

**ARTERIAL STIFFNESS AND ENDOTHELIAL  
DYSFUNCTION IN PATIENTS WITH CORONARY  
ARTERY DISEASE**

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## **DECLARATION**

Except where otherwise stated, this thesis, and the data presented therein is entirely the result of my own efforts. This work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution.

Except where otherwise stated, and to the best of my knowledge, this work contains no material previously published or written by another person.

Bushra Saeed Ilyas

## **PRESENTATIONS**

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

**Ilyas, B.S.**, Markie, D., Newby, D.E., Webb, D.J. Arterial stiffness and renal function in patients with coronary artery disease. Poster presentation at the Cardiovascular symposium 2006, Edinburgh. (Third prize for postgraduate posters).

**Ilyas, B.S.**, Markie, D., Newby, D.E., Webb, D.J. Determinants of arterial stiffness in patients with coronary artery disease. Oral presentation at ARTERY-6 2006, Athens.

## **ABSTRACT**

**Background:** Arterial stiffness and endothelial dysfunction are implicated in the pathogenesis of atherosclerosis. Both are present in patients with hypercholesterolaemia and diabetes mellitus, and are markers of future cardiovascular events in patients with coronary artery disease (CAD), hypertension and end-stage renal failure. The structural components, elastin and collagen, which influence skin elasticity are also responsible for the elasticity of arteries.

**Aims:** To investigate: 1. The association between skin elasticity and arterial elasticity in healthy subjects. 2. The determinants of arterial stiffness in patients with CAD, particularly renal function. 3. The determinants of endothelial dysfunction in patients with CAD. 4. The association between arterial stiffness and endothelial dysfunction in patients with CAD. 5. The survival of subjects from cardiovascular morbidity and mortality as determined by the severity of CAD, renal function, arterial stiffness and endothelial function.

**Methods:** Skin elasticity was measured in the arm, leg and back using a suction device which measures the vertical deformation of skin. Arterial stiffness was assessed using pressure pulse wave velocity (PWV), pulse wave analysis (PWA) and digital volume pulse (DVP) analysis. Endothelial function was determined non-invasively using PWA with the administration of glyceryl trinitrate (endothelium-independent vasodilator) and salbutamol (endothelium-dependent vasodilator). The study in CAD was a cohort study with longitudinal follow up for a median of 18 months. Adverse clinical events were determined through the Information and Statistics Division of the NHS and the General Register Office in Scotland. Renal

function was assessed using serum creatinine concentration ( $[\text{creat}]_{\text{sr}}$ ) and estimated glomerular filtration rate (eGFR) by using creatinine clearance calculated using the Cockcroft & Gault equation. Subjects with a history of renal disease were excluded. The primary-endpoint was a composite of hospitalisation and mortality due to cardiovascular causes.

**Results:** 1. Arterial elasticity and skin elasticity were only weakly associated. 2. Arterial stiffness was determined by age, heart rate, central systolic blood pressure and  $[\text{creat}]_{\text{sr}}$  ( $R^2=0.38$ ,  $P < 0.001$ ). Arterial stiffness was negatively associated with eGFR ( $R^2=0.30$ ,  $P < 0.001$ ), even within the normal range. 3. Endothelium-independent changes in the augmentation indices (AIs) were determined by age, body mass index and mean blood pressure ( $R^2=0.09$ ,  $P < 0.001$ ). Endothelium-dependent changes in AIs were weakly explained by mean blood pressure ( $R^2=0.02$ ,  $P < 0.001$ ) but not associated with hypercholesterolaemia, as previously reported, or renal function. However, the presence or severity of CAD did not explain the variance in arterial stiffness or endothelial function measures. 4. Endothelium-independent and dependent changes in AIs were positively correlated. In addition, endothelium-independent changes in AIs were lower in subjects with stiffer arteries ( $r = 0.20$ ,  $P < 0.01$ ). 5. Subjects with a high number of diseased coronary vessels ( $P < 0.001$ ), a low eGFR ( $P < 0.01$ ), or a PWV above the median ( $P < 0.05$ ) had a higher risk of developing adverse clinical events. Endothelial function, however, did not appear to predict a poor outcome.

**Conclusion:** In healthy subjects, skin elasticity is an unreliable marker of arterial elasticity. An important finding in the CAD study was that renal function was a

determinant of arterial stiffness in patients without a history of renal disease. In this treated group of subjects, traditional cardiovascular risk factors did not determine arterial stiffness or endothelial dysfunction and there was no association between arterial stiffness and endothelial dysfunction. Moreover, endothelial function measured using PWA, with the administration of GTN and salbutamol, is not a useful test in patients with CAD on drug treatment. However, the presence and severity of CAD, renal function, as well as the stiffness of arteries, are predictive of a shorter time to fatal and non-fatal cardiovascular outcomes.

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## ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
AIx	Augmentation index
AIx75	Augmentation index normalised to a heart rate of 75 beats per minute
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP	Augmentation pressure
ARA	Angiotensin II receptor antagonist
AUC	Area under the curve
$\beta$ -blocker	$\beta$ -adrenoreceptor antagonist
BMI	Body mass index
BP	Blood pressure
bpm	Beats per minute
CAD	Coronary artery disease
CABG	Coronary artery bypass graft
CCB	Calcium channel blocker
CCS	Canadian Cardiovascular Society
CF-PWV	Carotid-femoral pulse wave velocity
cGMP	Guanosine 3',5'-cyclic monophosphate
CHD	Coronary heart disease
CHF	Congestive heart failure
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DVP	Digital volume pulse
ECG	Electrocardiogram
ECM	Extracellular matrix
EDHF	Endothelium-derived hyperpolarizing factor
EDS	Ehlers-Danlos syndrome
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
ET-1	Endothelin-1
FBF	Forearm blood flow
FMD	Flow-mediated dilatation
GC	Guanylyl cyclase
GFR	Glomerular filtration rate
GTN	Glyceryl trinitrate
HDL-C	High density lipoprotein cholesterol
HR	Heart rate
HRT	Hormone replacement therapy
IHD	Ischaemic heart disease
LDL-C	Low density lipoprotein cholesterol
L-NMMA	N <sup>G</sup> monomethyl-L-arginine
LVH	Left ventricular hypertrophy
MAP	Mean arterial blood pressure
MDRD	Modification of diet in renal disease

MI	Myocardial infarction
mmHg	Millimetres of mercury
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NO	Nitric oxide
NOS	Nitric oxide synthase
oxLDL	Oxidised low-density lipoprotein
PAIx	Peripheral augmentation index
PP	Pulse pressure
PTCA	Percutaneous transluminal coronary angiography
PWA	Pulse wave analysis
PWV	Pulse wave velocity
PXE	Pseudoxanthoma elasticum
RAAS	Renin-angiotensin aldosterone system
RI	Reflection index
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SI	Stiffness index
SPSS	Statistical package for social scientists
TG	Triglycerides
TIMP	Tissue inhibitors of matrix metalloproteinases
t-PA	Tissue plasminogen activator
UV	Ultraviolet
VLDL	Very low-density lipoprotein

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## CHAPTER 1: INTRODUCTION

## 1.1 BACKGROUND

Coronary heart disease (CHD) is the leading cause of morbidity and mortality among adults in Europe and North America. Cardiovascular disease (CVD) accounts for about 233,000 deaths a year (38% of all deaths) in the United Kingdom [Petersen *et al.* 2005]. Coronary artery disease (CAD) and stroke are the main types of CVD and account for about half and one-quarter of all CVD deaths respectively. CHD is a common cause of premature death (age less than 75 years) accounting for 22% of deaths in men, and 12% in women. About 270,000 people suffer a heart attack each year, with under a third of them dying before they reach hospital. Although the mortality rate from CHD has declined over the last few years, the rate of morbidity due to heart disease has increased. It is estimated that that about 1.2 million people currently living in the U.K. have had a heart attack and around 2 million suffer from angina. CVD is a common cause of death among the elderly, and the rate of death increases with age. Changes in blood pressure regulation, cardiovascular function, circulatory haemodynamics and lipid metabolism lead to CVD. In addition, stiff arteries [McLeod *et al.* 2004] and endothelial dysfunction [Chan *et al.* 2003; Wu *et al.* 2005] have been found in subjects with atherosclerotic heart disease.

### 1.1.1 ATHEROSCLEROSIS

Atherosclerosis is a disease of large and medium-sized elastic arteries. It is characterised by inflammation of the vascular wall, endothelial dysfunction and an accumulation of cholesterol, lipids, calcium and cellular debris within the intima of the vessel wall [Orford *et al.* 2005]. Plaque deposits may grow and obstruct the lumen, or break-up and release material into the blood stream [Hansson 2005]. The

presence of plaque can result in altered blood flow and cardiovascular remodelling, which in turn may lead to myocardial infarction (MI), stroke, and peripheral vascular disease. A number of interrelated processes contribute to atherosclerosis [Loscalzo *et al.* 1996; Orford *et al.* 2005]. These include vascular dysfunction, smooth muscle cell migration and proliferation, cellular inflammation, thrombogenicity of the blood vessel wall, and activation of fibrinolysis and coagulation.

A popular theory of how atherosclerosis develops is the “response-to-injury” theory whereby injury of endothelial cells leads to inflammation and fibroproliferation of the vascular wall [Hansson 2005]. Factors that can lead to endothelial injury include oxidized low-density lipoprotein (oxLDL) cholesterol, hyperglycaemia, hyperhomocysteinemia and by-products of cigarette smoke [Ross 1999]. Circulating monocytes infiltrate the intima of the vessel wall, and these tissue macrophages act as scavenger cells, taking up low-density lipoprotein cholesterol (LDL-C) and forming foam cells which are characteristics of the initial stages of atherosclerosis [Berliner *et al.* 1995]. Atherosclerosis is initially characterised by the development of a fatty streak which is caused by the build-up of serum lipoproteins within the intima of the vessel wall [Ross 1993]. Progressive build-up of lipid-rich macrophages, T-lymphocytes, and the migration and proliferation of smooth muscle cells leads to the development of a fibrous cap [Libby *et al.* 2005; Stary *et al.* 1994]. In addition, reduced endothelium-derived nitric oxide (NO) bioavailability increases proliferation and leads to plaque maturation.

Atherosclerotic plaques have an increased tendency to occur at points where there is branching of blood vessels and high curvature [Ross 1999]. They are also more

likely to occur at areas of geometric irregularity in blood vessels and at points where there are sudden changes in blood velocity or direction of flow [Stary *et al.* 1992]. In particular, atherosclerosis is most commonly found in the coronary arteries, the major branches of the thoracic and abdominal aorta, and the large conduit vessels of the lower extremities [Glagov *et al.* 1992].



## 1.2 ARTERIAL STIFFNESS

Arterial stiffness describes the rigidity of the arterial walls. About 2,000 B.C. the famous Chinese "Yellow Emperor" Huang Ti noted an association between salt and a "hardened pulse" [He *et al.* 2002]. In 1827 Bright described a relationship between the hardening of arteries, albuminuria, cardiac hypertrophy and the risk of death from stroke and cardiac failure [Nichols *et al.* 1998]. Fifty years later Mahomed [Mackenzie *et al.* 2002; Nichols *et al.* 1998] was the first to describe changes in the contour of the arterial pressure pulse with age and in patients with hypertension. He noted that hypertensives and older people had an increase in the amplitude of the late systolic peak and little or no diastolic variation. This is illustrated in Figure 1-1. In 1922 Bramwell and Hill [Nichols *et al.* 1998] carried out the earliest in-vivo studies in which they made non-invasive assessments of the wave foot of sphygmograms to calculate pressure pulse wave velocity. This technique re-emerged in the late 1970's.

In healthy people, stiffening of the arteries with age is accompanied by an isolated elevation in systolic blood pressure (SBP), which averages 25-35 mmHg between the third and eighth decades of life [Nichols *et al.* 1998]. This increase in arterial pressure after-load affects cardiac function. For example, many individuals between the third and ninth decades have an increase in their left ventricular mass which may be partly due to increased arterial pressure and/or vascular stiffness. This has important implications as left ventricular hypertrophy has been shown to be a major independent risk factor for morbid cardiovascular events including MI and cardiac death [Brown *et al.* 2000; Deague *et al.* 2001].

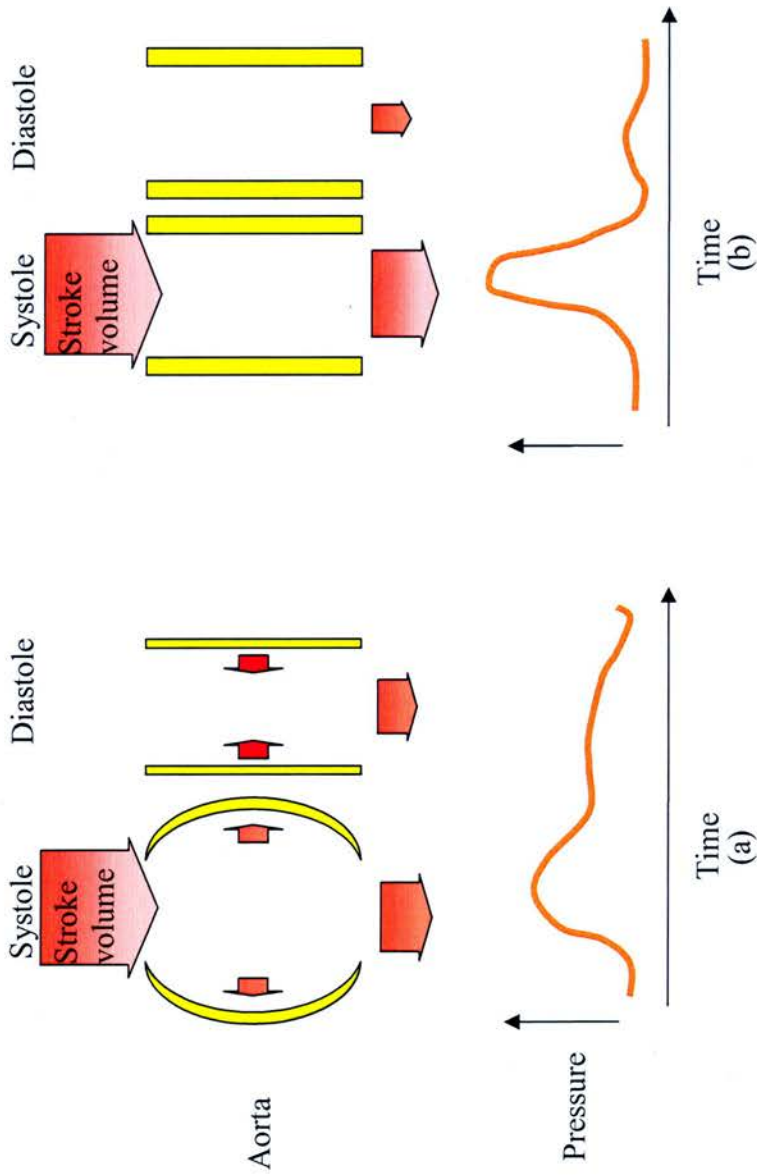


Figure 1-1 Pressure pulse waves in elastic and stiff arteries.

(a) In a normal elastic aorta, blood is ejected with each cardiac stroke and a small amount is retained by the aorta during systole. When the elastic aorta recoils, this small volume of blood is propelled forward into the peripheral circulation and backwards into the coronary arteries during diastole. (b) Stiffening of the aorta causes a loss of elastic recoil and an increase in vascular resistance which in turn results in most of the stroke volume being propelled into the circulation during systole with reduced flow during diastole. An increase in resistance also leads to an increase the cardiac work required with each stroke volume.

### 1.2.1 PHYSIOLOGY OF THE ARTERIES

The arterial wall comprises three layers: the tunica intima, the media and the adventitia [Loscalzo *et al.* 1996; Nichols *et al.* 1998]. This is illustrated in Figure 1-2. In simple terms, the tunica intima comprises an elastic membrane lining and smooth endothelium [Loscalzo *et al.* 1996]; the tunica media comprises elastic fibres and smooth muscle cells; and the tunica adventitia comprises connective tissue, elastin and collagen fibres. The distribution of elastin and collagen varies along the aorta [Nichols *et al.* 1998]. Elastin is the main component in the proximal aorta, whereas in the distal aorta, the collagen-to-elastin ratio is reversed with collagen being more dominant in the peripheral arteries [Avolio *et al.* 1998; Nichols *et al.* 1998]. Elastic fibres in the aorta form thick concentric lamellae in the tunica media with interlaminar connecting fibres scattered radially through the vessel wall [Arteaga-Solis E *et al.* 2000] whereas collagen fibres are arranged circumferentially [Nichols *et al.* 1998]. Several neurohumoral factors, for example angiotensin II and aldosterone, modulate collagen accumulation [Zieman *et al.* 2005]. Chemical modification of collagen, through processes such as breakdown, cross-linking, or protein glycation can cause an increase in arterial stiffness [Tayebjee *et al.* 2003]. Irregular distribution of collagen along the vascular wall results in discontinuities in their distribution along the walls mainly at vessel bifurcations.

The high elastin-to-collagen ratio in the walls of the large arteries allows them to expand and recoil with pulsatile flow [Loscalzo *et al.* 1996]. This ratio decreases towards the periphery. Arteries are found to stiffen with age due to an accumulation of fibre, intimal and medial thickening, calcium deposits in the extracellular matrix

(ECM) and breakdown of the elastic laminae [London 1995]. These changes are more commonly found in the aorta and are caused by cyclical stress over time. The diameters of the common carotid artery, abdominal aorta, femoral artery, and brachial artery are known to increase with age. However, the percentage change in diameter is found to decrease [Bortolotto *et al.* 1999a].

The elasticity of a given vascular segment is determined by its distending pressure which is, in turn, determined by mean arterial pressure (MAP) [Bank *et al.* 1996]. An increase in the distending pressure causes an increase in the recruitment of the inelastic collagen fibres which leads to a decrease in vascular elasticity [Lakatta *et al.* 2003]. Collagen and elastin are connected by smooth muscle cells. Therefore, changes in smooth muscle tone can affect the contribution of collagen and elastin to arterial stiffness. Smooth muscle tone is affected by the nervous system, hormones, drugs and locally produced vasoactive substances [Nichols *et al.* 1998; Safar ME *et al.* 1986].

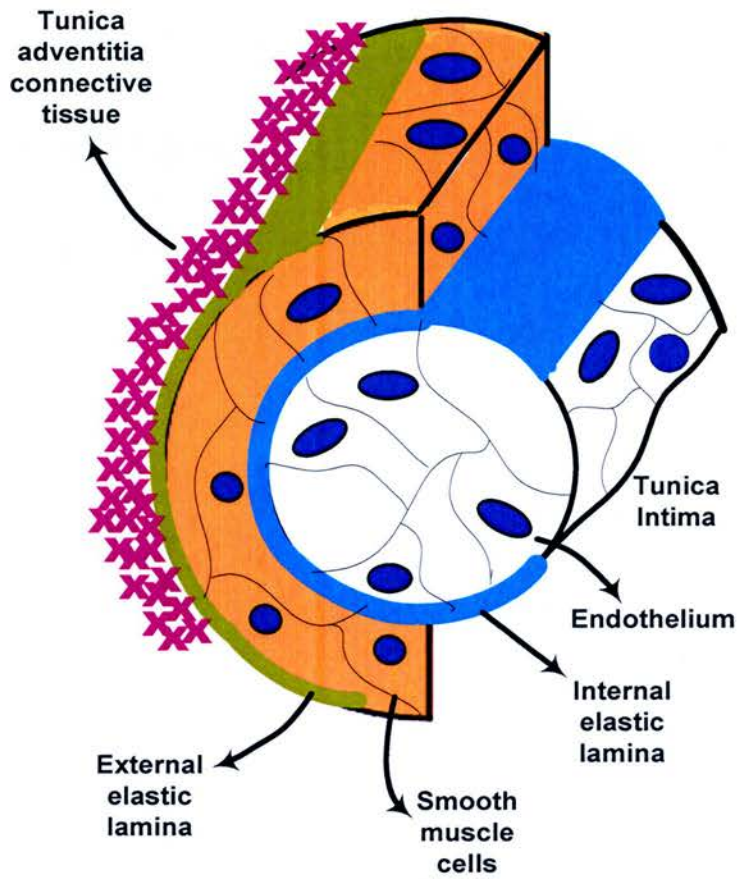


Figure 1-2 The arterial wall.

Schematic of an artery and its constituent layers: the tunica intima, tunica media and tunica adventitia. The tunica intima comprises a layer of endothelial cells lined by an elastic membrane. The tunica media comprises elastic fibres and smooth muscle cells while the tunica adventitia comprises connective tissue, elastin and collagen fibres.

### 1.2.2 ARTERIAL SYSTEM

The oldest model of the arterial system is the Windkessel system [Nichols *et al.* 1998]. This system is analogous to the inverted air-filled dome of a fire engine which works by changing pulsatile flow from a hand activated pump into a steady stream. The Windkessel theory models the circulation as a central reservoir (the large arteries) into which the heart pumps, and from which blood travels to the tissues through non-elastic conduits (peripheral arteries). It is now well known that during the systolic component of pulsatile flow, energy is absorbed by the large elastic arteries. This leads to a reduction in the work the heart requires to propel blood forward [London 1995].

### 1.2.3 ARTERIAL PRESSURE WAVEFORM

The arterial pressure waveform is a composite of the forward and reflected pressure waves created by ventricular contraction [Glasser *et al.* 1997]. Pressure waves are mainly reflected from branch points or junctions between low resistance conduit arteries and high resistance arterioles. This leads to changes in the arterial pressure waveform as shown in Figure 1-3. Other factors play an important role, for example, differences in arterial properties between two sites and the presence of arterial lesions. In elastic vessels the reflected waves arrive back at the aortic root during diastole and this augments diastolic pressure thereby improving coronary artery perfusion. As vessels become stiffer the velocity of the pressure wave increases [Nichols *et al.* 1998]. This leads to the reflected wave arriving earlier at the central arteries and augmenting SBP with a consequent decrease in diastolic blood pressure (DBP). Arterial stiffness also causes an increase in the amplitude of the reflected

wave which further augments central SBP. Pulse pressure (PP), a surrogate measure of arterial stiffness, is an independent predictor of cardiovascular mortality, and is a stronger predictor than either SBP or DBP in older subjects [Franklin *et al.* 1999]. Recent data have shown arterial stiffness to be an additional and independent risk factor [Blacher *et al.* 1999b].

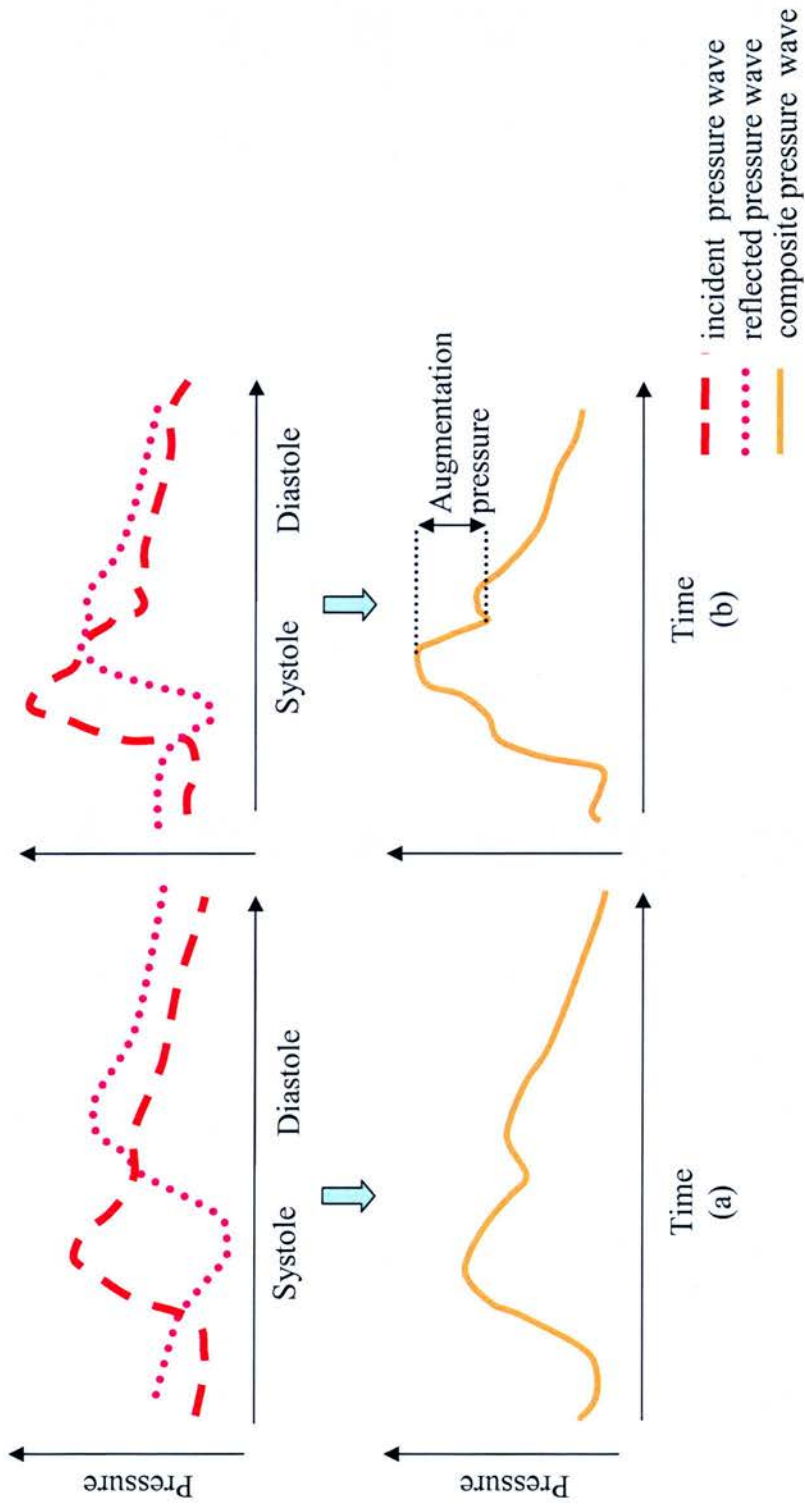


Figure 1-3 Forward and reflected pressure waves in arteries. Schematic of pressure waveforms in (a) an elastic artery and (b) a stiff artery. In elastic vessels the reflected wave returns to the aortic root during diastole. An increase in vessel stiffness increases pulse wave velocity which causes the reflected pressure wave to return during late systole.



