex statistical analysis was advised by an independent statistician employed by Nottingham University and by our colleagues conducting cooling study. The power calculation is based on detecting a significant change in increased vasocontractility in response to vasoconstrictors and / or decreased vasorelaxation in response to endothelium-dependent vasodilators from the baseline. A sample size would appear to be sufficient to detect a significant difference between the groups. The sample size is also similar to the previous studies that using similar technique (Morris et al., 2001, Luksha et al., 2011). However, not all the arterial segments that dissected from the fat samples were included in the analysis, arteries those did not respond to KPSS and / or U46619 were discarded.

Once each experiment has completed, increased in contraction for each drug stimulation was recorded from the Lab Chart, then all data was transferred to the excel file. Increased in milliNewtons (mN) from the base line for each agent in each artery (R_{max}) was calculated as following:

R_{max} = contraction per stimulation (mN) - base line (mN)

However, the percentage relaxation from the preconstrictor state (R_{max}) for each vasodilator drug was calculated as following:

Δ = preconstriction stable contraction (mN) - base line (mN)

R = relaxation per stimulation (mN)

% relaxation = preconstriction stable contraction (mN) – R / Δ × 100

All continuous variables were tested for normality using their histograms and normality tests. Contractile responses were expressed as an increased contraction in milliNewtons (mN) above the isometric baseline and related to vessel segment length (mm), which described the developed wall tension (mN/mm). The maximum contractile response (R_{max}) for each agent in all groups were calculated and drug potency (EC_{50}) value (defined as the concentration of drug required to produce 50% of the maximum response), while the response for vasodilators BK, Ach, and SNP was expressed as maximum % relaxation from the preconstriction state. All data were expressed as mean with standard error of the mean (mean ± SEM). The differences were tested at multiple dose-response time points for all data. The comparison between HD and control groups, obese and control groups was carried out using students *t*-test for unpaired observations and the differences were considered significant when $P < 0.05$. PWV and blood pressure data of each HD and obese patient were correlated with the response of each vessel size to each vasoconstrictor and vasodilator drug. PWV data was expressed as mean ± SEM and the analysis was undertaken using either SPSS V12 and / or GraphPad Prism-5 software. The comparison of *in vivo* haemodynamic measurements of PWV and blood pressure with *ex vivo* responses of all sized arteries to different stimuli was undertaken using spearman's rho correlation coefficient. The correlation was considered significant at $*P = 0.05$, and $**P = 0.01$ levels (2-tailed). Arterial segments that dissected from obese patients at six month following bariatric surgery were experimented to the same vasoactive agents that experimented in obese patients at baseline. Differences in the arterial responses of obese patients before and after surgery were determined either by

paired *t*-test or repeated measures of two-way analysis of variance (ANOVA) with Bonferroni's post tests for multiple comparisons.

Chapter 3
Results of Haemodialysis patients

Chapter 3: The effect of HD on vascular function in isolated subcutaneous arteries.

3.1 Introduction

HD patients are characterised by a wide range of both structural and functional abnormalities of the cardiovascular system. These include small vessel vascular structure and function. There is strong emerging evidence that the HD procedure itself causes significant systemic circulatory stress (Burton et al., 2009). This circulatory stress interacts with complex haemodynamic factors causing perfusion anomalies that accelerate end organ damage in a wide range of vulnerable vascular beds (McIntyre, 2010). Fixed structural abnormalities of the vascular tree develop during the lifetime of a progressive CKD patient. These include coronary arterial calcification and other drivers of reduced arterial compliance. These changes are the result of fixed vascular structural change but importantly additional functional microvascular abnormalities are potentially superimposed onto these processes (Sigrist and McIntyre, 2008). These may be more important in determining the propensity to multi organ based demand ischaemia.

It is unclear to what degree the observed abnormalities of microvascular function are related to the uremic milieu and exposure of relatively normal vasculature to a variety of functionally modifying factors, and to what degree there are intrinsic defects in pressor response in these vessels. Vascular endothelium plays an important role in the regulation of vascular function and tone through production and release of a wide range of vasoactive substances including endothelin, nitric oxide (NO), and thromboxane A2 (Stankevicius et al., 2003)

Arterial hyperreactivity to some vasoconstrictors such as noreadrenalin (NA) and endothelin-1 (ET-1) has been shown in isolated subcutaneous arteries from uremic CKD patients (Morris et al., 2001). However recent studies observed similar contractile response to NA and ET-1 between the isolated arteries of ESRD (patients starting PD) and controls (Luksha et al., 2012). In fact, there are no data available about the effect of HD on the isolated arterial response to different vasoconstrictors.

Vascular endothelium plays a crucial role in the regulation and control of vascular tone through release of various vasoactive substances. Vasorelaxation is mediated by the release of endothelium-derived relaxants including nitric oxide (NO) and prostaglandin I2 (Stankevicius et al., 2003). Both substances are known as potent endothelium vasodilators (Tormakangas et al., 2006). Vascular endothelium would seem a reasonable target for different factors that ends by alterations in their function and structure.

Different mechanisms have been proposed to cause endothelial dysfunction in HD patients, which is largely due to a defect in the endothelial NO bioavailability (Baylis, 2008). Several uremia-associated factors (discussed in details in section 1.4.3) including oxidative stress and reactive oxygen species (Hasdan et al., 2002, Ferraro et al., 2003), homocysteine (Bostom and Culleton, 1999) and plasma ADMA (Vallance et al., 1992) are found in high levels in the plasma of HD patients and may also contribute to endothelial dysfunction. However, the exact underlying mechanisms involved remain unclear. Endothelial dysfunction has been reported to occur in various stages of CKD even in the early stages (Go et al., 2004). This may explain the accelerated rate of cardiovascular diseases associated with renal impairment.

In uremic patients, some *in vivo* and *ex vivo* studies have suggested that functional alterations in blood vessels are primarily based on impaired NO bioavailability resulting in impaired endothelium-dependent vasodilatation. Such defect has been observed in other conditions, namely hypertension (Taddei and Salvetti, 2002), hyperlipidaemia (Warnholtz et al., 2001) and diabetes (van Etten et al., 2002). Early *in vivo* studies have demonstrated that NO-dependent flow-mediated vasodilation is decreased in HD patients (van Guldener et al., 1998, Holvoet et al., 1996). Such impairment was also observed *in* peripheral vessels of uremic CKD (pre-dialysis and post peritoneal dialysis (PD) patients) using forearm plethysmography (Morris et al., 2000, Annuk et al., 2001) .

Most of the limited number of initial *ex vivo* studies to date (Morris et al., 2001, Luksha et al., 2011, Luksha et al., 2012), have examined small isolated arteries (using myography) removed from uremic patients selected to be undergoing a surgical procedure relating to their ongoing care needs (PD catheter insertion and renal transplant) and challenged with a limited suite of vasoactive agents (comprising of NA, ET-1, Ach, and SNP).

Some reports have shown that the HD procedure itself may effects on endothelial function. Miyazaki *et al.* has demonstrated that a single haemodialysis session significantly blunted endothelium-dependent vasodilatation measured by non-invasive flow-mediated dilation (FMD) in the brachial artery (Miyazaki et al., 2000). Moreover, the HD procedure can acutely impairs endothelial function assessed by FMD in children's brachial artery (Lilien et al., 2005). The Lilien's study observed decreased in FMD in HD children compared to healthy control children. It was also observed that HD

induces further decreases in FMD when measured during an HD session. Therefore, it remains unknown whether the HD *per se* causes endothelial dysfunction. However, the underlying mechanism may be multifactorial.

Vascular remodelling and arterial stiffening in uremic patients have been identified in epidemiological studies as an independent risk factors that contribute to increased cardiovascular mortality in ESRD patients (Guerin et al., 2005, London, 2000). Such vascular alterations (increased media-to-lumen ratio and vascular smooth muscle hypertrophy), are frequently found in patients with hypertension (Mulvany, 2002), diabetes mellitus (Hadoke et al., 2000), obesity (Rizzoni et al., 2012), and sever renovascular hypertension (Brunner et al., 2005). These vascular changes develop rapidly in uremic CKD patients and believed to be responsible for increased incidence of cardiovascular risks and all-cause mortality including ischemic heart disease, left ventricular hypertrophy, congestive heart failure, and sudden death, particularly those receiving dialysis therapy (Blacher et al., 2001, Guerin et al., 2008). In addition, vascular calcification and associated cardiovascular dysfunction have been demonstrated in patients with CKD stage 4, HD, and patients on PD with significant differences between all groups (Sigrist et al., 2006). This represents a fixed vascular structural change onto which microvascular abnormalities are potentially superimposed (Sigrist and McIntyre, 2008).

Measurement of pulse wave velocity (PWV) is another helpful method to assess the vascular function. Large vessel compliance (arterial stiffness), as measured by increased PWV, is higher in patients on dialysis (and with non-dialysis dependant CKD) compared with the general population (Shinohara et al., 2004). Increased PWV is associated with

elevated cardiovascular morbidity and mortality risks in patients with CKD stage 5 (Blacher et al., 1999). A strong association has been observed between PWV and abdominal aorta calcification in HD patients (Raggi and Bellasi, 2007). Arterial stiffness was also demonstrated in HD patients undergoing transplantation compared with non-dialysis controls (Chung et al., 2010). This change in compliance is partially due to material alterations in the conduit arteries, however it is also blood pressure (BP) and endothelial dysfunction related, and modifiable by long-term treatment with angiotensin converting enzyme inhibitors (ACEIs) in CKD (Agata et al., 2004) and non-uremic hypertensives (Ong et al., 2011). It remains uncertain whether the isolated large arteries of uremic patients had similar relaxation response to small arteries.

3.2 Aims

In view of this background literature, we hypothesised that uremia may induces microvascular dysfunction in HD patients characteristically through enhanced vasocontractility and impaired endothelium-dependent vasodilatation of isolated different-sized arteries of HD patients in response to various stimuli.

This defect was observed previously in isolated arteries (small sizes) obtained from uremic CKD patients (non HD), investigating a limited suit of vasoactive stimuli. Therefore, the current project is novel in that, firstly we extend this principle of investigation to include different-sized arteries isolated from a homogenous uremic group of patients who purely on HD and testing them to various stimuli including NA, ET-1, U46619, AngII, vasopressin, BK, Ach, and SNP, with comparison to appropriate matched non uremic control arteries, isolated from an identical sampling site, using wire

myography. Secondly, to establish whether these vessels exhibit impaired endothelial-dependent vasodilation, as previously suggested (Morris et al., 2001, Luksha et al., 2011, Luksha et al., 2012). However, it remains uncertain whether the isolated large arteries of HD patients had similar contractile and relaxation responses to small arteries. The study also intended to characterize the relation between *ex vivo* vascular changes and *in vivo* parameters, correlating *ex vivo* myography data with *in vivo* results of pulse wave velocity (PWV) and blood pressure (BP) for each patient.

3.3 Materials and Methods

3.3.1 Participants and subcutaneous tissue biopsies

Subcutaneous fat samples were obtained from 11 HD patients (8 males; average age 62.3±15.6), who were within the first 90 days of starting dialysis. Control fat samples were obtained from 26 appropriately consented healthy volunteers (24 males; average age 63.9±11.17) without documented renal disease, who underwent either elective laparoscopic or abdominal surgery (elective hernia repair). The ethical approval was sought and granted by the Derbyshire Research Ethics Committee and the local NHS R&D department. Informed written consent was obtained in accordance with Good Clinical Practice guidelines according to the principles expressed in the Declaration of Helsinki. Different-sized arteries (small < 600 μm and large > 600 μm) were dissected from each sample and mounted as a ring preparation on wire myography. Details on

obtaining, transfer of fat samples for experimental study and the techniques of arterial dissection and mounting on wire myography are described in section 2.4.3.

3.3.2 Preparation of vasoactive agents

Vasoconstrictors and vasodilators that have been used to investigate the vascular function in isolated arteries of HD patients were include NA, ET-1, AngII, U46619, vasopressin, BK, Ach, and SNP. Purchases and preparation of these agents and other physiologic salt solutions are described in details in section 2.3.2 and 2.3.3

3.3.3 Protocol of experiment

Following mounting, arteries were kept for 15-30 minutes to be acclimatized and then subjected to normalization process. The general protocol of experiment is detailed in section 2.5. The internal diameter of arteries was calculated as described in section 2.4.3. For vasoconstrictor protocol, following a wash out of vessels and re-establish the base line, cumulative-concentration response curves were constructed for the following vasoconstrictors; NA (10^{-10} mol/L - 10^{-4} mol/L), ET-1 (10^{-12} mol/L - 10^{-6} mol/L), U46619 (10^{-12} mol/L - 10^{-6} mol/L), AngII (10^{-12} mol/L - 10^{-6} mol/L), and vasopressin (10^{-12} mol/L - 10^{-6} mol/L) starting with the lowest concentration of the drug allowing 3-5 minutes per stimulation as detailed in chapter 2 and Table 6.

For vasodilator protocol, separate protocol was carried out for vasodilators. Following washout period and re-establishment of the baseline (as described before in the general protocol), arteries were first contracted by stimulating them to a combination

of (100 nmol/L U46619 and 1 nmol/L ET-1). Once the peak steady contraction had reached, cumulative-concentration response curves for different vasodilators including; bradykinin (BK), acetylcholine (Ach), and sodium nitroprusside (SNP) were constructed starting with the lowest concentration of the drug to the highest concentration, which was 100 pM (10^{-10} molar) to 100 μM (10^{-4} molar) for all vasodilators allowing 3-5 minutes per stimulation (as described in section 2.5.2 and Table 6). There were some differences in the baseline relaxation starting points between both groups (but not significant), however, we used a combination of low concentration of (100 nmol/L U46619 and 1 nmol/L ET-1) for preconstriction until got a steady contraction before starting vasorelaxation response curves.

3.3.4 In vivo haemodynamic measurements

Carotid-to-femoral arterial PWV was measured using an oscillometric device (Vicorder, Skidmore Medical Ltd, Bristol, UK). Brachial artery blood pressure was measured in the non-fistula arm using an oscillometric device (Datex-Ohmeda, GE Healthcare, USA). PWV is calculated by the software by dividing arterial transit time by measured distance. The mean of 2 measurements was recorded (as described in section 2.5.3).

3.3.5 Statistical analysis

Contractile responses were expressed as an increased contraction in milliNewtons (mN) above the isometric baseline. Details on Lab Chart data calculation are described in

section 2.6. The maximum contractile response (R_{max}) for each agent in all groups were calculated and EC_{50} value (defined as the concentration of drug required to produce 50% of the maximum response), while the response for vasodilators BK, Ach, and SNP was expressed as maximum % of relaxation from the preconstriction state. All data were expressed as mean with standard error of the mean (mean ± SEM) and the analysis was using GraphPad Prism-5 software. All continuous variables were tested for normality using their histograms and normality tests. The differences were tested at multiple dose-response time points for all data The comparison between all groups was performed by using students *t*-test and differences were considered significant when $P < 0.05$. We correlated the PWV and blood pressure data of each patient with each vasoactive drug in each vessel size. All PWV and blood pressure data was expressed as mean ± SEM. Relationships between in vivo and ex vivo data was determined using spearman's rho correlation coefficient, which considered significant at $*P = 0.05$, and $**P = 0.01$ levels (2-tailed). All statistical analysis was undertaken using GraphPad Prism-5 software and SPSS V12.

Table 7 Characteristics of HD and control patients

Characteristic	HD (N=11)	Control (N=26)	*P* value
Age (*years*)	62.3 ± 15.6	53.9 ± 11.2	0.936
Sex	M = 8, F = 3	M = 24, F = 2	NS
Systolic BP (*mm Hg*)	141.6 ± 10.6	134.2 ± 16.5	0.781
Diastolic BP (*mm Hg*)	78.4 ± 10.5	80.6 ± 9.5	0.892
MAP	110.05 ± 8.1	98.7 ± 7.1	0.356
Creatinine (*µmol L*)	NA	84.8 ± 5.9	NS
eGFR (*ml min 1.73 m²*)	NA	84.4 ± 5.2	NS
BMI	27.5 ± 2.8	26.2 ± 2.3	0.746
Smoker (*n*)	3	10	NS
IHD	2	1	-
CVA	0	1	-

Abbreviation: N, number of patients; eGFR, estimated glomerular filtration rate; BP, blood pressure; MAP, main arterial pressure; BMI, body mass index; IHD, ischemic heart disease; CVA, cerebrovascular accident; NA, non-applicable; NS, non-significant.

3.4 Results

The background characteristics of HD patients and control group are described in (Table 7). There was no difference in the important characteristics of HD and non-HD patients other than uremic status Age and sex were similar between both groups, and

there was no statistically significant difference in systolic and diastolic blood pressure between HD and controls.

3.4.1 Vascular size

The total number of all small vessels investigated per vasoactive agents was 48 in HD (08 arteries were discarded) and 72 in control groups (11 arteries were discarded) (Table 8), whereas in large arteries, it was 42 in HD (03 arteries were discarded) and 65 in controls (07 arteries were discarded) (Table 9). The diameter of small vessels in HD patients was ranged between 192 μm – 580 μm, while in normal controls was between 220 μm – 575 μm, whereas the size of large vessels was (612 μm – 922 μm in HD and 610 μm – 886 μm in controls). In vessels investigated per vasocontractile agent, the average internal diameter (ID) was similar between HD and control groups, it was 472.6 ± 42.4 μm in HD (n = 30) *versus* 449.3 ± 78.7 μm in control (n = 45), $P = 0.821$. Similarly, there was no significant difference in the size of large vessels between both groups, it was 738.4 ± 96.2 μm (n = 27) *versus* 716.5 ± 88.6 μm (n = 40) in HD and controls respectively, $P = 0.869$.

In vessels that investigated per vasodilator drugs, there was a similarity in the vessel size between HD and controls. The average ID of all small arteries was 478 ± 81.2 μm in HD (n = 18), and 512.3 ± 44.7 μm in controls (n = 27), $P = 0.691$. While in large arteries, the average ID was 761.5 ± 62.3 μm (n = 15) and 744.8 ±91.2 μm (n = 25) in HD and controls respectively, $P = 0.896$.

Table 8 The internal diameter of small arteries size in HD and controls
All small arteries size of the used experiments testing the effects of HD on all vasoactive agents.

	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenalin Number L°	6 436.6 ± 68.1	10 401.3 ± 65.1	0.728
Endothelin-1 Number L°	6 425.3 ± 82.1	9 471.1 ± 53.6	0.633
U4 Number L°	6 381.1 ± 81.4	9 424.4 ± 78.1	0.716
Angiotensi II Number L°	6 458.8 ± 29.2	8 435.5 ± 39.2	0.686
Vasopressin Number L°	6 472.3 ± 32.9	9 453.7 ± 67.3	0.835
Bradykinin Number L°	6 537.5 ± 47.8	9 521.2 ± 46.6	0.817
Acetylecholine Number L°	6 482.6 ± 44.6	9 504.2 ± 60.1	0.797
Sodium nitruprosside Number L°	6 474.8 ± 41.8	9 519.4 ± 41.8	0.482

Abbreviation: n, number of patients; HD, haemodialysis; Number, is the number of small arteries used for concentration-response curves to all vasoactive agents. L_0 is the normalized internal diameter of arteries; U4, thromboxane A2. Data are expressed mean ± SEM and the comparison is by t-test.

Table 9 The internal diameter of large arteries size in HD and controls
All small arteries size of the used experiments testing the effects of HD on all vasoactive agents.

	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenaline			
Number	6	9	
L_o	735.8 ± 78.3	683.8 ± 42.2	0.532
Endothelin-1			
Number	5	9	
L_o	770.4 ± 80.5	749.2 ± 92.6	0.871
U4			
Number	6	8	
L_o	733.8 ± 84.6	702.1 ± 42.8	0.724
Angiotensi II			
Number	5	7	
L_o	676.8 ± 54.4	693.1 ± 66.2	0.861
Vasopressin			
Number	5	7	
L_o	724.1 ± 68.1	681.1 ± 57.5	0.635
Bradykinin			
Number	5	8	
L_o	722.6 ± 63.8	715.2 ± 41.1	0.922
Acetylecholine			
Number	5	8	
L_o	785.1 ± 76.7	710.8 ± 55.9	0.447
Sodium nitruprosside			
Number	5	9	
L_o	720.2 ± 77.7	729.1 ± 79.5	0.944

Abbreviations: n, number of patients; HD, haemodialysis; Number, is the number of large arteries used for concentration-response curves to all vasoactive agents. L_o is the normalized internal diameter of arteries; U4, thromboxane A2. Data are expressed as mean ± SEM, and the comparison is by t-test.

3.4.2 Vascular function

3.4.2.1 Effects of HD on the KPSS contractile response in different-sized vessels

The contractile response to KPSS in all size arteries (in vasoconstrictor and vasodilator experiments) was measured. In all small-sized arteries, the difference in the maximum KPSS contraction between both groups was not statistically significant. This response was 9.1 ± 5.6 mN in HD (n = 48), and 6.9 ± 1.8 mN in controls (n = 72), P = 0.663. Similarly, there was no significant difference in the average of maximum KPSS between both groups in all large arteries, it was 14.1 ± 4.2 mN in HD (n = 42), and 13.0 ± 6.5 mN in controls (n = 63), P = 0.899. However, from the bar chart (Figure 17), we observed that, largest diameter arteries had higher maximal contractile responses to KPSS in each group.