#### 1.2.4 MEASURING ARTERIAL STIFFNESS

There are various methods of measuring arterial stiffness. A number of techniques give information on systemic arterial stiffness, while others give information on local stiffness of a particular vessel.

##### 1.2.4.1 Pulse pressure

The blood pressure waveform with time is an interaction between the steady component of mean arterial blood pressure (MAP) and the pulsatile component of PP [Safar *et al.* 2003]. PP is the difference between SBP and DBP by intermittent ventricular ejection from the heart. PP is influenced by several cardiac and vascular factors, for example, cardiac output, large-artery stiffness and wave reflection [Safar *et al.* 2003]. Both SBP and DBP increase with age [Franklin *et al.* 1997]. However, beyond the age of 50–60 years DBP begins to decline. PP has been shown to increase with age and may be the best index of cardiovascular risk amongst the various components [Franklin *et al.* 1999]. In 1922 Bramwell and Hill recognised PP as a valuable surrogate marker of arterial stiffness.

Peripheral PP can be readily measured using a standard sphygmomanometer [Mackenzie *et al.* 2002] and is one of the simplest surrogate measure of arterial stiffness. However, measurement of PP alone is not an accurate measure of arterial stiffness. This is because the pressure wave is amplified as it travels from the aorta to the periphery and therefore PP measurements in the periphery may not accurately show changes in the central arteries [Nichols *et al.* 1998]. This is illustrated in Figure 1-4.

Central PP may be a more accurate predictor of risk than peripheral PP as the former contributes most to the development of left ventricular hypertrophy and it is also a predictor of cardiovascular mortality [Deague *et al.* 2001]. In addition, carotid intima medial thickness, a marker of cardiovascular risk [Simons *et al.* 1999], is more closely associated with carotid rather than brachial PP [Boutouyrie *et al.* 1999; Mackenzie *et al.* 2002].

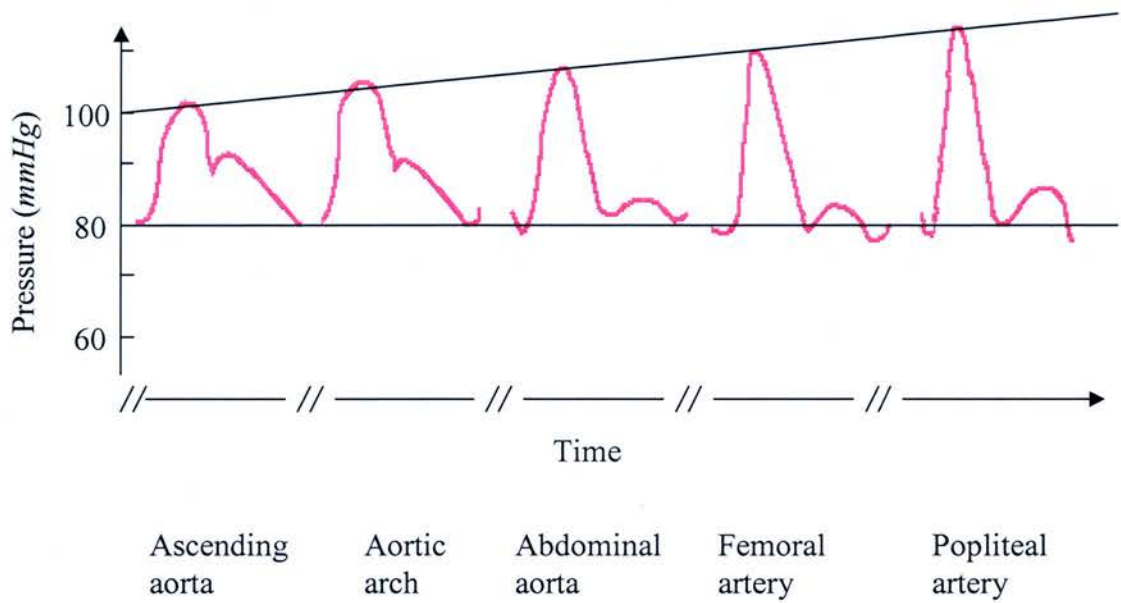


Figure 1-4 Pressure waves in the central and peripheral arteries. Schematic of changes in pressure waveforms as pressure waves travel towards peripheral arteries. The waveform for each arterial site describes the interaction of the incident and reflected pressure waves. (Adapted from Nicholls WW, O'Rourke M. McDonald's Blood Flow in Arteries: Theoretical, Experimental, and Clinical Principles.1998)

#### 1.2.4.2 Pulse wave velocity

Pulse wave velocity (PWV) is the speed at which the forward pressure wave is transmitted from the aorta through the vascular tree [Safar *et al.* 2003]. It is calculated by measuring the time taken for the arterial waveform to pass between two points a measured distance apart (Figure 2.2 and 2.3). This can be carried out by taking readings from the two sites simultaneously, or “gating” separate recordings to a fixed point in the cardiac cycle, usually the R wave of the ECG [Wilkinson *et al.* 2004]. The foot-to-foot methodology is most commonly used to detect the start of a waveform as this avoids the confounding influence of wave reflection [Mackenzie *et al.* 2002; Wilkinson *et al.* 1998]. PWV is calculated as an average of 10 consecutive waveforms. There is an increase in PWV with age. This increase is larger in the aorta than in peripheral vascular beds. This is due to a greater disorganisation of the arterial media in central elastic arteries. The elastic arteries are prone to fatigue and fracture of elastin fibres as a consequence of greater pulsatile strain [Avolio AP *et al.* 1985; Avolio *et al.* 1983].

Invasive and non-invasive methods can be used to measure characteristics of either the pressure or flow waves. PWV has been shown to increase with stiffness according to the Moens-Korteweg equation,  $PWV = \sqrt{(Eh/2\rho R)}$ , where E is Young's modulus of the arterial wall, h is wall thickness, R is arterial wall radius at the end of diastole, and  $\rho$  is the density of blood [Nichols *et al.* 1998]. From this equation we know that PWV increases in narrower blood vessels as seen in the peripheral blood vessels. The aorta and its first branches are responsible for most of the pathophysiological effects found in stiffer arteries. Therefore, PWV is most

commonly measured from differences in pulse wave travel in the carotid and femoral arteries to give a measure of central aortic stiffness.

The technique of PWV has been validated and is reproducible. The principle is relatively simple and the technique can easily be learnt and applied in both a research and clinical setting. Moreover, outcome data show that PWV is an independent predictor of cardiovascular risk in both patients with hypertension [Blacher *et al.* 1999a] and those with end-stage renal disease [Blacher *et al.* 2003]. Measurements of the pressure wave can be made non-invasively and estimated measures of the distance travelled by the pressure wave can be made from the body surface. The PWV measurements become less accurate, however, if the recording points are very close together, and the technique is, therefore, limited to use on larger arteries. PWV is a measure of a distinct pathway through the vascular system rather than systemic arterial stiffness.

#### 1.2.4.3 Ultrasound

Ultrasound is another method of measuring arterial stiffness, but its use is limited to the larger and more accessible arteries [Mackenzie *et al.* 2002]. This technique is commonly used in the brachial, femoral and carotid arteries and the abdominal aorta, and distensibility or compliance measurements are recorded. Images of the vessel wall are captured with each cardiac cycle, and the maximum and minimum cross-sectional areas of the vessel are calculated [Mackenzie *et al.* 2002]. One of the benefits of ultrasound imaging is that it is a non-invasive technique. However, there are some limitations to its use. One such limitation is that the bulky machines are not portable. There are also difficulties in detecting small changes in vessel diameter due

to its limited resolution (minimum scatter spacing at which it can discriminate closely spaced scattered echoes) with changes in graduations detectable to 0.1 mm. In addition, operators need to be trained to image blood vessels accurately. Blood pressure measured simultaneously with ultrasound imaging is usually carried out in the brachial artery or finger [Oliver *et al.* 2003]. This may not be an appropriate measure of blood pressure due to the phenomenon of pressure wave amplification especially when assessing distensibility of the central arteries [Oliver *et al.* 2003].

#### 1.2.4.4 Magnetic resonance imaging derived indices

Magnetic resonance imaging (MRI) is a non-invasive technique that measures vascular distensibility and compliance [Mackenzie *et al.* 2002]. Most MRI studies carried out on humans have involved taking measurements from the aorta. Studies using MRI have shown age to be inversely proportional to aortic distensibility and aortic distensibility has been shown to be lower in hypertensive patients [Mackenzie *et al.* 2002; Resnick *et al.* 1997]. In addition, arterial compliance, as measured by MRI, has been shown to be lower in patients with CAD [Mohiaddin RH *et al.* 1989]. The MRI machine is very expensive and provides more detailed images than ultrasound imaging. MRI scanners are not portable and patients need to be placed inside them even though only a small part of the body is being assessed. This makes them unsuitable for use in a clinical setting as a test of endothelial function [Mackenzie *et al.* 2002].

#### 1.2.4.5 Arterial waveform analysis

Numerous methods of analysing the arterial waveform have been developed including the vasculograph and, more recently, non-invasive applanation tonometry.

Arterial pressure or blood volume waveforms can be analysed to measure arterial stiffness.

#### 1.2.4.5.1 Arterial pressure waveform analysis

In healthy people, the reflected pressure wave returns during the diastolic phase [Nichols 2005; Yasmin *et al.* 1999]. However, an increase in PWV causes the forward wave to be transmitted quicker. This leads to the reflected wave returning earlier and augments the forward wave during systole [O'Rourke *et al.* 2004]. Early wave reflection distorts the favourable shape of the ascending aortic pressure wave causing a relative increase in pressure during systole and a relative decrease in pressure during diastole. This early wave reflection distorts arterial function, not only by increasing aortic and left ventricular pressure but also by decreasing aortic pressure during diastole [Nichols 2005].

Peripheral artery pressure can be measured using the technique of applanation tonometry [Nichols *et al.* 1998]. The arterial pressure waveform is calibrated to measure brachial blood pressure. Peripheral pressure wave analysis can be used to derive the central aortic waveform from a peripheral artery, for example the radial artery using a validated transfer function [O'Rourke *et al.* 1996; Pauca *et al.* 2001]. From the derived central aortic waveform, an average of 10 consecutive waveforms, the augmentation index (Aix) can be calculated [Nichols *et al.* 1998]. Aix is the proportion of central PP that results from arterial wave reflection and is used as a measure of systemic arterial stiffness as shown in Figure 1-5. Aix is determined by a number of factors including the PWV, intensity of wave reflections, and the elasticity and diameter small muscular arteries [O'Rourke *et al.* 2004].

Applanation tonometry is a simple non-invasive technique. The tonometer is the size of a pen which is connected to a personal computer. It is a portable system, which makes it suitable for use in both clinical and research settings. It is a useful technique whereby characteristics of blood pressure in the central arteries can be determined [Pauca *et al.* 2001]. It also allows continuous measurements, which makes it a useful tool for investigational drug studies. One of the main limitations of this technique is a generalised transfer function is used to derive central parameters from peripheral parameters [Oliver *et al.* 2003]. The pressure applied through the tonometer and the angle at which it is held can affect the shape and quality of the pressure waveforms, which in turn will affect the calculation of augmentation indices.

Pressure pulse contour analysis is another method of measuring arterial stiffness non-invasively [Cohn *et al.* 1995; McVeigh *et al.* 1999], which involves tonometry of the radial artery. However compliance is derived using a modified Windkessel model of the circulation and an assessment of the diastolic pressure decay. This method can be used to measure large-vessel and peripheral compliance. Pressure waveform analysis can also be carried out, more distally, using a servocontrolled pressure cuff on the digital artery as in the Finapres system [Millasseau *et al.* 2000].



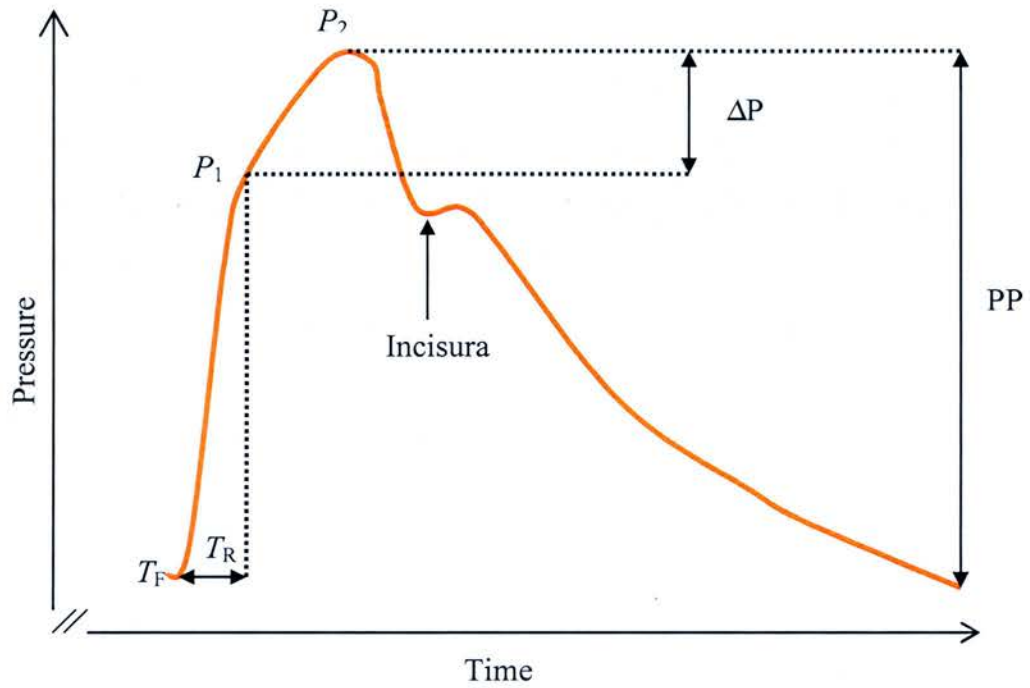


Figure 1-5 Central pressure waveform.

A schematic of a central pressure waveform from which the augmentation index can be calculated. The augmentation index is calculated as the difference between peaks  $P_2$  and  $P_1$  ( $\Delta P$ ) and this is expressed as a percentage of the PP.  $T_F$  is the foot of the wave, and  $T_R$  is the time between  $T_F$  and the point of inflection.

#### 1.2.4.5.2 Digital volume pulse waveform analysis

The digital volume pulse (DVP) waveform can be recorded using the technique of photoplethysmography [Chowienczyk *et al.* 1999]. The blood volume pulse waveform has similar characteristics to the pressure pulse waveform [Millasseau *et al.* 2000]. This technique resembles that of pulse oximetry, where a small infra-red light transmitting device is attached to the index finger and an averaged waveform is produced from a recording of 10 consecutive waveforms. The DVP is formed as a result of the pressure wave transmitted (systolic component) along a direct path from the aortic root to the finger [Millasseau *et al.* 2000]. The second part of the waveform is formed by a pressure wave which has been transmitted from the ventricle along the aorta to the lower body, and reflected back along the aorta to the finger (diastolic component). The dichrotic notch is produced by the overlapping of the incident and reflected waves and is associated with either the second peak or the point of inflection of the overlapping waves [Chowienczyk *et al.* 1999]. The timing of the diastolic component relative to the systolic component depends on the PWV of the pressure waves in the large arteries and aorta. The height of the subject is divided by the time between the first peak and the dichrotic notch to obtain a stiffness index (SI) [Millasseau *et al.* 2002] which has been shown to strongly correlate with aortic PWV [Millasseau *et al.* 2000] (Figure 2.4). The reflection index (RI) is the height of the second peak of the DVP expressed as a percentage of the waveform peak and is a measure of the tone of the small or medium arteries.

The DVP technique has the advantages of being relatively simple, less operator-dependent, less expensive and more portable. The photoplethysmogram is able to reject ectopic beats and artefacts while recording fast sequential measurements. The

photoplethysmographic technique is, however, affected by damping of the peripheral pulse and temperature-dependent changes in the peripheral circulation. A study [Bortolotto *et al.* 2000] comparing photoplethysmography of the finger with PWV found the latter to correlate more closely with those parameters which influence vascular compliance, namely age and atherosclerosis. Further work is therefore needed to verify the relationship between the DVP and central pressure waveforms.

#### 1.2.4.6 Reproducibility of PWV, AIx and DVP

##### 1.2.4.6.1 PWV

Arterial stiffness assessed non-invasively by measurement of pulse wave velocity (PWV), is a simple and reproducible method. This was shown by Wilkinson *et al.*, who carried out a reproducibility study of aortic and brachial PWV in 24 subjects (10 controls, 8 hypertensives, and 6 hypercholesterolaemics), with a mean age of 40 years [Wilkinson *et al.* 1998]. Measurements were made by 2 observers randomly at least 2 minutes apart on the same day. From Bland-Altman plots the mean  $\pm$  SEM within-observer differences for aortic and brachial PWV were  $0.07 \pm 0.24$  m/s and  $0.14 \pm 0.07$  m/s respectively (the SD of the differences for aortic and brachial PWV was 1.17 m/s and 0.82 m/s respectively;  $P=0.78$ ). The mean  $\pm$  SEM between-observer difference was  $-0.30 \pm 0.26$  m/s and  $-0.44 \pm 0.23$  m/s for aortic and brachial PWV (the SD of the differences for aortic and brachial PWV was 1.25 m/s and 1.09 m/s respectively;  $P=0.68$ ). These results showed good reproducibility of the technique.

#### 1.2.4.6.2 AIx

The reproducibility of AIx was analysed in a group of 33 subjects (5 controls, 12 diabetics and 16 hypertensives) with a mean age of 51 years [Wilkinson *et al.* 1998]. The mean  $\pm$  SEM within-observer difference was found to be  $0.49 \pm 0.9\%$  ( $P=0.36$ ) and the between-observer difference,  $0.23 \pm 0.7\%$  ( $P=0.73$ ). The SD of the within-observer differences was 5.37% while the difference for between-observers was 3.80%. This showed AIx to be reproducible not only over time but also between two different operators.

#### 1.2.4.6.3 DVP

A generalised transfer function has been derived by Millasseau *et al.* that predicts the pressure pulse from the volume pulse across a wide age range in both normotensive and hypertensive subjects [Millasseau *et al.* 2000]. The transfer function can be applied to predict the pressure pulse after GTN administration, when large changes in volume and pressure are seen. They went on to show [Millasseau *et al.* 2002] that SI correlated strongly with carotid-femoral PWV, as measured using applanation tonometry, and that both the SI and PWV correlated independently with age and blood pressure. This indicates that both the SI and PWV are influenced by similar factors.

### 1.2.5 ARTERIAL STIFFNESS AND CARDIOVASCULAR FUNCTION

In elastic arteries the reflected pressure wave returns during the diastolic phase of blood pressure and therefore enhances coronary blood flow. As large vessels stiffen, the limited ability of the aorta to stretch means the reflected pressure waves arrive earlier during systole resulting in a late systolic peak in arterial pressure. This

process leads to larger systolic and PPs. Moreover, the heart has to work harder to pump blood into the circulation against a higher pressure gradient. This has a detrimental effect as it leads to left ventricular hypertrophy of the heart as it tries to maintain cardiac stroke volume.

AIx has been shown to increase with both MAP [Wilkinson *et al.* 2001], and with age [Kelly R *et al.* 1989], and it is inversely proportional to heart rate [Gatzka *et al.* 2001a]. AIx has also been shown to be higher in patients with risk factors for atherosclerosis, such as patients with type I diabetes [Wilkinson *et al.* 2000b] and hypercholesterolaemia [Wilkinson *et al.* 2002d], despite similar peripheral blood pressure when compared to controls. In a study by London *et al.* a high carotid AIx was found to predict mortality in patients who had normal PWV measures [London *et al.* 2001]. This highlights the importance of assessing other characteristics of the arterial pressure waveform. In young subjects peripheral arterial stiffness exceeds central arterial stiffness. This is reversed, however, with increasing age. This is due to a decrease in pressure wave amplification and reflection but an increase in harmful forward pressure wave into the microcirculation [Mitchell *et al.* 2004b].

Development of arterial plaque can affect vascular function and lead to atherothrombotic complications. It is known that plaque develops at locations of low shear stress [Ross 1999]. However, as the size of the plaque grows into the lumen it is exposed to higher shear stress which can lead to destabilisation of the fibrous cap and plaque rupture [Hansson 2005]. The shoulder regions of the plaque are especially vulnerable to rupture. In addition, pulsatile stress can cause plaque instability. Lovett *et al.* have shown PP to positively correlate with carotid plaque rupture and plaque

ulceration [Lovett *et al.* 2003]. Therefore, an increase in PP due to the stiffening of large arteries can lead to plaque destabilisation and rupture.

#### 1.2.6 ARTERIAL STIFFNESS AND CARDIOVASCULAR RISK

An increase in arterial stiffness may have several important consequences. An increase in PP or aortic stiffness is associated with an increase in the risk of coronary events, stroke, all-cause and cardiovascular mortality [Blacher *et al.* 1998; Domanski *et al.* 1999; Gatzka *et al.* 1998; Laurent *et al.* 2001; Mitchell *et al.* 1997]. Arterial stiffness measured by PWV, carotid elastic modulus, or the ratio of stroke volume to PP, independently predicts all-cause and CVD mortality in patients with end-stage renal failure (ESRF) and essential hypertension [Asmar *et al.* 1995; Blacher *et al.* 1998; Laurent *et al.* 2001]. In addition, brachial PP has been shown to be an independent risk factor for CHD and is associated with all-cause and CVD mortality [Benetos *et al.* 1997]. However, brachial PP is an indirect index of stiffness of the large central arteries to PP amplification [Franklin *et al.* 1999; Nichols *et al.* 1998; O'Rourke *et al.* 1999]. This is supported by findings in which central PP determines left ventricular load [Saba *et al.* 1993] and is associated with the degree of carotid intima-media thickness [Boutouyrie *et al.* 1999], which is itself a predictor of cardiovascular risk. In addition, central arterial stiffness has been shown to be higher in patients with CAD, diabetes mellitus, stroke, hypertension, hypercholesterolaemia and ESRD [Laurent *et al.* 2001; Laurent *et al.* 2003; Schram *et al.* 2004; Weber *et al.* 2004; Wilkinson *et al.* 2002d].

In newly diagnosed patients with CAD a higher left ventricular mass and stiffer proximal aorta, as measured by echocardiography, has been found compared to

control subjects [Gatzka *et al.* 1998]. Moreover, Hirai *et al.* have shown in patients with a previous MI and who have a higher number of diseased coronary blood vessels to have higher proximal aortic stiffness [Hirai T *et al.* 1989]. More recently, by measuring AIx, London *et al.* showed arterial wave reflections to be a major predictor of mortality in ESRF patients [London *et al.* 2001]. These results were further supported by others who found that patients with CAD and early wave reflections had stiffer arteries. Early wave reflections were also found to be related to the severity of CAD, particularly in patients less than 60 years of age [Nurnberger *et al.* 2002; Weber *et al.* 2004].

### 1.3 ENDOTHELIAL FUNCTION

The endothelium plays an important role in regulating vascular tone and blood flow through the arteries. Endothelial function and the release of endothelium-derived factors are necessary to maintain and regulate tissue perfusion pressure. In 1980, work by Furchgott & Zawadzki showed that relaxation of blood vessels by acetylcholine requires the presence of endothelial cells. Ach acts on muscarinic receptors, on these endothelial cells, and causes the release of a substance resulting in vascular smooth muscle relaxation [Furchgott *et al.* 1980]. The vasodilator produced by the endothelium in response to Ach was named 'endothelium derived relaxing factor' which was later identified as NO [Palmer *et al.* 1987]. More recently, studies have shown the presence of abnormal endothelium-dependent vasomotion in patients with atherosclerosis and its associated risk factors [Bonetti *et al.* 2003; Brunner *et al.* 2005]. Abnormalities in endothelial function are now known to be an early pathophysiological manifestation of hypertension, diabetes and atherosclerosis [Gimbrone 1999].

#### 1.3.1 THE ENDOTHELIUM

The endothelium is a single cell thick layer which lines the lumen of blood vessels. In total, the endothelial lining weighs ~1.8 kg and has a surface area of ~700 m<sup>2</sup> [Luscher *et al.* 1997]. The endothelial lining cells are ideally positioned to directly influence cells within the vessel wall and circulating blood components. The endothelium regulates a large number of processes, for example, smooth muscle cell proliferation, production of free radicals, platelet aggregation, leukocyte adhesion, haemostasis, thrombosis, inflammation, and immune responses [Deanfield *et al.*



2005; Widlansky *et al.* 2003]. Vascular tone is controlled by endothelial cells via the secretion of vasodilator substances, such as prostacyclin and nitric oxide (NO), and contracting substances, such as endothelin. Vascular tone is also regulated by norepinephrine, serotonin, inactivation of bradykinin and conversion of angiotensin I to angiotensin II.

### 1.3.2 ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction describes an impairment of the normal biochemical processes carried out by endothelial cells. Endothelial dysfunction commonly precedes the development of coronary atherosclerotic lesions and thrombotic events (e.g., acute myocardial infarction (AMI) and unstable angina) [Celermajer *et al.* 1992; Shah 1920]. Endothelial dysfunction is characterised by an impairment of endothelium-dependent vasodilatation, inflammation, proliferation of smooth muscle cells, and a procoagulatory state [Endemann *et al.* 2004].

NO is the main vasodilator released by the endothelium [Endemann *et al.* 2004]. The enzyme eNOS, which is stimulated by calcium, catalyses the conversion of L-arginine to L-citrulline and NO [Moncada *et al.* 1993]. NO activates soluble guanylate cyclase, in the smooth muscle cells, which leads to an increase in cyclic guanosine 5-monophosphate (cGMP) and subsequent vasodilatation of the arterial wall. This is illustrated in Figure 1-6. NO is responsible for inhibition of monocyte-endothelium cell interactions, smooth muscle cell proliferation and migration, and platelet activation. Other relaxing factors include endothelium derived hyperpolarising factor (EDHF), prostacyclin, C-type natriuretic peptide, 5-hydroxytryptamine (serotonin; 5-HT), adenosine triphosphate (ATP), substance P,

and acetylcholine [Shepherd 1995; Shimokawa 1999]. The endothelium also releases contracting factors like endothelin-1 (ET-1), angiotensin II, thromboxane A<sub>2</sub>, prostaglandin H<sub>2</sub>, superoxide anions, and ATP [Endemann *et al.* 2004]. Basal release of NO has been found in both conduit and resistance vessels in the human coronary circulation [Quyyumi *et al.* 1995]. Basal vascular tone is also likely to be mediated by endothelin, angiotensin II and prostacyclin [Harris *et al.* 2004]. Basal blood flow is another factor that can influence vascular tone. An increase in blood flow promotes a continuous release of endothelium-derived relaxing factors [Bassenge *et al.* 1988] while the presence of hypoxia, thrombin, norepinephrine and stretch promote the release of endothelium-derived contracting factors [Gimbrone 1999].

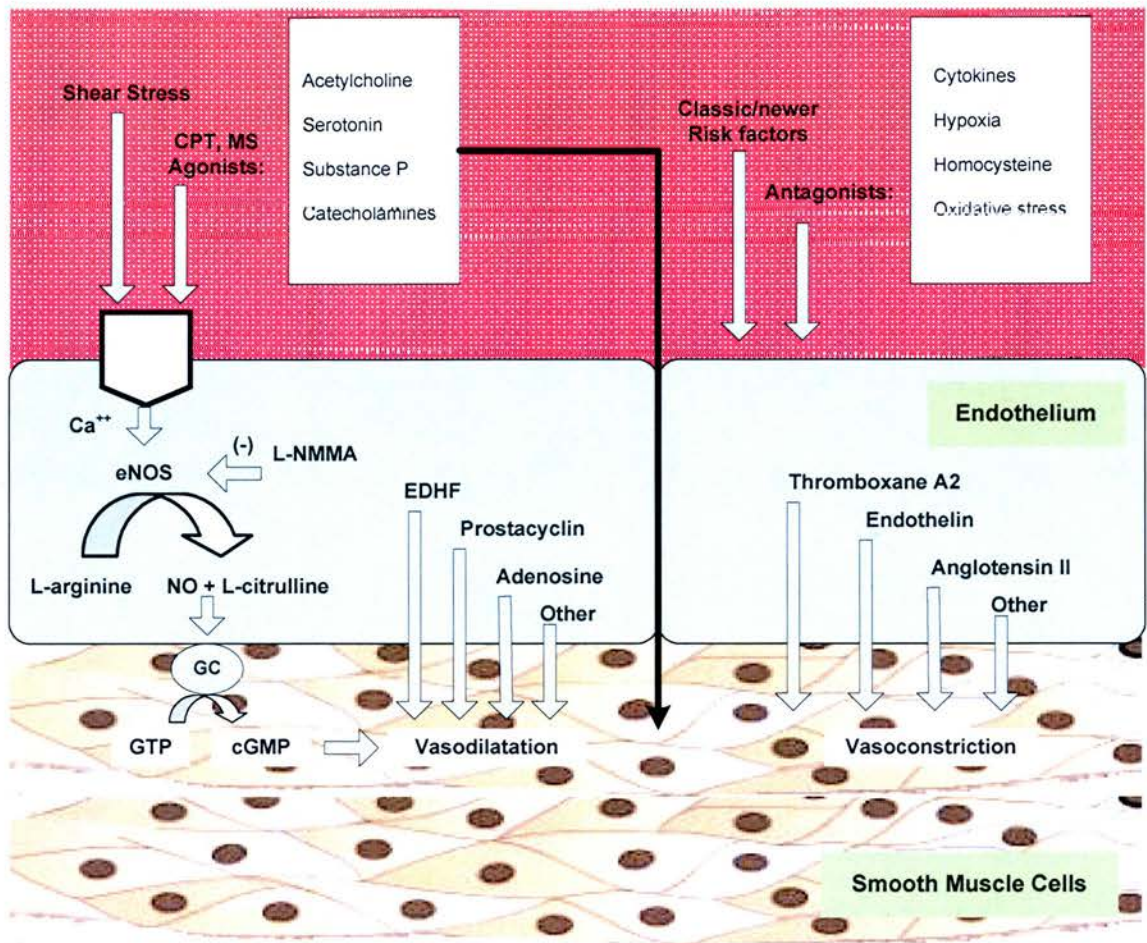


Figure 1-6 The vascular endothelium and smooth muscle cells.

Schematic of the interaction between the vascular endothelium and smooth muscle cells in regulating vascular tone. In normal coronary arteries infusion of Ach causes vasodilatation, however, in the presence of endothelial dysfunction Ach acts directly on the smooth muscle cells and causes vasoconstriction. CPT denotes cold pressor test; eNOS, endothelial nitric oxide synthase; GC, guanyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; L-NMMA, N<sup>G</sup>-monomethyl-L-arginine; and MS; mental stress; NO, nitric oxide. Adapted from Tousoulis et al. Evaluating endothelial function in humans: A guide to invasive and non-invasive techniques.2005 [Tousoulis *et al.* 2005]

### 1.3.3 ASSESSMENT OF ENDOTHELIAL FUNCTION

Endothelial function is measured by exposing blood vessels to an endothelium-dependent vasodilating stimulus followed by measurement of the vasodilatory response. Endothelial function of the coronary circulation is assessed by invasive techniques while the peripheral circulation can be assessed by either invasive or non-invasive techniques.

#### 1.3.3.1 Invasive techniques

##### 1.3.3.1.1 Intracoronary infusions

Coronary artery endothelial function is commonly assessed by intracoronary infusion of acetylcholine (ACh), which acts on muscarinic receptors found on endothelial cells, causing the release of NO and consequently dilatation of the coronary artery [Tousoulis *et al.* 2005]. Acetylcholine dilates normal coronary arteries. However, in patients with risk factors for atherosclerosis, and those with CAD, infusion of acetylcholine results in an impaired vasodilatory response or paradoxical vasoconstriction [Tousoulis *et al.* 1998; Widlansky *et al.* 2003]. Other vasoactive substances that can be used as a test of coronary endothelial function include bradykinin, serotonin and substance P.

##### 1.3.3.1.2 Intrabrachial infusions

Endothelial function in the coronary arteries has been shown to closely correlate with peripheral endothelial function [Jambrik *et al.* 2004]. Intrabrachial infusion of vasoactive agents, such as ACh and sodium nitroprusside, are used as endothelium-dependent vasodilators [Deanfield *et al.* 2005] and endothelium-independent



vasodilators respectively. Forearm blood flow (FBF) can be measured using strain-gauge plethysmography or FMD via measurement of change in brachial artery diameter ultrasound. Both these methods are described in detail in Section 1.3.3.2.1 and 1.3.3.2.2. This methodology has fewer complications compared to intracoronary infusions and the brachial artery is easily accessible in comparison to the coronary arteries.

The disadvantage of using an invasive technique to assess endothelial function is the risk associated with invasive procedures and the cost/complexity of carrying out invasive tests. Invasive endothelial function testing, especially intracoronary vascular function testing, is not an appropriate screening tool in a clinical setting. This has led to the development of non-invasive techniques of evaluating endothelial function [Kuvin *et al.* 2003].

#### 1.3.3.2 Non-invasive techniques

Non-invasive techniques of measuring peripheral endothelial function testing provide an opportunity to evaluate large patient populations. Various non-invasive techniques available are discussed below.

##### 1.3.3.2.1 Flow mediated dilatation

High-resolution ultrasound, to image the brachial artery during reactive hyperaemia, can be used to assess peripheral vascular function [Anderson *et al.* 1995b]. This technique measures FMD. [Corretti *et al.* 2002; Sorensen KE *et al.* 1995]. Arterial blood supply is interrupted by the use of a cuff inflated to suprasystolic pressure which causes ischaemia of the forearm. Release of the cuff induces reactive

hyperaemia (which is an increase in blood flow due to increased shear stress) causing an increase in NO production and consequent vasodilatation of the brachial artery. Endothelial function is measured as the magnitude of the change in vessel diameter from the baseline period to the peak during reactive hyperaemia [Landmesser *et al.* 2002; Rubenfire *et al.* 2000; Rubenfire *et al.* 2002].

#### 1.3.3.2.2 Strain-gauge plethysmography

Another non-invasive method of assessing endothelial function is the evaluation of changes in FBF to hyperaemia [Tousoulis *et al.* 2005]. Changes in FBF to reactive hyperaemia represents endothelium-dependent vasodilatation whereas changes in FBF to GTN represents endothelium-independent vasodilatation. Vasodilatation due to hyperaemia and GTN is calculated as the percentage change in flow from baseline to maximum flow. Strain-gauge plethysmography does not require highly trained personnel compared to assessment of FMD.

#### 1.3.3.2.3 Arterial wave reflections

Newer non-invasive techniques of assessing endothelium-dependent vasodilatation in the peripheral circulation include applanation tonometry or finger photoplethysmography. Administration of vasodilators, such as glyceryl trinitrate, have a profound effect on the pressure waveform, reducing wave reflection, augmentation index, central systolic pressure and arterial stiffness, without causing detectable changes in peripheral vascular resistance or peripheral blood pressure [Kelly RP *et al.* 1990]. Salbutamol, a  $\beta_2$ -adrenergic receptor agonist, has been shown to lower both the inflection point of the DVP measured by finger photoplethysmography [Chowienzyk *et al.* 1999] and AIx measured by applanation tonometry [Wilkinson

*et al.* 2002b]. These actions of salbutamol are mediated, at least in part, through the NO pathway [Dawes *et al.* 1997; Hayward *et al.* 2002]. Furthermore, in patients with hypercholesterolaemia, who are known to have impaired endothelial function, the effect of salbutamol on AIX was blunted. This finding correlated with acetylcholine-induced vasodilatation in the forearm using strain gauge plethysmography [Wilkinson *et al.* 2002b]. Patients with CAD have also been found to have a smaller decrease in AIX after the administration of salbutamol when compared with healthy controls [Hayward *et al.* 2002].

#### 1.3.4 ENDOTHELIAL DYSFUNCTION AND CARDIOVASCULAR FUNCTION

Changes in one or more functions of the endothelium can lead to endothelial dysfunction. The endothelium, for example, may cause oxidation of low-density lipoprotein (LDL) [Ohara *et al.* 1993] which, when produced, can directly injure the endothelium [Ross 1993]. Endothelial injury promotes the development of atherosclerosis by increasing the adhesiveness of platelets, monocytes and T-lymphocytes to the endothelium. Endothelial injury also causes an increase in the permeability of the endothelial layer and increases production of cytokines [Ross 1999]. Monocytes and lymphocytes enter the intima of the artery. The monocytes are activated to macrophages which absorb the oxidised LDL (oxLDL) and form foam cells. An increase in vasoconstrictors, such as endothelin and angiotensin II, cause further damage by promoting smooth muscle cell proliferation which leads to the development of plaque [Drexler 1998]. Therefore, the presence of endothelial dysfunction is an indicator of the presence of vascular damage even though structural changes may not be apparent [Davignon *et al.* 2004].

Endothelial function is affected by many factors [Cooke JP 1997; Drexler *et al.* 1999; Minor R.L.Jr *et al.* 1990; Russo *et al.* 2002], including higher LDL concentrations [Anderson *et al.* 1995a], higher plasma homocysteine concentrations [Stuhlinger *et al.* 2001], presence of hypertension [Panza JA *et al.* 1990] or diabetes mellitus [Tilton *et al.* 1997], oestrogen deficiency [Taddei *et al.* 1996], and increase in free radicals due to smoking [Celermajer DS *et al.* 1993]. Impairment of endothelial-dependent vasodilatation can be caused by a decrease in NO production, a decrease in NO bioavailability, an increase in oxidative stress or a decrease of EDHF [Bolad *et al.* 2005; Cai *et al.* 2000]. Vascular aging, for example, is associated with a decrease in endothelium-dependent relaxations which may, in part, be related to a decrease in basal and stimulated release of NO, or a reduced expression of the endothelial synthase gene [Barton *et al.* 1997; Tschudi *et al.* 1996].

A number of interventions have been shown to restore endothelium-dependent vasodilatation such as lipid lowering therapy, angiotensin converting enzyme (ACE) inhibitors, antioxidants, anti-hyperglycaemia, diet and exercise [Davignon *et al.* 2004]. Statins, for example, have been shown to improve vasodilatation of the coronary arteries [Treasure *et al.* 1995]. Statins are known to decrease production of ET-1 and to increase endothelial nitric oxide synthase (eNOS) expression [Mraiche *et al.* 2005]. Oestrogen therapy in post-menopausal women has also been found to improve endothelial function [Guetta *et al.* 1997]. Infusion of intracoronary estradiol, in postmenopausal women with atherosclerosis or CAD, enhanced ACh-induced coronary blood flow. This is thought to be due to an increase in the bioavailability of NO.



Although a number of drugs have been shown to improve endothelial function in experimental studies, these have not always been translated in-vivo. In addition, several approaches have been shown to improve endothelial function and cardiovascular risk, but not consistently. For example, various oestrogen [Gerhard *et al.* 1998] and antioxidant [Timimi *et al.* 1998] preparations have been shown to improve endothelial function. However, they have not shown any improvement in patient outcomes [The Heart Outcomes Prevention Evaluation Study Investigators 2000]. Atherosclerosis is, however, a multifactorial disease and other causative factors may obscure any positive benefits of a particular drug therapy. In addition, the inability of drugs to work at relevant sites, for example, the inability of antioxidants to work at the vessel wall, may be misinterpreted as being ineffective.

#### 1.3.5 ENDOTHELIAL DYSFUNCTION AND CARDIOVASCULAR RISK

Endothelial dysfunction in untreated hypertensive patients has been shown to be a marker of future cardiovascular events, even after adjustment of risk factors for atherosclerosis [Perticone *et al.* 2001b]. This study highlights the relationship between endothelial dysfunction and cardiovascular events in patients who have risk factors for atherosclerosis. Studies have also demonstrated that treatment of cardiovascular risk factors, known to lead to endothelial dysfunction, is associated with a decrease in cardiac events [Pedersen 2004]. Endothelial dysfunction may lead to cardiac events due to myocardial ischaemia in the absence of CAD [Hasdai *et al.* 1997; Zeiher *et al.* 1995]. Acceleration of coronary atherosclerosis, due to endothelial dysfunction, can result in obstructive CAD and lead to cardiac events [Hansson 2005].

In a study, by Suwaidi et al., subjects with CAD who had impaired endothelial function were found to be more likely to experience cardiovascular events than those subjects with normal endothelial function or mild dysfunction [Suwaidi *et al.* 2000]. These findings were supported by another study [Schachinger *et al.* 2000] in which patients with poor coronary endothelial function experienced a larger number of cardiovascular events, mainly revascularisation procedures. Patients with poor endothelial function comprised patients who had enhanced constrictor responses to cold pressor testing and acetylcholine, and a lower flow-dependent dilatation and nitroglycerin-induced vasodilatation. In addition, they found that patients who had impaired endothelium-independent vasodilatation to GTN had a poor prognosis. This indicates that impairment of smooth muscle function can contribute to a reduced vasodilatation of the coronary arteries. Halcox et al. reported that epicardial and microvascular coronary endothelial dysfunction predicted cardiovascular events, including sudden cardiac death, MI, and cerebral infarction in patients with and without CAD [Halcox *et al.* 2002b]. However, they did not find endothelium-independent vasodilatation of the epicardial and microvascular arteries to be predictive of cardiovascular events. These studies indicate that endothelial function testing provides valuable prognostic information. However, some studies have shown coronary endothelial function testing to be of no benefit [Asselbergs *et al.* 2004] .

Extending observations from coronary endothelial function studies, peripheral endothelial function is predictive of future cardiovascular events. For example, patients with impaired peripheral vasodilatory responses have been shown to experience a larger number of cardiovascular events [Heitzer *et al.* 2001; Perticone *et al.* 2001b], require revascularisation procedures, and impaired responses are

predictive of postoperative outcomes even after correcting for other cardiovascular risk factors [Neunteufl *et al.* 2000].

#### 1.4 ENDOTHELIAL FUNCTION AND ARTERIAL STIFFNESS

Arterial stiffness and endothelial dysfunction have been shown to co-exist in patients with cardiovascular risk factors, for example in diabetes [De Vriese AS *et al.* 2000; Wilkinson *et al.* 2000b] and smokers [Liang *et al.* 2001; Zeiher *et al.* 1995]. Both arterial stiffness and endothelial dysfunction have also been found in children with low birth weight [Martin *et al.* 2000], severe obesity [Tounian *et al.* 2001], and in familial hypercholesterolaemia [Yacine *et al.* 2000].

Animal studies have demonstrated that the vascular endothelium contributes to the regulation of arterial stiffness. For example, PWV has been shown to be higher with the administration of L-monomethyl- $N^G$ -arginine (L-NMMA), due to the inhibition of basal endothelial NO production [Wilkinson *et al.* 2002e]. L-NMMA has also been shown to inhibit reductions in PWV with the administration of ACh, an endothelium-dependent vasodilator [Wilkinson *et al.* 2002e]. In addition, endothelin (ET-1), a vasoconstrictor acting via the  $ET_A$  receptor, has been shown to cause an increase in PWV [McEniery *et al.* 2003]. Moreover, selective blockade of the  $ET_A$  receptor by BQ-123 led to a decrease in PWV. These studies highlight that arterial distensibility is increased with an increase in NO bioavailability or a decrease in endothelial-derived vasoconstrictors. In both of the above-mentioned studies drugs infused through the catheter exposed the arterial segment under study to the drug. However, infusion of drugs through the sheath did not expose the arterial segment to the effects of the drug because this was placed distal to the pressure sensors. This methodology allowed exclusion of indirect drug effects such as reflex activation and

changes in blood flow. Moreover, the local administration of drugs did not alter mean arterial pressure or heart rate therefore excluding any possible systemic effects.

Studies in vivo have shown that inhibition of basal NO production reduces brachial artery elasticity [Kinlay *et al.* 2001] and increases AIx [Wilkinson *et al.* 2002a]. In addition, administration of ACh reduces PWV of the iliac artery in healthy subjects compared to patients with congestive heart failure (CHF). An increase in vessel wall shear stress increases both endothelium-dependent vasodilatation and arterial distensibility. However, these responses are impaired in CHF patients [Ramsey *et al.* 1995]. Moreover, drugs which reduce arterial stiffness have also been shown to reduce endothelial dysfunction, for example, with the use of ACE inhibitors [Joannides *et al.* 2001]. Impaired endothelial function and an increase in AIx have been found in patients with growth hormone deficiency [Smith *et al.* 2002]. Both these measures improved, without any changes in blood pressure, with growth hormone replacement therapy.

## 1.5 ARTERIAL ELASTICITY AND SKIN ELASTICITY

Arteries are known to become stiffer, and are described as being less elastic, with both age and disease. A decrease in elasticity of the skin is also found with advancing age. It is of interest that both the arteries and the skin derive their mechanical properties from the structural components elastin and collagen. A study carried out by Uiterwaal *et al.* showed school children who had a lower PP to have higher skin extensibility [Uiterwaal *et al.* 2003]. More recently, a study [Elvan-Taspnar *et al.* 2005] in women with a history of pre-eclampsia showed them to have both stiffer arteries and stiffer skin (reduced elasticity of the skin) compared to controls.

Abnormalities in skin and vascular collagen have been found to occur in patients with cervico-cerebral artery dissections, intracranial aneurysms and in patients with heritable connective tissue disorders, such as Ehlers Danlos syndrome (EDS) Type IV and pseudoxanthoma elasticum (PXE). Patients with spontaneous cervico-cerebral artery dissections have ultra-structural defects in collagen fibrils and fragmented elastic fibres within the reticular dermis, indicating the presence of defects within the extra-cellular matrix of arteries and skin [Brandt *et al.* 2001]. In addition, patients with intracranial aneurysms have been found to have alterations in the connective tissue of the reticular dermis [Grond-Ginsbach *et al.* 2002]. In patients with EDS type IV there are defects in normal collagen cross-linking and reduced collagen content, specifically type III collagen, and these patients typically feature hyperextensible skin and easy rupturing of the large arteries [Pope *et al.* 1975]. Patients with PXE also feature increased elasticity of both the skin and arteries, for

example, as seen in the carotid artery due to an increase in fragmentation of elastic fibres and an accumulation of proteoglycans [Kornet *et al.* 2004]. These studies indicate that stiffness of connective tissues occurs both in the arteries and the skin. If, connective tissue changes in the skin represent stiffening of arteries and cardiovascular risk, skin elasticity has the potential to be used as an external marker of arterial elasticity and as a predictor of cardiovascular risk.

#### 1.5.1 STRUCTURAL COMPOSITION OF ARTERIES

Large arteries (aorta, carotids, iliofemorals) are composed of both elastin [Avolio *et al.* 1998] and collagen. In comparison the smaller muscular arteries (brachial, radial, coronary, renal, and mesenteric arteries) consist of a lower elastin-to-collagen ratio [Champion *et al.* 1998]. Both collagen and elastin give the aorta its tensile strength and stiffness. Elastic lamellae, formed from elastin, are found between smooth muscle cells while collagen, mainly Type I and Type III collagen, is found as a fine meshwork which surrounds smooth muscle cells or as bundles between smooth muscle cells and the elastic lamellae [Loscalzo *et al.* 1996]. On histological examination the intima of stiffened vessels has shown increased collagen, broken elastin molecules, abnormally distributed endothelial cells, and infiltration of vascular smooth muscle cells, macrophages, and mononuclear cells [Johnson *et al.* 2001; Lakatta 2003; Zieman *et al.* 2005]. The ECM composition of arteries changes with both age or blood pressure [Glagov *et al.* 1992; Lim *et al.* 2004b] with a decrease in elastin and an increase in collagen causing blood vessels to become stiffer [Johnson *et al.* 2001]. The physiological composition of arteries is described

in detail in Section 1.2.1 and a more detailed schematic of the structure of an artery is illustrated in Figure 1-7.



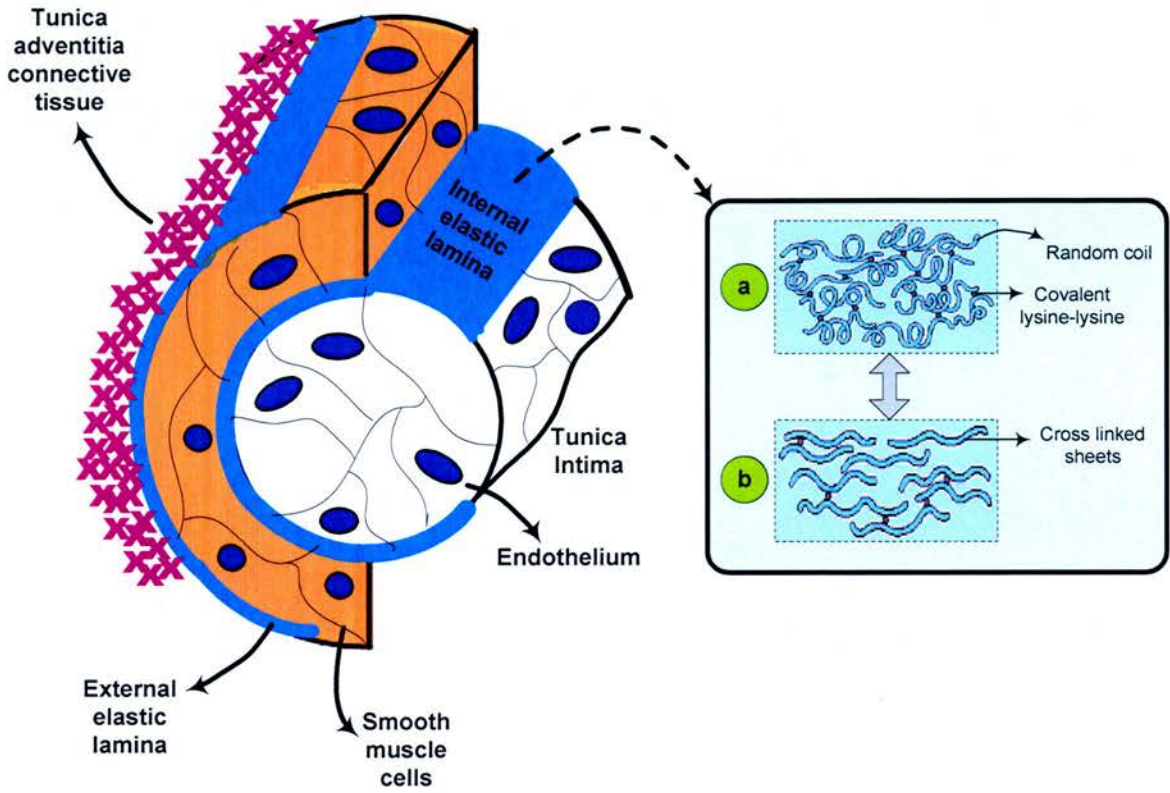


Figure 1-7 Elastin and collagen in an artery.