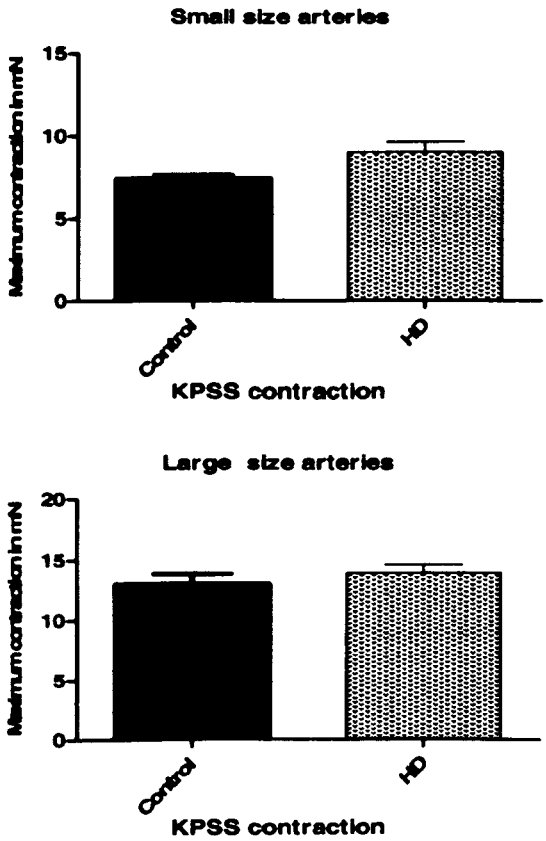

Figure 18 The maximum KPSS response in different-sized vessels of HD and controls

Abbreviations: KPSS, Potassium physiologic salt solution; HD, haemodialysis

3.4.2.2 Effects of HD on the contractile response of different-sized arteries to different vasoconstrictors

In small vessels, results for the maximum contractile response of small arteries to all vasocontractile agents (R_{max}), and the potency of drug (EC_{50}) are described in Table 10. The maximum contractile responses of small vessels (mN) to ET-1, U46619, AngII

and vasopressin were greater in HD compared to controls, being highly significant in U46619, AngII, and vasopressin ($P < 0.0001$ in each drug) as showed in Figures 19 C, D, and E. In NA response curve, the contractile response was similar between the two groups (Table 10 and Figure 19 A). Our results also showed that, the highest contractile response in HD group was in response to vasopressin (15.1 mN ± 0.3), whereas in controls was in response to U46619 (9.3 mN ± 0.3). The lowest contractile response in HD group was in response to NA (5.7 mN ± 0.1), while in control group was in response to AngII (4.8 mN ± 0.2). The potency of each vasocontractile agent (NA, ET-1, U4, AngII and vasopressin) was similar between both groups (no significant difference in the EC_{50} between HD and controls in each type of drug (see Table 10 and Figure 19).

In large vessels, details for all vasoconstrictor results of large arteries in both groups are showed in (Table 11 and Figure 20). Similarly, the contractile response to different vasoconstrictors in large arteries was greater in HD than controls. This contraction was highly significant in response to U46619, Ang II, and vasopressin as showed in Figures 20 C, D, and E. In NA and ET-1, the response was higher in HD compared to controls with ($P = 0.022$ and $P = 0.082$ respectively). Unlike small arteries, large arteries of HD patients require significantly less concentration of U46619 and vasopressin than controls to give 50 % of maximum contraction (see Table 11, and Figures 20 C and E). From these response curves, we observed that the potency of NA, End-1, and Ang II in large arteries was similar in both groups (no statistical difference between the two groups). Moreover, we observed that the response of isolated large vessels of HD patients to vasopressin was the highest in the group of vasoconstrictors (16.8 mN ± 0.4), whereas in control group the highest vasocontraction was in response to

U46619 (12.4 mN ± 0.4). The lowest contractile response in both groups was in response to AngII (11.9 ± 0.3 mN in HD group and 6 ± 0.2 mN in controls).

Table 10 The maximum response of small arteries and Potency of all vasoconstrictors in HD and controls

R_{max}	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenaline	5.7 ± 0.1 mN	5.6 ± 0.1 mN	0.6875
Endothelin-1	11.1 ± 0.2 mN	8.9 ± 0.6 mN	0.0155
U46619	14.1 ± 0.3 mN	9.3 ± 0.3 mN	< 0.0001
Angiotensin II	7.3 ± 0.2 mN	4.8 ± 0.2 mN	< 0.0001
Vasopressin	15.1 ± 0.3 mN	7.0 ± 0.4 mN	< 0.0001
EC_{50}			
Noradrenaline	7.7 ± 0.1	8.2 ± 0.1	0.085
Endothelin-1	9.9 ± 0.1	10.3 ± 0.1	0.051
U46619	8.3 ± 0.0	8.3 ± 0.1	0.951
Angiotensin II	9.2 ± 0.0	9.4 ± 0.1	0.316
Vasopressin	10.5 ± 0.1	10.1 ± 0.2	0.224

Abbreviations; n, number of patients; R_{max}, maximum response in mN; EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}); HD, haemodialysis; U46619, thromboxane A2. Data are mean ± SEM, and comparison is by t-test.

Table 11 The maximum response of large arteries and Potency to all vasoconstrictors in HD and controls

R_{max}	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenaline	13.1 ± 0.3 mN	11.1 ± 0.5 mN	0.022
Endothelin-1	12.7 ± 0.3 mN	11.7 ± 0.3 mN	0.082
U46619	16.0 ± 0.4 mN	12.4 ± 0.4 mN	0.0002
Angiotensin II	11.9 ± 0.3 mN	6.0 ± 0.2 mN	< 0.0001
Vasopressin	16.8 ± 0.4 mN	11.3 ± 0.5 mN	< 0.0001
EC_{50}			
Noradrenalin	7.4 ± 0.3	7.7 ± 0.1	0.091
Endothelin-1	9.4 ± 0.1	9.8 ± 0.2	0.061
U46619	9.5 ± 0.4	8.9 ± 0.4	0.008
Angiotensin II	9.6 ± 0.1	9.6 ± 0.6	0.861
Vasopressin	10.3 ± 0.1	9.3 ± 0.1	0.001

Abbreviations; n, number of patients; R_{max}, maximum response in mN; EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}); HD, haemodialysis; U46619, thromboxane A2. Data are mean ± SEM, and comparison is by t-test.

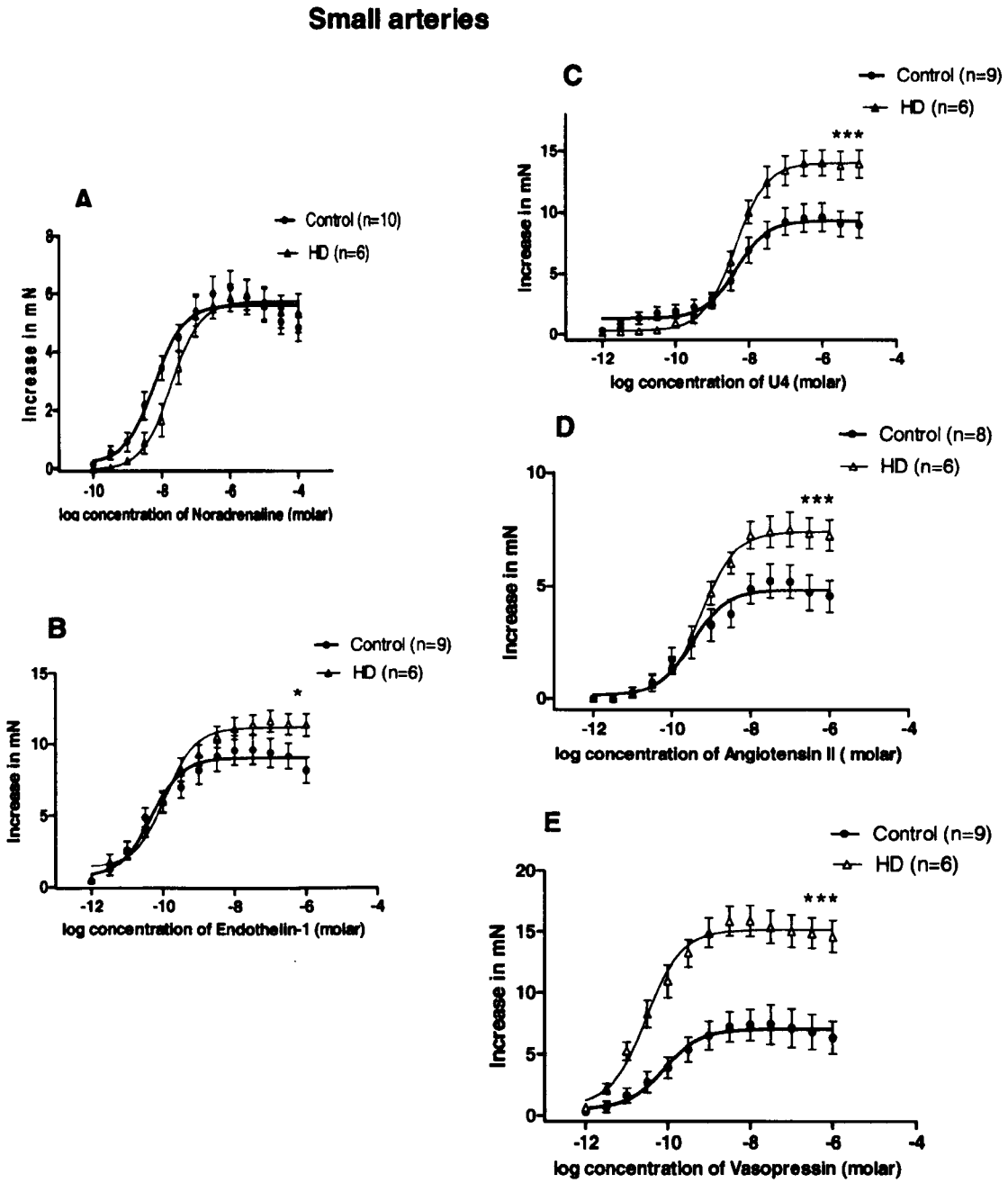

Figure 19 Concentration-response curves for vasoconstrictors in small vessels of HD and controls

Data are expressed as mean ± SEM. NA (A), ET-1 (B), U4 (thromboxane A2) (C), AngII (D) and vasopressin (E). The differences were tested at multiple dose-response time points. Comparison is by students *t*-test, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

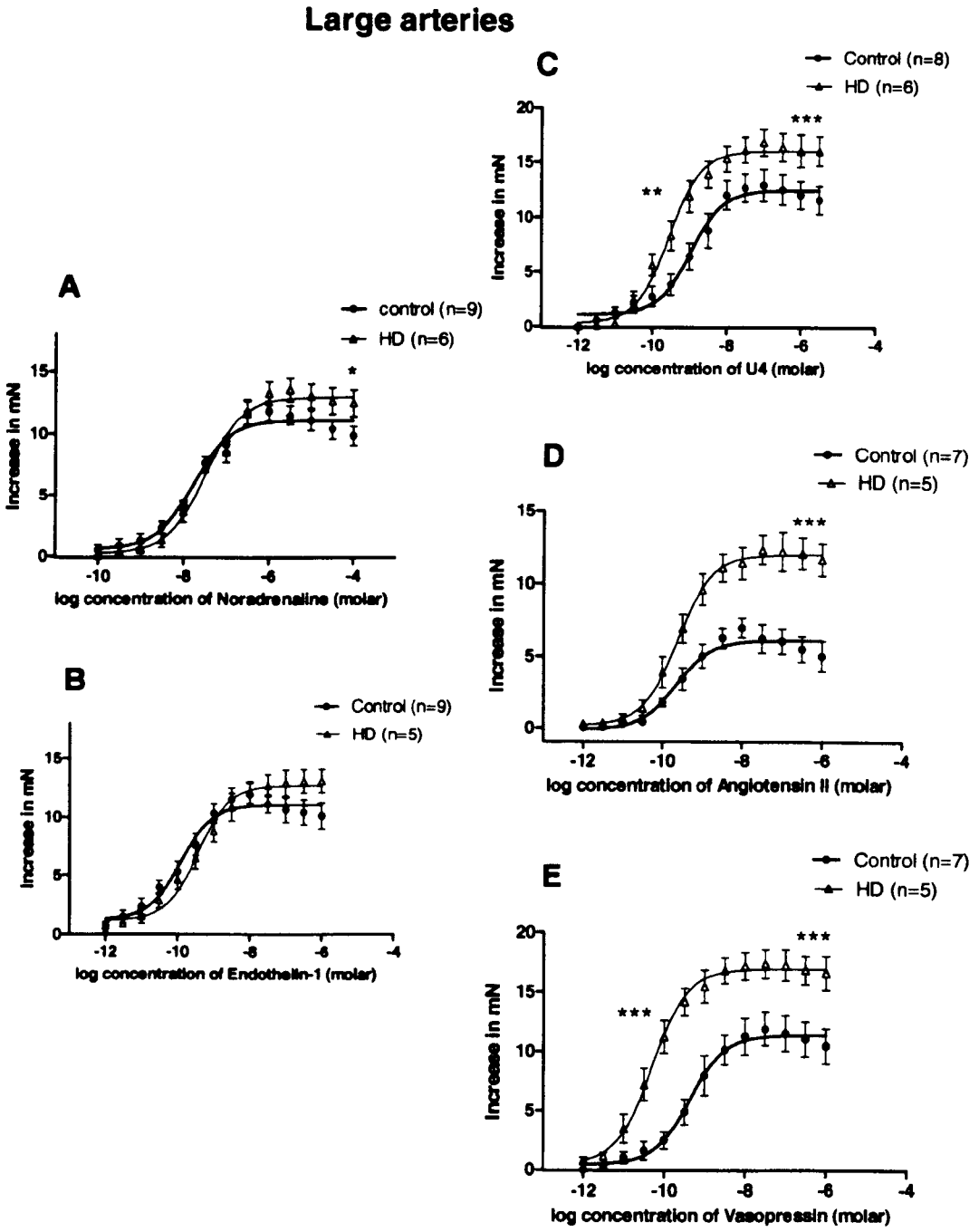

Figure 20 Concentration-response curves for vasoconstrictors in large vessels of HD and controls

Data are expressed as mean ± SEM. NA (A), ET-1 (B), U4 (thromboxane A2). (C), AngII (D) and vasopressin (E). The differences were tested at multiple dose-response time points. Comparison is by students *t*-test, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

3.4.2.3 Effects of HD on the contractile response of different-sized arteries to vasoconstrictors-KPSS

The contractile response of small and large vessels to different vasoconstrictors was also expressed as a percentage of maximum KPSS-induced contraction in an effort to normalise the data. In HD patients the percentage maximum contractile response of small arteries to all vasoconstrictors (NA, ET-1, U46619, AngII, and vasopressin) was significantly higher compared to controls (Table 12). In both groups the highest % of maximum contractile response of small vessels was in response to U46619 (144.3 ± 3.3 % KPSS in HD and 120.9 ± 3.1 % KPSS in controls), while the lowest % of contraction was in response to AngII (78.9 ± 2.1 % KPSS in HD and 65.3 ± 2.1 % KPSS in controls). However, the potency of all vasoconstrictors was similar in both groups.

In large vessels, the % of maximum KPSS-induced contraction to all vasoconstrictors was significantly greater in HD group compared to controls (Table 13). In HD and controls, the contractile response to vasopressin was the highest in the group (135.8.0 ± 3.1 % KPSS in HD, and 113.2 ± 3.7 % KPSS in controls, $P < 0.05$), whereas the lowest % was in response to AngII (85.3 ± 2.5 % KPSS and 74.6 ± 1.9 % KPSS in HD and controls respectively, $P < 0.05$). However, vasopressin and U46619 was less potent in large arteries of control group compared to HD patients. The potency of vasopressin was (9.3 ± 0.1 in control *versus* 10.2 ± 0.1 in HD, $P < 0.001$), while in U46619 was (8.9 ± 0.1 in controls *versus* 9.6 ± 0.1 in HD, $P < 0.05$). The EC_{50} of NA, ET-1, and AngII was similar between the two groups.

Table 12 The % maximum KPSS –induced contraction in small arteries of HD and controls

% Max KPSS	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenalin	108.9 ± 2.5 %	89.9 ± 2.8 %	0.0004
Endothelin-1	134.5 ± 4.5 %	102.5 ± 2.7 %	< 0.0001
U46619	144.3 ± 3.3 %	120.9 ± 3.1 %	0.0003
Angiotensin II	78.9 ± 2.1 %	65.3 ± 2.1 %	0.001
Vasopressin	137.0 ± 3.5 %	90.6 ± 3.1 %	< 0.0001

Abbreviations; % Max KPSS, percentage of maximum KPSS contraction; Data are mean ± SEM, and comparison is by t-test.

Table 13 The % maximum KPSS-induced contraction in large arteries of HD and controls

% Max KPSS	HD *(n = 11)*	Control *(n = 26)*	*P* value
Noradrenalin	107.4 ± 2.9 %	80.4 ± 2.5 %	< 0.0001
Endothelin-1	102.9 ± 3.1 %	96.8 ± 2.7 %	0.007
U46619	118.2 ± 3.3 %	99.5 ± 3.6 %	0.003
Angiotensin II	85.3 ± 2.5 %	74.6 ± 1.9 %	0.006
Vasopressin	135.8.0 ± 3.1 %	113.2 ± 3.7 %	0.001

Abbreviations; % Max KPSS, percentage of maximum KPSS contraction; Data are mean ± SEM, and comparison is by t-test.

3.4.2.4 Effects of HD on the vasorelaxation response of different-sized arteries to different vasodilators

In small arteries, details for the percentage of maximum relaxation (R_{max}) and the potency of drug (EC_{50}) to all vasodilator agents are described in Table 14. The potency of all vasodilator drugs (BK, Ach and SNP) was similar between the two groups; however, the % of relaxation in response to BK and Ach was significantly lower in HD compared to controls (see Figures 21 A and B). In Ach group, the % of relaxation was 36.2 ± 1.2 % in HD, and 53.8 ± 1.3 % in controls ($P < 0.0001$). While in BK group, it was 35.6 ± 0.7 % and 40.0 ± 0.6 % in HD and controls respectively ($P < 0.05$). Relaxation to SNP was similar between the two groups as described in Table 14 and Figure 21 C.

In large vessels, the percentage of maximum relaxation to all vasodilator agents (R_{max}) and the potency of drug (EC_{50}) are described in Table 15. Similarly, the results of large arteries showed no statistically significant difference in the potency of all vasodilator drugs (EC_{50}) between HD and control groups; however significantly lower relaxation response curves to BK and Ach were obtained in HD patients compared to controls. The % relaxation of large arteries to Ach was 38.7 ± 1.8 % in HD and 65.6 ± 1.4 % in controls ($P < 0.0001$), while in the BK group, it was 46.2 ± 0.9 % in HD and 52.8 ± 0.8 % in controls ($P < 0.0001$). A similar relaxation response curve to SNP was observed in both groups (64.2 ± 1.9 % in HD and 67.5 ± 1.3 % in controls), see Figure 22 C).

Table 14 The % relaxation of small arteries and Potency to vasodilators in HD and controls

R_{max}	HD	Control *(n = 26)*	*P* value
Bradykinin	35.6 ± 0.7 %	40.1 ± 0.6 %	0.001
Acetylcholine	36.2 ± 1.2 %	53.8 ± 1.3 %	< 0.0001
Sodium nitroprusside	59.4 ± 1.2 %	62.4 ± 1.3 %	0.134
EC_{50}			
Bradykinin	6.7 ± 0.1	6.9 ± 0.1	0.187
Acetylcholine	7.3 ± 0.1	7.1 ± 0.1	0.142
Sodium nitroprusside	7.2 ± 0.0	7.0 ± 0.3	0.155

Abbreviations: R_{max}, maximum percentage of relaxation (%); EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM, and comparison is by students *t*-test.

Table 15 The % relaxation of large arteries and Potency to vasodilators in HD and controls

R_{max}	HD *(n = 11)*	Control *(n = 26)*	*P* value
Bradykinin	46.2 ± 0.9 %	52.8 ± 0.8 %	< 0.0001
Acetylcholine	38.7 ± 1.8 %	65.6 ± 1.4 %	< 0.0001
Sodium nitroprusside	64.2 ± 1.9 %	67.5 ± 1.3 %	0.160
EC_{50}			
Bradykinin	7.1 ± 0.2	7.2 ± 0.1	0.880
Acetylcholine	7.6 ± 0.6	7.4 ± 0.2	0.164
Sodium nitroprusside	7.0 ± 0.3	7.2 ± 0.3	0.147

Abbreviations: R_{max}, maximum percentage of relaxation (%); EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM, and comparison is by students *t*-test.

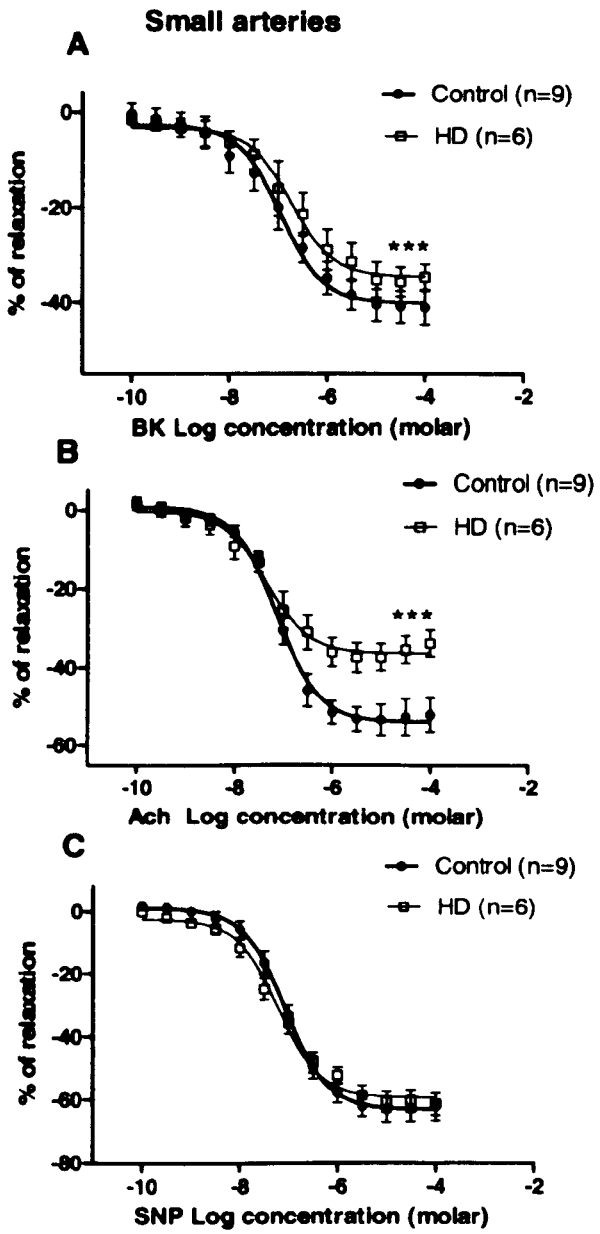

Figure 21 Concentration-response curves for vasodilators in large vessels of HD and controls

Data are expressed as mean ± SEM. BK (A), Ach (B), and SNP (C). The differences were tested at multiple dose-response time points. Comparison is by students *t*-test, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

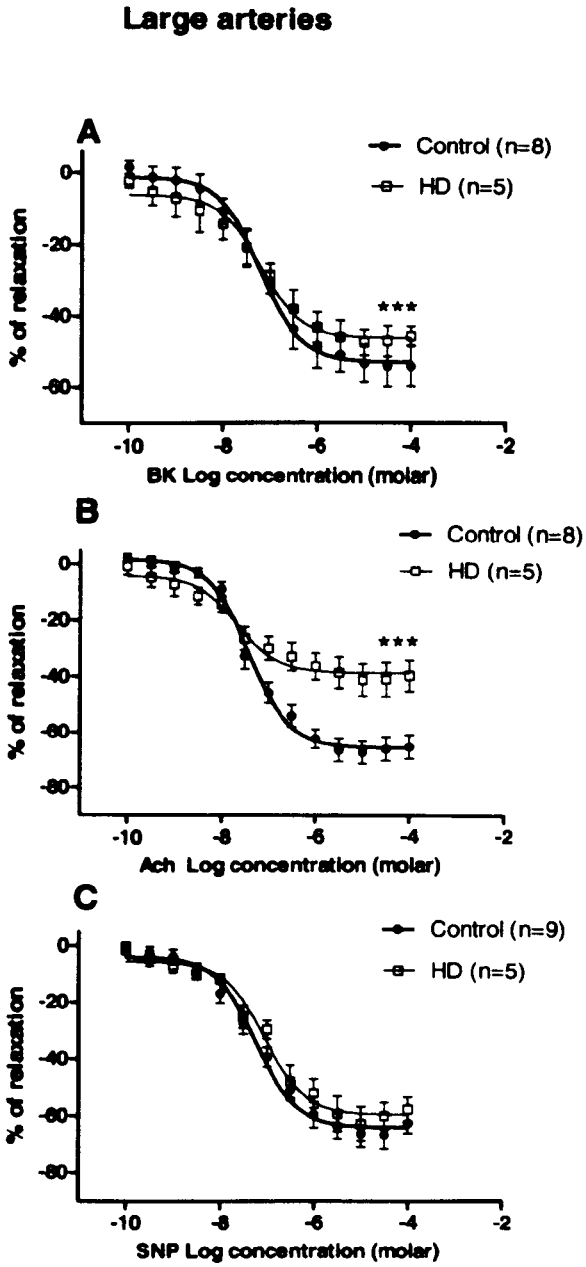

Figure 22 Concentration-response curves for vasodilators in large vessels of HD and controls

Data are expressed as mean ± SEM. BK (A), Ach (B), and SNP (C). The differences were tested at multiple dose-response time points. Comparison is by students *t*-test, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

3.4.2.5 Effects of HD on different vasoactive response in each vessel size

To investigate the pattern of the vascular response in different-sized vessels of HD and control individuals, we compared the arterial response in small arteries with those large sizes in each group (Table 16 and 17). In both HD and controls, the contractile response (R_{max}) to all vasoconstrictors was significantly higher in large arteries than small ones. In addition, our results showed that larger size arteries from HD patients significantly relax more to BK and SNP than small arteries. This variable relaxation response when comes in Ach stimulation a trend of more relaxation appears in large arteries compared to small ones, though the difference was not statistically significant. In control group, significantly higher relaxation responses to BK and Ach were obtained in large vessels compared to small vessels, with no significant relaxation difference was observed in response to SNP.

Table 16 The maximum responses of small and large sized vessels to different vasoactive agents in HD.

R_{max}	Small	Large	*P* value
Noradrenalin	5.7 ± 0.1 mN	13.1 ± 0.3 mN	< 0.0001
Endothelin-1	11.1 ± 0.2 mN	12.7 ± 0.3 mN	0.0004
U46619	14.1 ± 0.3 mN	16.0 ± 0.4 mN	0.002
Angiotensin II	7.3 ± 0.2 mN	11.9 ± 0.3 mN	< 0.0001
Vasopressin	15.1 ± 0.3 mN	16.8 ± 0.4 mN	0.004
Bradykinin	35.6 ± 0.7 %	46.2 ± 0.9 %	< 0.0001
Acetylcholine	36.2 ± 1.2 %	38.7 ± 1.8 %	0.250
Sodium nitroprusside	59.4 ± 1.2 %	64.2 ± 1.9 %	0.043

Abbreviations; R_{max}, maximum response in mN; %, percentage of relaxation; Data are mean ± SEM, and comparison is by t-test.

Table 17 The maximum responses of small and large sized vessels vessels to different vasoactive agents in controls.

R_{max}	Small	Large	*P* value
Noradrenalin	5.6 ± 0.1 mN	11.1 ± 0.5 mN	< 0.0001
Endothelin-1	8.9 ± 0.6 mN	11.7 ± 0.3 mN	0.003
U46619	9.3 ± 0.3 mN	12.4 ± 0.4 mN	< 0.0001
Angiotensin II	4.8 ± 0.2 mN	6.0 ± 0.2 mN	0.001
Vasopressin	7.0 ± 0.4 mN	11.3 ± 0.5 mN	< 0.0001
Bradykinin	40.1 ± 0.6 %	52.8 ± 0.8 %	< 0.0001
Acetylcholine	53.8 ± 1.3 %	65.6 ± 1.4 %	< 0.0001
Sodium nitroprusside	63.4 ± 1.3 %	65.8 ± 1.7 %	0.272

Abbreviations; R max, maximum response in mN; %, percentage of relaxation; Data are mean ± SEM and comparison is by t-test.

3.4.3 Correlation of ex vivo data with in vivo haemodynamic measurements of HD patients

Haemodynamic measurements including Pulse wave velocity (PWV), systolic blood pressure (SBP) and diastolic blood pressure (DBP) for each individual HD patient were measured in vivo as described in section 3.3.4. Tables 18 and 19 illustrated the relationship between haemodynamic measurements in HD patients with the maximum vasoactive response and with the percentage of maximum contractile-KPSS response respectively. PWV was significantly correlated with the maximum contractile response of large arteries to vasopressin ($r = 0.829$, $P = 0.042$), see Figure 23.1 (B). However, when the data was expressed as the percentage of maximum KPSS response, a positive correlation was also seen between PWV and percentage of maximum contractile response of small arteries to vasopressin ($r = 0.886$, $P = 0.019$). However, the correlation of PWV with the percentage response of large vessels to vasopressin was lost. The correlation between PWV and the other vasoactive agents was not statistically significant.

With regard to the correlation of *ex vivo* myography data with blood pressure, a significant inverse correlation was observed between the DBP of HD patients and the response of large vessels to SNP ($r = -0.974$, $P = 0.016$), see Figure 23.2 (D). There was also a negative correlation between DBP and the percentage of contractile response of small arteries to vasopressin-KPSS responses ($r = -0.829$, $P = 0.042$). However, there was no significant correlation between SBP and the response of isolated arteries of HD patients to vasoactive agents.

17. 1 Demographic details of in vivo measurements of HD patients

Characteristic	HD (N= 11)	Control (N= 26)	*P* value
Age (*years*)	62.3 ± 15.6	63.9 ± 11.2	0.936
Sex	M = 8, F = 3	M = 24, F = 2	NS
Systolic BP (*mm Hg*)	141.6 ± 20.6	134.2 ± 16.5	0.781
Diastolic BP (*mm Hg*)	78.4 ± 10.5	80.6 ± 9.5	0.892
MAP	110.05 ± 8.1	98.7 ± 7.1	0.356
PWV (m/s)	8.4 ± 2.1	-	-
Smoker (*n*)	3	10	NS
DM	2	1	
IHD	2	1	-
CVA	0	1	-

Abbreviation: PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus, IHD, ischemic heart disease; CVA, cerebrovascular accident; (m/s), meter/second.

Table 18 Correlations of *in vivo* data with *ex vivo* vasoactive responses of isolated different-sized vessels of HD patients to different stimuli.

Vasoactive Agents	Vessel size	PWV		SBP		DBP	
		r value	*P* value	*r* value	*P* value	*r* value	*P* value
Noradrenaline	*Small*	0.376	0.497	0.492	0.355	-0.347	0.497
	Large	0.200	0.783	0.000	1.050	0.300	0.683
Endothelin-1	*Small*	0.142	0.802	-0.200	0.713	0.314	0.563
	Large	-0.300	0.683	0.000	1.050	0.205	0.783
U46619	*Small*	0.144	0.802	-0.463	0.355	-0.463	0.355
	Large	0.232	0.658	0.025	1.000	0.000	1.000
Angiotensin II	*Small*	0.428	0.419	0.025	1.000	-0.257	0.658
	Large	0.100	0.950	-0.800	0.133	0.200	0.783
Vasopressin	*Small*	0.257	0.658	0.542	0.297	-0.142	0.802
	Large	0.829	0.042*	-0.600	0.241	-0.314	0.563
Bradykinin	*Small*	-0.231	0.658	-0.057	0.919	0.376	0.497
	Large	0.000	1.000	0.800	0.133	0.200	0.783
Acetylcholine	*Small*	-0.771	0.102	-0.142	0.802	0.314	0.563
	Large	-0.100	0.950	0.000	1.050	-0.153	0.783
Sodium nitroprusside	*Small*	0.543	0.297	0.257	0.658	-0.428	0.419
	Large	-0.300	0.683	0.000	1.050	-0.974	0.016*

Abbreviation: PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; r value, correlation coefficient; small, *Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed)

Table 19 Correlation of *in vivo* data with the % maximum vasoconstrictor-KPSS induced response of HD patients

Correlation of PWV, SBP, and DBP with the % maximum vasoconstrictor-KPSS induced response of isolated different-sized vessels of HD patients to different vasoconstrictors.

Vasoconstrictor -KPSS Agents	Vessel size	PWV		SBP		DBP	
		r value	*P* value	*r* value	*P* value	*r* value	*P* value
Noradrenaline	*Small*	0.314	0.563	0.200	0.713	-0.200	0.713
	Large	0.700	0.233	0.200	0.783	0.200	0.783
Endothelin-1	*Small*	0.371	0.497	0.371	0.497	0.542	0.297
	Large	0.300	0.683	-0.100	0.950	-0.820	0.133
U46619	*Small*	0.428	0.419	0.600	0.241	0.428	0.419
	Large	0.828	0.058	0.142	0.802	-0.028	1.000
Angiotensin II	*Small*	0.314	0.563	0.085	0.919	-0.428	0.419
	Large	0.900	0.083	-0.400	0.516	-0.410	0.516
Vasopressin	*Small*	0.886	0.019*	-0.543	0.297	-0.829	0.042*
	Large	-0.371	0.497	0.200	0.713	0.600	0.208

Abbreviation: PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; r value, correlation coefficient; s; *Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).

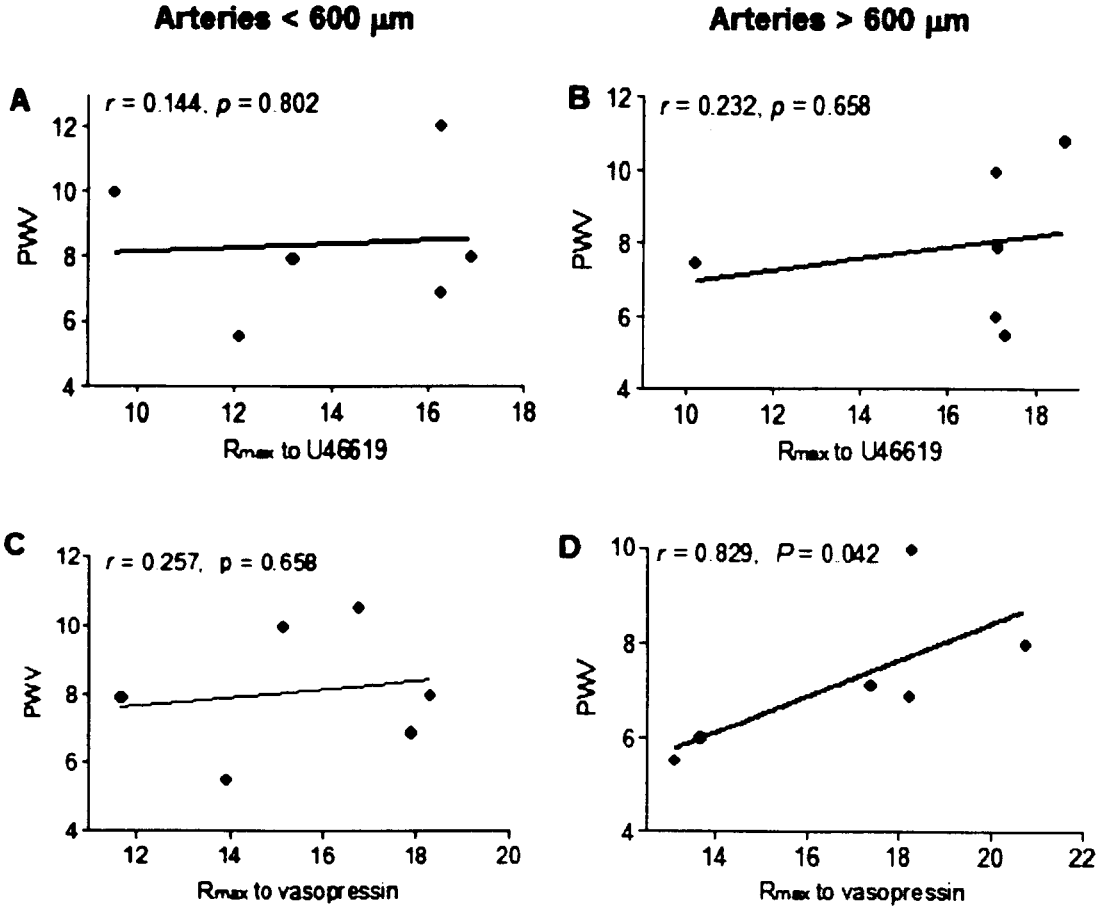

Figure 23.1 Correlation Plots of Pulse Wave Velocity (PWV) of HD patients with the maximum contractile response (R_{max}) of small vessels (A) and large vessels (B) in response to vasopressin.

Panels C and D describe the correlation of PWV with the R_{max} of small and large vessels in responses to U46619 respectively. Correlation coefficient (*r*) and P values are shown in each panel.

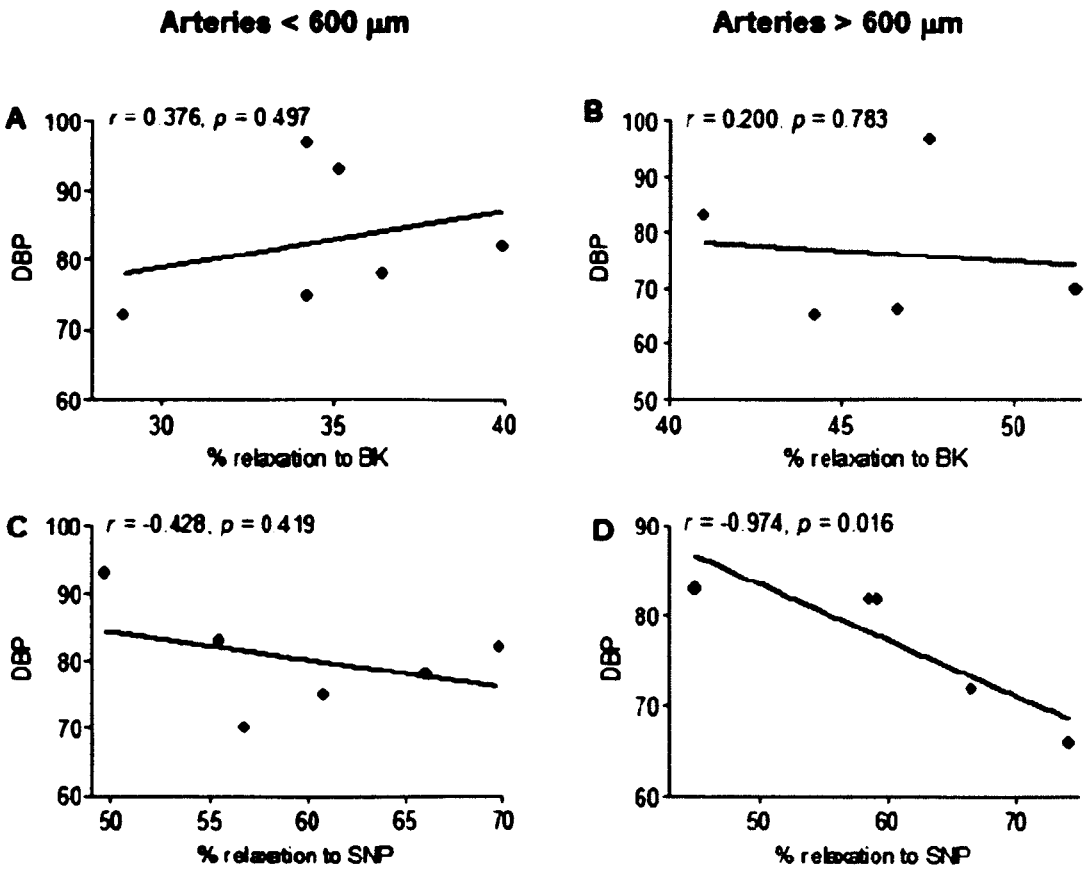

Figure 24.2 Correlation Plots of diastolic blood pressure (DBP) of HD patients with the % relaxation responses of small vessels (A) and large vessels (B) to BK.

The correlation of DBP with the % relaxation of small and large vessels to SNP are shown in curves (C and D) respectively. In each panel, correlation coefficient (r) and P values are shown.

3.5 Discussion

Endothelial dysfunction is a crucial element in the pathophysiology of increased cardiovascular risk amongst CKD patients, including those on HD. Limited human studies reported to date (Morris et al., 2001, Luksha et al., 2011) have examined the effect of uraemia on vascular function in isolated subcutaneous arteries. These studies were conducted on small arteries isolated from ESRD patients (with a very wide range of dialysis vintages), investigating a limited suit of vasoactive agents. The current study was therefore conducted on subcutaneous different-sized arteries isolated from HD patients investigating different vasoconstrictors and vasodilators.

The present study provides a number of key findings. Firstly there is a markedly increased responsiveness of isolated arteries from HD patients to different vasoconstrictors even with low drug concentrations. Secondly, impaired endothelium-dependent vasodilatation, while preserved endothelium-independent vasodilatation were observed in subcutaneous arteries of HD patients. Thirdly, different-sized arterial responses were correlated to clinically relevant *in vivo* assessment of cardiovascular system function.

The data published to date on enhanced vasocontractile responses of isolated arteries have been somewhat contradictory. Increased contractile responses of isolated subcutaneous arteries have been reported before in CKD patients (not HD), characterised by greater and more prolonged response to NA and ET-1 (Morris et al., 2001). These vascular responses have not been replicated in subsequent study (Luksha et al., 2011). In later study similar contractile response of small arteries to NA, ET-1, and AngII was observed between uremic and control groups. This is in contrast to our study with

consistently observed significant increments in the arterial contraction in isolated different-sized arteries of HD patients in response to NA, ET-1, U46619, AngII, and vasopressin. Small and larger isolated arteries from HD patients show significant enhanced contractile response to U46619, AngII, and vasopressin compared with controls. In small arteries, we did not demonstrate significant differences in the potency of pressor agents; however the overall contractile response was markedly higher in HD patients compared to controls. Furthermore, the large arteries of our HD patients had higher sensitivity to U46619 and vasopressin than control values and were characterized by statistically significant difference in the EC_{50}. When all vasocontractor data were expressed as a percentage of KPSS response, the difference in the contractile response between both groups was maintained with greater response in HD compared to controls in all vasoconstrictors. Similarly, large arteries of HD patients showed highly sensitivity to vasopressin and U46619. This may reflects enhanced vascular response secondary to vascular changes such as arterial stiffness which has been demonstrated previously in HD patients (Shinohara et al., 2004, Chung et al., 2010) or probably vascular hypertrophy that described in experimental uraemia (Amann et al., 1995b). in addition to that, higher levels of vasopressin plasma concentration have been observed in HD patients than that of normal controls (Nord and Danovitch, 1979).

The data derived from our study are consistent with a previous report on isolated subcutaneous arteries with more sustained contraction at the highest concentration of NA and ET-1 was observed in patients with ESRD (Morris et al., 2001), but is in contrast with the earlier myography study by Aalkjaer *et al*, in which a similar response to NA and AngII was observed between controls and patients with chronic intermittent dialysis

(Aalkjaer et al., 1986), also in contrast with the recent study that showed no differences in response to NA, ET-1, and AngII between uremic and control patients (Luksha et al., 2011). Our population was unselected (no survivor or transplant bias) and all had been exposed to a very similar and short period of HD (<90 days), furthermore a larger number of arterial segments were studied with the benefits of robust normal volunteer comparators.

In addition to excessive pressor response of isolated arteries from HD patients, all showed significant decreased in the vasorelaxation. This was exclusively endothelium-dependent vasodilatation (to BK and Ach). This defect was observed regardless of vessel sizes in HD patients. Vasorelaxation response to SNP (endothelium-independent vasodilator) was preserved in HD group. Previous studies on isolated subcutaneous arteries of uremic ESRD patients have demonstrated impaired vasodilatory function in response to Ach (Morris et al., 2001, Luksha et al., 2011). In both studies Ach response was significantly blunted among uremic patients with normal SNP-induced relaxation. Furthermore, recent study by Luksha *et al.* found that the percentage relaxation and the sensitivity (EC_{50}) to both BK and Ach were reduced in isolated small arteries of patients commencing PD (Luksha et al., 2012). Similar results have been reported previously in vivo using non-invasive techniques such as Doppler ultrasound measurements of brachial artery or dorsal hand vein in adult HD patients (Hand et al., 1998), as well as, in study using forearm plethysmography in patients with chronic renal failure (Morris et al., 2000). Conversely, Cupistis *et al.* showed similar Ach-induced vasorelaxation of skin microcirculation (measured by laser Doppler flowmetry) in non-hypertensive uremic

patients (as compared to controls) with reduced vasorelaxation response to Ach (Cupisti et al., 2000).