Schematic of the structure of arteries and changes in the orientation of elastin in the arterial wall when the artery is (a) relaxed and (b) dilated. Elastic fibres are folded in concentric rings and are connected axially by elastin. Collagen and elastin fibres are found in the medial layer. At low strains the elastin lamellae unfold allowing the aorta to deform easily, whereas at higher strains collagen is sufficiently stretched for it to take most of the stress.

### 1.5.2 STRUCTURAL COMPOSITION OF SKIN

The skin plays a vital role by regulating body temperature, preventing water loss, synthesising chemicals, and excreting waste materials. The skin consists of three layers: the epidermal, dermal and subcutaneous layers. This is illustrated in Figure 1-8. The epidermis is composed of stratified epithelial cells, keratinocytes, melanocytes, and, Langerhans and Merkel cells. The dermis is a thicker layer composed of supporting matrix or ground substance. It is the ECM that contains the protein fibres elastin and collagen. The dermis also contains fibroblasts, mast cells and histiocytes (monocyte/macrophage). The dermis has variable thickness in different parts of the body. For example it is 5mm in thickness on the back and thighs, and 1 mm on the eyelids.

In the skin, the mechanical and structural function is determined by collagen while its elasticity is determined by elastin [Tzaphlidou 2004]. The dermal layer of the skin is composed of 2 layers: the papillary layer and the reticular layer. Collagen in the reticular layer consists of about 70% Type I collagen and 15% Type III collagen [Silver *et al.* 2001a]. These ratios are reversed in arteries [Champion *et al.* 1998]. Type I collagen has thick fibrils which are closely packed, whereas, Type III collagen exists as loose network of thin striated fibrils. Both elastin and collagen are more compact with advancing age due to a decrease in spaces between the fibres. The elastic fibres are also fragmented, affecting the elastic recovery of skin, and collagen bundles are more randomly distributed [Tzaphlidou 2004].

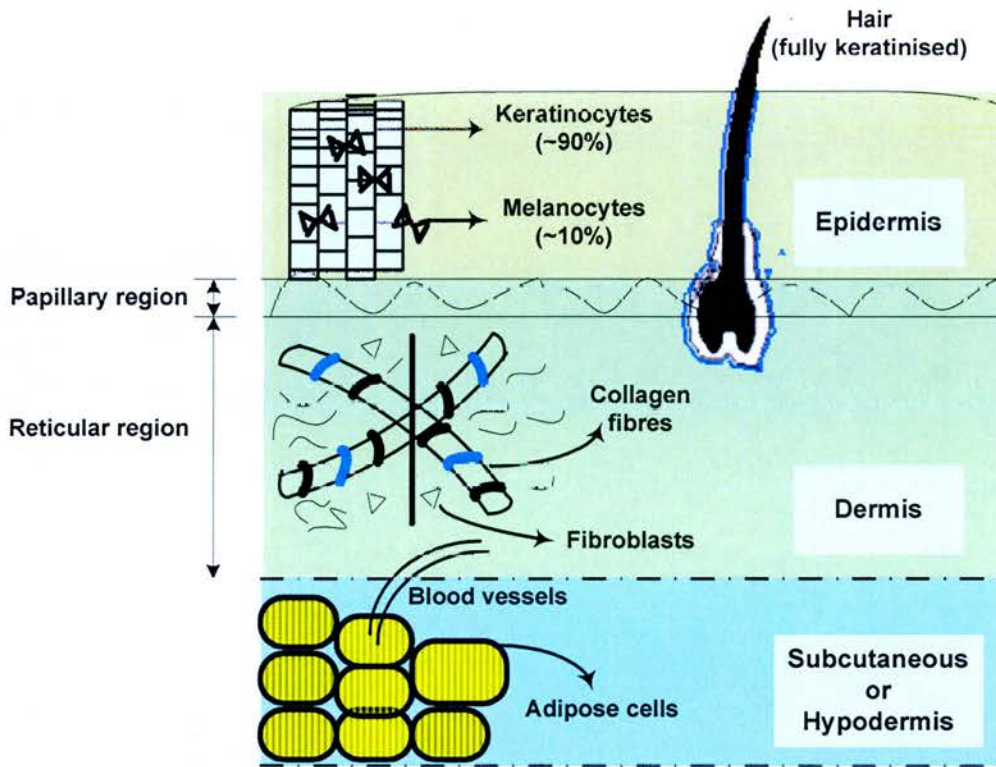


Figure 1-8 Schematic of the skin which consists of the epidermis, dermis and hypodermis.

The dermis consists of elastin and collagen which gives skin its elasticity and resilience. Collagen fibres in the skin are randomly arranged, and deform in the direction of the applied stress.

### 1.5.3 FACTORS AFFECTING SKIN ELASTICITY

The skin is affected by both intrinsic (pathological or genetically determined) and extrinsic (external or environmental) factors. Intrinsic factors include age, gender, female hormones, and enzymatic breakdown of elastin or collagen while extrinsic factors include exposure to ultraviolet light and humidity.

#### 1.5.3.1 Intrinsic factors

With advancing age, the elastic fibres are fragmented with a more randomised distribution of collagen fibres and an increase in cystic spaces [Balin *et al.* 1989]. In addition, skin thickness has been shown to increase between the ages of 0 and 20-30 years, in both men and women, but fall thereafter [Escoffier *et al.* 1989]. Findings from a study by Leveque *et al.* showed that skin thickness in women decreased earlier (30-40 years) than that in men (40-50 years) [Leveque JL *et al.* 1980].

Genetic and endocrine factors have also been shown to affect skin elasticity [Dunn *et al.* 1983]. For example, changes in female hormones during the menstrual cycle alter skin elasticity [Berardesca E *et al.* 1989]. Post-menopausal women have a larger decrease in skin elasticity, due to a significant decrease in collagen, compared to pre-menopausal women [Maheux *et al.* 1994; Sumino *et al.* 2004b]. Hormonal receptors, including oestrogen receptors, have been found in human skin which may explain changes in skin during the menstrual cycle and menopause [Sator *et al.* 2001].

There are a number of enzymes which degrade elastin and collagen mainly the serine proteases, cysteine proteases, and MMPs which result in connective tissue abnormalities [Elsner *et al.* 1990]. MMPs and TIMPs regulate the degradation of collagen, elastin, and other components of the ECM [Champion *et al.* 1998; Kingwell *et al.* 2001]. MMP-1, MMP-8, MMP-13 (collagenases 1,2 and 3) are the principal MMPs capable of initiating the degradation of fibrillar collagens I, II, III, and V. MMP-2 and MMP-9 are important in the final degradation of fibrillar collagens while MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-12 are capable of degrading elastin [Lakatta 2003].

#### 1.5.3.2 Extrinsic factors

The effect of high exposure to sunlight on skin elasticity has been widely studied. Solar radiation varies during different seasons and causes ultraviolet erythema (sunburn) and fragmentation and destruction of elastic tissue fibres [Serup *et al.* 1995]. Photodamaged skin is characterised by degraded elastic fibres and dysregulation of elastin [Jenkins 2002], fragmentation and clumping of collagen fibres [Fligiel *et al.* 2003; Varani *et al.* 2001], and cross-linking of collagen fibres [Bernstein *et al.* 1996]. The total collagen content of sun-damaged skin is 20% less than normal exposed skin [Tzaphlidou 2004]. In a study by Fisher *et al.*, ultraviolet light radiation was shown to increase the synthesis of MMP, causing degeneration of collagen fibres [Fisher *et al.* 1997]. The effect of temperature and humidity is known to affect the stratum corneum [Serup *et al.* 1995]. However, there is currently no data on any effects temperature and humidity may have on dermal skin elasticity.

## 1.6 HYPOTHESES

The following specific hypotheses will be addressed in this thesis:

### **Hypothesis 1**

*Skin elasticity is a marker of arterial stiffness, independent of age, in healthy volunteers.*

A number of techniques have been developed to assess arterial stiffness. However, their use is limited in the clinical setting due to the invasive nature of the techniques or inaccessibility of the equipment. Non-invasive measurement of carotid-femoral PWV is a validated technique used to assess stiffness of the arteries or their loss of elasticity. However, carotid-femoral PWV is not a practical methodology that can be applied very simply in a clinical setting. Therefore, this provided us with a basis for developing a simple technique whereby an external marker of arterial stiffness can be measured. Both the skin and arteries are made of elastin and collagen, which gives them their characteristic elasticity. If the elasticity of the skin and arteries are found to degrade at the same rate, there is a potential of using a non-invasive measurement of skin elasticity as a marker of arterial stiffness, especially in patients with CAD, hypertension or diabetes.

This hypothesis was addressed by aiming to:

- a) establish the reproducibility of measurements of skin elasticity in healthy volunteers for the arm, leg and back regions of the body (Chapter 3),

b) evaluate the relationship between skin elasticity and arterial elasticity, independent of age (Chapter 4).

## **Hypothesis 2**

*Arterial stiffness assessed by PWV, augmentation indices and DVP are similarly associated with clinical risk, in patients with CAD.*

A number of non-invasively derived measures are thought to represent arterial stiffness and are potential markers of the stiffening of arteries. This provided us with a basis to evaluate information derived from these measures in addition to that of a known marker of arterial stiffness.

This hypothesis was addressed by aiming to determine the association between the augmentation indices and DVP measures with PWV and clinical risk factors (Chapter 5).

## **Hypothesis 3**

*Arterial stiffness, assessed using non-invasive techniques, is determined by traditional cardiovascular risk factors and renal function, in patients with CAD.*

Previous studies have shown that patients with impaired renal function who experienced cardiovascular events have stiffer arteries [Blacher *et al.* 2003]. Therefore, this provided us a basis for evaluating whether arterial stiffness in patients with CAD was related to renal function.

This hypothesis was addressed by aiming to:

a) validate the use of the Colin<sup>®</sup> 7000, an automated tonometric device in conjunction with the SphygmoCor<sup>®</sup> Mx Aortic Blood Pressure Monitoring System, as an alternative device for measuring augmentation values (Chapter3).

b) evaluate the determinants of arterial stiffness, including renal function (Chapter 5).

#### **Hypothesis 4**

*Endothelial dysfunction, assessed using the non-invasive technique of pressure wave analysis, is determined by cardiovascular risk factors, in patients with CAD.*

A simple non-invasive test of endothelial function has recently been developed [Wilkinson *et al.* 2002b]. However, this model has not been assessed to determine its usefulness in clinical risk assessment. This provides the basis for the evaluation of this simple non-invasive technique as a potential tool in assessing clinical risk.

This hypothesis was addressed by aiming to:

a) establish the reproducibility of changes in augmentation indices to inhaled salbutamol, an endothelium-dependent vasodilator, as a test of endothelial function in patients with CAD (Chapter 3),

b) determine whether endothelial function, evaluated using the technique of PWA, is a valid tool for use as a simple non-invasive technique to assess cardiovascular risk (Chapter 6).



## **Hypothesis 5**

*Arterial stiffness is associated with endothelial dysfunction, in patients with CAD.*

Stiffening of arteries and endothelial dysfunction has been shown to play a role in atherosclerotic heart disease [Celermajer *et al.* 1992; Lovett *et al.* 2003]. Both arterial stiffness and endothelial dysfunction are predictors of adverse cardiovascular events [Gatzka *et al.* 1998; Suwaidi *et al.* 2000].

This hypothesis was addressed by aiming to assess the relationship between non-invasive measurements of arterial stiffness and endothelial function (Chapter 6).

## **Hypothesis 6**

*The severity of CAD, renal function, arterial stiffness, and endothelial function are prognostic indicators of fatal and non-fatal outcomes, in patients with CAD.*

Finally, this thesis aimed at investigating the predictors of outcomes in patients with CAD (Chapters 5 & 6).

## CHAPTER 2: METHODOLOGY

## 2.1 INTRODUCTION

In order to address the aims of this research, non-invasive techniques, that have the potential of being readily used in a clinical setting, were employed in the following chapters. The use of non-invasive techniques allow measurements to be made easily in a wider group of patients without the added risk of invasive procedures, for example injury to the median nerve or the terminal artery [Tousoulis *et al.* 2005] when measuring vascular function in the brachial artery. Arterial stiffness can be assessed indirectly from measurements of the pressure and volume pulse waves. Similarly, the non-invasive measurement of pressure waves, in combination with systemic drug administration, provides a method of indirectly measuring endothelial function. The use of non-invasive techniques also allows us to more easily study the relationship between arterial stiffness and skin elasticity in healthy subjects. In this Chapter I discuss the non-invasive techniques used in the studies presented in later chapters.

## 2.2 GENERAL STUDY REQUIREMENTS

### 2.2.1 ETHICAL CONSIDERATIONS

All studies were undertaken in accordance with the Declaration of Helsinki from the World Medical Association. The study protocols were approved by the local research ethics committee and written informed consent obtained from each participant prior to each study.

### 2.2.2 STUDY PARTICIPANTS

All subjects abstained from alcohol for 24 hours and tobacco and caffeine-containing drinks for at least five hours before each study. Studies were performed in a quiet temperature controlled room maintained within the range of 22 – 25 °C and a humidity level in the range of 40 – 50%.

### 2.2.3 BLOOD PRESSURE MEASUREMENTS

Blood pressure was measured in the dominant arm using a validated [O'Brien *et al.* 1996] oscillometric technique (HEM-705CP, Omron Corporation, Japan) or an integrated automated sphygmomanometer (Colin CBM-7000, Colin Medical Technology Corporation, Japan), depending on the technique used to measure pressure.

## 2.3 ARTERIAL STIFFNESS IN PATIENTS WITH CORONARY ARTERY DISEASE

### 2.3.1 MEASUREMENT OF ARTERIAL STIFFNESS

Arterial stiffness was measured using three non-invasive techniques: measurement of pressure PWV; determination of augmentation indices from the arterial pressure waveforms obtained by PWA and determination of the SI and RI from the arterial volume waveforms by DVP analysis. An overview of the techniques used to measure arterial stiffness is shown in Figure 2-1.

#### 2.3.1.1 Pulse wave velocity

PWV is measured using an arterial tonometer. The tonometer, a piezo-resistive pressure sensor (Millar SPT-301, Millar Instruments, Texas, USA) is connected to a blood pressure monitoring device (SphygmoCor<sup>®</sup> Mx, AtCor Medical, Sydney, Australia). The tonometer flattens, but does not occlude, the artery with gentle pressure. With flattening the circumferential pressures are equalised and a high fidelity pressure waveform is obtained. The SphygmoCor<sup>®</sup> system software permits on-line recording of the waveform. PWV is determined by sequential measurements of the pressure pulse waveform at the site of maximal arterial pulsation at the carotid and femoral arteries.

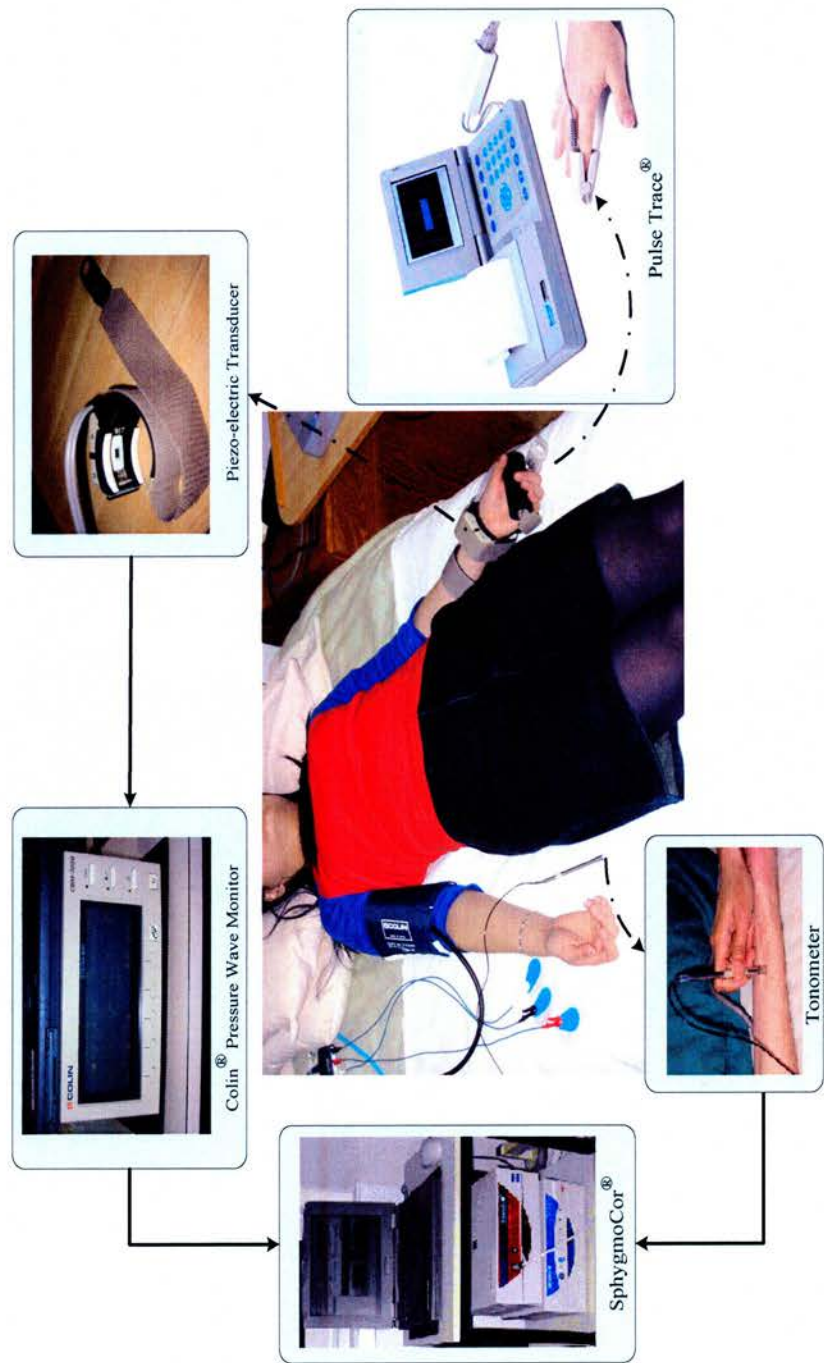


Figure 2-1 Study set-up for arterial stiffness and endothelial function. Schematic overview of the techniques used to measure arterial stiffness and endothelial function in Chapters 5 and 6 respectively.

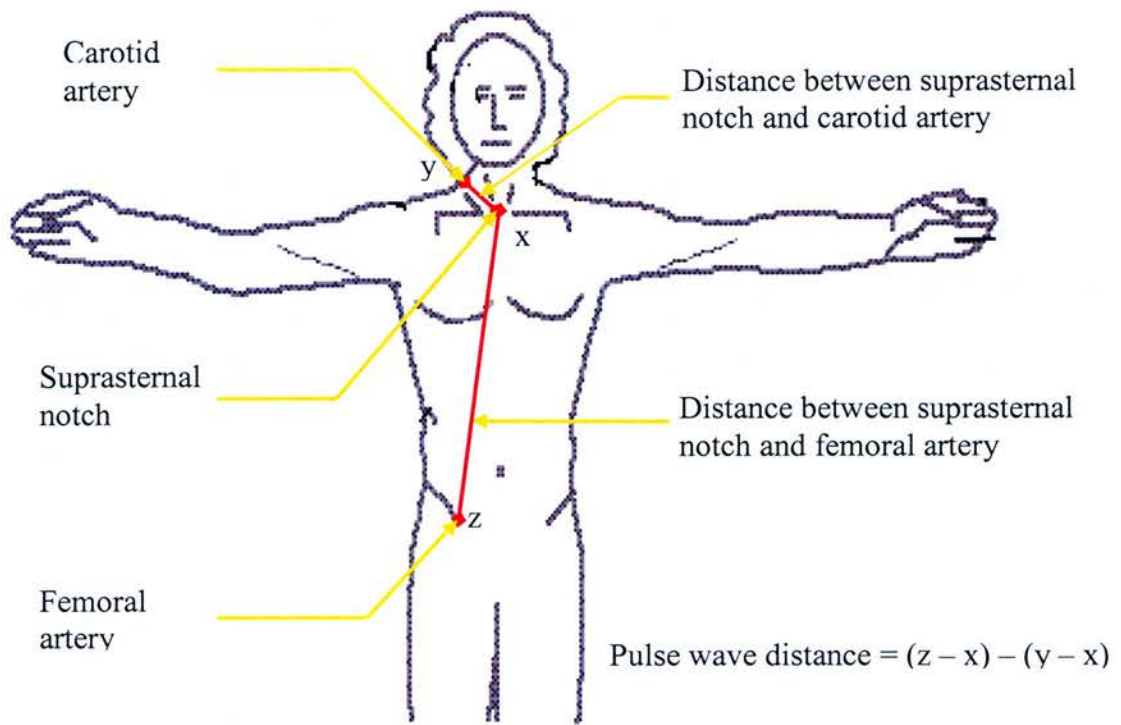


Figure 2-2 Aortic pulse wave velocity distance measurements.  
Schematic of sites of carotid and femoral pulse and distance measurements.

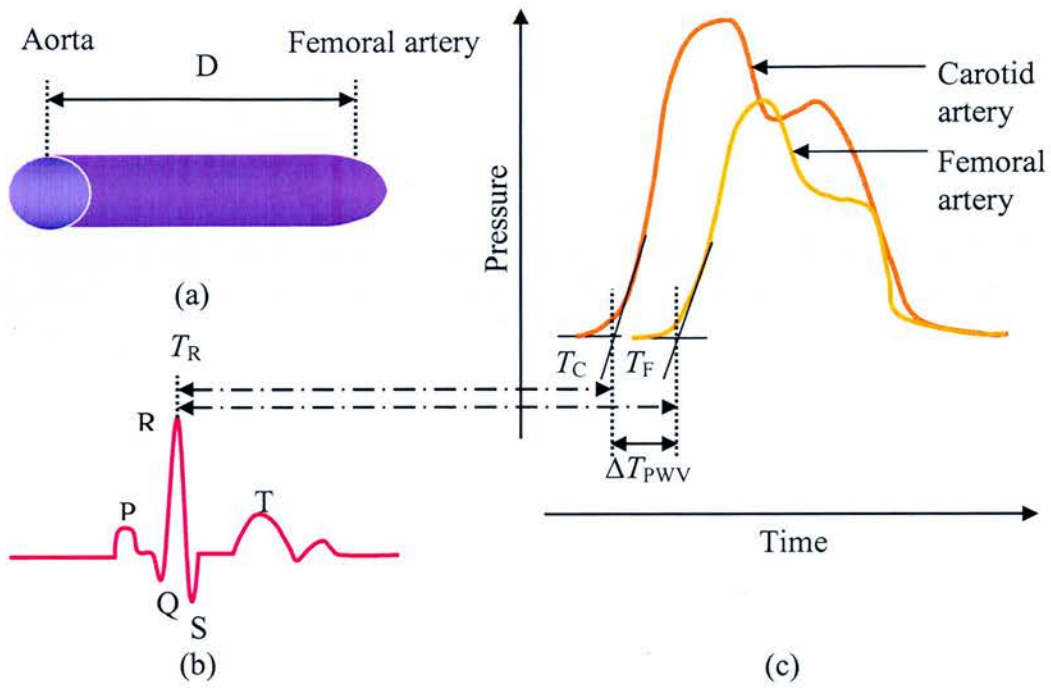


Figure 2-3 Gating aortic pressure waves to the electrocardiogram. Subfigure (a) represents the distance travelled by the pressure pulse wave and (b) and (c) represent the calculation of the time taken for the pressure wave to travel from the aorta to the femoral artery.