This impaired relaxation response could be due to NO dysfunction or decreased ability of vascular endothelium to produce and release vasodilator substances such as endothelium-derived hyperpolarizing factor (EDHF). These vasodilators act by stimulating release of both NO and NO-independent factor (possibly EDHF), from the endothelium of human resistance arteries (McIntyre et al., 1998), However, the exact mechanism underlying endothelial changes is still unclear. Circulating uremic factors in HD patients may responsible for these changes. These retained toxins have being widely considered and include elevated plasma homocysteine level (Mallamaci et al., 2002), increase reactive oxygen species (Ferraro et al., 2003, Hasdan et al., 2002), and elevated plasma levels of nitric oxide synthase inhibitors such as ADMA in dialysis patients (Vallance et al., 1992, Kielstein et al., 2002). The manner of endothelial dysfunction has previously been determined in other conditions including diabetes (van Etten et al., 2002) and hypertension (Taddei and Salvetti, 2002). However, in our study diabetic patients were excluded and both controls and HD patients had similar systolic and diastolic blood pressure. The arteries were examined after being removed from exposure to humeral consequences of CKD.

An interesting observation in our data is that the contractile and relaxation responses varied from small to large arteries. Previous data on diabetic patients have shown greater contractile response to NA and ET-1 observed in large than small arteries (Hadoke et al., 2000). However, the present study extends this principle to include various stimuli. We showed that the contractile response to NA, ET-1, U46619, AngII,

and vasopressin was significantly higher in large arteries compared to small vessels in each group. Moreover, our results showed that larger size arteries from HD patients significantly relax more to BK and SNP than smaller size. This variable relaxation response when it comes in Ach stimuli a trend of more relaxation appears in large arteries compared to small, though the difference was not statistically significant. This response has not been reported in uremic patients before; though Hadok *et al* conversely observed that different-sized vessels of diabetic patients had similar response to BK and SNP (Hadoke et al., 2000). Therefore, these observations may indicate presence of functional heterogeneity between small and large resistance arteries obtained from subcutaneous fat of different human tissue. These vascular alterations, particularly damage of large arteries, are the major contributory factor to the high cardiovascular morbidity of patients with ESRD (Blacher et al., 2002).

This study represents the first attempt to systematically examine the association between in vivo cardiovascular performance and ex vivo arterial function. PWV as a measure of arterial stiffness positively correlated with a greater response to vasopressin. These findings reflect the aberrant haemodynamic response of isolated arteries in HD patients which can be characterised by an exaggerated vasocontractile response and deficient relaxation. However, we did not observe any significant correlation between PWV and the vascular response to the other vasoconstrictors.

In addition, the present study compared the *ex vivo* vasocontractor and vasodilator data with the *in vivo* measurement of BP in HD patients. It was found that the DBP was inversely correlated with the contractile response of small arteries to vasopressin, as well as, negatively correlated with the vasorelaxation response of large arteries to SNP. The

existence of overhydration in those dialysis patients which have been identified as hypertensive is far from universal. Wabel and co-workers measured pre-dialysis SBP and fluid status in 500 HD patients by bioimpedance monitoring (compared to a matched healthy population). Only 15% of patients fitted the stereotype of fluid overload with hypertension. 13% of patients had hypertension despite dehydration and 10% had fluid overload despite normal BP or hypotension (Wabel et al., 2008). Similar findings were seen in another study using the same methodology in 639 patients using PD. These studies highlight that although physicians often estimate hydration status by BP, the two factors are often dissociated in the setting of the profound physiological derangement characteristic of dialysis dependant CKD. A wide variety of potential other pathophysiological processes characteristic of dialysis patients may contribute to generating hypertension without recourse to simple fluid overload, and enhanced pressor response of abnormal uremic resistance vessels appears important.

In summary, this study demonstrated additional insights into the effect of HD on vascular function characterized by incremental increased in vasocontractility and blunted endothelium-dependent vasodilatation. The detailed mechanistic responses underlying these changes are still unclear. However, exaggerated pressor response and endothelium based failure of relaxation are associated with in vivo measurements of markers of cardiovascular performance we already know to be important in determining HD patient survival. In addition this defective vascular response may be important in sensitising HD patients to recurrent cumulative ischaemic end organ injury driven by the circulatory stress of HD.

Chapter 4
Results of obese patients

Chapter 4: Eeffects of obesity on vascular function in isolated subcutaneous arteries.

4.1 Introduction

The increasing incidence and prevalence of obesity across developed countries worldwide has an enormous impact on elevated cardiovascular risk (Frisbee, 2007). Obesity is frequently associated with a number of well-recognised risk factors including insulin resistance, oxidative stress, and inflammatory conditions which can damage the endothelium (Meyers and Gokce, 2007, Karalis et al., 2009). Changes in the microcirculation of isolated arteries including vascular wall thickening and a reduction in the internal lumen have been recognised in obesity milieu (Grassi et al., 2010b). It also well demonstrated in essential and secondary hypertension (Mulvany, 1990, Schiffrin, 2004, Levy et al., 2008), and type II diabetes mellitus (Levy et al., 2008, Rizzoni et al., 2001a). These alterations may contribute to an increase in peripheral resistance. Not only the vascular structure altered in obesity but also functional responses, especially endothelium-dependent function. The exact mechanisms by which obesity can cause endothelium dysfunction are unclear, however several factors may possibly responsible for obesity-related endothelial alterations, including insulin resistance, inflammatory conditions (Zeidan et al., 2005), oxidative stress and reactive oxygen species (Karalis et al., 2009), activation of renin-angiotensin system and sympathetic nervous system (Grassi et al., 2009). These obesity-associated factors may contribute to the development of cardiovascular disease (Poirier et al., 2006).

Impaired endothelial function as measured by vasodilator response to Ach has been demonstrated in human small resistance arteries of hypertensive (Cupisti et al., 2000), diabetic (Rizzoni et al., 2001b), and uremic(Luksha et al., 2011) patients. However, few data are available about the functional study of isolated arteries from severely obese patients undergo bariatric surgery. Several *in vivo* studies have examined the vascular function in obesity. For example, significantly greater vascular response to AngII infusion has been observed in obese patients compared to non-obese subjects using venous occlusion plethysmography (Nielsen et al., 2004). Other studies have also examined the vascular function in human forearm vessels using non-invasive methods (Perticone et al., 2001, Sciacqua et al., 2003).These studies observed reduction in the endothelium-dependent vasorelaxation in response to Ach in obese patients compared with healthy controls. In animal models, Boustany-Kari *et al.* observed enhanced vascular reactivity of coronary arteries to phenylephrine and impaired endothelium-dependent vasodilatation of coronary blood flow in obese rats (Boustany-Kari et al., 2007).

Limited *ex vivo* human studies have examined vascular remodelling and vascular function in obese patients, especially those without diabetes or hypertension. Recently, Grassi *et al.* evaluated vascular structure and function in 17 severely obese patients compared to 16 non-obese individuals. Small resistance arteries were dissected from abdominal subcutaneous fat and conducted using wire myography (Grassi et al., 2010b).The study observed that media thickness and media-to-lumen ratio were significantly greater in obese subjects than in non-obese subjects. It also showed

impaired Ach-induced endothelial-dependent vasodilatation in obese patients. In subsequent study the author observed that media thickness and media-to-lumen ratio were markedly greater in obese patients compared to lean controls, and Ach-induced vasodilatation was impaired in obese patients compared to lean controls (Grassi et al., 2010a). Impaired endothelium-dependent vasorelaxation has also been observed in the subcutaneous arteries of severely obese patients (De Ciuceis et al., 2011). This study compared three groups of patients: hypertensive non-obese, severely obese and non-obese controls. It observed that Ach-induced vasodilatation was significantly reduced in obese and hypertensive patients compared to normotensive non-obese controls. The exact mechanisms of endothelial dysfunction in obese patients are still understood, however several factors were advocated to explain the obesity-related endothelial alterations, including insulin resistance, inflammatory conditions, oxidative stress and reactive oxygen species (Karalis et al., 2009, Zeidan et al., 2005).

In addition, some studies proposed that different mediators associated with obesity such as free fatty acid (FFAs), tumour necrosis factor alpha (TNF- α,), interleukin (IL)-6, resistin, and leptin, have been shown to have a direct and / or indirect effects on the vascular endothelium . For example leptin has been demonstrated to cause direct effect on the endothelial production of NO (Winters et al., 2000). These trigger factors may cause vascular alterations through thickening of intima and media of the vascular wall (Ouchi et al., 2003).

Large vessel compliance (arterial stiffness), as measured by increased pulse wave velocity (PWV), is higher in obese patients compared with the general population. PWV

measured in the upper limbs was increased significantly in obese people (n = 27) compared to non-obese controls (n = 25) independent of age, gender, and blood pressure (Toto-Moukouo et al., 1986). In the overall population, a significant positive correlation was observed between PWV and the degree of obesity ($r = 0.85$, $P < 0.001$) (Toto-Moukouo et al., 1986). It has also been shown that obesity is associated with increase in aortic stiffness, independent of age and blood pressure level (Sutton-Tyrrell et al., 2001). This change in the arterial elasticity has been shown associated with impaired endothelium-dependent vasodilatation (Arcaro et al., 2002). The pathophysiological mechanism of vascular stiffness in obese people is still largely unknown. However, adipocytes have an elevated lipolytic activity that results in increased free fatty acids release and insulin resistance (Safar et al., 2006). Indeed, obese patients with high levels of leptin have been shown to be correlated with decrease in arterial distensibility (Singhal, 2005).

Patients undergoing bariatric surgery have decreased overall mortality compared to those who do not have surgery (Sjostrom et al., 2007). Weight loss post bariatric surgery has been shown to improve a number of cardiovascular risk factors including left ventricular relaxation (Leichman et al., 2008), and left ventricular hypertrophy (Ikonomidis et al., 2007). Marked decrease in body weight and BMI, together with reduction in the blood glucose level, serum cholesterol, triglycerides and plasma lipids have been observed in one-year follow-ups of severely obese patients who had undergone bariatric surgery (De Ciuceis et al., 2011). However, very few data are presently available about improvement of vascular function in isolated vessels from

obese patients following surgery. On the other hand, several *in vivo* studies have established that, reduction in the body weight and changes in the lifestyle can improve endothelial function. Sasaki et al observed enhanced forearm blood flow in response to Ach-induced endothelium-dependent vasodilatation in obese patients following diet-induced weight loss (Sasaki et al., 2002). Raitakari *et al.* showed that weight loss with low calorie diet can improve flow-mediated vasorelaxation in obese patients (Raitakari et al., 2004). Similar findings have been demonstrated by Hamdy *et al.* who observed improvement in the macrovascular endothelial function in obese patients following six months weight loss and exercise (Hamdy et al., 2003). Sciaqua *et al.* demonstrated that energy-restricted diet induce a significant and clinically improvement in Ach-mediated vasodilatation of forearm vessels in obese healthy individuals (Sciacqua et al., 2003). Moreover, significant reduction in media thickness and media-to-lumen ration was also observed in this group of patients after one year following bariatric surgery (De Ciuceis et al., 2011), however a few number of patients had improvement in endothelium-dependent vasorelaxation in response to Ach following the surgery. Also, a marked improvement in Ach-mediated vasodilatation has observed in dorsal hand vein of obese patients after weight loss induced by bariatric surgery (Vazquez et al., 2005). Persistent reduction in the body weight following bariatric surgery seems to improve vascular function and reduces vascular structural alterations; however the responsible mechanisms for that are still not understood. Some studies (Intengan and Schiffrin, 2001, Savoia and Schiffrin, 2007) have proposed the possible roles of oxidative stress and inflammation in the development of both endothelial dysfunction and vascular alterations.

4.2 Aims

In light of the literature, we hypothesized that obesity can alters vascular structure and function. Such alteration was observed previously in isolated arteries (small sizes) obtained from obese patients, investigating a limited suit of vasoactive stimuli. (Georgescu et al., 2011, De Ciuceis et al., 2011). Therefore, the current project was focused to extend this principle of investigation to include different-sized arteries isolated from severely obese patients who were undergoing bariatric surgery with comparison to appropriate matched non obese control arteries. This was to understand the vascular reactivity in obese patients through measurement of their responses to different stimuli, and to establish whether obesity alters the vascular function by impaired endothelium-dependent vasodilatation and preserved endothelium-independent function. The study was also aimed to assess changes that might underlie altered vascular responses following bariatric surgery (decrease in weight), and whether reduction in weight will improve endothelial function. We also intended to characterize the relation between *ex vivo* vascular responses and *in vivo* assessment of vascular function in obese patients.

4.3 Materials and Methods

4.3.1 Participants and subcutaneous fat samples

Subcutaneous fat samples were obtained from 12 obese patients (7 males; average age 38.7 ± 10.9), who were severely obese (average BMI 54.2 ± 6.1) undergoing bariatric

surgery. Control fat samples were obtained from 26 appropriately consented healthy volunteers (24 males; average age 63.9±11.17) without documented obesity and diabetes mellitus, who underwent elective surgery (elective hernia repair). The ethical approval was sought and granted by the Derbyshire Research Ethics Committee and the local NHS R&D department. Informed written consent was obtained in accordance with Good Clinical Practice guidelines according to the principles expressed in the Declaration of Helsinki. Details on obtaining baseline fat samples and at six month after bariatric surgery described in methodology chapter section 2.2.3. In the present study, different size arteries (small < 600 μm and large > 600 μm) were dissected from each sample and mounted as a ring preparation on wire myography as previously described in section 2.4.3.

Four abdominal subcutaneous fat samples were also obtained from six month follow-up post bariatric surgery patients. Arteries were dissected from these samples in each patient and investigated to the same vasoactive agents that used in the baseline ones. Details on the biopsy procedure transfer of fat samples for experimental study and the techniques of arterial dissection and mounting on wire myography are described in section 2.4.

4.3.2 Preparation of vasoactive agents

Vasoconstrictor and vasodilator agents that used per experiments in this project to investigate the vascular function in obese subjects were include NA, ET-1, AngII,

U46619, vasopressin, BK, Ach, and SNP. Details of the purchases and preparation of these agents and the physiologic solutions are described in detail in section 2.3.

4.3.3 Protocol of experiment

Following mounting, arteries were kept for 15-30 minutes to be acclimatised and then subjected to normalisation process. The general protocol of experiment is described in detail in the methodology chapter section 2.5. The internal diameter of arteries was calculated as described before in section 2.4.3.

For the vasoconstrictor protocol, following a wash out of vessels and re-establish the base line, cumulative-concentration response curves were constructed for the following vasoconstrictors; NA (10^{-10} mol/L - 10^{-4} mol/L), ET-1 (10^{-12} mol/L - 10^{-6} mol/L), U46619 (10^{-12} mol/L - 10^{-6} mol/L), AngII (10^{-12} mol/L - 10^{-6} mol/L), and vasopressin (10^{-12} mol/L - 10^{-6} mol/L) starting with the lowest concentration of the drug allowing 3-5 minutes per concentration.

Separate protocol was carried out for experiments testing vasodilator drugs. Following washout period and re-establishment of the baseline (as described before in the general protocol), arteries were first contracted by stimulating them to a combination of (100 nmol/L U46619 and 1 nmol/L ET-1). Once the peak steady contraction had reached, cumulative-concentration response curves for different vasodilators including; bradykinin (BK), acetylcholine (Ach), and sodium nitroprusside (SNP) were constructed starting with the lowest concentration of the drug to the highest concentration, which was

100 pM (10^{-10} molar) to 100 μM (10^{-4} molar) for all vasodilators allowing 3-5 minutes per stimulation (as described in section 2.5.2 and Table 6).

4.3.4 In vivo haemodynamic measurements

Carotid-to-femoral arterial PWV was measured using an oscillometric device (Vicorder, Skidmore Medical Ltd, Bristol, UK). Brachial artery blood pressure was measured in the non-fistula arm using an oscillometric device (Datex-Ohmeda, GE Healthcare, USA). PWV is calculated by the software by dividing arterial transit time by measured distance. The mean of 2 measurements was recorded (as described in section 2.5.3).

4.3.5 Statistical analysis

Contractile responses were expressed as an increased contraction in milliNewtons (mN) above the isometric baseline. The maximum contractile response (R_{max}) for each agent in all groups were calculated and EC_{50} value (defined as the concentration of drug required to produce 50% of the maximum response), while the response for vasodilators BK, Ach, and SNP was expressed as maximum % of relaxation from the preconstriction state. Details on Lab Chart data calculation are described in section 2.6. All data were expressed as mean with standard error of the mean (mean ± SEM) and the analysis was using GraphPad Prism-5 software. The differences were tested at multiple dose-response time points for all data. All continuous variables were tested for normality using their

histograms and normality tests. The comparison between all groups was performed by using students *t*-test and differences were considered significant when $P < 0.05$. In regard to correlation of in vivo with ex vivo data, we correlated the PWV and blood pressure data of each patient with each vasoactive drug in each vessel size. All PWV and blood pressure data was expressed as mean ± SEM. Relationships between in vivo and ex vivo data was determined using spearman's rho correlation coefficient, which considered significant at $*P = 0.05$, and $**P = 0.01$ levels (2-tailed). All statistical analysis was undertaken using GraphPad Prism-5 software and SPSS V12.

Differences in the arterial responses of obese patients before and after surgery were determined either by paired *t*-test or repeated measures of two-way analysis of variance (ANOVA) with Bonferroni's post tests for multiple comparisons.

4.5 Results

The background characteristics of obese and control groups are described in (Table 20). The mean age of obese patients was younger than that of controls, and sex were similar between both groups. As expected, BMI was significantly higher in obese patients compared with controls. There was no significant difference in systolic and diastolic blood pressure between both groups. Patients and controls had similar renal function and smoking habits. Five patients were on angiotensin converting enzyme inhibitors (ACEIs), and two patients were on angiotensin receptor blockers (ARB).

4.5.1 Vascular size

The total number of all small vessels tested in all vasoactive agents was 53 in obese (07 arteries were discarded) and 72 in control groups (11 arteries were discarded), whereas the total number of all large arteries was 47 in obese (05 arteries were discarded) and 65 in controls (07 arteries were discarded) (Table 21). In small vessels used per vasocontractile agent, the average arterial size in obese patients was ranged between 235 μm – 577 μm in diameter, whereas in normal controls was between 220 μm – 575 μm. In small arteries, the internal diameter (ID) was similar between obese and control groups 440.7 ± 82.9 μm in obese (n = 33), and 447.6 ± 76.4 μm in control groups (n = 45), P = 0.951. Similarly, there was no significant difference in the size of large vessels between both groups, it was 730.2 ± 94.3 μm (n = 30) and 716.5 ± 78.6 μm (n = 40) in obese and controls respectively, P = 0.911. In vessels that investigated per vasodilator drugs, there small arteries size was similar between obese and controls with average ID (μm) 492 ± 53.4 μm in obese (n = 20), and 517.6 ± 45.1 μm in control groups (n = 27), P = 0.715. While the average ID of large arteries tested per vasodilator drugs was 738.3 ± 81.1 μm (n = 17) and 717.7 ± 45.1 μm (n = 25) in obese and controls respectively, P = 0.812.

Table 20 Characteristics of obese and control patients

Characteristic	Obese (N= 12)	Control (N= 26)	*P* value
Age *(year)*	38.7 ± 10.9	63.9 – 11.2	0.074
Sex	M = 7, F = 5	M = 24, F = 2	-
Systolic BP *(mm Hg)*	137.5 = 12.7	134.2 = 16.5	0.899
Diastolic BP *(mm Hg)*	81.2 = 10.5	80.6 = 9.5	0.969
MAP	100.0 = 9.3	98.7 = 7.1	0.915
Creatinine *(μm L)*	82.9 = 11.3	84.8 = 5.9	0.870
eGFR (ml min^{-1}1.73 m^{-2})	78.1 = 11.4	84.4 = 5.2	0.564
BMI	54.2 = 6.1	26.2 = 2.3	< 0.0001
Smoker	4	10	NS
DM	3	1	NS
HTN	4	3	NS
IHD	2	1	NS
CVA	0	1	NS

Abbreviation: N, number of patients; eGFR, estimated glomerular filtration rate; BP, blood pressure; MAP, main arterial pressure; BMI, body mass index; DM, diabetes mellitus; HTN, hypertension; IHD, ischemic heart disease; CVA, cerebrovascular accident; NA, non-applicable; NS, non-

Table 21 The internal diameter of small and large arterial sizes in obese and controls All arteries size of the used experiments testing the effects of obesity on vasoactive agents.

	Obese Small	Control Small	*P* value
Noradrenaline Number L_o	^{7} 419.4 ± 62.3	^{10} 401.3 ± 65.1	0.84
Endothelin-1 Number L_o	^{7} 502.2 ± 85.1	^{9} 471.1 ± 101.6	0.82
U4 Number L_o	^{7} 452. 4 ± 98.5	^{9} 424.4 ± 129.1	0.86
Angiotensi II Number L_o	^{6} 396.8 ± 101.3	^{8} 435.5 ± 39.2	0.85
Vasopressin Number L_o	^{6} 484.6 ± 67.4	^{9} 453.7 ± 67.3	0.69
Bradykinin Number L_o	^{7} 478.8 ± 26.4	^{9} 521.2 ± 46.6	0.47
Acetylecholine Number L_o	^{6} 486.9 ± 82.4	^{9} 504.2 ± 60.1	0.86
Sodium nitruprosside Number L_o	^{7} 482.2 ± 21.3	^{9} 519.4 ± 41.8	0.48
	Obese Large	**Control Large**	***P* value**
Noradrenaline Number L_o	^{6} 735.8 ± 78.3	^{9} 683.8 ± 42.2	0.53
Endothelin-1 Number L_o	^{6} 770.4 ± 80.5	^{9} 749.2 ± 92.6	0.87
U46619 Number L_o	^{7} 733.8 ± 84.6	^{8} 702.1 ± 42.8	0.73
Angiotensi II Number L_o	^{6} 686.8 ± 54.4	^{7} 693.1 ± 66.2	0.94
Vasopressin Number L_o	^{5} 724.1 ± 68.1	^{7} 681.1 ± 57.5	0.63
Bradykinin Number L_o	^{5} 738.6 ± 52.9	^{8} 715.2 ± 41.1	0.86
Acetylecholine Number L_o	^{6} 692.8 ± 45.8	^{8} 710.8 ± 55.9	0.92
Sodium nitruprosside Number L_o	^{6} 748.6 ± 62.5	^{9} 729.1 ± 79.5	0.68

Number, is the number of large arteries that used per concentration-response curve to all vasoactive agents. L_o is the normalized internal diameter of arteries. Data are expressed as mean ± SEM and the comparison is by t-test.

4.5.2 Vascular function

4.5.2.1 Effects of obesity on the KPSS contractile response.

The maximum KPSS contraction in all each-sized arteries was similar between control and obese patients. In small size vessels, the KPSS contraction was 9.1 mN ± 6.2 in obese (n = 53), and 6.9 mN ± 1.8 in controls (n = 72), $P = 0.701$. Similarly, in all large arteries the difference in the maximum KPSS response between both groups was not significant, it was 15.4 mN ± 7.9 in obese (n = 47) and 13.0 mN ± 6.5 in controls (n = 65), $P = 0.890$. However, we observed that arteries with large diameter size had a higher maximal contractile response to KPSS in each groups (Figure 24).

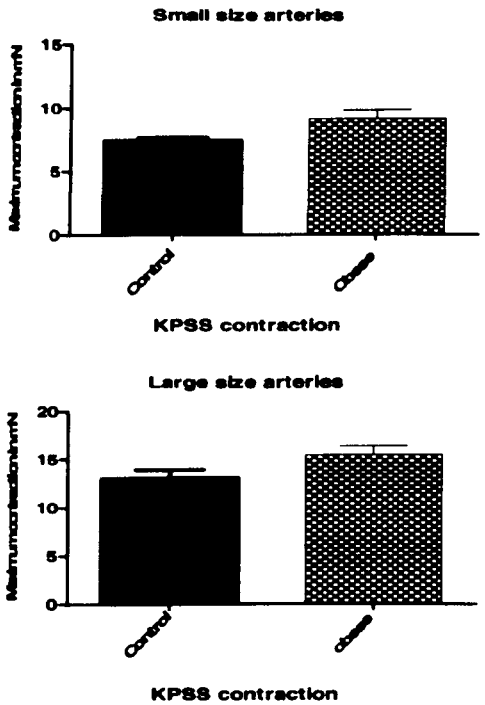

Figure 25 The maximum KPSS contraction in small and large arteries of obese and controls

4.5.2.2 Effects of obesity on the contractile response of different-sizes arteries to different vasoconstrictors

In small vessels, results for the maximum contraction of arteries (R_{max}), and the potency of drug (EC_{50}) to all vasocontractile agents in small arteries are presented in Table 22. Greater contractile response to all vasoconstrictors was observed in small arteries isolated from obese patients compared to controls. This contraction was highly significant in response to U46619 and vasopressin ($P<0.0001$) (see Figures 25 C, D and E). Significantly greater contractile responses to NA, ET-1 and AngII were also observed between both groups (P = 0.015, 0.006 and 0.004 respectively). The highest contractile response in each group was observed in response to U46619 (R_{max}, 12.7 mN ± 0.3 in obese and 9.3 mN ± 0.3 in control groups), while the lowest contractile response was observed in response to NA and AngII. The potency of NA, ET-1, AngII and vasopressin was similar between the two groups. However, small vessels of obese patients were highly potent to U4 than small vessels of control group (EC_{50}, 9.0 molar ± 0.1 in obese and 8.3 molar ± 0.1 in controls, P = 0.002) (see Table 22 and Figure 25 C).

In large arteries, Table 23 and Figure 26 showed the results for all vasoconstrictors of in both groups. Greater contractile responses to all vasoconstrictors were obtained in large arteries of obese individuals compared to controls. The difference in the contractile response to NA, ET-1, U46619 and AngII was highly statistically significant between both groups with $P < 0.0001$ in each drug. A significant greater contractile response to vasopressin was also observed in large vessels of obese patients than controls (P = 0.001). These results also showed that the highest contractile response obtained in both groups was in response to U46619 (17.3 mN ± 0.4 in obese and 12.4

mN ± 0.4 in controls), whereas the lowest response was in AngII response (8.4 mN ± 0.3 and 6.0 mN ± 0.2 in obese and controls respectively), as described in Table 23. Our data also observed that large arteries of obese patients were highly potent to U46619 and vasopressin than large vessels of controls, characterized with significantly less concentration of both drugs were required to produce 50 % of the maximum response in compared with controls (P = 0.001 and P = 0.019 respectively), see Figures 26 C and E. The EC_{50} of the other vasoconstrictors (NA, ET-1, and AngII) was similar.

Table 22 The maximum response of small arteries and Potency of all vasoconstrictors in obese and controls.

R_{max}	Obese *(n = 12)*	Control *(n = 26)*	*P* value
Noradrenalin	6.2 ± 0.1 mN	5.6 ± 0.1 mN	0.015
Endothelin-1	10.3 ± 0.2 mN	8.9 ± 0.6 mN	0.006
U46619	12.7 ± 0.3 mN	9.3 ± 0.3 mN	<0.0001
Angiotensin II	7.4 ± 0.1 mN	4.8 ± 0.2 mN	0.004
Vasopressin	11.1 ± 0.4 mN	7.0 ± 0.4 mN	<0.0001
EC_{50}			
Noradrenalin	7.9 ± 0.2	8.2 ± 0.1	0.171
Endothelin-1	10.4 ± 0.1	10.3 ± 0.1	0.519
U46619	9.0 ± 0.1	8.3 ± 0.1	0.002
Angiotensin II	9.1 ± 0.1	9.4 ± 0.2	0.132
Vasopressin	9.9 ± 0.1	10.1 ± 0.2	0.638

Abbreviations: R_{max}, maximum response in mN; %, percentage of relaxation; EC_{50}, potency of drug; (expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM, and comparison is by students t-test.

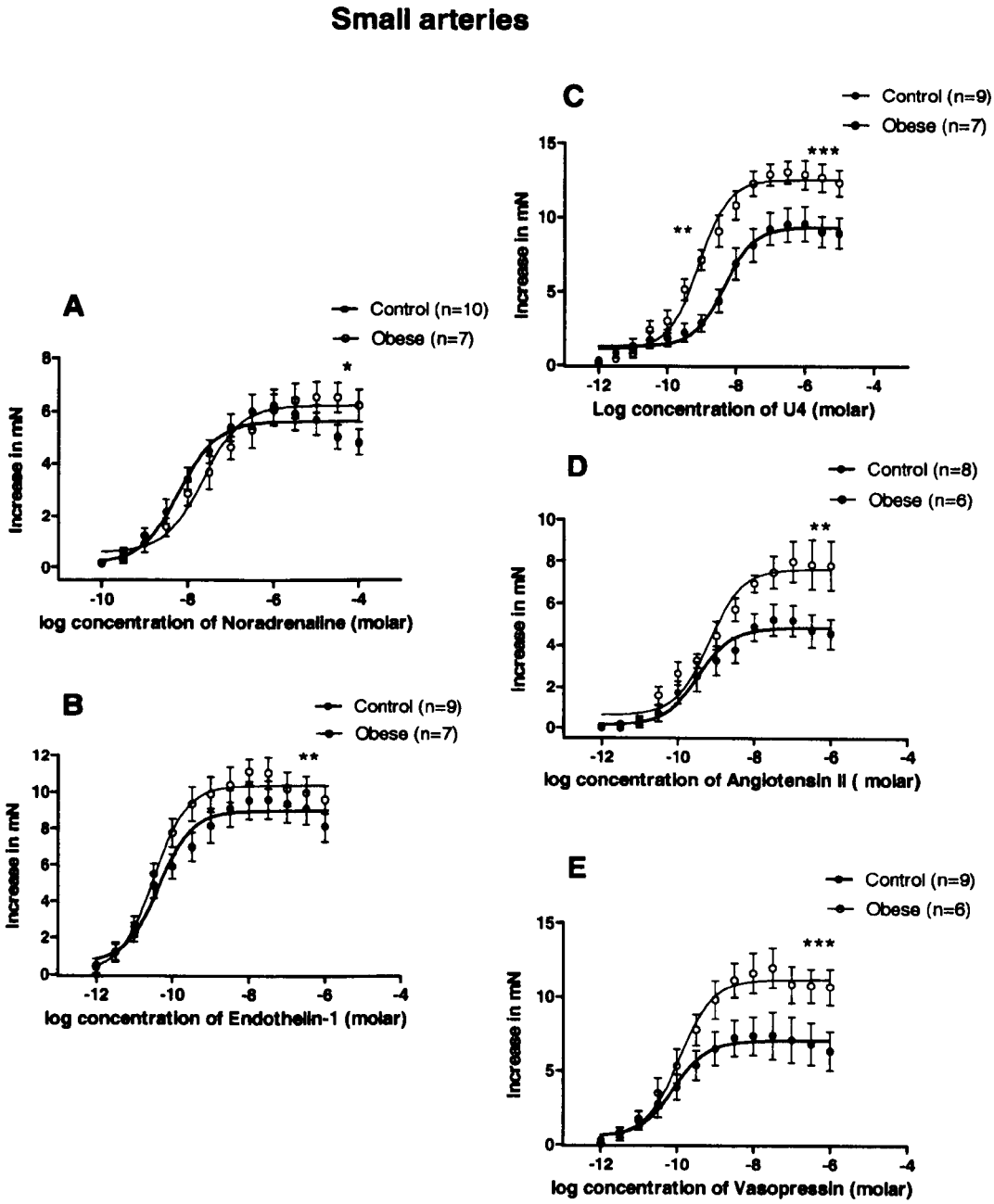

Figure 26 Concentration-response curves for vasoconstrictors in small vessels of obese and controls

Data are expressed as mean ± SEM. NA (A), ET-1 (B), U4 (thromboxane A2) (C), AngII (D) and vasopressin (E). The differences were tested at multiple dose-response time points for all data. Comparison is by students *t*-test, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

Table 23 The maximum response of large arteries and Potency of all vasoconstrictors in obese and controls

R_{max}	Obese *(n = 12)*	Control *(n = 26)*	*P* value
Noradrenaline	15.3 ± 0.3 mN	11.1 ± 0.5 mN	<0.0001
Endothelin-1	14.6 ± 0.3 mN	11.7 ± 0.3 mN	<0.0001
U46619	17.3 ± 0.4 mN	12.4 ± 0.4 mN	<0.0001
Angiotensin II	8.4 ± 0.3 mN	6.0 ± 0.2 mN	0.0002
Vasopressin	14.5 ± 0.4 mN	11.3 ± 0.5 mN	0.001
EC_{50}			
Noradrenaline	7.4 ± 0.1	7.7 ± 0.1	0.117
Endothelin-1	10.1 ± 0.1	9.8 ± 0.2	0.129
U46619	9.8 ± 0.1	8.9 ± 0.4	0.001
Angiotensin II	9.5 ± 0.3	9.6 ± 0.6	0.714
Vasopressin	9.9 ± 0.1	9.3 ± 0.1	0.019

Abbreviations: R max, maximum response in mN; %, percentage of relaxation; EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50} Data are mean ± SEM, and comparison is by students *t*-test.

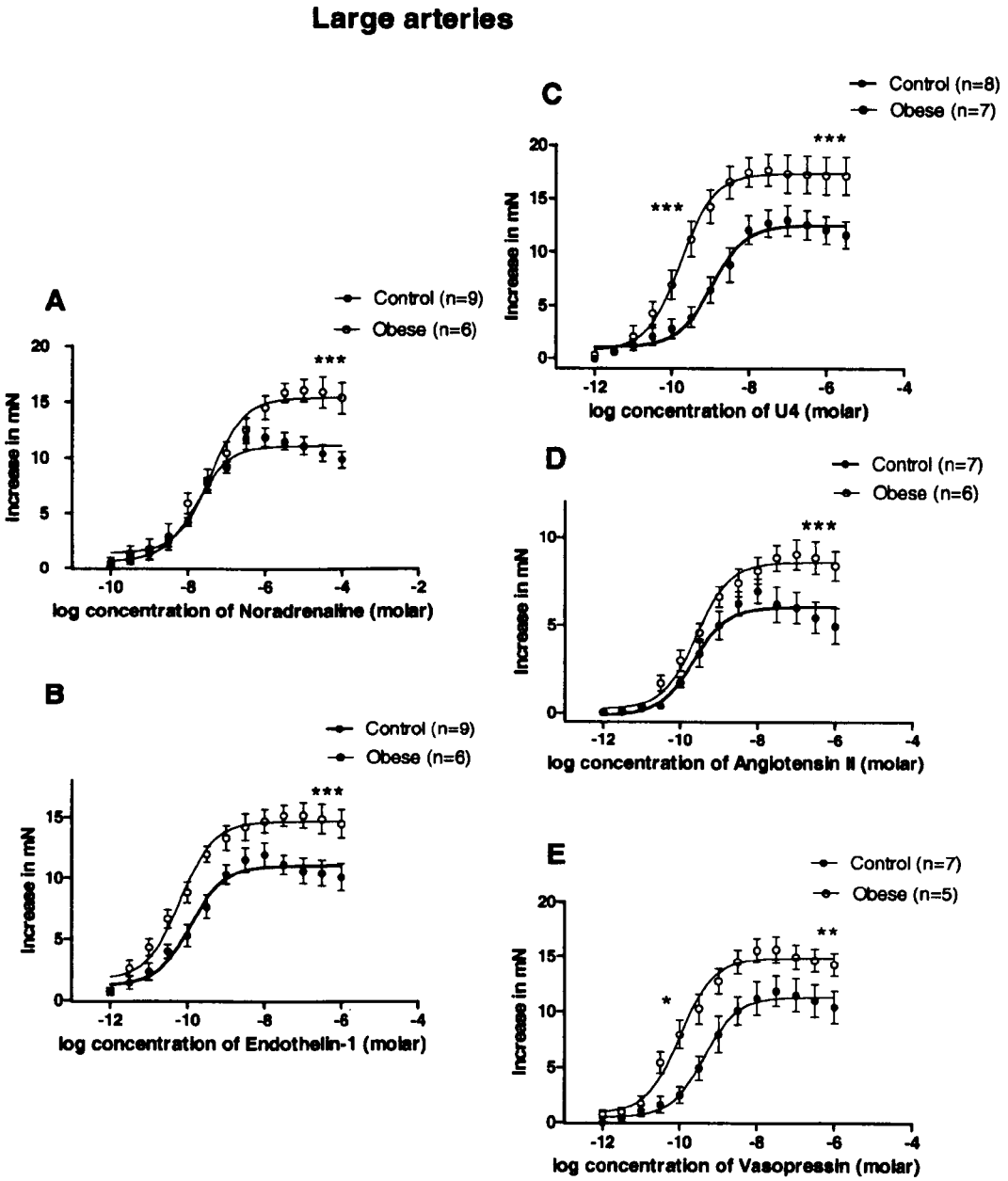

Figure 27 Concentration-response curves for vasoconstrictors in large vessels of obese and controls

Data are expressed as mean ± SEM. NA (A), ET-1 (B), U4 (thromboxane A2) (C), AngII (D) and vasopressin (E). The differences were tested at multiple dose-response time points for all data Comparison is by students *t*-test, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

4.5.2.3 Effects of obesity on the contractile response of different-sized arteries to vasoconstrictors-KPSS

Vasocontractile response of both sized-vessels to different vasoconstrictors was assessed as a percentage maximum KPSS-induced contraction in an effort to normalise the data. The percentage of maximum contractile response was maintained as a greater response of all sized vessels to all vasoconstrictors was observed in obese patients compared to controls. In obese patients the maximum contractile response of small arteries to ET-1, U46619, AngII, and vasopressin was significantly higher compared to controls (Table 24), while it was not significant in response of NA. In obese group, the highest % maximum contractile response of small vessels was in response to vasopressin (138.2 ± 3.6 % KPSS). In control group, it was in response to U46619 (120.9 ± 3.1 % KPSS). In both groups, the lowest % of contraction was in response to AngII (84.8 ± 2.4 % KPSS in obese *versus* 65.3 ± 2.1 % KPSS in controls). However, the potency of all vasoconstrictors was similar between both groups.

In large vessels, statistically significant difference in the % maximum contractile response to ET-1, U46619, AngII, and vasopressin has obtained with greater response in obese group compared to controls (Table 25), whereas no difference has observed in NA response. In obese and controls, the vasopressin contractile response was the highest in the group (125.6 ± 3.6 % KPSS in obese and 113.2 ± 3.7 % KPSS in controls, P = 0.043). Whereas in obese patients, the lowest % was in response to NA (85.2 ± 2.6 % KPSS), and in controls, it was in response to AngII (74.6 ± 1.9 % KPSS). In obese group, large arteries were highly potent to vasopressin and U46619 than controls. In vasopressin response, the potency was (9.3 ± 0.1 in control and 9.8 ± 0.12 in obese, P = 0.023), while

in U46619 (8.9 ± 0.1 in control versus 9.7 ± 0.3 in obese, P = 0.012). Similar drug potency of NA, ET-1, and AngII was obtained between the two groups.

Table 24 The % maximum KPSS –induced contraction in small arteries of obese and controls

% Max KPSS	Obese *(n = 12)*	Control *(n = 26)*	*P* value
Noreadrenaline	92.5± 3.4 %	89.9 ± 2.8 %	0.564
Endothelin-1	114.1 ± 2.4 %	102.5 ± 2.7 %	0.009
U46619	136.6 ± 3.7 %	120.9 ± 3.1 %	0.006
Angiotensin II	84.8± 2.4 %	65.3 ± 2.1 %	< 0.0001
Vasopressin	138.2 ± 3.6 %	90.6 ± 3.1 %	< 0.0001

Abbreviations: % Max KPSS, percentage of maximum KPSS contraction; Data are mean ± SEM, and comparison is by t-test.

Table 25 The % maximum KPSS-induced contraction in large arteries of obese and controls

% Max KPSS	Obese *(n = 12)*	Control *(n = 26)*	*P* value
Noradrenaline	85.2 ± 2.6 %	80.4 ± 2.5 %	0.280
Endothelin-1	105.4 ± 2.6 %	96.8 ± 2.7 %	0.015
U46619	110.8 ± 3.6 %	99.5 ± 3.6 %	0.048
Angiotensin II	101.4 ± 1.4 %	74.6 ± 1.9 %	< 0.0001
Vasopressin	125.6 ± 3.6 %	113.2 ± 3.7 %	0.043

Abbreviations; % Max KPSS, percentage of maximum KPSS contraction; Data are mean ± SEM, and comparison is by students *t*-test.

4.5.2.4 Effects of obesity on the vasorelaxation response of different-sized arteries to different vasodilators

In small arteries, results for the percentage relaxation (R_{max}) of small arteries and the potency of drug (EC_{50}) to all vasodilators (BK, Ach, and SNP) in the two groups are described in Table 26 and Figure 27. There were some differences in the baseline relaxation starting points between both groups (but not significant), however, we used a combination of low concentration of (100 nmol/L U46619 and 1 nmol/L ET-1) for preconstriction until got a steady contraction before starting vasorelaxation response curves. There was no significant difference in the potency of these drugs between obese and controls. However, the % of relaxation in response to Ach and BK (endothelium-dependent vasodilators) was significantly lower in obese patients compared to controls (Figures 27 A and B). The % relaxation in response to Ach was 49.4 % ± 0.6 and 53.8 % ± 1.3 in obese and controls respectively ($P = 0.021$). While in BK, it was 37.4 % ± 0.7 in obese and 40.1 % ± 0.6 in controls, $P = 0.008$. Relaxation to SNP was similar in the two groups (62.7 % ± 1.6 in obese and 62.4 % ± 1.3 in controls, $P = 0.886$).

In large arteries, Table 27 and Figure 28 illustrate the percentage maximum relaxation (R_{max}) of larger arteries to all vasodilators including Ach, BK, and SNP, and the potency of these drugs (EC_{50}). Despite similar observation in the vasodilator drug potency in large vessels of obese and control groups, the maximum vasorelaxation response to BK and Ach obtained in obese patients was also significantly less than that obtained from controls (Figures 28 A and B). The % relaxation in Ach group was 56.7 % ± 1.8 in obese and 65.6 % ± 1.4 in controls, $P < 0.0001$. The % relaxation in BK group

was 48.8 % ± 0.9 in obese and 52.8 % ± 0.8 in controls, P = 0.009. The Relaxation response to SNP was similar between both groups (Figure 28 C).