The distance ( $D$ ) travelled by the pulse wave was measured over the body surface as the direct line distance between the suprasternal notch and each of the recording sites (Figure 2-2). The system software subtracts the distance between the carotid artery and the suprasternal notch from that between the suprasternal notch and the femoral artery. This final distance is an approximation to the difference in distance between the carotid and femoral artery measurement points. The transit time was calculated by referencing the carotid and femoral waves to the peak of the R-wave on a simultaneously recorded electrocardiogram (ECG). The transit time is determined by subtracting the time interval between the systolic R-wave and carotid systolic up-stroke from the time interval between the systolic R-wave and the femoral systolic up-stroke. The transit time,  $\Delta T_{PWV}$ , is given by

$$\Delta T_{PWV} = (T_F - T_R) - (T_C - T_R)$$

where  $T_F$  and  $T_C$  are the times of the feet of the femoral and carotid pressure waves respectively and  $T_R$  is the time of the peak of the R-wave. The foot of a wave is identified as the intersection of two tangents: one tangent to the last part of the preceding wave and the other to the upstroke of the next wave (Figure 2-3). PWV is calculated as the difference,  $D$ , in carotid to femoral path length divided by the transit time,  $\Delta T_{PWV}$ , as given by below. The influence of respiration is reduced by averaging over one respiratory cycle which is equivalent to averaging 10 consecutive pressure waveforms.

$$PWV \text{ m/s} = D / \Delta T_{PWV}$$

### 2.3.1.2 Stiffness Index and Reflection Index

A non-invasive high fidelity photo-plethysmograph transducer (Micro Medical, Gillingham, Kent, United Kingdom) using infrared light measures changes in finger blood flow to produce a volume waveform [Mackenzie *et al.* 2002]. It was placed on the index finger of the left hand whilst the subject was lying supine. Temperature controlled heating pads at the bottom and top of the sensor minimise poor quality signals from vasoconstricted and poorly perfused subjects. Two indices are calculated from the DVP. The first, SI, is said to be a measure of large artery stiffness and is calculated as the subject's height,  $H$ , divided by the time,  $\Delta T_{DVP}$ , between the systolic and diastolic peaks of the DVP as below (see also Figure 2-4).

$$SI \text{ (m/s)} = H/\Delta T_{DVP}$$

The second, the RI, is a measure of reflection calculated as

$$RI \text{ (\%)} = (b/a) \times 100$$

where  $a$  is the height of the incident wave and  $b$  is the height of the reflected wave.

The height of the reflected wave is affected by the tone of the small blood vessels.

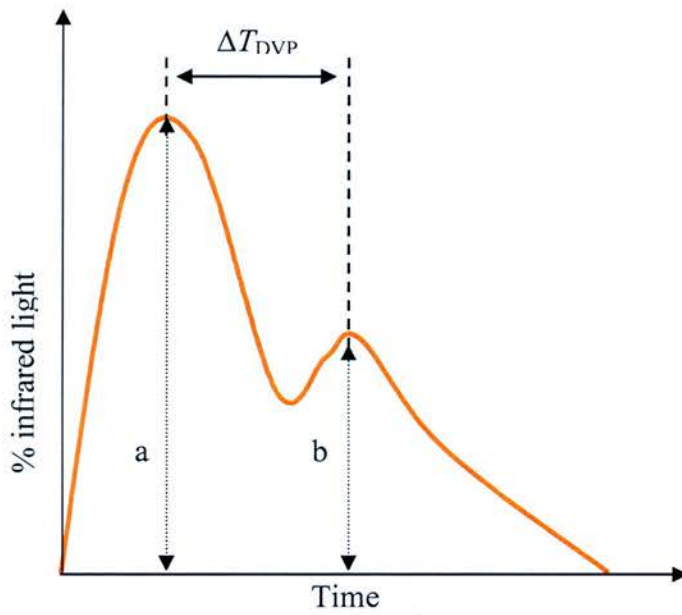


Figure 2-4 Digital volume pulse waveform. Schematic of the DVP waveform in the finger from which the SI and RI are calculated.

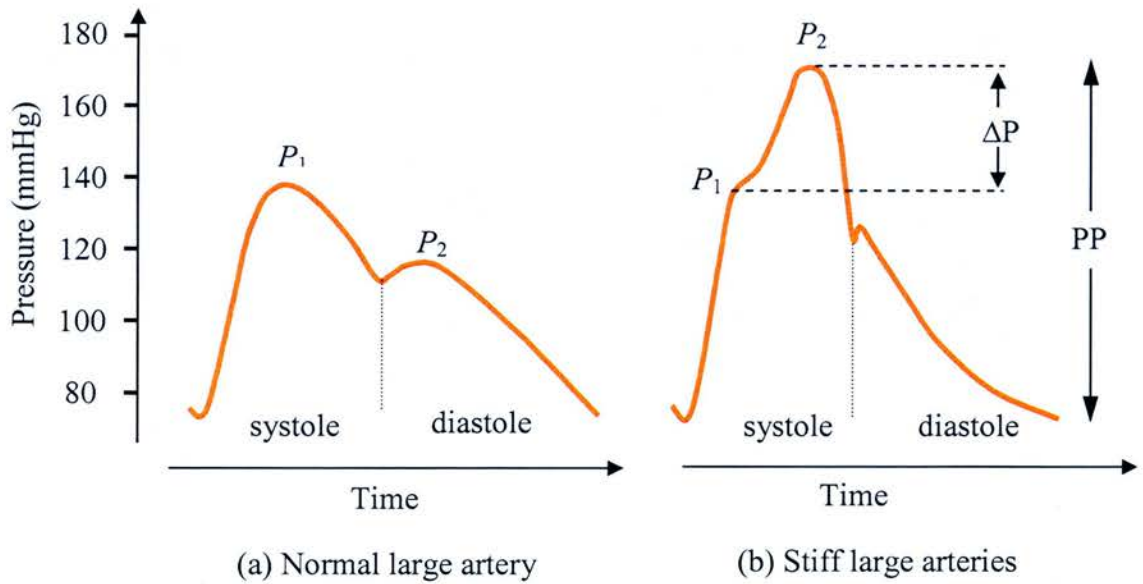


Figure 2-5 Aortic pressure waveforms in elastic and stiff arteries. Schematic of the aortic pressure waveform derived from the radial artery waveform (a) in a person with normal arteries and (b) in a person with stiff large arteries from which AIx is calculated.

### 2.3.1.3 Augmentation Index

Systemic arterial stiffness was determined by pressure PWA. Radial artery waveforms were analysed using a high fidelity micromanometer (SPC-301, Millar Instruments). The radial artery is usually identified by palpation and recorded at the level of the right wrist (at the styloid process of the radius); the arm is slightly extended and the hand externally rotated. The probe is held still for at least 10 seconds (the time of one respiratory cycle) so that consecutive pressure waves can be analysed. A continuously calibrated blood pressure waveform of the ascending aorta is derived in real time from a recording of the radial artery blood pressure waveform. Quantitative aortic data (such as systolic pressure, diastolic pressure, PP, augmentation pressure, and ejection duration) are derived from the radial artery waveform using the SphygmoCor<sup>®</sup> system software version 6.31 (Mx Aortic Blood Pressure Monitoring System, AtCor Medical, Sydney, Australia). The SphygmoCor<sup>®</sup> software uses a validated transfer function [Pauca *et al.* 2001] to derive AIx and central blood pressures. AIx is calculated as the difference between the first (**P1**) and second (**P2**) peaks, expressed as a percentage of the PP as shown in the equation below (see also Figure 2-5).

$$\text{AIx \%} = [(P2 - P1) / (CSBP - CDBP)] \times 100$$

where **P2** is the pressure at the 2nd peak in systole, **P1** is the pressure at the 1st peak in systole, CSBP is the central systolic pressure, and CDBP is the central diastolic pressure. The SphygmoCor<sup>®</sup> system software also calculates AIx corrected to a heart rate of 75 beats per minute (AIx75) by reducing AIx by 4.8 for every 10 beat/minute increase in heart rate [Wilkinson *et al.* 2000a; Wilkinson *et al.* 2002c]. The

peripheral augmentation index (PAIx) is not provided by SphygmoCor<sup>®</sup> and so it was calculated manually as shown below

$$\text{PAIx \%} = [(P2 - P1) / (\text{PSBP} - \text{PDBP})] \times 100$$

where PSBP is the peripheral systolic pressure, and PDBP is the peripheral diastolic pressure.

## 2.4 ENDOTHELIAL FUNCTION IN PATIENTS WITH CORONARY ARTERY DISEASE

### 2.4.1 MEASUREMENT OF ENDOTHELIAL FUNCTION

Endothelial function was measured using an automated tonometric device containing piezo-electric pressure sensors (Colin<sup>®</sup> 7000, Colin Medical Technology Corporation, Japan) coupled to a SphygmoCor<sup>®</sup> system (SphygmoCor<sup>®</sup> Mx Aortic Blood Pressure Monitoring System, AtCor Medical, Australia). This was used to obtain continuous measurements of the pressure waveforms in the radial artery. The Colin<sup>®</sup> has an integrated automated microphonic sphygmomanometer that measures SBP and DBPs which are used to calibrate the radial pressure pulse wave. The central and peripheral augmentation indices were obtained as detailed in Section 2.3.1.3. A dose of 400  $\mu\text{g}$  of salbutamol (Ventolin Evohaler<sup>®</sup>, Allen & Hanbury, Middlesex, Uxbridge), a  $\beta_2$ -adrenoceptor agonist, was administered to assess endothelium-dependent vasodilation [Wilkinson *et al.* 2002b]. A dose of 500  $\mu\text{g}$  of glyceryl trinitrate (Glyceryl trinitrate, Alparma, Barnstaple, Essex, U.K.) was administered to assess endothelium-independent vasodilatation. The technique used to measure endothelial function is shown in Figure 2-1.

## 2.5 SKIN ELASTICITY AS A MARKER OF ARTERIAL STIFFNESS

### 2.5.1 TECHNIQUES OF MEASURING SKIN ELASTICITY

The mechanical behaviour of skin can be assessed by either inducing a surface deformation or by applying a load, and by assessing the resisting force of the skin or skin deformation respectively. There are a number of available techniques that can be used to study the mechanical properties of skin. Examples of these are torsion, levarometry (elevation), ballistometry and suction [Elsner *et al.* 1990].

#### 2.5.1.1 Torsion

Torsion involves attaching a disk on to the skin which is rotated with a pre-determined torque and by measuring the degree of rotation [Elsner *et al.* 1990]. The torque moves the skin under the disc, without any brake from the subcutaneous tissue, and causes the skin to elongate in a twisted fashion. Upon torque application there is an immediate elastic deformation ( $U_e$ ) followed by a creeping viscoelastic deformation ( $U_v$ ). Release of the torque is associated with an immediate recovery ( $U_r$ ). This method is most commonly used for assessing properties of the stratum corneum.

#### 2.5.1.2 Levarometry

Levarometry involves applying a perpendicular pull to the skin without a guard ring [Elsner *et al.* 1990]. A circular piece of Perspex, about 0.5 cm in diameter, is attached to the skin by double sided adhesive tape. The circular piece of Perspex is connected to a counterbalanced measuring rod which has a net pressure of less than

1g/cm<sup>2</sup>. Elevating weights used vary between 5 to 40 g/cm<sup>2</sup>. This method is used as a measure of the slackness of skin.

#### 2.5.1.3 Ballistometry

Ballistometry is a technique which involves the use of a small stylus or impacting mass that strikes the skin at a low force and measuring the indentation and resilience of the skin. When the stylus strikes the skin surface the elastic component of the skin begins to restore some of the kinetic energy of the falling object. Subsequent release of this kinetic energy by the skin provides the rebound. The ground substance in skin slows compression and rebound and the largest rebound is found with the largest ratio of the elastic to viscous component.

#### 2.5.1.4 Suction

The technique of suction involves a small dome of skin being sucked into a measurement cup, attached to a probe, by a known vacuum and the displacement of the skin is measured. The skin around the opening of the probe is kept in position by an external guard ring attached to the probe. The depth of skin penetration is measured by an optical system. During application of a vacuum force there is an initial fast phase determined by the elastic component, followed by a viscoelastic phase and finally a viscous phase. When the vacuum force is released to 0 mbar of negative pressure, there is a fast initial drop in the elastic phase, followed by the viscoelastic and the plastic component.



## 2.5.2 METHODOLOGY USED TO MEASURE SKIN ELASTICITY IN THIS RESEARCH

Skin elasticity was measured in this thesis using a non-invasive suction device (Cutometer® MPA 580, Courage and Khazaka, Köln, Germany) with a hand held probe linked to a computer. The Cutometer® consists of a micro-processor, an air evacuation system and a probe with an 8 mm diameter suction cup [Courage et al. 2003]. The probe is applied perpendicular to the skin surface and measures the vertical deformation of the skin as a function of time [Dobrev 2002]. A constant controlled vacuum force of 450 mbar is applied for 5 seconds after which the probe's vacuum is reduced to 0 mbar for 3 seconds allowing the skin to return to its original position. A probe with a small aperture, e.g. 2 mm in diameter, causes a disproportionate deformation of the skin whereby only the epidermis and outer dermis can unfold and undergo strain. Therefore, a larger aperture i.e. 8 mm in diameter, was chosen for the full thickness of the skin to deform [Dobrev 2002]. The system software calculates a number of absolute and relative parameters from the waveform of skin deformation with time. The absolute parameters are dependent on skin thickness. However, the ratios of the absolute parameters (relative parameters) are independent of skin thickness and were, therefore, used for analysis. Elasticity measurements were made at the following five regions: the back, above the right shoulder blade; the dorsal and ventral forearm, midway between the elbow and the wrist; the anterior half of the upper arm, midway between the shoulder and the elbow; and, the anterior third of the upper leg, midway between the groin and the knee. An overview of the techniques used to measure skin elasticity and arterial stiffness is given in Figure 2-6.

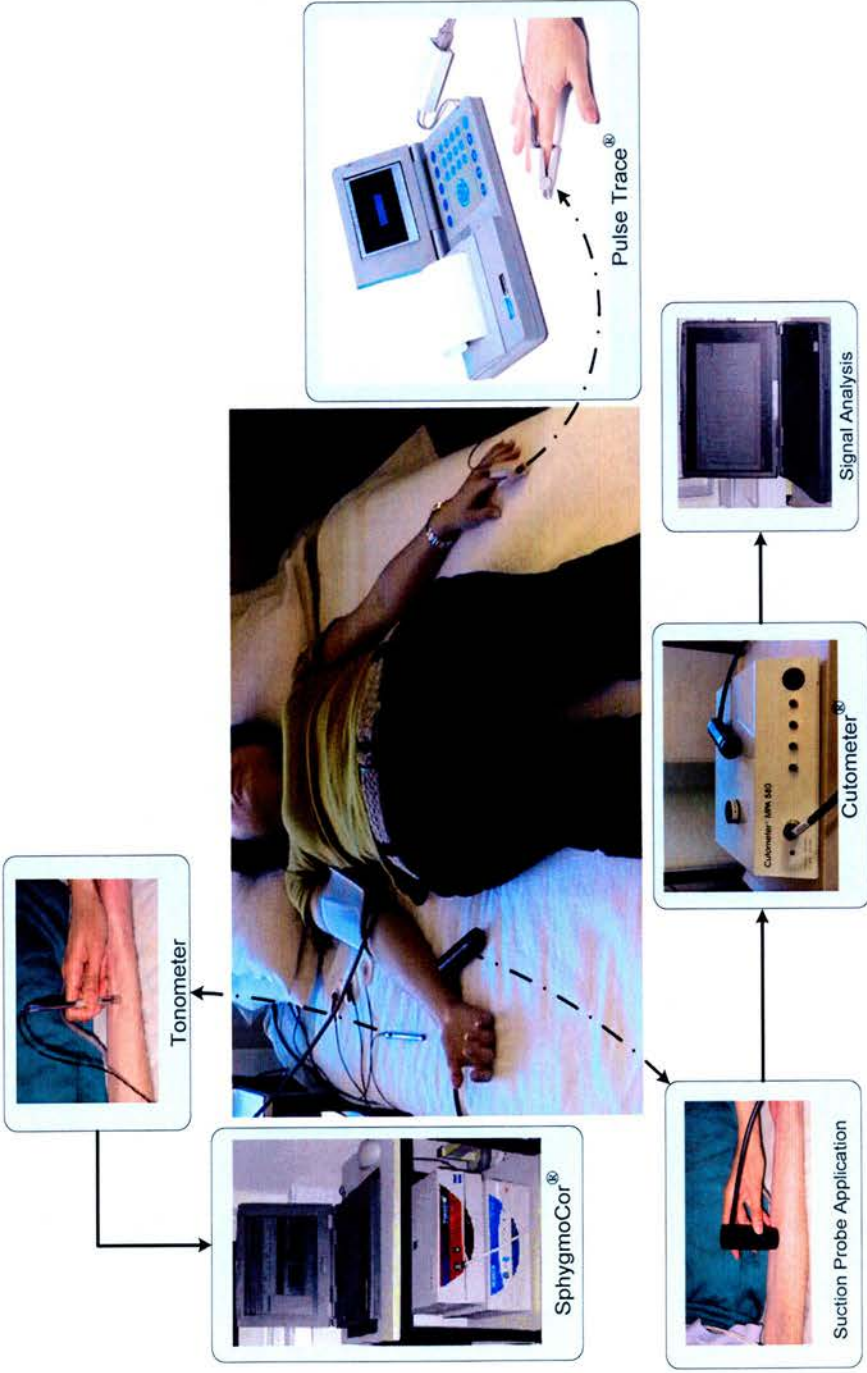


Figure 2-6 Schematic overview of the techniques used to measure skin elasticity and arterial stiffness.

## 2.6 OUTCOMES

Follow-up time was calculated as days between the first study visit and date of the first cardiovascular event or date of last follow-up for censored subjects. Due to the initial short follow-up up to July 2005 only a small number of deaths were expected. Therefore, the primary pre-defined outcome of this study was a composite end-point [Anavekar *et al.* 2004; Beddhu *et al.* 2002; Santopinto *et al.* 2003] consisting of death, AMI, stroke and hospitalisation due to CHD while the post-hoc outcome studied was future coronary intervention (CABG or PTCA).

Follow-up data for hospitalisations due to an AMI, CHD admission, stroke, and coronary intervention came from the Scottish Morbidity Record for all inpatient and daycase discharges (SMR 01). Individual diagnoses were classified according to the 10<sup>th</sup> International Classification of Disease 10<sup>th</sup> revision (ICD-10) codes. The codes for CHD are I20-I25; AMI, I21-I22; angina, I20 and I249; cardiac failure, I50; cerebrovascular disease, I60-I69 and G45; and stroke, I61-I64. CHD admissions were defined as admissions relating to cardiovascular death, non-fatal MI, recurrent hospitalisation for myocardial ischaemia, revascularisation procedures or hospitalised angina.

Operations codes used were based on Office for Populations Census and Surveys Version 4<sup>th</sup> Revision Classification of Surgical Operations and Procedures (OPCS4) codes. The codes for CABG were K40-K46; angioplasty, K49 and K50.1; and angiography, K63 and K65. Revascularisation procedures 6 months after study

participation were included in the analysis while revascularisation procedures during this period were assumed to be planned elective procedures.

Deceased subjects were confirmed by death certificates from the General Register Office and all other subjects were considered to be alive at the end of the follow-up period. Cardiovascular death was defined as fatal MI or sudden death in the presence of CHD and the absence of other life threatening disease.

## 2.7 BIOCHEMICAL BLOOD ANALYSIS

Venous blood was obtained on the day of the study and collected into an EDTA tube (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany). Clinical biochemistry analyses were performed by the regional clinical laboratories. The Vitros<sup>®</sup> 950 Chemistry Products system (Ortho-Clinical Diagnostics Inc., Rochester, New York) was used for analysis of serum concentrations of total cholesterol, high density lipoproteins cholesterol (HDL-C), triglycerides (TG) and serum creatinine. Due to differing methods of measuring serum creatinine concentrations between the Royal Infirmary and Western General Hospital there is a potential for a bias. However as this bias is only significant for high serum creatinine concentrations and the majority of subjects with CAD had normal serum creatinine concentrations the differences in measurements of serum creatinine concentration are not expected to confound results.

### 2.7.1 TOTAL CHOLESTEROL CONCENTRATION

Total cholesterol was analysed using the Vitros<sup>®</sup> CHOL slide based on an enzymatic method similar to that of Allain et al. at 37°C [Allain *et al.* 1920]. A 10  $\mu$ L drop of patient sample is placed on the slide which contains the Triton X-100 surfactant. This surfactant aids the disassociation of the cholesterol and cholesterol esters from the lipoprotein complexes. The hydrolysis of the cholesterol esters to cholesterol is catalysed by cholesterol ester hydrolase. The next step involves the oxidation of free cholesterol by cholesterol oxidase to form cholestenone and hydrogen peroxide. A leuco dye is oxidised by hydrogen peroxide with the help of peroxidase to form a coloured

dye. The density of this coloured dye is proportional to the cholesterol concentration in the sample and is measured by reflectance spectrophotometry. The lower limit of sensitivity for this test following the NCCLS Protocol EP5-T2 [Wayne 1992] is 1.29 mmol/L. The within day variability (SD) for a mean concentration of 3.9 mmol/L is 0.07 and for a mean concentration of 6.4 mmol/L is 0.13. The correlation coefficient for the accuracy of this test following the NCCLS Protocol EP9-A [Wayne 1995] in comparison to that of Abell-Kendall is 1.0 [Abell *et al.* 1952].

### 2.7.2 TRIGLYCERIDE CONCENTRATION

Triglyceride concentration was analysed using the Vitros<sup>®</sup> TRIG slide based on an enzymatic method described by Spayd *et al.* [Spayd *et al.* 1978]. A drop of the patient's blood sample is placed on the slide containing Triton X-100 which aids the disassociation of TG from the lipoprotein complexes. The triglyceride molecules are hydrolysed by lipase and give rise to glycerol and fatty acids. Glycerol is phosphorylated by glycerol kinase with the aid of adenosine triphosphate. This is followed by the oxidation of L- $\alpha$ -glycerophosphate to dihydroxyacetone phosphate and hydrogen peroxide by the aid of L- $\alpha$ -glycerol phosphate oxidase. Finally, a leuco dye is oxidised by hydrogen peroxide in the presence of a peroxidase catalyst. The density of the dye formed is proportional to the triglyceride concentration and is measured by reflectance spectrophotometry. The lower limit sensitivity of this test is 0.11 mmol/L. The within-day variability (SD) for a mean concentration of 1.24 mmol/L was 0.01 and for a concentration of 2.62 mmol/L was 0.02 based on the NCCLS Protocol EP5 [Wayne 1999]. This correlation coefficient of this test has an accuracy of 0.99 using the NCCLS

Protocol EP9 [Wayne 1995] when analysed against a method of Fossati and Prencipe [Fossati *et al.* 1982].

### 2.7.3 HIGH DENSITY LIPOPROTEIN CHOLESTEROL

The patients sample is pre-treated with Vitros<sup>®</sup> Magnetic HDL-Cholesterol reagent and the HDL cholesterol is measured by the Vitros CHOL slide. LDL and very low-density lipoprotein (VLDL) are precipitated by dextran sulphate and magnesium chloride allowing HDL to separate out. A magnetic field is applied to remove the precipitated lipoproteins from the supernate containing HDL. Approximately 10 $\mu$ L of the pre-treated patients sample is placed on the slide and reacts to produce hydrogen peroxide. The hydrogen peroxide reacts with the leuco dye to produce a blue dye complex from which the amount of reflectance is calculated. The lower limit sensitivity for this test is 0.08 mmol/L. The within-day variability (SD) for a mean concentration of 0.81 mmol/L is 0.019 and for a concentration of 1.35 mmol/L is 0.047 based on the NCCLS Protocol EP5-T2 [Wayne 1992]. The correlation coefficient for the accuracy of this test is 1.00 in comparison to the Vitros<sup>®</sup> HDL cholesterol (Individual Tube) method.

### 2.7.4 SERUM CREATININE CONCENTRATIONS

A drop of the patients sample is placed on the Vitros<sup>®</sup> CREA slide. Creatinine is hydrolysed by the reagent and is converted to sarcosine and urea by creatinine aminohydrolase. In the presence of sarcosine oxidase, sarcosine is oxidised to glycine formaldehyde and hydrogen peroxide. This is followed by the oxidation of the leuco dye

by a peroxide catalyst to produce a coloured product. After the addition of the patient's sample, the slide is incubated. Endogenous creatinine in the sample is oxidised during the initial reaction phase and the resulting change in reflection density is measured. The concentration of creatinine present in the sample is proportional to the rate of change in reflection density. This test can detect serum concentration ranges between 4 – 1238  $\mu\text{mol/L}$ . The with-in day variability (SD) for a mean concentration 81  $\mu\text{mol/L}$  is 0.7 and for a mean concentration of 499  $\mu\text{mol/L}$  is 4.1. The correlation coefficient for the accuracy of this test is 0.99 in comparison to analysis by High Performance Liquid Chromatography [Ambrose *et al.* 1983].

#### 2.7.5 LOW DENSITY LIPOPROTEIN CHOLESTEROL

Low density lipoprotein cholesterol (LDL-C) concentration was calculated by the Friedewald equation [Friedewald *et al.* 1972]

$$C_{\text{LDL}} (\text{mg/dL}) = C_{\text{plasma}} - C_{\text{HDL}} - \text{TG}/5$$

where,  $C_{\text{LDL}}$  denotes LDL-C concentration;  $C_{\text{plasma}}$ , total cholesterol concentration;  $C_{\text{HDL}}$ , HDL-C concentration; and TG the triglyceride concentration.



## 2.8 DATA ANALYSIS

Statistical analysis was performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). All results are expressed as mean  $\pm$  standard deviation (SD). Data were examined by two tailed Student's *t*-test, analysis of variance (ANOVA), analysis of covariance (ANCOVA) and regression analysis where appropriate. The Bonferroni correction was used for all post-hoc comparisons calculated as  $\alpha = 0.05/k$  ( $\alpha$  is the alpha value for each comparison and *k* is the number of comparisons carried out). Stepwise linear regression was used for multivariate analysis. Cumulative survival and event free probabilities were determined using the Kaplan-Meier product-limit method and compared by the Mantel (log-rank) test. All testing was two-sided and significance was taken at the 5% level.