Table 26 The % relaxation of small arteries and potency of all vasodilators in obese and controls

R_{max}	Obese *(n = 12)*	Control *(n = 26)*	^{p} value
Bradykinin	37.4 ± 0.7 %	40.1 ± 0.6 %	0.008
Acetylcholine	49.4 ± 0.6 %	53.8 ± 1.3 %	0.021
Sodium nitroprusside	62.7 ± 1.6 %	62.4 ± 1.3 %	0.886
EC_{50}			
Bradykinin	6.8 ± 0.1	6.9 ± 0.1	0.427
Acetylcholine	6.9 ± 0.2	7.1 ± 0.1	0.117
Sodium nitroprusside	6.8 ± 0.1	7.1 ± 0.3	0.100

Abbreviations: R_{max}, maximum response in mN; %, percentage of relaxation; EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM, and comparison is by t-test

Table 27 The % relaxation of large arteries and potency of all vasodilators in obese and controls

R_{max}	Obes *(n = 12)*	Contr *(n = 26)*	*P* value
Bradykinin	48.8 ± 0.9 %	52.8 ± 0.8 %	0.009
Acetylcholine	56.7 ± 1.8 %	65.6 ± 1.4 %	< 0.0001
Sodium nitroprusside	66.7 ± 0.2 %	67.5 ± 1.3 %	0.629
EC_{50}			
Bradykinin	7.4 ± 0.3	7.2 ± 0.1	0.131
Acetylcholine	7.2 ± 0.1	7.4 ± 0.2	0.365
Sodium nitroprusside	7.4 ± 0.2	7.2 ± 0.3	0.646

Abbreviations: R_{max}, maximum response in mN; %, percentage of relaxation; EC_{50}, potency of drug (expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM, and comparison is by t-test

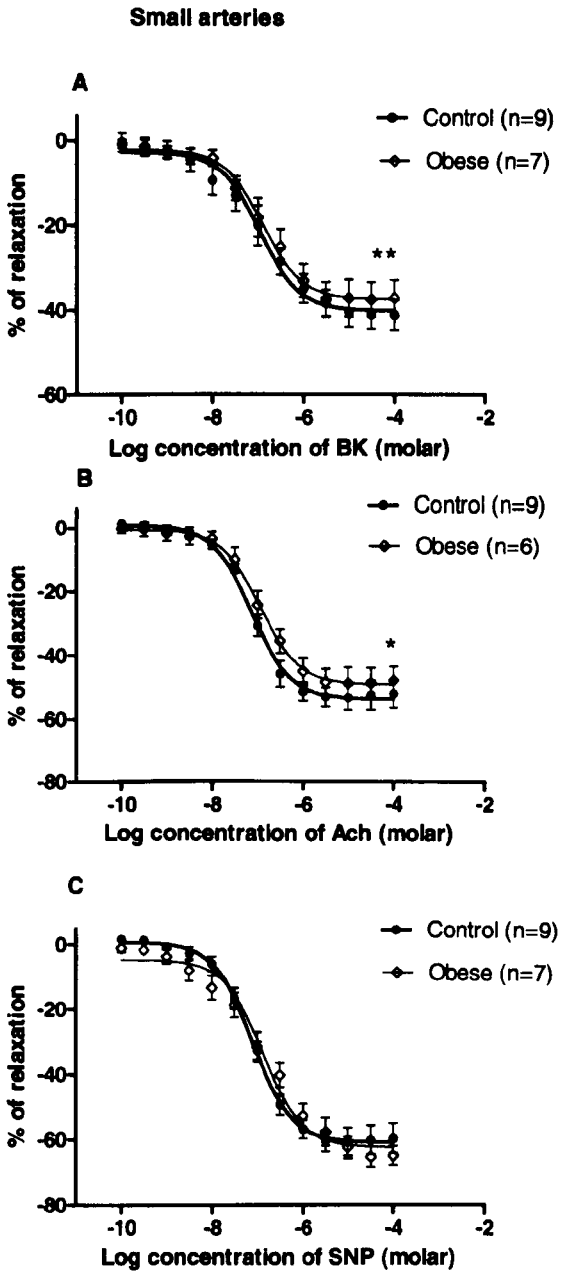

Figure 28 Concentration-response curves for vasodilators in small vessels of obese and controls

Data are expressed as mean ± SEM. BK (A), Ach (B), and SNP (C). The differences were tested at multiple dose-response time points for all data Comparison is by students *t*-test, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

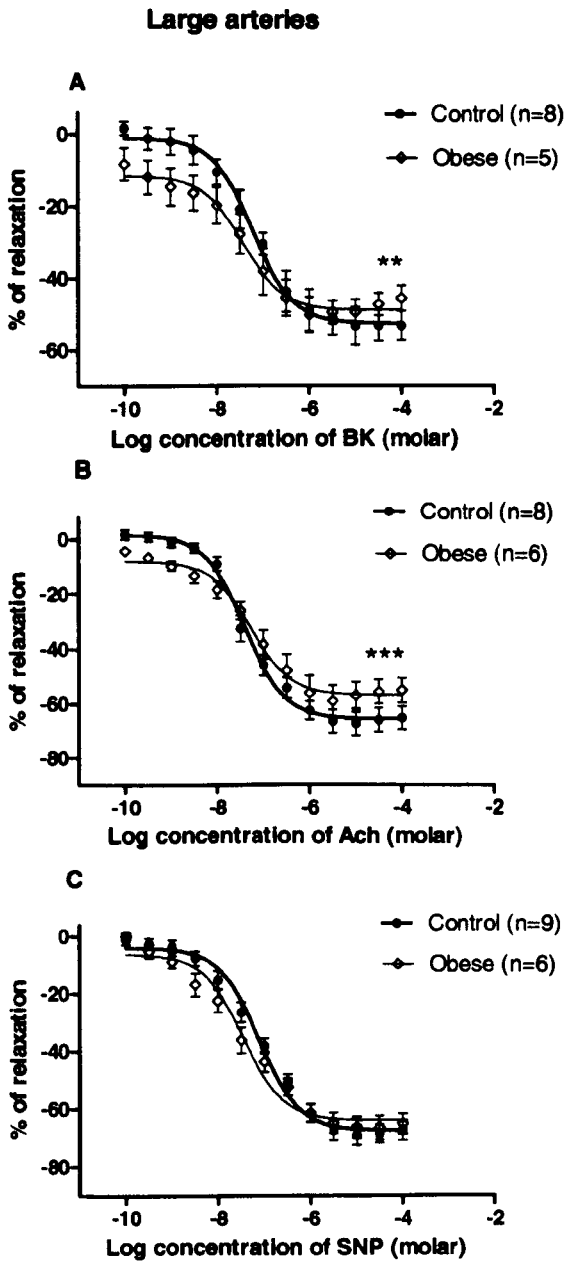

Figure 29 Concentration-response curves for vasodilators in large vessels of obese and controls

Data are expressed as mean ± SEM. BK (A), Ach (B), and SNP (C). The differences were tested at multiple dose-response time points for all data Comparison is by students *t*-test, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

4.5.2.5 Effects of obesity on different vasoactive response in each vessel size

To investigate the pattern of the vascular response in different vessel size in obese patients, we analyzed the arterial response in small and large arteries. The contractile response (R_{max}) to all vasoconstrictors was significantly higher in large vessels compared with small ones ($P < 0.0001$ in each drug response). In vasorelaxation responses, our results observed that the percentage relaxation to all endothelium-dependent and endothelium-independent vasodilators was significantly higher in large than small arteries, this difference being highly statistically significant in response to BK ($P < 0.0001$) see Table 28.

Table 28 The maximum responses of all sized-vessels to different vasoactive agents in obese patients

R_{max}	Small	Large	*P* value
Noradrenaline	6.2 ± 0.1 mN	15.3 ± 0.3 mN	<0.0001
Endothelin-1	10.3 ± 0.2 mN	14.6 ± 0.2 mN	<0.0001
U46619	12.7 ± 0.3 mN	17.3 ± 0.4 mN	<0.0001
Angiotensin II	7.4 ± 0.1 mN	8.4 ± 0.3 mN	0.004
Vasopressin	11.1 ± 0.4 mN	14.5 ± 0.4 mN	<0.0001
Bradykinin	37.4 ± 0.7 %	48.8 ± 0.9 %	<0.0001
Acetylcholine	49.4 ± 0.6 %	56.7 ± 1.8 %	0.004
Sodium nitroprusside	62.7 ± 1.6 %	66.7 ± 0.2 %	0.038

Abbreviations: R_{max}, maximum response in mN; %, percentage of relaxation; Data are mean ± SEM, and Comparison is by students *t*-test.

4.5.3 Effects of weight loss on the vascular function in obese patients following bariatric surgery

The study investigated the vascular function in isolated small arteries obtained from obese patients at six months after bariatric surgery. The results from six month samples were compared with those before surgery (baseline samples) for each vasoactive agent in each patient. Background data on the total number of small arteries used per vasoactive agent and their internal diameter (ID) in all obese patients following surgery are illustrated in Table 29. The vessel size in each vasoactive drug was similar between pre-surgery and post-surgery groups. The average ID was 502.2 ± 58.3 μm in pre-surgery group, while it was 495.4 ± 62.6 μm in post-surgery group. In KPSS response, there was no significant difference in the maximum KPSS contraction in obese patients before and after surgery. The total average KPSS contraction of small arteries was 9.3 ± 5.6 mN (n = 26) in obese patients before surgery, and 8.0 ± 2.9 mN (n = 26) after surgery, $P = 0.875$ (Figure 29).

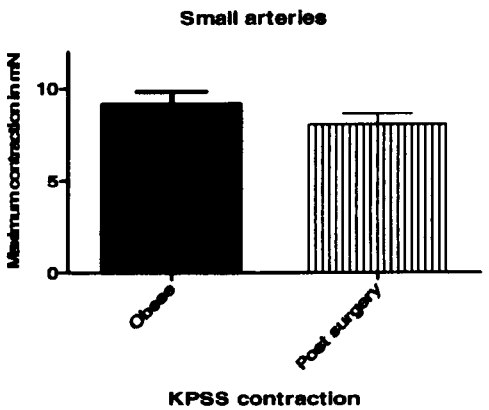

Figure 30 The maximum KPSS response in small arteries (Obese *vs* Post surgery).

Tables 30 and 31 described results of the average maximum response (R_{max}) of small arteries to all vasoactive agents and the potency of these agents (EC_{50}) in obese individuals before and after surgery. In regard to the effect of surgery on the vascular response to different stimuli, a similar vasocontractile response of small vessels to NA, ET-1, U46619, AngII, and vasopressin was obtained in obese patients before and after surgery. However a trend of maximum contractile response to each drug was obtained in pre-surgery group compared with those post-surgery (Figure 30). We also observed that the highest contractile response obtained in both groups was in response to U46619 (13.5 ± 0.3 mN pre-surgery *vs* 11.6 ± 0.4 mN post-surgery group) and vasopressin (11.5 ± 0.4 mN pre-surgery *vs* 10.3 ± 0.3 mN post-surgery group), whereas the lowest arterial contraction in both groups was observed in response to NA (6.5 ± 0.1 mN pre-surgery *vs* 6.1 ± 0.2 mN post-surgery patients). Similarly, there was no significant difference in the potency (EC_{50}) of each vasocontractile agent observed between the two groups (Table 31).

In the vasorelaxation response of isolated vessels to BK, Ach, and SNP (Figure 31), our results observed that the average % of vascular relaxation in obese patients before and after surgery was not statistically significant, though the response to BK and Ach was slightly more in post-surgery patients compared with those before surgery. In BK response, the % of vasorelaxation was 37.3 ± 0.8 % in pre-surgery and 40.6 ± 1.1 % in post-surgery group, $P < 0.240$, while in Ach response; it was 49.1 ± 0.9 % and 51.8 ± 1.8 % in pre-surgery and post-surgery groups respectively, $P < 0.324$. The % of vasorelaxation in response to SNP was 63.1 ± 2.0 % in pre-surgery and 62.7 ± 1.6 % in

post-surgery group, $P < 0.924$. The potency of each vasodilator drug was similar between both groups. These results were also analysed using two-way repeated measures of ANOVA to find out the effect of surgery on the vascular response. We observed that no significant difference in the potency and the maximum response of small vessels to NA, U46619, AngII, and vasopressin was obtained between the two groups. However, the contractile response to ET-1 was statistically significant ($P = 0.043$).

Table 29 The internal diameter of small arteries of obese patient's pre and post- surgery

	Pre-surgery *(n = 4)*	Post-surgery *(n = 4)*	*P* value
Noradrenaline Number L_0	4 492.7 ± 53.3	4 461.5 ± 76.8	0.604
Endothelin-1 Number L_0	3 489.7 ± 72.6	3 522.6 ± 35.3	0.837
U4 Number L_0	3 458.2 ± 68.8	3 517.2 ± 48.4	0.668
Angiotensi II Number L_0	3 511.4 ± 93.4	3 538.9 ± 88.3	0.689
Vasopressin Number L_0	3 509.8 ± 73.9	3 463.8 ± 112.3	0.885
Bradykinin Number L_0	3 525.6 ± 38.5	3 492.6 ± 74.5	0.462
Acetylecholine Number L_0	4 482.3 ± 76.5	4 504.8 ± 64.3	0.759
Sodium nitruprosside Number L_0	3 548.6 ± 47.5	3 511.3 ± 21.3	0.357

Number is the number of small arteries that complete the concentration-response curve. L_0 is the normalized internal diameter of arteries. Data are expressed as mean ± SEM and the comparison is by students *t*-test.

Table 30 The maximum vasoactive response of small arteries in obese patient's pre and post-surgery

R_{max}	Pre-surgery ($n = 4$)	Post-surgery ($n = 4$)	*P* value	**P* value
Noradrenaline	6.5 ± 0.1 mN	6.1 ± 0.2 mN	0.277	0.679
Endothelin-1	11.5 ± 0.4 mN	9.1 ± 0.3 mN	0.114	0.043
U46619	13.5 ± 0.3 mN	11.6 ± 0.4 mN	0.261	0.457
Angiotensin II	8.5 ± 0.2 mN	7.9 ± 0.2 mN	0.138	0.084
Vasopressin	11.5 ± 0.4 mN	10.3 ± 0.3 mN	0.371	0.551
Bradykinin	37.3 ± 0.8 %	40.6 ± 1.1 %	0.240	0.143
Acetylcholine	49.1 ± 0.9 %	51.8 ± 1.8 %	0.324	0.390
Sodium nitroprusside	63.1 ± 2.0 %	62.7 ± 1.6 %	0.924	0.301

Abbreviations: *n*, number of patients; R_{max}, average maximum response of all patients; %, average percentage of relaxation; Data are mean ± SEM, and Comparison is by paired t-test (*P*), and 2-way ANOVA (**P*).

Table 31 The potency of all vasoactive agents in small arteries of obese patient's pre and post-surgery

EC_{50}	Pre-surgery ($n = 4$)	Post-surgery ($n = 4$)	*P* value
Noradrenaline	7.6 ± 0.1	7.3 ± 0.2	0.251
Endothelin-1	10.5 ± 0.1	10.2 ± 0.6	0.580
U46619	9.3 ± 0.3	9.1 ± 0.7	0.705
Angiotensin II	8.9 ± 0.1	9.1 ± 0.2	0.504
Vasopressin	9.3 ± 0.5	9.7 ± 0.5	0.187
Bradykinin	6.8 ± 0.2	6.9 ± 0.2	0.767
Acetylcholine	6.9 ± 0.1	6.8 ± 0.1	0.647
Sodium nitroprusside	6.9 ± 0.1	7.0 ± 0.1	0.521

Abbreviations: *n*, number of patients; EC_{50}, average potency of drug (; expressed as the negative logarithm of the EC_{50}). Data are mean ± SEM and comparison is by paired t-test.

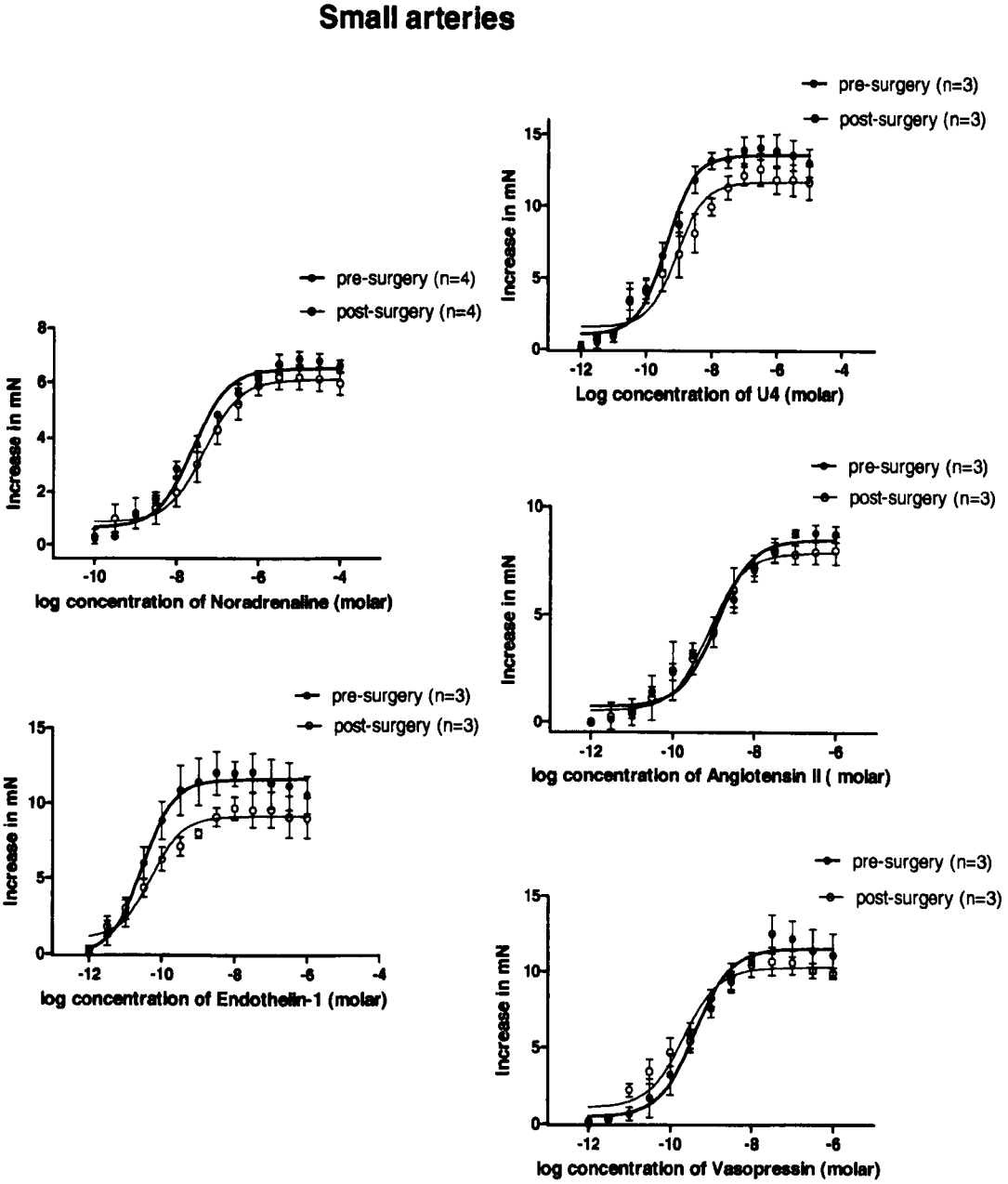

Figure 31 Concentration-response curves for vasoconstrictors in obese patients (pre-surgery *vs* post-surgery)

The differences were tested at multiple dose-response time points for all data. Data are expressed as mean ± SEM. Comparison is by paired *t*-test.

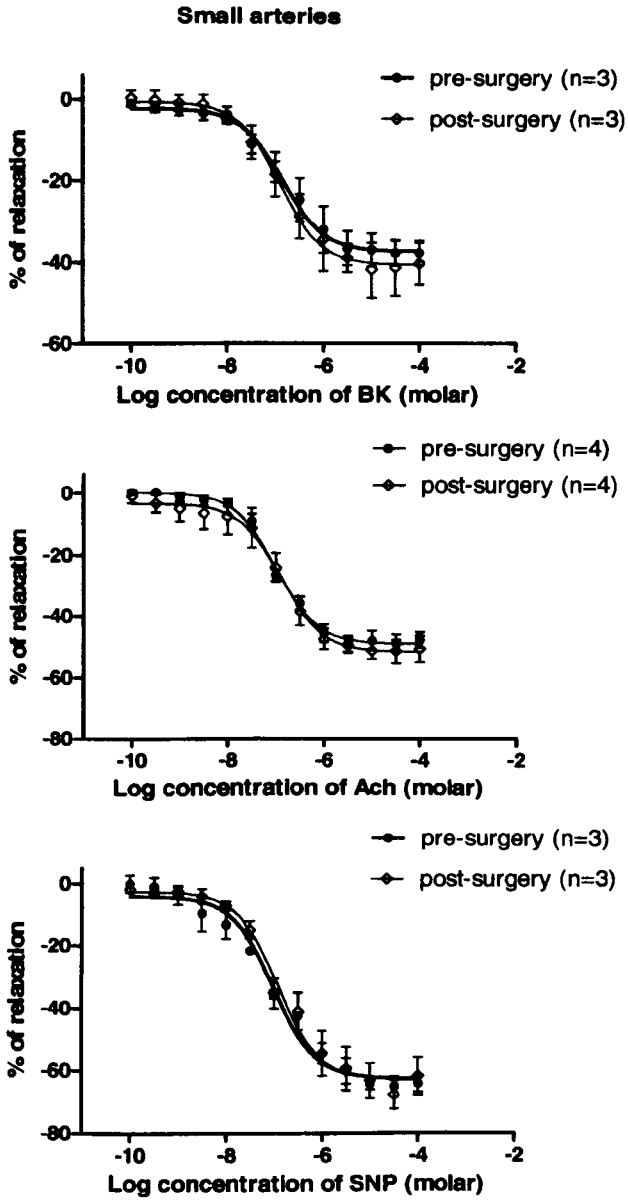

Figure 32 Concentration-response curves for vasodilators in obese patients (pre-surgery *vs* post-surgery)

The differences were tested at multiple dose-response time points for all data. Data are expressed as mean ± SEM. Comparison is by paired *t-test.*

4.5.4 Correlation of ex vivo data with in vivo haemodynamic measurements of obese patients

Haemodynamic measurements including Pulse wave velocity (PWV), systolic blood pressure (SBP) and diastolic blood pressure (DBP) for each individual (obese patients pre-surgery and post-surgery) were measured *in vivo* as described in section 3.3.4. Tables 32 and 33 illustrated the relationship between haemodynamic measurements in obese patients with the maximum vasoactive response and with the percentage of maximum contractile-KPSS response respectively. PWV was significantly correlated with the maximum response of large arteries to U46619 ($r = 0.928$, $P = 0.006$) see Figure 33 B. It is also positively correlated with the maximum response of small arteries to vasopressin ($r = 0.885$, $P = 0.033$) (Figure 33 C). These correlations lost when the data was expressed as a percentage of maximum KPSS response (Table 33). The correlation of PWV with other vasoactive agents was not statistically significant.

With regard to the correlation of *ex vivo* myography data with blood pressure, a positive correlation was obtained between SBP of obese patients and the response of large vessels to U46619 ($r = 0.785$, $P = 0.048$) (Figure 34 B). However, there was no significant correlation between DBP and the vascular response of isolated vessels in obese patients. In obese patients following surgery, our results observed no significant correlations between *ex vivo* vasoactive response and *in vivo* measurement of PWV and blood pressure, however a number of correlated points were small.

Table 32 Correlations of *in vivo* data with *ex vivo* vasoactive responses of isolated different-sized vessels of obese patients to different stimuli.

Vasoactive Agents	Vessel size	PWV		SBP		DBP	
		r value	*P* value	*r* value	*P* value	*r* value	*P* value
Noradrenaline	*Small*	0.000	0.461	0.250	0.594	-0.414	0.353
	Large	0.714	0.136	0.608	0.241	0.028	1.000
Endothelin-1	*Small*	0.214	0.661	0.285	0.556	-0.285	0.556
	Large	0.314	0.563	-0.028	1.000	0.028	1.000
U46619	*Small*	0.464	0.302	0.392	0.395	0.607	0.166
	Large	0.928	0.006**	0.785	0.048*	0.036	0.963
Angiotensin II	*Small*	0.371	0.497	0.347	0.497	0.142	0.802
	Large	0.771	0.102	-0.637	0.175	0.314	0.563
Vasopressin	*Small*	0.885	0.033*	0.376	0.497	0.521	0.297
	Large	0.600	0.350	0.153	0.783	0.500	0.450
Bradykinin	*Small*	-0.357	0.444	-0.428	0.353	-0.321	0.497
	Large	-0.300	0.683	0.600	0.350	-0.359	0.516
Acetylcholine	*Small*	-0.485	0.355	-0.714	0.136	-0.231	0.658
	Large	-0.371	0.497	-0.347	1.497	0.600	0.241
Sodium nitroprusside	*Small*	-0.571	0.200	-0.126	0.782	0.702	0.088
	Large	-0.600	0.241	-0.666	0.175	0.405	0.419

Abbreviation: PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; r value, correlation coefficient; small, *Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed)

Table 33 Correlation of *in vivo* data with the % maximum vasoconstrictor-KPSS induced response of HD patients

Correlation of PWV, SBP, and DBP with the % maximum vasoconstrictor-KPSS induced response of isolated different-sized vessels of HD patients to different vasoconstrictors.

Vasoactive-KPSS Agents	Vessel size	PWV		SBP		DBP	
		r value	*P* value	*r* value	*P* value	*r* value	*P* value
Noradrenaline	*Small*	-0.464	0.302	0.200	0.713	-0.200	0.713
	Large	0.314	0.563	0.200	0.783	0.200	0.783
Endothelin-1	*Small*	-0.071	0.906	0.371	0.497	0.542	0.297
	Large	-0.542	0.297	-0.100	0.950	-0.820	0.133
U46619	*Small*	0.571	0.200	0.600	0.241	0.428	0.419
	Large	0.607	0.166	0.142	0.802	-0.028	1.000
Angiotensin II	*Small*	0.711	0.136	0.085	0.919	-0.128	0.119
	Large	0.485	0.355	-0.400	0.516	-0.410	0.516
Vasopressin	*Small*	-0.142	0.802	-0.085	0.919	-0.200	0.713
	Large	-0.300	0.683	0.200	0.713	0.142	0.802

Abbreviation: PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; r value, correlation coefficient; s; *Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).

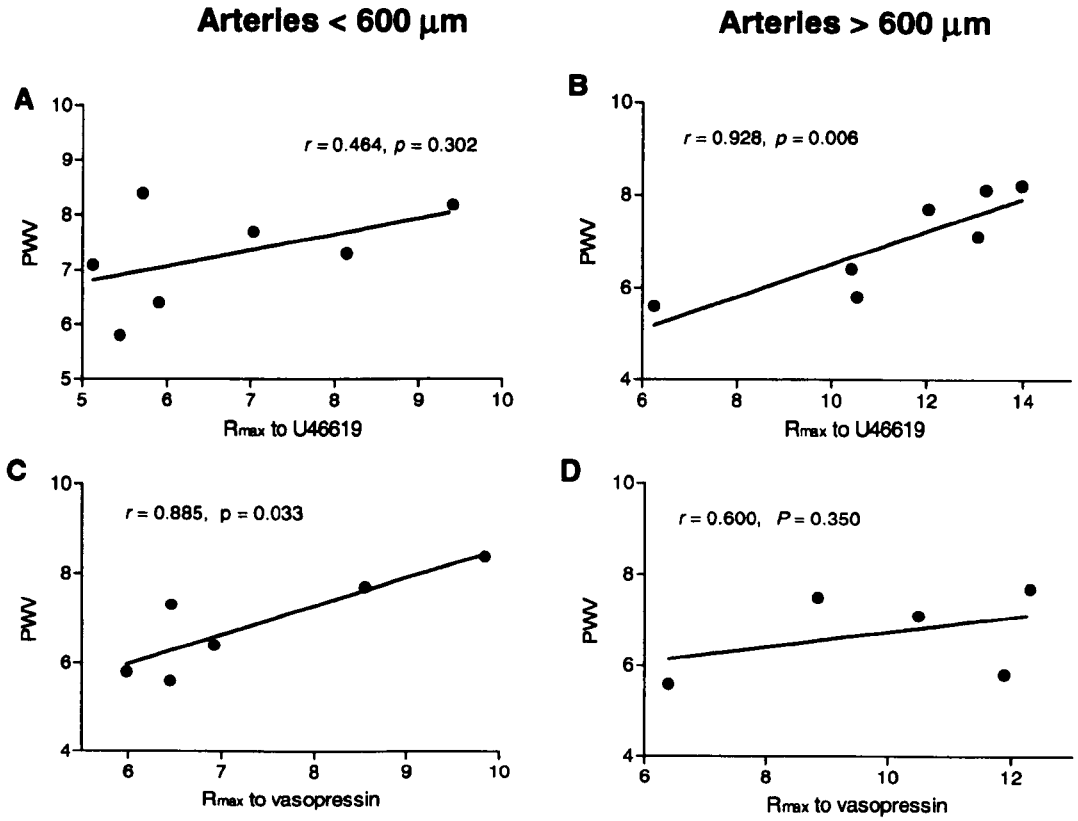

Figure 33 Correlation Plots of Pulse Wave Velocity (PWV) of obese patients with the maximum contractile response (R_{max}) of small vessels (A) and large vessels (B) to U46619.

Panels C and D describe the correlation of PWV with the R_{max} of small and large vessels in responses to vasopressin respectively. Correlation coefficient (r) and P values are shown in each panel.

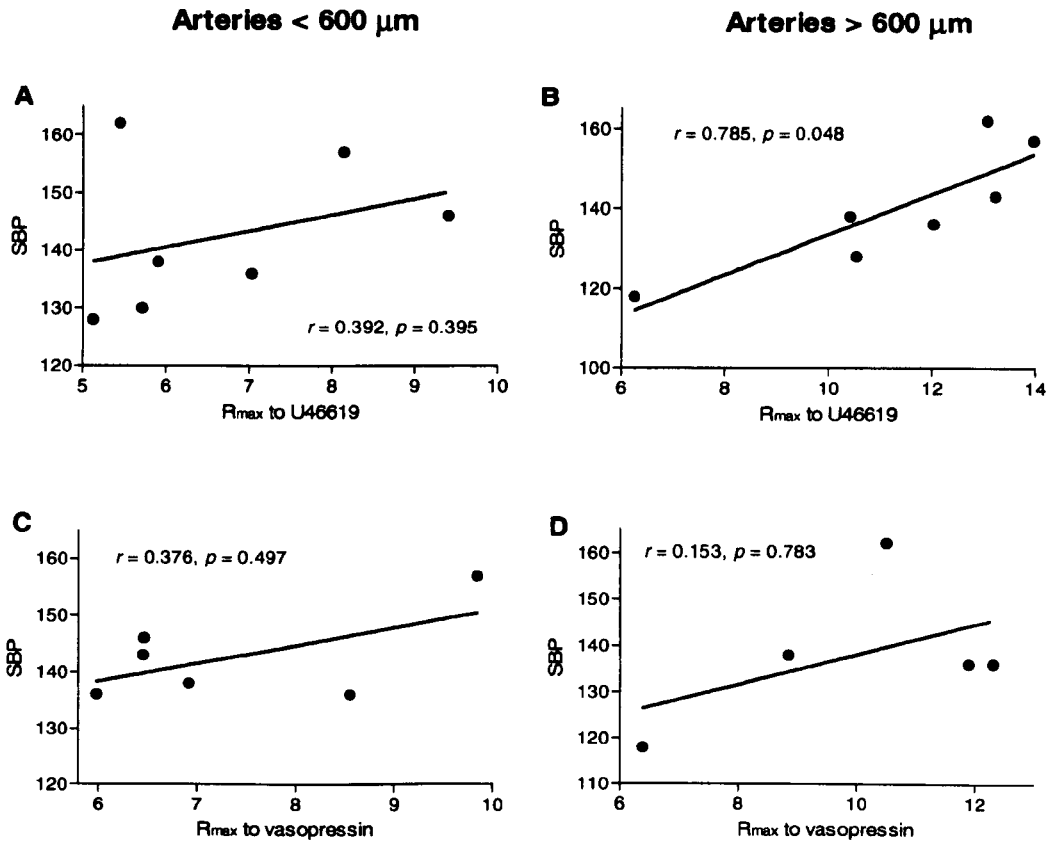

Figure 34 Correlation Plots of systolic blood pressure (SBP) of obese patients with the R_{max} of small vessels (A) and large vessels (B) to U46619.

The correlation of SBP with the R_{max} of small and large vessels to vasopressin are shown in curves (C and D) respectively. Correlation coefficient (r^2) and P values are shown in each panel.

4.8 Discussions

Impaired endothelium-dependent function has been demonstrated in isolated vessels of obese patients (Grassi et al., 2010b, Georgescu et al., 2011). These studies were mainly focused on the Ach-induced vasorelaxation. This chapter reports interesting key findings on the effects of obesity on the vascular function of isolated subcutaneous arteries obtained from obese patients who have undergone bariatric surgery. Firstly, hyper-responsiveness of these arteries to different vasoconstrictors compared to control. Secondly, impaired endothelium-dependent vasodilatation in response to Ach and BK was observed in subcutaneous different-sized arteries of obese patients. Thirdly, there was no significant improvement in the vascular function in a small group of patients six months after bariatric surgery.

4.8.1 Isolated arterial function in response to vasoconstrictors

In light of the literature, greater contractile response to NA and ET-1 has been observed in isolated vessels of diabetic (Hadoke et al., 2000) and uremic (Morris et al., 2001). However, few data are available on the effect of obesity on the vasocontractility of isolated vessels. Recent studies showed significant enhancement in the vascular contraction (in response to NA) of isolated arteries in obese patients and obese patients with diabetes compared to controls (Georgescu et al., 2011). In contrast, De Ciuceis *et al.* observed no significant difference in the vasocontractile response of isolated vessels to NA between obese, hypertensive obese, and control groups (De Ciuceis et al., 2011). Therefore, to address that, we extend this principle of increased vascular reactivity in

isolated vessels of obese patients by testing different-sized arteries to various stimuli including NA, ET-1, U46619, AngII, and vasopressin.

The current study demonstrates that obesity can produce a significant vascular hyper-reactivity to different vasoconstrictors. Although, the potency (EC_{50}) of some vasoconstrictors in small and large vessels was similar between the two groups, isolated vessels of obese patients were significantly more constricted than that of controls. This vasoconstriction was highly significant in response to U46619, AngII, and vasopressin than that observed in NA, ET-1. These findings were similar to the response of isolated small arteries from HD obtained in chapter 3. This may indicate that isolated vessels from HD and obese patients are highly responsive to these drugs and / or these agents are potent vasoconstrictors. However the isolated vessels of obese patients were highly potent to U46619 and vasopressin. This may indicate that subcutaneous vessels of obese patients are highly sensitive in response to these agents. Furthermore, the vascular response was maintained when the data expressed as a percentage of KPSS response, where significantly higher percentage of vasoconstriction in response to all vasoconstrictors showed in obese patients compared to controls.

The exact mechanism by which the isolated vessels of obese patients showed increment vascular contractility has not been known yet, however one proposed reason could be due to the vascular alteration such as vascular hypertrophy and stiffness. Recently, alteration in the vascular function in obese patients has been shown to be accompanied with changes in the vascular structure including increase media thickening and media-to-lumen ratio (Grassi et al., 2010b, De Ciuceis et al., 2011). Moreover, these

structural changes together with impaired endothelium-dependent function in response to Ach have also been recently demonstrated in isolated small arteries of obese patients (Rizzoni et al., 2012), and patients with obesity and metabolic syndrome (Grassi et al., 2010a). All these findings might suggest presence of a mechanistic links between functional abnormalities and structural changes among this risk group. However, the present findings suggest presence of vascular dysfunction in obese patients characterised by exaggerated vascular contractility in obesity, which probably could be secondary to an alteration in the vascular structure that have been demonstrated previously.

Recent data on obesity-related vascular hyper-reactivity of human gluteal subcutaneous vessels clarified that both anti-contractile function of subcutaneous vessels and increase in the NO function are reduced among this group (Greenstein et al., 2009). Other authors suggested that some factors are amplified in obesity; these include insulin resistance, sympathetic nervous system activity and rennin-angiotensin system activity (Grassi et al., 2009). These factors responsible for increased the vascular contractility in obese individuals either through direct arterial response and reduced compensatory vasodilator response in obese patients (Kataoka et al., 2001) or by increased adrenergic and sympathetic activity (Landsberg, 2001).

Our data also observed variability in the vasoreactive response in isolated different-sized vessels of obese patients. Functional heterogeneity in isolated different range of vessel sizes has been demonstrated previously in diabetic patients with greater contractile response in large arteries compared with large veins and small arteries (Hadoke et al., 2000). The author observed no difference in the relaxation response to

BK and SNP between the three categories of vessels, however large arteries did not produce an active relaxation to Ach compared with small vessels. In the animal model, variable contractile response from the highest to the lowest vasocontraction has observed in large, medium, and small isolated vessels from normal animals using isovolumic myography method (Lu and Kassab, 2011). However, there are no data published on the vasoreactive response of isolated different-sized vessels from subcutaneous fats of obese patients. The results presented in this chapter showed significantly greater contractile response to all vasoconstrictors in large arteries than that observed in small vessels. Furthermore, the relaxation response to all vasodilators was significantly more in large arteries.

4.8.2 Isolated arterial function in response to vasodilators

The current chapter also demonstrated impaired endothelium-dependent function in obesity characterized by a significant decrease in BK and Ach-induced vasorelaxation in obese patients. The effect of obesity and obesity-associated factors on the endothelial function has been reported in several studies investigating isolated small resistance vessels from subcutaneous fats of obese people. In sever obese humans, both Ach-induced vasodilatation (Grassi et al., 2010b) and BK-mediated vasorelaxation (Sato et al., 2002) in subcutaneous small resistance arteries (diameter ranged between 150 μm – 450 μm) were reduced compared with controls. Moreover, marked reduction in the vasorelaxation response to Ach in isolated subcutaneous arteries has been observed in patients with obesity alone and obesity together with diabetes mellitus (Georgescu et al., 2011). Recently, significant reduction in the vasodilatation response to Ach was observed

in isolated small subcutaneous vessels of normotensive and hypertensive obese patients compared to controls (De Ciuceis et al., 2011). In both studies only small vessels were investigated either in response to Ach alone or Ach compared with SNP. However to address that issue, the present study was focused to investigate the vascular function in different-sized vessels by testing them to different vasodilators (BK, Ach, and SNP). Interestingly, the results presented here show that there is a marked impairment in the vasorelaxation response of different-sized vessels of obese patients to both endothelium-dependent vasodilators (BK and Ach), while similar vasorelaxation response to SNP has observed in both groups. Therefore, impaired endothelial function in obesity does not only affect large arteries (Levy et al., 2008) it also impairs small vessels as well. Our findings are in keeping with the recent results of human obesity described previously in this section and with the results comparing isolated small vessels of severely obese patients with controls using pressurized myoraphy (Grassi et al., 2010b), in which endothelium-dependent function in response to Ach was impaired. Since BK mediated it's action through stimulation of endothelial cells to release either NO (Michel and Vanhoutte, 2010) or EDHF (Bellien et al., 2008), Ach also promotes it's action through stimulation of the endothelium release of EDHF (Garland et al., 2011). Thus impaired endothelial function could possibly due to a defect in NO or EDHF pathway. On the other hand, Sivitz *et al.* observed that *in vivo* vascular responses to both Ach and nitroprusside were impaired to approximately the same extent in the forearm vessels of obese patients (Sivitz et al., 2007), suggesting this defect does not reflect a specific abnormality in the endothelium-dependent function. However there is a general

agreement that obese individual alone or with other chronic diseases such as diabetes and hypertension have impaired endothelium-dependent function.

The association between obesity and vascular dysfunction has been widely discussed; however, the exact pathophysiological mechanisms by which obesity can cause impaired endothelial function are still unclear. Many proposed that insulin resistance can impair NO vasodilator function through reduce the expression of NO synthase (eNOS) in obese human endothelial cells (Georgescu et al., 2011). Also, enhanced production of tumour necrosis factor (TNF) in obesity can down-regulate the expression of eNOS and up-regulate ET-1 in human extracellular matrix (Jonk et al., 2007). Furthermore, presence of some hormonal factors such as insulin resistance (Gustafson et al., 2007) and increase sympathetic activity (Grassi et al., 2004), might have a role in obesity-related endothelial dysfunction. Other metabolic mediators in obesity field might also have multiple adverse effects on the vascular function; these include leptin, adipokines, TNF-α, and excess release of reactive oxygen species (Knudson et al., 2008). These factors can impair endothelial function through stimulation of vascular smooth muscle hypertrophy (Zeidan et al., 2005, Knudson et al., 2008). In conditions other than obesity, an early data suggested impaired endothelium-dependent vasodilatation in diabetic (Johnstone et al., 1993) and hypertensive patients (Panza et al., 1990, Taddei et al., 1993). In our study, however we did not exclude the diabetes from obese patients, while we exclude it from the control group. Therefore, we can suggest that either the circulating factors associated with obesity and / or other co-morbidities are most likely the contributory factor responsible for endothelial dysfunction.

In obesity, increased in the arterial stiffness and decreased in elasticity as measured by elevated PWV has been shown to impair endothelium-dependent function (Arcaro et al., 2002). In the current study, PWV as a measure of arterial stiffness positively correlated with a vascular response of isolated different-sized vessels of obese patients to vasopressin and U46619. Moreover, the present study also observed significantly positive correlation between SBP of obese patients and the vascular response to U46619. These findings reflect the aberrant response of isolated vessels of obese patients which can be characterised by an exaggerated vasocontractile response of isolated vessels to different stimuli. This change in compliance is partially due to material alterations in the conduit arteries; however it is also BP and endothelial dysfunction related.

In this chapter, we also measured the vascular reactivity in obese patients at six months following bariatric surgery. Limited studies have previously examined the effect of weight loss on endothelial vasodilatory function in isolated vessels of obese patients. Recently, De Ciuceis *et al.* observed improvement of Ach-mediated endothelial vasodilatation in isolated subcutaneous arteries in a few number (4 of the total 8 recruited for surgery) of obese patients following bariatric surgery (De Ciuceis et al., 2011). However, the current results did not show significant differences in the contractile and relaxation response of the recruited small number of obese patients before and after surgery though a trend of higher contractile response has observed in obese patients before surgery compared with those post-surgery. On the other hand, greater decrease in the body weight with pronounced improvement in endothelium-dependent function of the

forearm vessels was observed previously in obese patients following gastric bypass surgery (Gokce et al., 2005). Conversely, vascular function in obese patients' brachial artery assessed by flow-mediated dilatation and nitroglycerin-mediated dilatation did not change after weight loss (Brook et al., 2004). Our data on isolated vessels did not demonstrate significant improvement in the endothelium-dependent function after surgery; however the corresponding *in vivo* results (data not shown) of the same patients showed significant improvement in the renal function characterized by marked improvement in eGFR and reduction in albuminuria.

Although the results provided about the effect of bariatric surgery on the vascular function did not achieve the statistical significance, particularly improvement in the endothelium-dependent function, the percentage of vasorelaxation response to BK and Ach appears more in post-surgery group compared with pre-surgery (but not significant). This finding might provide some suggestions about the possibility of improved endothelium-dependent function in obese patients with persistent weight loss induced by bariatric surgery. Therefore, a large study with long-term follow-up is helpful to confirm such improvement. Since impaired endothelial function in obese patients is associated with increased future cardiovascular risks (Gokce et al., 2005), this kind of surgical intervention might be associated with long-term clinical benefits.

In summary, obesity is associated with marked functional alterations in isolated subcutaneous arteries of obese patients which are characterized by significant increment responsiveness of subcutaneous resistance arteries to a wide range of vasoconstrictor stimuli, and impaired endothelium-dependent vasodilatation in response to BK and Ach.

Similar findings were observed in chapter 3, in which similar endothelial dysfunction was observed in HD patients, indicating that vascular endothelium in HD and obese patients would seem a reasonable target for uremic and obesity-associated factor respectively. These changes highlights the elevated risks of cardiovascular, cerebral and renal events reported in these risk groups. Improvement in the renal function and BMI was observed after decreased in weight induced by bariatric surgery; however we did not observe a significant improvement in the endothelial function in obese patients at six month after surgery assessed by the response of isolated small arteries to endothelium-dependent vasodilators (Ach and BK) investigating a small number of patients. Therefore, further longer studies with large number of participants are needed to confirm that.