## CHAPTER 3: DEVELOPMENT OF METHODOLOGY

This chapter discusses the reproducibility of two techniques employed in the following chapters. First, the reproducibility of measurements of skin elasticity in different regions of the body was assessed. This study was carried out to determine which skin elasticity ratios and which skin regions were most reproducible. Second, I aimed to assess the reproducibility changes in augmentation indices following the administration of inhaled salbutamol as a non-invasive test of endothelial function. This chapter is also aimed at validating the use of an automated sphygmomanometer, Colin<sup>®</sup> 7000, in comparison to a more commonly used tonometric device, the Millar micromanometer.

## 3.1 REPRODUCIBILITY OF MEASUREMENTS OF SKIN ELASTICITY

### 3.1.1 INTRODUCTION

The Cutometer<sup>®</sup> MPA 580, as discussed in Chapter 1, is an appropriate tool for measuring skin elasticity in our group of subjects. This is because the Cutometer<sup>®</sup> allows the determination of dermal skin elasticity with a larger probe and it is a simple technique that can be readily used in a clinical setting. The reproducibility from this device has not previously been studied. Therefore, this study was undertaken to evaluate the reproducibility of the skin elasticity ratios R2 (Ua/Uf), R5 (Ur/Ue), R6 (Uv/Ue) and R7 (Ur/Uf) for the dorsal, ventral and upper arm as well as the upper leg and back [Serup *et al.* 1995].

Elasticity of the skin and arteries is structurally determined by elastin and collagen in the dermal and medial layers respectively. Change in elasticity of the arteries has been found in patients with connective tissue disorders such as Ehlers-Danlos syndrome type IV, PXE, and Marfans syndrome. Patients with Ehlers-Danlos syndrome have reduced collagen content in the skin and aorta and, more specifically, they lack type III collagen [Pope *et al.* 1975]. Reduced collagen content in patients results in fragile and hyperextensible connective tissues and patients are, therefore, more prone to rupturing of the arteries. Patients with PXE, on the other hand, have fragmented elastic fibres and accumulations of calcium and proteoglycans in the skin and also in their carotid arteries [Kornet *et al.* 2004]. This results in increased skin elasticity as well as thicker and more elastic carotid arteries.

### 3.1.2 METHODS

#### 3.1.2.1 Study Subjects

Healthy volunteers were recruited through advertisements at the local churches and at the Western General Hospital, in Edinburgh. Ten healthy volunteers attended on 2 occasions 5 days apart. The exclusion criteria were that subjects could not be on: any long term medications, corticosteroids or recent antibiotic therapy. Also, subjects were excluded if they had a skin disorder or had recent exposure to high levels of ultraviolet (UV) light.

#### 3.1.2.2 Measurements

Subjects were asked not to use cosmetics, moisturisers or moisturising baths on the day of the study. Skin elasticity was measured on the back, arm, and leg using a non-invasive suction device with a hand held probe linked to the Cutometer<sup>®</sup> MPA 580 (Courage and Khazaka, Köln, Germany). This system consists of a micro-processor and an air evacuation system with an 8mm diameter suction cup probe. The measuring probe is held in position by an adhesive guard ring to reduce lateral displacement of the skin as much as possible.

Elasticity measurements on the back were made above the right shoulder blade. Three measurements were made on the arm: two were made on the dorsal and ventral aspect of the right forearm, midway between the elbow and the wrist; and one was made on the inner side of the upper arm, midway between the shoulder and the elbow. Measurements

of the leg were made on the upper anterior third of the leg between the knee and the groin.

#### 3.1.2.3 Study Protocol

Subjects were rested for 10 minutes and then measurements of skin elasticity were taken in the following order: dorsal arm, ventral arm, upper arm, upper leg and back (

Figure 3-1). Each set of measurements consisted of 10 cycles, each cycle of which comprised the skin being suctioned for 5 seconds and released for 3 seconds. These sets of measurements were only made once to avoid the phenomenon of creep which is an increase in deformation of the skin as a function of time when a constant stress is applied.

#### 3.1.2.4 Data Analysis

Statistical analysis was performed using Microsoft Excel 2002. The R2, R5, R6, and R7 ratios of skin elasticity measurements were examined by Bland-Altman plots and two tailed Student's paired *t*-test. All results are expressed as mean  $\pm$  SD. Statistical significance was taken at the 5% level.

Skin elasticity protocol

	Rest						
			UA	DA	VA	UL	B
Recording			↓	↓	↓	↓	↓
B.P.	*	*	*	*	*	*	*
Time (min) →	0	10	20		30		40

Figure 3-1 Study protocol for the reproducibility of skin elasticity measurements.

### 3.1.3 RESULTS

The study group comprised of 8 women and 2 men with a mean age of  $33 \pm 8$  years (range 28 – 52). Subjects attended a second visit after a mean time of  $5 \pm 1$  days (range 4 – 6 days). Table 3-1 shows the four mean skin elasticity ratios taken at the five body regions for the first and second visits. Bland-Altman plots for the reproducibility in measurements of R2, R5, R6 and R7 for the various body regions are shown in Figures 3.2 - 3.6. The data shown in the table and figures is summarised in the following sections.

#### 3.1.3.1 Back

The R6 skin elasticity ratio had the least difference, with one outlying subject. This was followed by the R5 and R7 ratios, after the R2 ratio, as having the largest difference between visits. The mean difference in R5, R6 and R7 was increased by one extreme outlying subject (Figure 3-2).

#### 3.1.3.2 Dorsal arm

The R6 ratio had the least mean difference and the least variability in measurements, except for one extreme outlying subject. Measures of R7 had the least mean differences and both the R2 and R5 values very highly variable (Figure 3-3).

#### 3.1.3.3 Ventral arm

The R2 and R7 measures had the least differences between visits. However, the R6 values were the most closely distributed, except for 1 extreme outlier. Measures of R5



were also closely distributed except for one slightly outlying subject and an extreme outlier (Figure 3-4)

#### 3.1.3.4 Upper Arm

The R6 ratio was the most reproducible with the least mean difference between visits, followed by the R5 and R7 measures. The R2, R6 and R7 were affected by one extreme outlying subject which, if excluded, would have reduced the mean differences and improved the reproducibility of these parameters (Figure 3-5).

#### 3.1.3.5 Upper Leg

The R6 parameter had the least mean difference between visits. However, the R2 parameters were the least variable, with one outlying subject. Measurements of R2 were close to the zero line. However, there were two outliers that increased the mean difference. The R7 measurement showed most variability. One outlying subject contributed to the increase in the mean difference for both R5 and R7 ratios (Figure 3-6).

Table 3-1 Skin elasticity ratios during two visits.  
 Summary of mean values and mean differences in skin elasticity ratios for the first and second visits.

Skin region	Parameter	First visit	Second visit	Mean of	Coefficient of
		mean $\pm$ SD, mm	mean $\pm$ SD, mm	differences $\pm$ SD, mm	
Back	R2	0.87 $\pm$ 0.09*	0.93 $\pm$ 0.05	0.06 $\pm$ 0.07	0.141
	R5	0.64 $\pm$ 0.13	0.72 $\pm$ 0.14	0.08 $\pm$ 0.14	0.275
	R6	0.28 $\pm$ 0.07	0.27 $\pm$ 0.03	-0.02 $\pm$ 0.06	0.127
	R7	0.50 $\pm$ 0.11	0.57 $\pm$ 0.11	0.07 $\pm$ 0.09	0.184
Dorsal Arm	R2	0.81 $\pm$ 0.06	0.84 $\pm$ 0.06	0.02 $\pm$ 0.06	0.123
	R5	0.58 $\pm$ 0.06	0.60 $\pm$ 0.07	0.02 $\pm$ 0.05	0.094
	R6	0.35 $\pm$ 0.03	0.36 $\pm$ 0.06	0.01 $\pm$ 0.05	0.097
	R7	0.43 $\pm$ 0.05	0.44 $\pm$ 0.06	0.01 $\pm$ 0.04	0.086
Ventral Arm	R2	0.81 $\pm$ 0.09	0.84 $\pm$ 0.04	0.03 $\pm$ 0.07	0.137
	R5	0.55 $\pm$ 0.10	0.58 $\pm$ 0.06	0.03 $\pm$ 0.06	0.12
	R6	0.31 $\pm$ 0.03	0.36 $\pm$ 0.05	0.05 $\pm$ 0.06	0.122
	R7	0.42 $\pm$ 0.07	0.43 $\pm$ 0.06	0.01 $\pm$ 0.04	0.076
Upper Arm	R2	0.87 $\pm$ 0.08	0.90 $\pm$ 0.06	0.03 $\pm$ 0.08	0.151
	R5	0.65 $\pm$ 0.12	0.67 $\pm$ 0.10	0.02 $\pm$ 0.13	0.262
	R6	0.28 $\pm$ 0.03	0.28 $\pm$ 0.03	-0.003 $\pm$ 0.02	0.033
	R7	0.51 $\pm$ 0.10	0.52 $\pm$ 0.07	0.02 $\pm$ 0.10	0.201
Upper Leg	R2	0.95 $\pm$ 0.04	0.97 $\pm$ 0.02	0.02 $\pm$ 0.03	0.052
	R5	0.81 $\pm$ 0.07	0.84 $\pm$ 0.06	0.02 $\pm$ 0.06	0.123
	R6	0.23 $\pm$ 0.04	0.23 $\pm$ 0.04	-0.003 $\pm$ 0.04	0.077
	R7	0.66 $\pm$ 0.06	0.68 $\pm$ 0.06	0.02 $\pm$ 0.06	0.112

N=10 \* P < 0.05

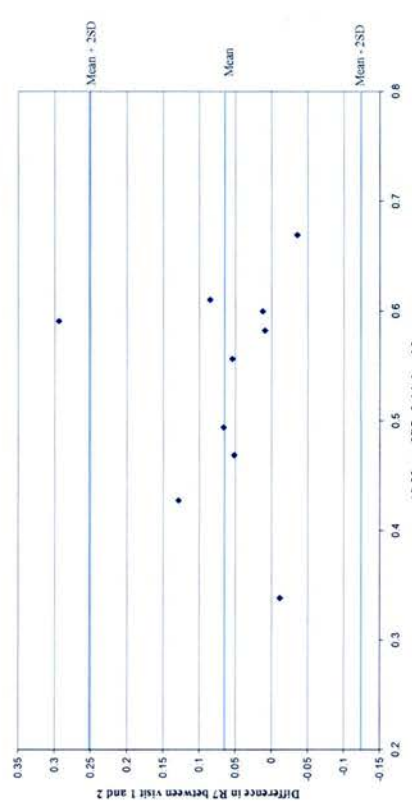
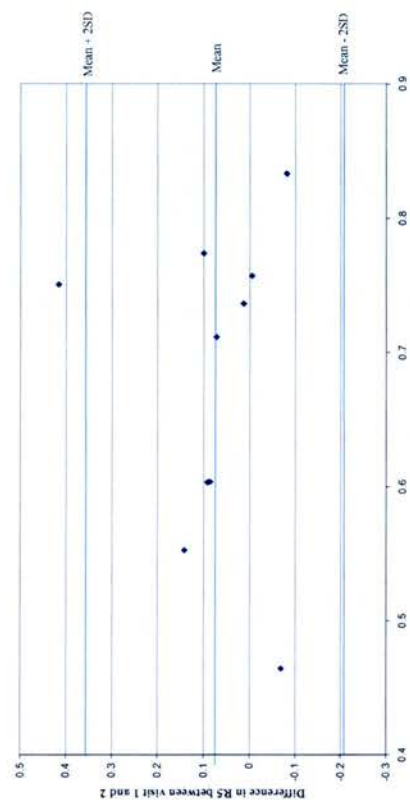
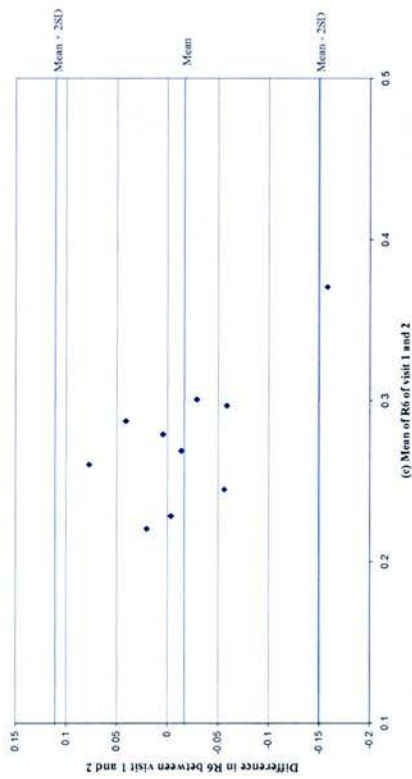
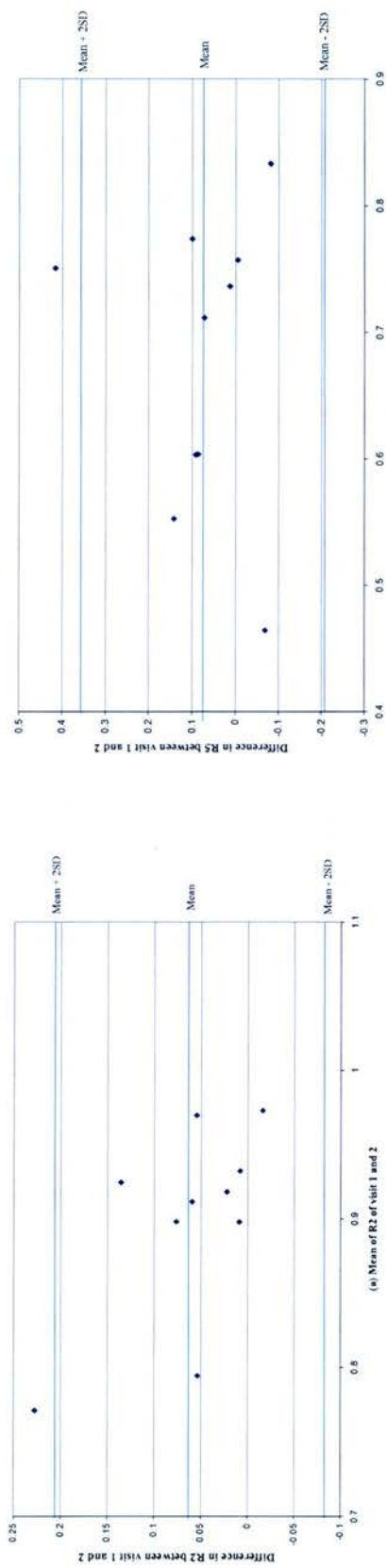


Figure 3-2 Bland-Altman plots of the skin elasticity ratios for the back: (a) R2 (b) R5 (c) R6 and (d) R7.

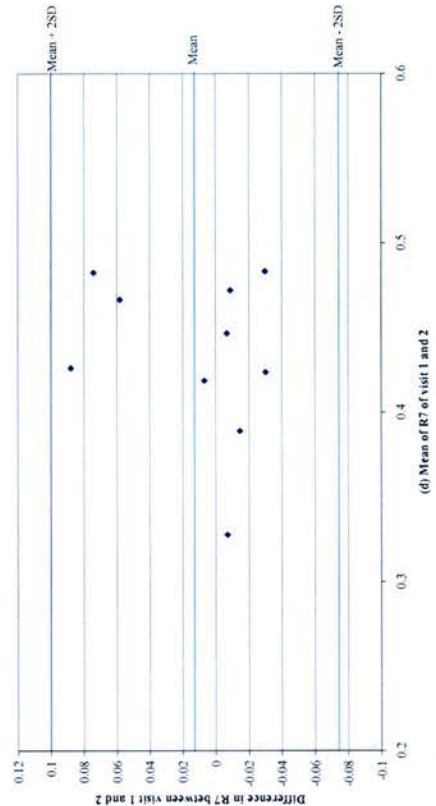
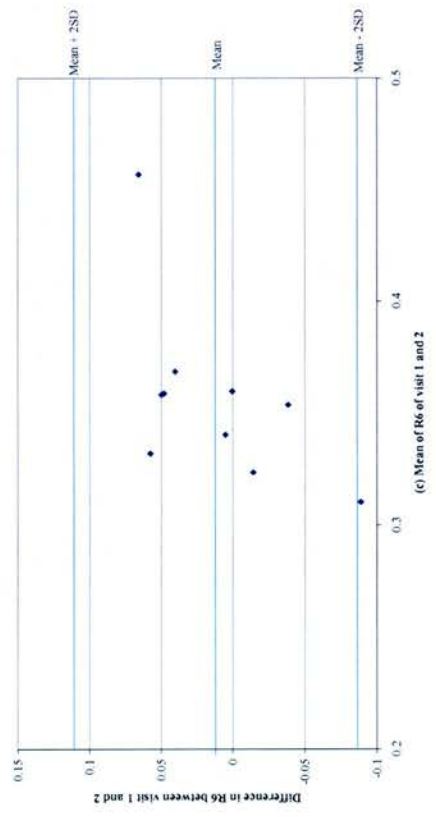
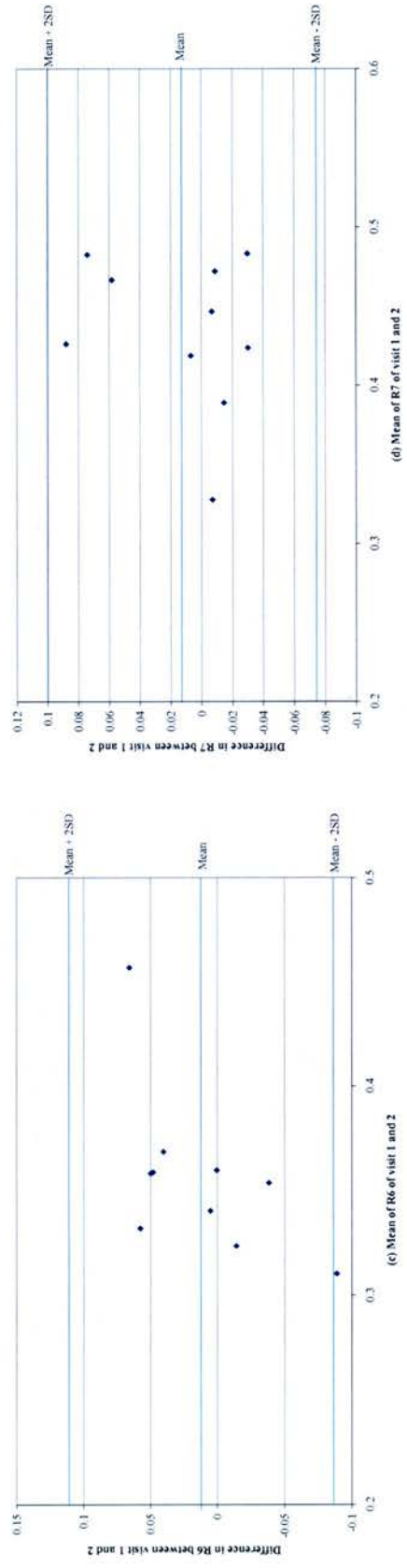
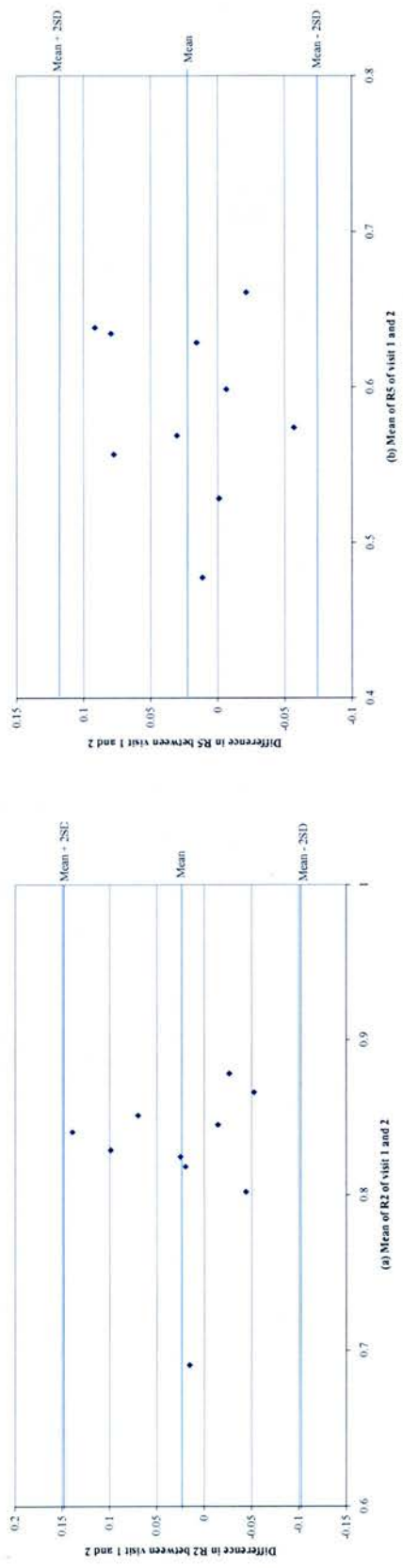


Figure 3-3 Bland-Altman plots of the skin elasticity ratios for the dorsal arm: (a) R2 (b) R5 (c) R6 and (d) R7.

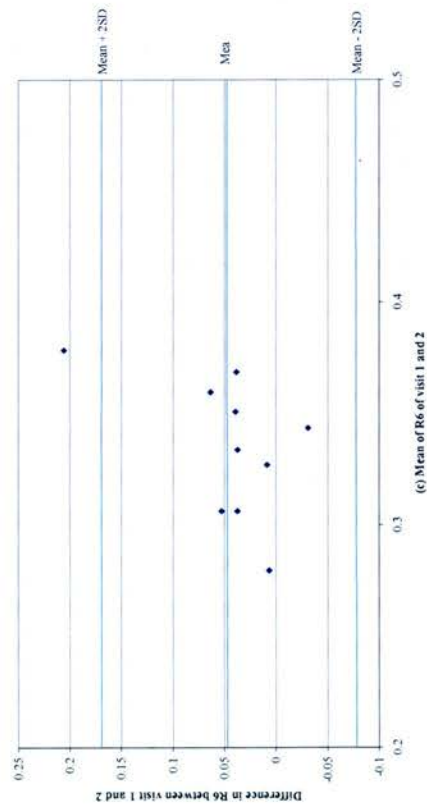
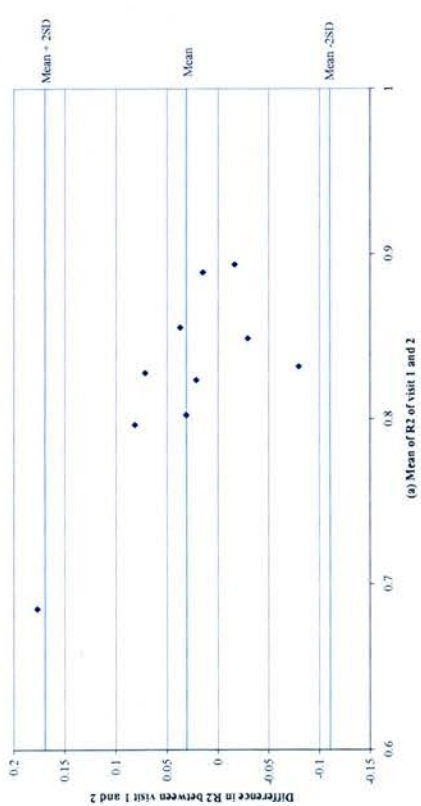
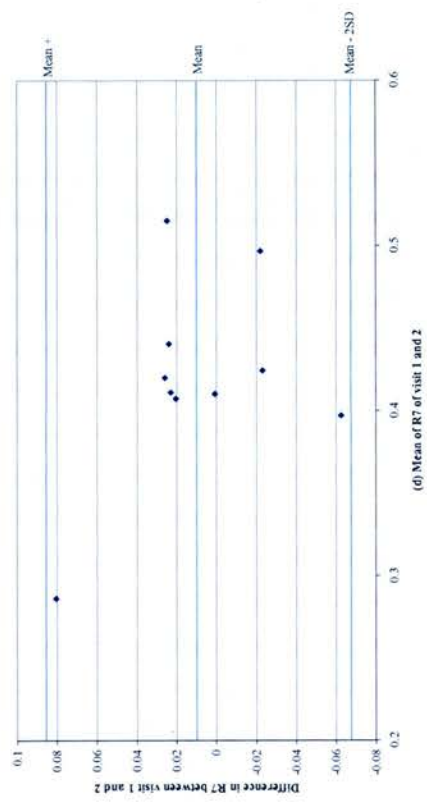
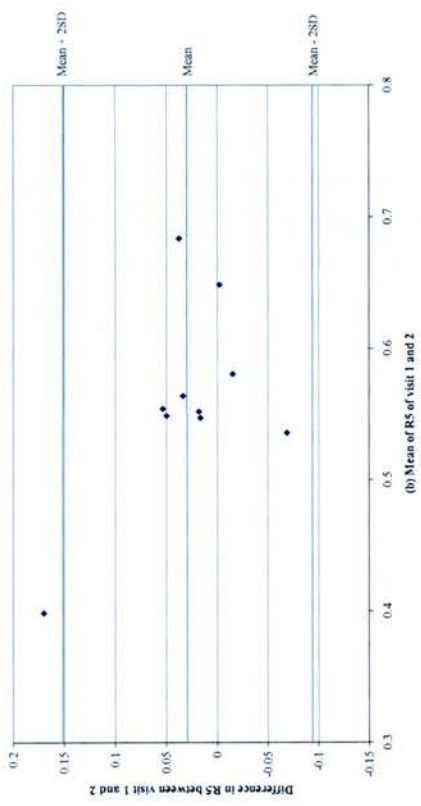


Figure 3-4 Bland-Altman plots of the skin elasticity ratios for the ventral arm: (a) R2 (b) R5 (c) R6 and (d) R7

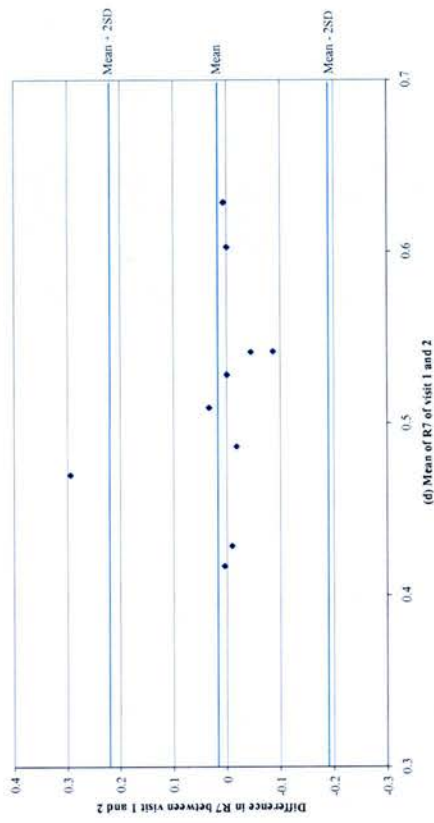
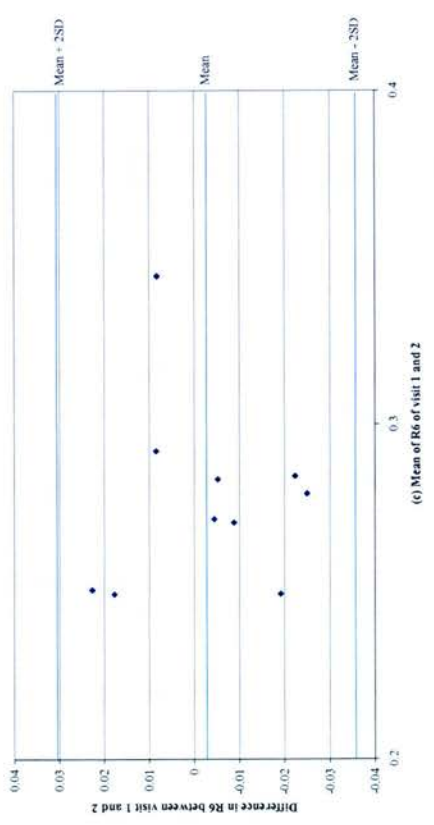
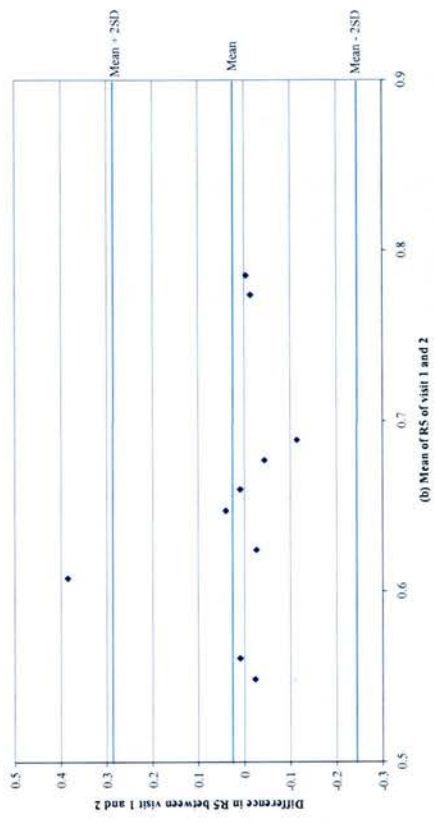
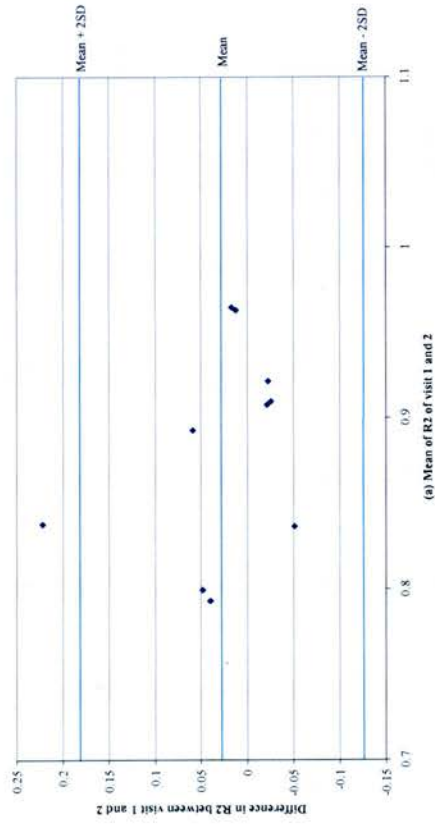


Figure 3-5 Bland-Altman plots of the skin elasticity ratios for the upper arm: (a) R2 (b) R5 (c) R6 and (d) R7.

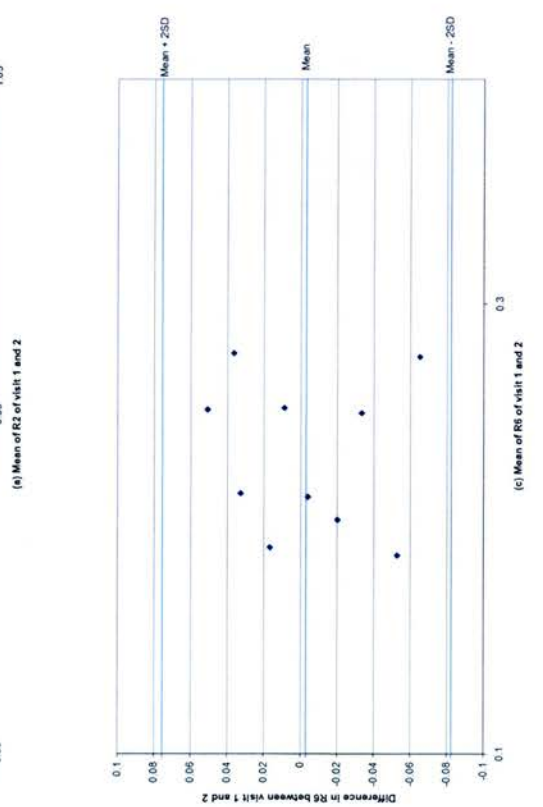
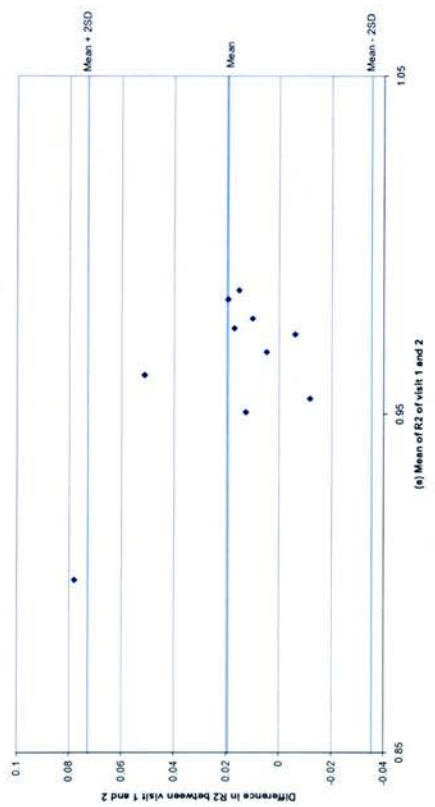
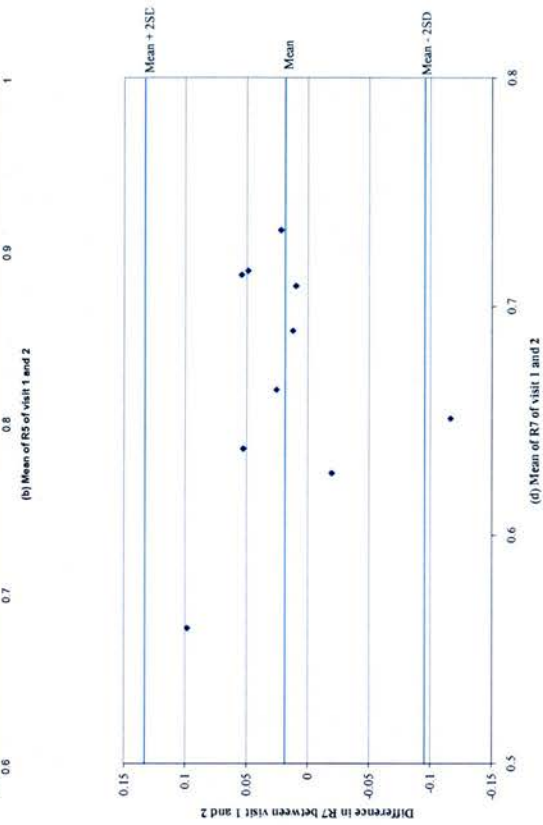
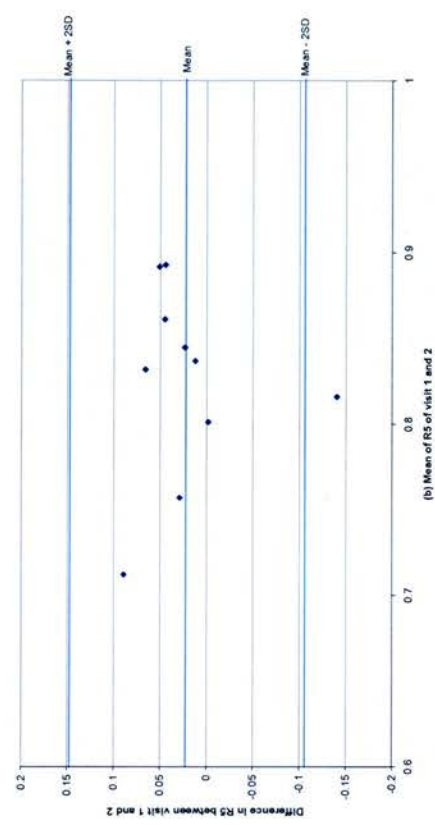


Figure 3-6 Bland-Altman plots of the skin elasticity ratios for the upper leg: (a) R2 (b) R5 (c) R6 and (d) R7.

#### 3.1.4 DISCUSSION

The R2, R5, R6 and R7 parameters were most reproducible in the upper arm and upper leg. Measurements in the upper arm had the least scatter while those of the upper leg had the least mean difference between measurements. Of the four ratios the R6 was the ratio most consistently reproducible for the different body regions.

The mean differences of the four ratios were increased by an outlying point for most of the measurements. Outlying points were found to be from different female subjects. With over two-thirds of the subjects studied being females, there is potential for changes in hormones to confound skin elasticity measurements. Oestrogen and other hormonal receptors have been noted to be present in human skin [Sumino *et al.* 2004a]. In addition, hormone replacement therapy has been shown to improve or maintain the skin's thickness, collagen content and elastic properties [Dunn *et al.* 1997; Sator *et al.* 2001]. The possible confounding effects of hormonal changes during the ovulation period was reduced by studying female subjects between days 7-28 of their menstrual cycle [Berardesca E *et al.* 1989].

Of the five regions of the body the measurement from the back showed the largest differences between visits and varied most. This can be attributed to the difficulty in taking measurements due to the movement of the body with respiration. Measurements of the dorsal arm were taken as a comparison to the ventral arm which is less exposed to the effects of ultraviolet radiation, but neither were found to be as reproducible as those from the upper arm and leg. Measurements from the ventral arm were difficult to record due to the presence of underlying veins which made it difficult to replicate the measurements during the second visit. In contrast,



measurements from the upper arm and leg were the most reproducible as these areas are least affected by extrinsic factors.

There are other factors that may play a role in the variability between skin measurements. One example is skin hydration which increases the elasticity of the skin. The skin's ability to acclimatise to the temperature and humidity levels of the study room may have varied between the two visits and may have, therefore, led to some differences in measurements. In a previous study [Sator *et al.* 2001], menopausal female subjects were required to rest for two hours under standard room conditions to allow them to adapt to the temperature and humidity levels. Subjects in our study rested for at least 20 minutes, which may not have been sufficiently long for their skin to adapt. However, if this technique is intended for use in a clinical setting, it would be impractical for patients to be rested for such a long duration.

#### 3.1.4.1 Study limitations

The main limitation of this study is the relatively small number of subjects in the older age group. Second, this study had a narrow age distribution of subjects which limited our understanding of the reproducibility of the skin elasticity measurement technique in older people. Third, the majority of subjects studied were women, which makes it difficult to determine whether men have more reproducible measurements due to the absence of cyclical changes in hormones, which might act as a confounding factor.

### 3.1.5 CONCLUSIONS

We conclude that the upper arm and upper leg were the regions of the skin that were most reproducible in this study while the R6 parameter was the most reproducible skin elasticity ratio.

## 3.2 VALIDATION OF THE USE OF THE COLIN<sup>®</sup> 7000 IN PATIENTS WITH CORONARY ARTERY DISEASE

### 3.2.1 INTRODUCTION

The non-invasive technique of arterial tonometry is increasingly being chosen as a method of measuring arterial pressure waves. The micromanometer is connected to a system software such as SphygmoCor<sup>®</sup> which derives central aortic pressure waveforms from peripheral arterial pressure waves using a transfer function [Karamanoglu *et al.* 1993; Pauca *et al.* 2001]. Central blood pressures and various arterial indices are then calculated from the central pressure waveforms. The micromanometer most commonly used with SphygmoCor<sup>®</sup> is the Millar micromanometer [McLeod *et al.* 2004; Weber *et al.* 2004; Wilkinson *et al.* 2002c; Wilkinson *et al.* 2002b]. Continuous measurements carried out using this micromanometer, however, depend on the accuracy with which the operator replaces the micromanometer probe at the same site after each consecutive measurement. An alternative device that can be used in conjunction with the SphygmoCor<sup>®</sup> system is the Colin<sup>®</sup> 7000. It is composed of an array of tonometers and has been designed in the form of a wrist band which is applied to the radial artery. The Colin<sup>®</sup> automatically detects the strongest point of the radial pulse for pressure wave recordings and is able to carry out measurements at predefined time intervals. The Millar micromanometer, in comparison, is more operator-dependent and is adversely affected by the placement of the probe at different angles to the radial artery during consecutive measurements. This study was aimed at validating the use of the Colin<sup>®</sup> against the more commonly used Millar tonometer in patients with CAD, for which there is no data to date. If the measurements recorded using the Colin are in

agreement with those from the Millar micromanometer, the Colin<sup>®</sup> would be a better alternative to use in studies that record large numbers of continuous measurements.

### 3.2.2 METHODS

#### 3.2.2.1 Study Subjects

Patients with CAD who took part in the endothelial function study (discussed in Chapter 6) were invited to take part in this study. The first fifteen subjects who agreed comprised the population for this study. Approval for all studies was obtained from the local research ethics committee and informed written consent obtained from each participant prior to the study.

#### 3.2.2.2 Measurements

Subjects were studied at least 2 hours after their morning medications and were asked to avoid caffeine and food 2 hours prior to the study. PWA was carried out using the SphygmoCor<sup>®</sup> system (SphygmoCor<sup>®</sup> Mx, AtCor Medical, Sydney, Australia, version 6.31). This system uses the technique of applanation tonometry, in which a high-fidelity micromanometer (SPT-301, Millar Instruments, Texas, USA) or a probe containing piezoelectric pressure sensors (Colin<sup>®</sup> 7000, Colin Medical Technology Corporation, Japan) is used to determine AIx and AIx75. Brachial blood pressure was measured using a validated oscillometric technique (HEM-705CP; Omron Corporation, Japan) in combination with the micromanometer or an automated blood pressure monitor which is incorporated into the Colin.

### 3.2.2.3 Study Protocol

The study protocol is detailed in Figure 3-7. This protocol consisted of the following sequence of measurements:

a blood pressure measurement followed by the mean of 3 augmentation measurements using the Millar micromanometer,

a blood pressure measurement followed by the mean of 3 augmentation measurements using the Colin<sup>®</sup>,

a blood pressure measurement, followed by the mean of 3 augmentation measurements using the Colin<sup>®</sup>,

a blood pressure measurement, followed by the mean of 3 augmentation measurements using the Millar micromanometer,

### 3.2.2.4 Data Analysis

Statistical analysis was performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). Data were examined by Bland-Altman plots and two tailed Student's t-test. All results are expressed as mean  $\pm$  SD. Statistical significance was taken at the 5% level.

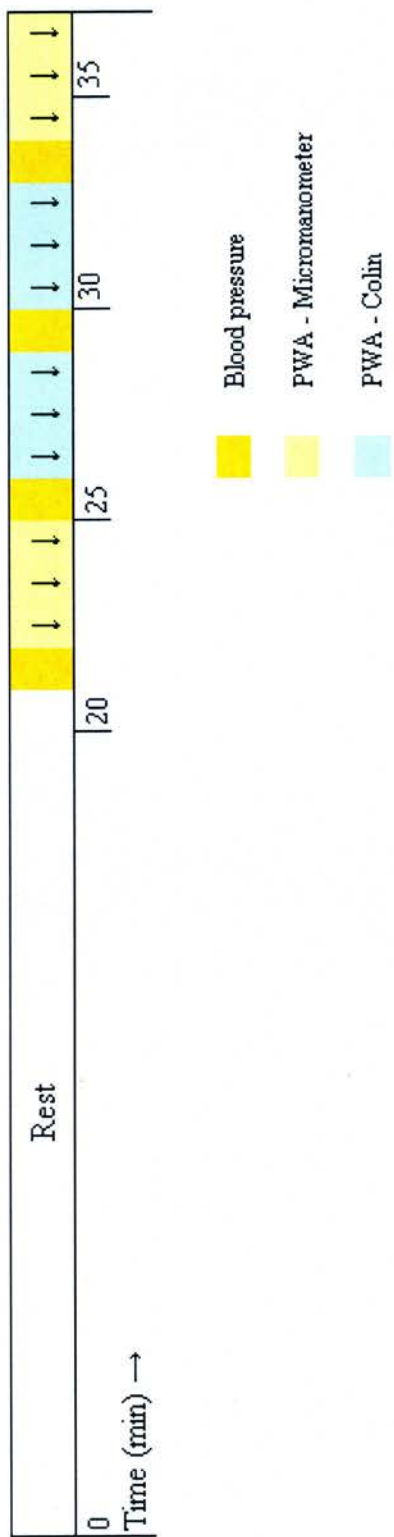


Figure 3-7 Protocol for the validation of the Colin<sup>®</sup> 7000. Schematic of the study protocol undertaken. The downward arrow, ↓, denotes the recording time of a measurement.

### 3.2.3 RESULTS

The group of subjects who took part in the validation study comprised 7 males and 8 females with a mean age of  $65 \pm 6.7$  years. Mean central blood pressure was  $124/70 \pm 15/12$ , and mean peripheral blood pressure was  $116/71 \pm 16/12$ .

Nine subjects were in CCS Class 1, while 5 subjects were in CCS Class 2, and 1 subject in CCS Class 3. Out of the 15 subjects, 1 had no coronary vessel disease, 6 had disease in 1 coronary vessel, 3 had 2-vessel disease and 5 patients had 3-vessel disease. The drug and medical history of all subjects is outlined in Table 3-2.

Table 3-2 Validation study subject's clinical and therapeutic profile

<b>Parameter</b>	<b>% (n)</b>
<b>Past medical history</b>	
Hypercholesterolaemia	93.9 (14)
Diabetes	6.7 (1)
Hypertension	40.0 (6)
Ischaemic heart disease	93.3 (14)
Myocardial Infarct	40 (6)
Heart failure	0
<b>Drug Therapy</b>	
Diuretics	6.7 (1)
$\beta$ -blocker	60 (9)
Nitrates	80 (12)
ACE inhibitors	26.7 (4)
Calcium channel blockers	33.3 (5)
Angiotensin receptor blocker	6.7 (1)
Nicorandil	33.3 (5)
Statin	86.7 (13)

Values are proportions. N=15



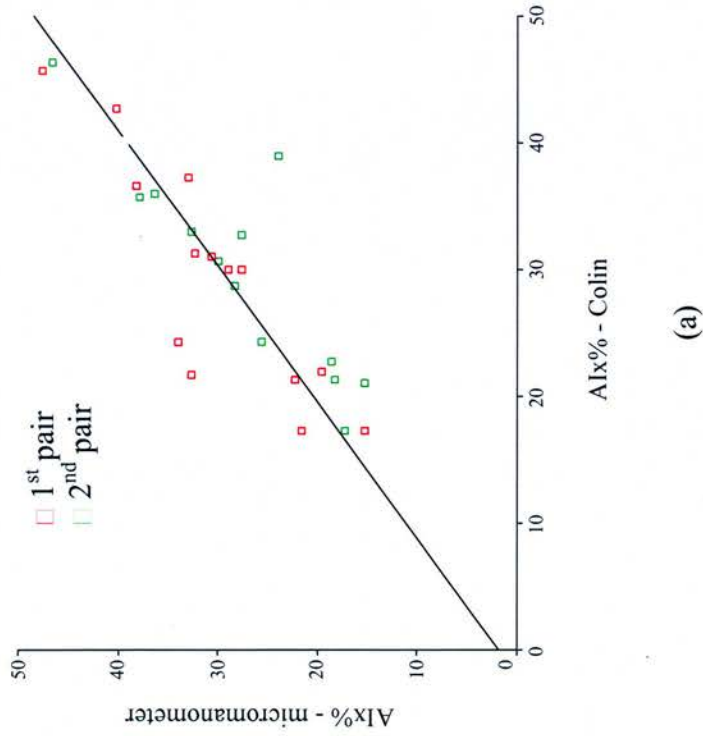
The mean baseline values, SDs and mean differences are shown in Table 3-3. There were no significant differences in the mean values of AIX and AIX75 between the first and second sets of recordings carried out using a micromanometer. Similarly, there were no differences between sets of recordings made using the Colin<sup>®</sup>. There were also no significant differences in the mean values or SDs of AIX or AIX75 between the micromanometer and Colin<sup>®</sup>.

There was a strong positive correlation in AIX ( $r = 0.87$ ,  $P < 0.001$ ) and AIX75 ( $r = 0.79$ ,  $P < 0.001$ ) on assessment of scatter plots of measurements from the micromanometer and the Colin<sup>®</sup> (Figure 3-8). The Bland-Altman plots for the reproducibility in measurements of AIX and AIX75 are shown in Figure 3-9. The mean of the differences for AIX and AIX75 was  $-0.2 \pm 4.6\%$  and  $-0.3 \pm 4.9\%$  respectively.

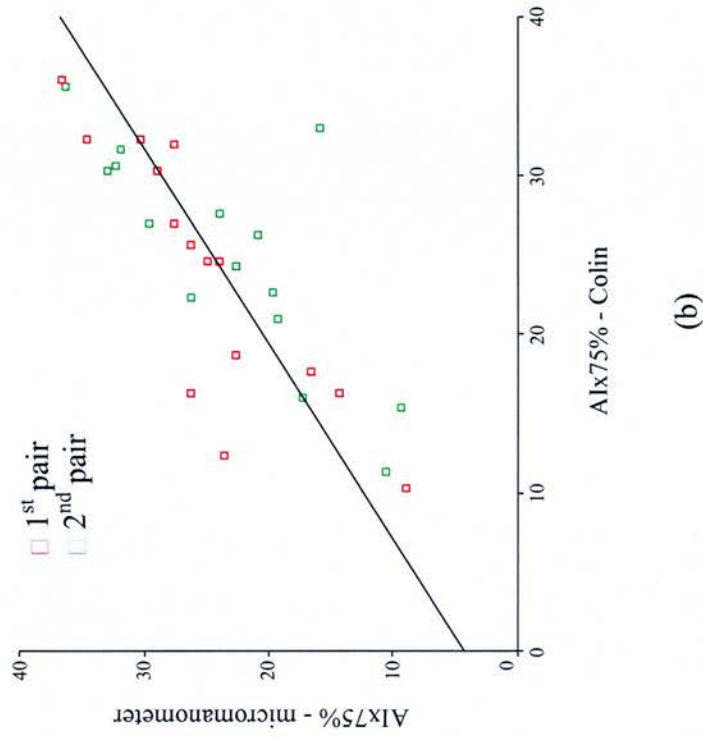
Table 3-3 Means and differences of Alx and Alx75 for the micromanometer and Colin® 7000.