Chapter 5
General discussion

Chapter 5: General discussion

Previous studies have identified impaired endothelium-dependent vasodilatation in isolated subcutaneous small vessels of both uremic (Morris et al., 2001, Luksha et al., 2011, Luksha et al., 2012) and obese patients (Georgescu et al., 2011, De Ciuceis et al., 2011). Several reports established that ESRD and obesity are associated with vascular alteration including functional and structural changes (Guerin et al., 2005, Grassi et al., 2010b, Rizzoni et al., 2012). Although, the effect of uremic on the vascular function in isolated subcutaneous resistance arteries was demonstrated in ESRD patients, particularly those on peritoneal dialysis (PD) and renal transplantation, very little information is available on the effect of HD on the isolated vascular function. Enhanced vascular contraction of isolated subcutaneous vessels from uremic patients in response to vasoconstrictors has been reported before in one study (Morris et al., 2001), though other studies have not (Luksha et al., 2011, Luksha et al., 2012). These studies demonstrated impaired endothelium-dependent vasodilatation in subcutaneous vessels of uremic patients.

Therefore, the main aim of the current project was to establish whether the isolated subcutaneous arteries of HD and obese patients show enhanced vascular response and impaired endothelium-dependent function, and whether the bariatric surgery improves vascular function. In obesity, exaggerated responses of isolated vessels to vasoconstrictors (Georgescu et al., 2011), and impaired endothelium-dependent function (Georgescu et al., 2011, De Ciuceis et al., 2011) have been reported before. We dissected different sized-vessels, assuming that the vascular response varies depending

on the size of the vessel; however lack of data to-date on the effect of uremia and obesity on the vascular response of isolated different sized vessels. Therefore, we extended the principle of vascular response in previous work to include investigation of isolated different-sized vessels from the three groups (HD, obese, and controls) by testing them to various stimuli.

In the first part of this project (chapter 3), we have examined the vascular function (using wire myography technique) in isolated different-sized vessels in a group of homogenous patients starting HD, and correlating the *ex vivo* myography data with *in vivo* haemodynamic measurements of PWV and BP. The major finding in this part of the project was that HD patients had impaired endothelium-dependent vasodilatation and enhanced vasocontractility. This vascular enhancement in response to vasopressin was significantly correlated to the *in vivo* measurement of PWV. The present data also observed an inverse correlation of diastolic BP of HD patients with the contractile response of small arteries to vasopressin, and with the vasorelaxation response of large arteries to SNP. In light of these findings, we can suggest that PWV as a measure of arterial stiffness positively correlated with a greater response to vasopressin. This may reflect the aberrant haemodynamic response of isolated arteries in HD patients which can be characterised by an exaggerated contractile response and deficient relaxation. The reason for that could be due to an alteration in the vascular structure including arterial calcification and thickening of muscular media that have been reported previously in this risk group of patients (Adragao et al., 2009). These findings, however strengthens an association between HD and increased conduit arterial stiffness.

The vascular findings in this project are consistent with previous studies highlighting impaired vasorelaxation response to BK and Ach (both endothelium-dependent vasodilators) in uremic patients (Morris et al., 2001, Luksha et al., 2011, Luksha et al., 2012). Similarly, these findings have also been demonstrated in human with severe obesity (Grassi et al., 2010b), and in patients with obesity alone or obesity with chronic diseases such as diabetes mellitus and hypertension (Georgescu et al., 2011, De Ciuceis et al., 2011). Only small isolated resistance vessels were investigated in all these studies examined a limited suite of vasoactive stimuli. In order to examine this further in the current study small and large isolated arteries from the three groups of participants were directly investigated to different vasoactive agents using wire myography. This will extend the study research area to a different range of vessel sizes and a wide range of stimuli.

We provide some major findings on the effect of both HD and obesity on the vascular function. In chapter 3, we demonstrated significant incremental increase in the arterial contraction in both small and large arteries of patients who are purely on HD. Such response has not been observed before in HD patients. However, this finding is in keeping with uremic study by Morris *et al.* 2001, at least in part with enhanced response of small vessels to NA and ET-1 in uremic patients compared to controls (Morris et al., 2001), but in contrast with the recent study of Luksha *et al.* 2011, in which uraemia had no effect on the response of small isolated vessels to NA, ET-1, and AngII (Luksha et al., 2011). However in both studies, impaired endothelium-dependent vasodilatation was in agreement with our observation that the vasorelaxation response to Ach and BK was significantly blunted in HD patients. This will support the concept of an impaired

endothelial function in the uremic milieu in all late stages of CKD including those on dialysis. Interestingly, similar results (enhanced vascular contractility and impaired endothelium-dependent function) were obtained in isolated different-sized vessels of obese patients (in chapter 4).

In obesity, previous studies that examined the endothelial function in isolated small vessels were focused on the assessment of Ach-induced relaxation in patients with obesity alone or associated with chronic diseases. In chapter 4, our data was in keeping with the results that show enhanced response to NA and ET-1 in isolated small vessels from patients with type 2 diabetes (Hadoke et al., 2000), and in patients either with obesity alone or obesity with diabetes (Georgescu et al., 2011). However it was in contrast with the findings observed that isolated vascular contractility in response to NA was not altered in obese and hypertensive obese patients (De Ciuceis et al., 2011). The vascular response in these studies has not been investigated with regard to different sized vessels and different stimuli. Therefore, to address this issue, the current project was examined the vascular function in different-sized vessels by testing them to various stimuli. In all vessel sizes of obese patients, we showed that the vasocontractile response to different vasoconstrictors was greatly enhanced and the endothelium-dependent relaxation to Ach and BK was significantly decreased. However, in effort to normalize the data, similar vasocontractile results in all vessel sizes in both HD and obese patients were observed when these results were expressed as a potentiated maximum KPSS response.

An interesting finding in this project was that small and large vessels of both HD and obese patients show highly contract to U46619 and vasopressin more so than the

other vasoconstrictors, while these vessels contract less to NA and AngII. Furthermore, large vessels of both groups are highly potent to U46619 and vasopressin, which could indicate that these vessels are highly sensitive to both vasoconstrictors. In light of these results, we highlighted presence of endothelial defect in both groups of patients characterized by failure of all sized vessels to relax in response to Ach and BK, while they normally relaxed to SNP. One proposed reason for such failure is alteration in the vascular structure such as vascular hypertrophy, thickening of vascular media, and arterial stiffness that have been reported recently in HD (Chung et al., 2010) and obesity (Grassi et al., 2010b). In obesity, it has also been found that alteration in the vascular structure can lead to vascular dysfunction appears as increased in the vascular contractility and reduced endothelium-dependent vasorelaxation (Georgescu et al., 2011). In regard to this explanation, there may be an association between changes in the vascular function and alteration in the structure of the vascular wall. This possibly was emphasized in our HD and obese patients by significant positive correlation between enhanced vasocontractility in response to vasopressin and PWV (in HD patients) and significant positive correlations between PWV and SBP with vasocontractile response to U46619 (in obese patients). These observations may indicate presence of alteration in the vascular wall structure, particularly conduit vessels, among this risk group of patients. This correlation may strengthen the link between ESRD and elevated arterial stiffness (Peralta et al., 2009). However, elevated peripheral vascular resistance and increased sympathetic activity in ESRD patients could also be enhance the vasoreactivity in this group of patients. These vascular alterations, particularly in large vessels, may predict cardiovascular mortality as described previously (Blacher et al., 2002).

The results in chapters 3 and 4 showed that different sized isolated arteries in both HD and obese patients fail to relax in response to endothelium-dependent vasodilators (Ach and BK). This particular impairment in subcutaneous vessels has not been published before. Previous *ex vivo* myography studies on uremic and obese patients have examined only small-sized isolated arteries (Morris et al., 2001, Luksha et al., 2011, Georgescu et al., 2011). In our HD and obese patients the size of the vessel was ranged from small to large arteries. In the current project, endothelial dysfunction in the microvasculature of HD and obesity was further strengthened by significantly blunted vasorelaxation response to BK and Ach, and preserved response to SNP in all vessel sizes.

Taken with the results in chapter 3 and chapter 4, we suggest that uremia and obesity can alter the vascular reactivity of subcutaneous isolated arteries by enhanced vasocontractility and impaired endothelial function. However, the exact underlying mechanism of endothelial dysfunction is unclear. Several reports have proposed that circulating uremic factors in HD patients, as well as obesity-related factors (mentioned in chapters 3 and 4) may be responsible for these changes. It is also important to note that ED can occur in other conditions such as hypertension (Taddei and Salvetti, 2002), diabetes mellitus (van Etten et al., 2002), and cigarette smoking (Heitzer et al., 1996), and seems to be controversial in hypertension. Our HD patients were non-diabetic, and they had similar BP with controls. There were no obvious cardiovascular diseases in HD patients, and the average BMI was normal in HD. Thus in uremic HD, it is more likely that uremic toxins, and not the existing co-morbidities, are the main factors responsible

for altered vascular function and ED. Therefore, the present findings may contribute to the explanation of the relationship between uraemia and vascular alteration.

In obesity, several reports have suggested that impaired NO bioavailability (Jonk et al., 2007) and alteration in the vascular structure (Zeidan et al., 2005) are the primary responsible factors that affect the endothelial function through different pathophysiological mechanisms involving metabolic and hormonal factors associated with obesity. These include insulin resistance, adipocytokines, and excess release of reactive oxygen species. We did not investigate these factors in this study; however incremental increase in the vasocontractility together with failure in the endothelium-dependent vasorelaxation in our obese patients may suggest alteration in the vascular structure or could probably a defect in NO function, where all sized vessels of obese patients were normally relaxed to NO donor (SNP). However, the exact underlying mechanism is still unclear. Therefore from the results obtained in chapters 3 and 4, we can suggest that there are some associated factors linked in HD and obese patients that alter the vascular function which, in our results, are characterized by vascular hyper-responsiveness and impaired endothelium-dependent vasodilatation.

In order to further investigate whether the vascular function will be improved in obese patients following bariatric surgery, small isolated arteries from the same patients were investigated to the same vasoactive drug after six month follow-up post-surgery. Improvement in the endothelium-dependent function in obese patients after bariatric surgery has been observed in few numbers of isolated subcutaneous arteries (De Ciuceis et al., 2011), as well as, in forearm vessel (Gokce et al., 2005). This is in contrast to the in vivo study by Brook *et al.* in which endothelium-dependent vascular function in

brachial artery did not change in obese patients following surgery (Brook et al., 2004). Therefore, the effect of surgery on the vascular function is still controversial. In chapter 4, results of obese patients following bariatric surgery did not significantly differ from the baseline results. However a noticeable trend of higher contractile response to all vasoconstrictors was observed in obese patients before surgery compared with the same patients after surgery. In regard to the vasorelaxation response, our results did not reveal a significant improvement in the endothelium-dependent function following surgery, though the percentage relaxation to Ach and BK appeared more in obese patients after surgery, but this difference was not statistically significant. This may likely indicate that reduction in the body weight might decrease the vasoreactivity and improve the vascular relaxation in obesity. However, further large studies with extensive follow-up are needed to confirm these results.

In regard to the difference in the vasoreactive response of different-sized vessels in all groups of participants, our data observed that the contractile response varies along the vascular tree. Previous data on diabetic patients have shown greater contractile response to NA and ET-1 observed more in large arteries than small (Hadoke et al., 2000). However, the present study extends this principle to include various vasoconstrictors in different subjects including HD, obese, and controls. In the present study, large arteries from all groups were significantly contract to all vasoconstrictors than small vessels. Moreover, in our obese patients, large arteries significantly relax to all vasodilators more so than small arteries. While in HD group, similar results were obtained in response to BK and SNP but not Ach. These findings have not been reported

before in uremic and obese patients; though Hadok *et al.* conversely observed similarity in the response of isolated different-sized vessels of diabetic patients to BK and SNP (Hadoke et al., 2000). Therefore, these observations might indicate presence of functional heterogeneity between small and large resistance arteries obtained from subcutaneous fat of different human tissue.

The focus of this project is investigating *ex vivo* vascular function in a high-risk group of subjects; HD and obese patients, and the conclusion from chapters 3 and 4 is that there is enhanced vascular contractility and impaired endothelium-dependent function. These findings suggesting that vascular endothelium in HD and obesity would seem a reasonable target for uremic and obesity-associated factor respectively. The exact underlying mechanism of impaired endothelial function in both groups is complex and yet unclear. It might be that there is impaired NO function or could probably be an alteration in the vascular structure, which has been reported previously in uraemia and obesity milieu, but with different associated factors involved in the complicated pathophysiological mechanisms.

5.1 Limitations and future work

The key aspect of this thesis was to investigate *ex vivo* vascular function in high-risk and selective patients, HD patients and severely obese individuals who had undergone bariatric surgery. The results in this project provide interesting findings on the effects of HD and obesity in isolated vascular function despite a small number of participants, which may limit the greater applicability of the results. The reason for recruiting a small number of HD patients is the nature of subcutaneous fat sampling with

extra incision required in this highly sensitive group of patients. Together with minor complications of the procedure such as minor bleeding and scar formation. All of which could result to either refuse the patients or withdraw them from the participation in the study. However, a large number of arterial segments from different-sized vessels were recovered from a homogenous group of incident dialysis patients, and a large number of control participants were included in the study. Since the mechanism of endothelial dysfunction in uraemia and obesity is multi-factorial, several reports have suggested that impaired NO function through elevated ADMA (Kielstein et al., 2004), hyperhomocystinaemia (Mallamaci et al., 2002) and excessive oxidative stress (Pawlak et al., 2004) play crucial roles in the mechanism of endothelial dysfunction in uraemia. Also, insulin resistance (Poirier et al., 2006), adipocytokines (Knudson et al., 2008) and excess reactive oxygen species (Silver et al., 2007) may be responsible for the same mechanism in obesity. However, we did not measure these circulating indices that could support our results.

Although we demonstrated an interesting positive correlation between the contractile response of small and large vessels in response to vasopressin with the *in vivo* measurements of PWV, a large number of patients had an additional association between *in vivo* hemodynamic measurements and the *ex vivo* functional findings. These would allow us to raise new possible mechanisms of vascular dysfunction, thus necessitating further study of the effect of vasopressin response on vascular function. Arterial function could have been affected either by the biopsy procedure or use of local anaesthetics, however, all arteries that completed the experiments were tested for their ability to

contract at least 5 mN either to a high KPSS or U46619, and the endothelium function was assessed using BK.

As a result of the relatively few number of obese patients evaluated post operatively, our results did not provide enough information about the effect of surgery on vascular function, which may also limit the discussion about the vascular response following surgery in obese patients. The reason for recruiting only small numbers of post-surgery obese patients could also be due to the nature of an extra incision needed to collect subcutaneous fat samples in patients who already had major surgery six months prior. Although these results are derived from a small HD and obese sample size, a large number of control participants were included in the study, and statistical significance was achieved between the patient and control groups. Endothelial dysfunction in ESRD and obesity are likely to occur in the earliest stages of the disease, thus, further studies are necessary to identify ways of preventing such a defect; this includes haemodynamic and biochemical investigation.

Despite these limitations, our study demonstrated that HD and obesity can alter the vascular function in isolated different-sized vessels through impaired endothelium-dependent vasodilatation and enhanced vasocontractility. The future aim would be to extend the myography study to further investigate the role of endothelium-dependent function through abrasion of the endothelial layer lining the isolated vessels using human hair. Also, by using NO synthase inhibitors, such as NG-nitro-L-arginine methyl ester (L-NAME), we could investigate the role of endothelium-derived NO. Moreover, the future work would be to extend the same investigations into these risk groups correlating *ex vivo* vascular response with biochemical measures of circulating factors that might be

responsible for endothelial dysfunction in HD and obese patients. A larger study would further investigate the association between the circulating markers and functional responses in the isolated vessels would be helpful in finding new insights into the underlying mechanisms. A morphological examination of isolated vessels, including measurement of vascular wall thickness and media-to-lumen ratio to determine their histological features, should be considered as well.

5.2 Conclusion

The overall aim of this thesis was to investigate the *ex vivo* vascular function in HD and obese patients using wire myography. This project provides new insights into the effect of HD and obesity on human resistance artery function. We show that HD and obesity affects endothelial function via incremental increase in the vascular contractile response to various stimuli and blunted dilatation response to endothelium-dependent agonists, while preserved endothelium-independent function in isolated different-sized vessels. The detailed mechanistic responses underlying these changes are still unclear. However, the failure of arterial relaxation is mediated by endothelial dysfunction. The association between HD and obesity with endothelial dysfunction in isolated arteries would be expected to accelerate the cardiovascular risk which impacts on cardiovascular morbidity and mortality. These findings further highlight the general state of endothelial dysfunction in both ESRD including those on dialysis and obesity with or without associated hypertension and diabetes mellitus. We therefore propose that development of cardiovascular disease in such risk patients is mediated, at least partly, by functional alterations at the level of microcirculation. An *ex vivo* vascular function of isolated

vessels from HD and obese patients were correlated to *in vivo* assessment of arterial function characterised by an exaggerated vasocontractile response and deficient relaxation, which in turn, strengthens an association between these risk groups and increased conduit arterial stiffness. Proper weight loss following bariatric surgery may improve the renal function and BMI; however, these are not accompanied by the improvement in the endothelial function though further large studies with longer time follow-up or increased the patient's number are necessary to establish the effect of surgery on improvement of endothelial function.

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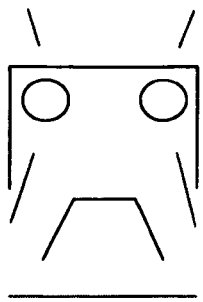
7 Appendix

7.1 Protocol for myograph calibration (Small jaws for resistance vessels)

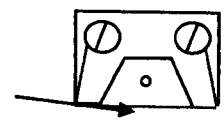
- Turn on myograph and turn on the heat

- Add 3-5 ml of distilled water to each chamber, and wait until 37°C

- Attach a wire to the 'transducer' jaw. Make sure the wire is on tight and there is no slack – it should look like below:

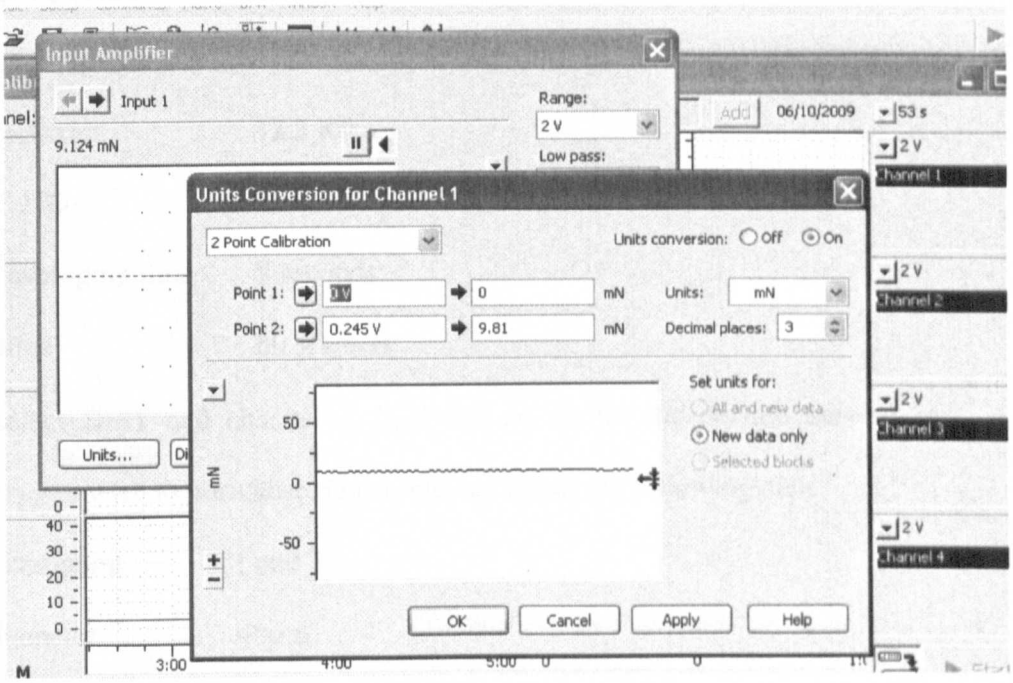


Aim to place the balance arm in this



- Open a chart file
- On channel 1, place the calibration bridge over the transducer jaw. Make sure that the tip of the balance arm is in the space between the jaw on the inside of the wire as shown in the diagram (think about the fact that the arm will press against the wire causing the change in tension to be recorded on the transducer)
- On channel 1, open 'input amplifier'
- Turn off the unit conversion (so amplifier is reading in V)

- On the myograph, so to the calibration screen and select the channel you want to calibrate by pressing F2.
- It will read 'No force on Force 1' – press F2 to continue and now read the 'V' value on the chart file – should be around 0.000V (no force should = no V)
- Next it will read '2 gram on Force 1' – place the 2 g weight on the transducer arm. When the reading is stable, press F2: cal and read the 'V' value on the chart file.
- Now go back to the Units Conversion section in 'Input Amplifier' and enter the value in V obtained at 0 force and the value obtained at with the weight – we measure in mN, which is 9.81mN. Make sure the units conversion is on before leaving the screen.



- The channel is now calibrated!

- Repeat for the other 3 transducers

7.2 Normalization of arteries by wire myography

- Turn on lab chart version 5 ensuring it is calibrated. Important to calibrate it before conducting the experiment if the myography has not been calibrated in the past two weeks.
- Mount the vessels as described before
- Once the vessels are mounted start the chart reader running
- From the top tool bar select DMT and normalization settings. Enter the following information below

Eye piece calibration	1
Target pressure	13.3 KPA
ICI / IC 100	0.9
Online averaging time	2 seconds
Delay time	60 seconds

- Then select DMT and channel 1, 2, 3 and so on for the desired amount of channels you wish to normalise. Once selected in put the following data

Tissue end point	1 and 3
Wire diameter	40μm

- Bring the jaws together until the reading is at a negative number >30 mN is fine

- Allow the first reading to be taken then move the jaw slightly apart so that the number on the myography screen is as close to zero as possible.
- Allow the normalization to begin, add tension giving the vessel around 2 mN tensions. Let the software time the normalization for 60 seconds. Once this has happened you will need to take note of the micrometer screw reading and add this to the programme.
- Keep adding tension until a pressure of 13.3 KPA has been reached. When this occurs then take the final micrometer reading and input it into the programme.
- The programme will then return the corresponding micrometer position that will give 13.3 KPA, the programme will also return the inner circumference of the vessel. Using this circumference it is thus possible to work out the diameter by dividing the circumference by π.