Parameters	Micromanometer		Colin		Mean of differences ± SD
	1 <sup>st</sup> pair mean ± SD	2 <sup>nd</sup> pair Mean	1 <sup>st</sup> pair mean ± SD	2 <sup>nd</sup> pair Mean	
<b>Mean</b>					
Alx	31.0 ± 8.8	29.5 ± 9.9	29.9 ± 9.1	31.0 ± 8.3	30.5 ± 8.6
Alx75	24.9 ± 7.3	23.3 ± 8.2	23.8 ± 8.1	25.0 ± 7.0	24.4 ± .4

N=15



(a)



(b)

Figure 3-8 Scatter plots of the micromanometer compared to the Colin<sup>®</sup> of (a) Alx% and (b) Alx75%

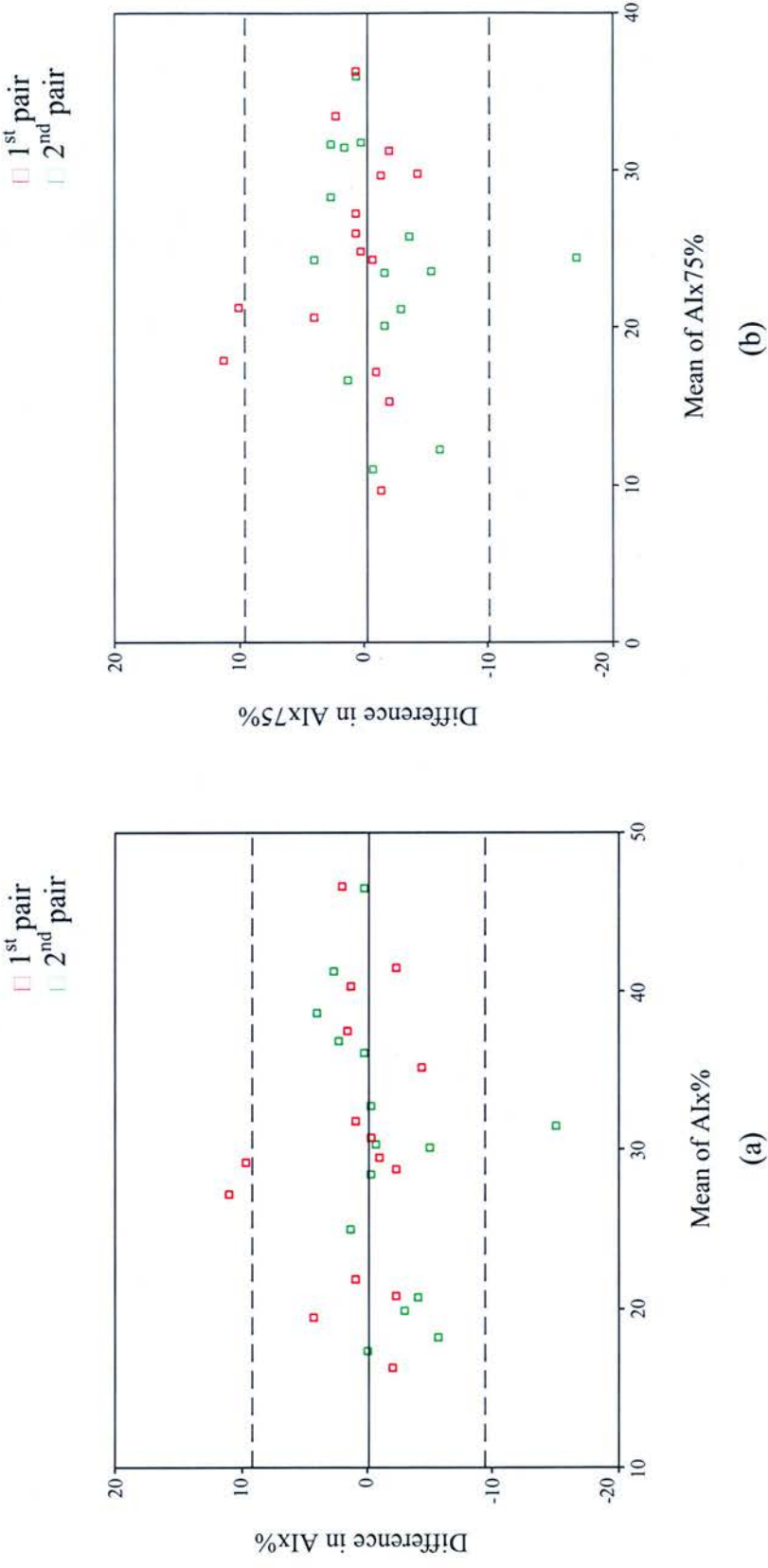


Figure 3-9 Bland-Altman plots for the micromanometer and Colin<sup>®</sup> of (a) Alx% and (b) Alx75%.

### 3.2.4 DISCUSSION

The augmentation values of the micromanometer were strongly correlated with those of the Colin<sup>®</sup>. There were no significant differences between these techniques, nor were there differences between repeated measurements of the individual techniques.

The strong correlation between the augmentation values measured using the Millar micromanometer and Colin<sup>®</sup> devices indicate that the Colin<sup>®</sup> can be used as an alternative device to the micromanometer. This conclusion is reinforced by our failure to find any significant differences in augmentation values measured for each device. The sequence of measurements (micromanometer, Colin<sup>®</sup>, Colin<sup>®</sup>, micromanometer) strengthens these findings by reducing any possible errors due to changes in blood pressure. Therefore, any variations in augmentation values are more likely to be caused by the variability in measures caused by the individual devices. However, the differences in augmentation values between devices were no greater than differences in the reproducibility of AIx, carried out using a micromanometer, in a study by Wilkinson et al. [Wilkinson *et al.* 1998]. In the latter study, the mean difference in AIx from a single device (Millar micromanometer) was  $0.49 \pm 5.4\%$ . Therefore, there is no greater variability between measurements from these 2 devices as compared to measurements from a single device.

#### 3.2.4.1 Limitations

This study was limited to a small number of subjects, nevertheless, there was a good agreement in augmentation values between the Millar micromanometer and Colin<sup>®</sup>.

### 3.2.5 CONCLUSIONS

The augmentation values derived using measurements from the Colin<sup>®</sup> are in good agreement with those of the micromanometer.

### 3.3 REPRODUCIBILITY OF THE SALBUTAMOL RESPONSE AS A TEST OF ENDOTHELIUM-DEPENDENT FUNCTION IN PATIENTS WITH CORONARY ARTERY DISEASE

#### 3.3.1 INTRODUCTION

Endothelial dysfunction has been shown to predict cardiovascular events in patients with CAD [Schachinger *et al.* 2000] as well as in patients with angiographically normal coronary arteries [Halcox *et al.* 2002b]. Impairment of endothelial function, as measured by flow-mediated dilatation (FMD), predicts events in patients with chest pain while a normal FMD in the brachial artery is associated with a low risk of cardiac events [Neunteufl *et al.* 2000].

A number of techniques have been used to study endothelial function in the coronary and peripheral vascular bed. The most reliable method for detection of endothelial dysfunction involves determining the change in coronary blood flow, coronary artery diameter, and coronary vascular resistance to intracoronary infusion of acetylcholine [Deanfield *et al.* 2005]. It is, however, expensive and has risks related to the procedure of angiography which makes it an unsuitable screening tool for the general population. An alternative reliable and reproducible technique is intrabrachial infusion of vasoactive substances during venous occlusion strain-gauge plethysmography. One of the drawbacks of using this technique is it is an invasive and time consuming technique that does not lend itself to large patient populations [Widlansky *et al.* 2003].

Attempts to overcome some of the problems faced by the above techniques have led to development of non-invasive techniques. Examples include high resolution

ultrasound to determine FMD and strain-gauge venous plethysmography during reactive hyperaemia. FMD of the brachial artery is a validated technique [Deanfield *et al.* 2005] and is well correlated with coronary endothelial vasodilator function [Anderson *et al.* 1995b]. However, investigators need to be trained in assessing FMD results compared to strain gauge venous plethysmography which is less observer dependent [Tousoulis *et al.* 2005].

The technique of PWA with the administration of GTN and salbutamol as a test of endothelial function is a relatively new non-invasive technique. The reflected arterial pressure wave, influenced by the resistance of peripheral vessel tone, is altered by GTN and salbutamol. GTN, an endothelium-independent vasodilator, is an exogenous source of NO while salbutamol, an endothelium-dependent vasodilator, generates NO through its action on the endothelium. Both GTN and salbutamol result in a reduction in stiffness via NO dependent pathways and can be used to assess endothelial function.

The GTN and salbutamol responses have been described in hypercholesterolaemics [Wilkinson *et al.* 2002b], CAD [Hayward *et al.* 2002] and patients with diabetes [Chowienczyk *et al.* 1999] and has been validated as a model of endothelial dysfunction through nitric oxide synthase inhibition [Chowienczyk *et al.* 1999; Dawes *et al.* 1997]. There are currently limited data on the reproducibility of this model with only one study [Hayward *et al.* 2002] to date carried out in healthy volunteers. This study is aimed at determining the reproducibility of this technique in patients with CAD.



### 3.3.2 METHODS

#### 3.3.2.1 Study Subjects

Subjects who had taken part in a large study looking at endothelial function in patients with CAD at the Western General Hospital, discussed in Chapter 6, were invited to take part in this study. The first twenty subjects who agreed to attend a second visit a week later comprised the reproducibility study population.

#### 3.3.2.2 Measurements

Subjects had all vasoactive medications withheld on the morning of the study and were studied after an overnight fast on both occasions. Brachial blood pressure was measured using a validated oscillometric technique (HEM-705CP; Omron Corporation, Japan). Peripheral pressure pulse waveforms were determined by a sensor containing piezoelectric pressure transducers (Colin<sup>®</sup> 7000; Colin Medical Technology Corporation, Japan) from which the central waveform was derived. AIx and AIx75 were calculated using the SphygmoCor<sup>®</sup> system software.

#### 3.3.2.3 Study Protocol

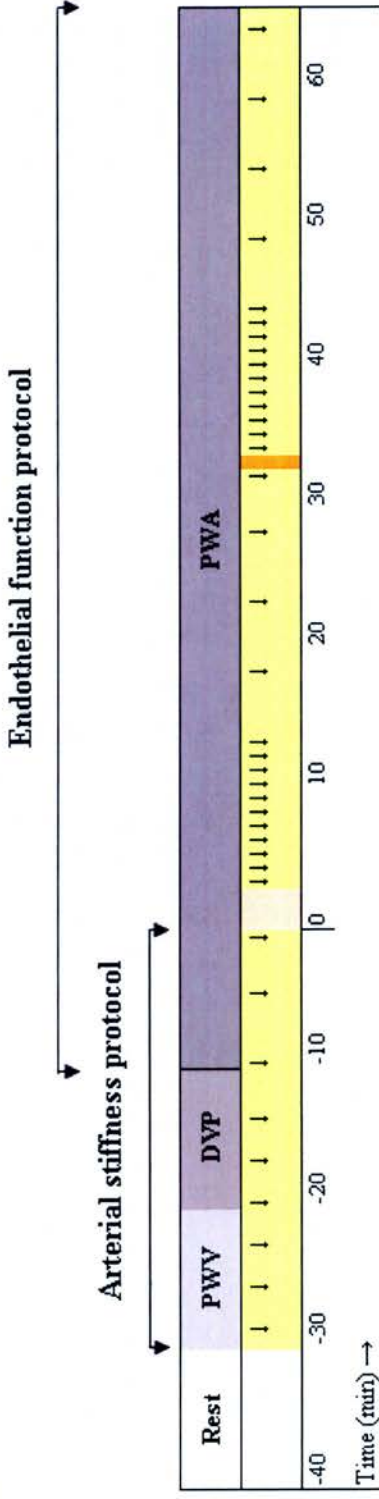
The study protocol is detailed in Figure 3-10. Subjects underwent arterial stiffness and vascular function measurements during their first visit. During their second visit subjects repeated only the test of endothelial function which comprised measurements of augmentation indices in response to salbutamol.

#### 3.3.2.4 Data Analysis

Statistical analysis was performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). Salbutamol responses

were determined by calculating peak changes and AUCs. Data were examined by Bland-Altman plots and two tailed Student's t-test. All results are expressed as mean  $\pm$  SD. Statistical significance was taken at the 5% level.

Visit 1:



Visit 2:

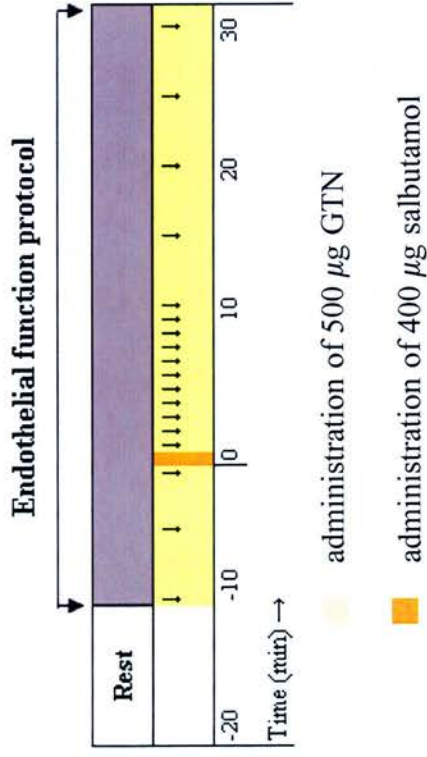


Figure 3-10 Protocol of salbutamol reproducibility study. Schematic of the study protocol undertaken. The downward arrows, ↓, denotes the recording time of a measurement.

### 3.3.3 RESULTS

The twenty subjects who took part in the reproducibility study comprised 18 men and 2 women with a mean age of  $61.7 \pm 7.7$  years. Mean central blood pressure was  $125/76 \pm 16/10$ , and mean peripheral blood pressure was  $132/75 \pm 16/10$ . Patients returned for a second visit after a mean time of  $8 \pm 2$  days. Fifteen patients comprised the Canadian Cardiovascular Society Angina classification (CCS) Class 1, while 4 patients were in CCS Class 2, and 1 patient in CCS Class 3. Out of the 20 patients, 6 had disease in 1 coronary vessel, 8 had 2-vessel disease and 6 patients had 3-vessel disease. The drug and medical history of all subjects is outlined in Table 3-4.

The mean baseline values, peak changes and area under the curves for responses to salbutamol are shown in Table 3-5. The Bland-Altman plots for the reproducibility in measurements of AIx and AIx75 are shown below in Figure 3-11. The mean of differences in peak responses for AIx and AIx75 was  $0.02 \pm 6.6\%$  and  $0.37 \pm 6.4\%$  respectively. No significant differences were found between visits ( $P < 0.49$ ;  $P < 0.40$ ). The time to peak effect of salbutamol was earlier in the first visit compared to that of the second visit. The mean difference in AUC between visits were  $37 \pm 198$  and  $40 \pm 184$  for AIx and AIx75 with no significant differences between visits ( $P < 0.21$ ;  $P < 0.17$ ) respectively.

Table 3-4 Salbutamol reproducibility study subject's clinical and therapeutic profile.

<b>Parameter</b>	<b>%</b>
<b>Past medical history</b>	
Hypercholesterolaemia	75
Diabetes	0
Hypertension	40
Ischaemic heart disease	95
Myocardial Infarct	45
Heart failure	0
<b>Drug Therapy</b>	
Diuretics	90
$\beta$ -blocker	70
Nitrates	85
ACE inhibitors	25
Calcium channel blockers	25
Angiotensin receptor blocker	5
Nicorandil	20
Diuretic	10
Statin	90

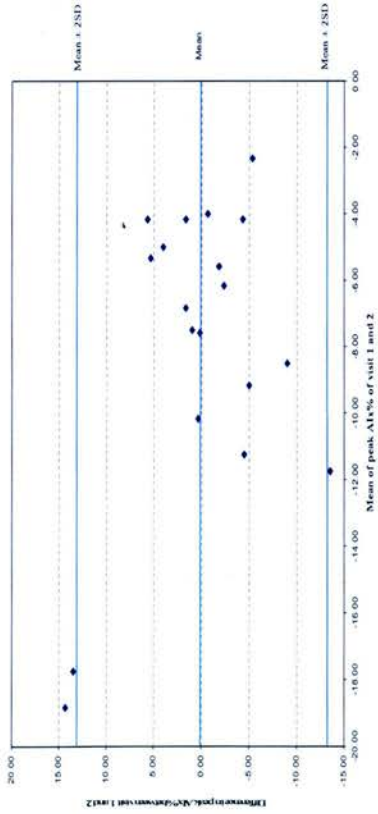
Values are proportions. N=20

Table 3-5 Summary of baseline augmentation measures, peak changes and area under the curve for salbutamol.

Parameters	Visit 1 mean $\pm$ SD	Visit 2 mean $\pm$ SD	Mean of differences $\pm$ SD
<b>Baseline values</b>			
Aix, %	35.0 $\pm$ 10.6*	31.4 $\pm$ 9.6	-1.8 $\pm$ 4.2
Aix75, %	25.1 $\pm$ 9.3	23.0 $\pm$ 7.9	-1.0 $\pm$ 3.9
<b>Peak changes</b>			
$\Delta$ Aix, %	-7.7 $\pm$ 6.5	-7.7 $\pm$ 4.4	0.02 $\pm$ 6.6
$\Delta$ Aix75, %	-6.4 $\pm$ 6.2	-6.1 $\pm$ 4.1	0.4 $\pm$ 6.4
<b>Time to peak effect</b>			
Aix, mins	12 $\pm$ 7	16 $\pm$ 9	-
Aix75, mins	10 $\pm$ 5*	16 $\pm$ 10	-
<b>AUC</b>			
AUC Aix%	3186 $\pm$ 145	3149 $\pm$ 124	37 $\pm$ 198
AUC Aix75%	3153 $\pm$ 137	3114 $\pm$ 112	40 $\pm$ 184

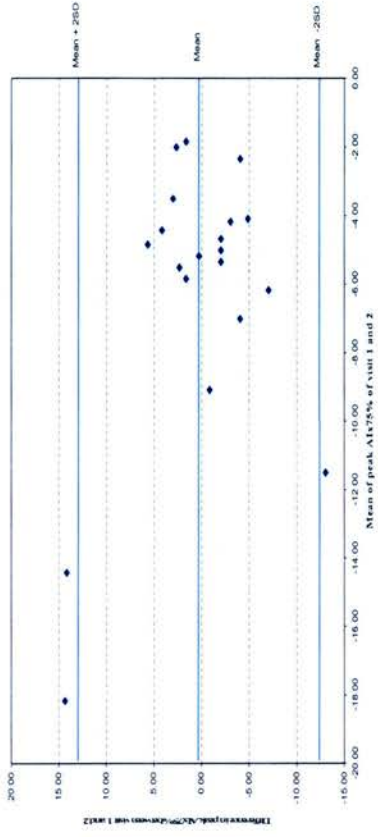
N=20 \* P < 0.05

Bland Altman plot of peak changes in A1x%.



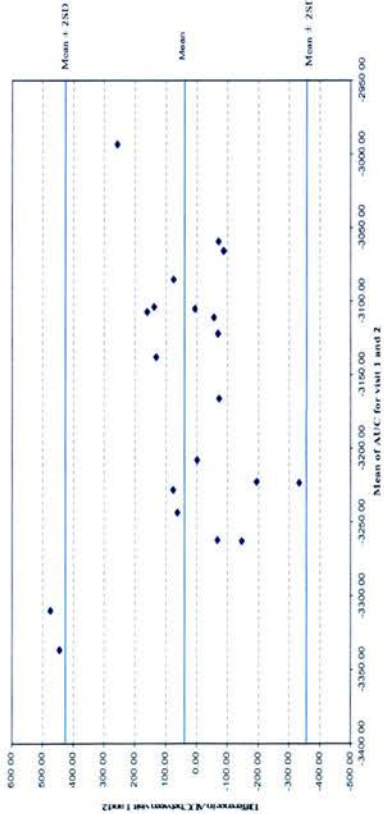
(a)

Bland Altman plot of peak changes in A1x75%.



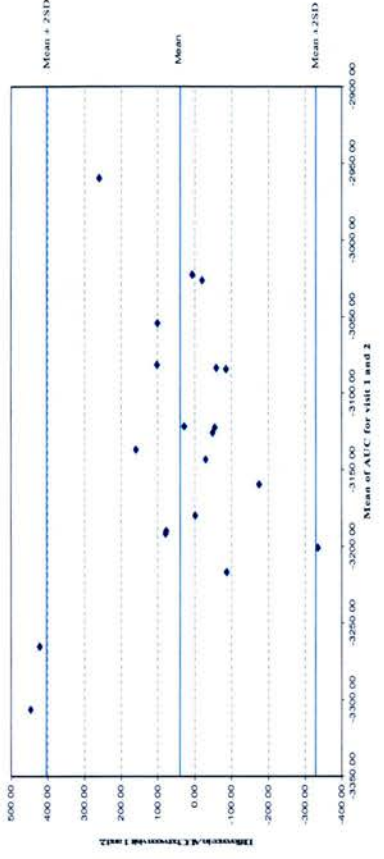
(b)

Bland Altman plot of the AUC for A1x%.



(c)

Bland Altman plot of the AUC for changes in A1x75%.



(d)

Figure 3-11 Reproducibility of changes in augmentation indices to salbutamol. Bland-Altman plots of (a) peak A1x, (b) peak A1x75, (c) AUC of A1x, (d) AUC of A1x75 in response to salbutamol

### 3.3.4 DISCUSSION

The peak effect of the salbutamol response was well reproduced during the second visit. The AUC of salbutamol, however, was not as well reproduced mainly due to the variability in responses between the 2 visits for three subjects.

The mean difference in peak response was  $0.02 \pm 6.6$  for AIx and  $0.37 \pm 6.4$  for AIx75. The mean difference in our study was lower than that previously reported by Wilkinson et al. [Wilkinson *et al.* 2002b] who found the mean difference in peak AIx to be  $-2.3 \pm 3.0\%$  in 13 healthy subjects. Prior to their study in 2002, Wilkinson et al. [Wilkinson *et al.* 1998] carried out a study of 33 subjects (5 controls, 12 diabetics and 16 hypertensives) in which 2 measurements of AIx were made. They found the mean difference to be  $0.49 \pm 5.37\%$ . Wilkinson's measurements of baseline AIx% were carried out sequentially on the same day. The mean baseline difference was slightly larger in our study population. However, the SDs were slightly smaller compared to those found by Wilkinson. This implies that the amount of variability found in baseline measurements of AIx and AIx75 are comparable to that found during the peak change in AIx and AIx75 to salbutamol. The mean difference in the AUC was large and patients were found to have a larger overall response to salbutamol during the second visit compared to the first. The large SDs, in our study, were mainly due to the measurements from three outlying patients. They may be attributed to the use of PWA with the administration of GTN and salbutamol being a poor technique or alternatively due to



polymorphisms of the human  $\beta_2$  adrenoreceptor resulting in variability of augmentation measures not determined by endothelial function [Green *et al.* 1994; Reihnsaus *et al.* 1993].

An increasing heart rate results in the reflected wave arriving during diastole, while a reduced heart rate would have led to the reflected wave arriving during the systolic phase [Davies *et al.* 2003]. In order to correct for the effect of heart rate on the variability of the data, changes in AIx corrected to a heart rate of 75 beats per minute were made. The peak changes in AIx75 had less variability than the peak change in AIx. However, correction for heart rate did not differentiate the AUC responses in AIx and AIx75. This may be partly due to the cumulative variability of the different time points when calculating AUCs for each subject confounding corrections of heart rate.

In the study by Wilkinson *et al.* [Wilkinson *et al.* 2002b], the peak change in AIx in 13 healthy subjects was  $-9.3 \pm 3.8$  (1<sup>st</sup> visit) and  $-11.6 \pm 3.8$  (2<sup>nd</sup> visit), while patients in our reproducibility study who had CAD were found to have a peak change in AIx of  $-7.7 \pm 6.5$  (1<sup>st</sup> visit) and  $-7.7 \pm 4.4$  (2<sup>nd</sup> visit). The minimum mean difference, based on the second visit of these 2 studies, required to detect endothelial dysfunction in patients with CAD is about 4%. Therefore based on these data, a power calculation indicates that a sample size of 85 subjects is required for an 80% power to detect a 4% difference in the peak responses to salbutamol.

#### 3.3.4.1 Limitations

A number of limitations are evident in this study, and these are as follows. First, subjects only undertook the endothelial function test protocol during their second visit. This may have led to a hangover effect of GTN on the salbutamol response during the first visit and therefore larger peak, as evidenced by the larger SDs, and the AUCs in augmentation indices in response to salbutamol. However, in a study by Greig et al. [Greig *et al.* 2005] the response to GTN persisted up to 25 minutes therefore eliminating the likelihood of a hangover effect of GTN in our subjects after 30 minutes. Second, patients may be better at their inhaler technique on their second visit and this “training effect” may explain the reduced variability in responses during the second visit. Third, the peak effect of salbutamol was expected to occur roughly around 11 minutes based on data from Wilkinson et al. [Wilkinson *et al.* 2002b] who found the mean peak salbutamol response to occur at  $11 \pm 3$  minutes in normocholesterolemics and  $12 \pm 3$  minutes in hypercholesterolaemics. Measurements were recorded every minute for the first 11 minutes followed by measurements every 5 minutes for a period of 30 minutes. The result from our group of subjects with CAD indicates that the mean peak response to salbutamol took place at  $13 \pm 8$  minutes for both AIX and AIX75. Therefore, there is the potential of underestimating peak effects which take place in between the 5-minute intervals. In addition the time to peak effect may have been underestimated were they to fall in between the 5 minute intervals. If these limitations had been avoided the precision of the endothelial response during both visits would have been better.

### 3.3.5 CONCLUSIONS

The Bland-Altman plots reflect that, on the whole, the daily variability was low, but there were a number of patients who had highly variable readings during the two visits which could adversely affect the interpretation of their response to salbutamol in a clinical setting. Variability in endothelial function measures may be due to the endothelial function test being a poor technique or human  $\beta_2$ -adrenoreceptor polymorphisms.

CHAPTER 4: SKIN ELASTICITY AS A MARKER OF ARTERIAL ELASTICITY IN  
HEALTHY VOLUNTEERS

#### 4.1 INTRODUCTION

Both arterial wall elasticity and skin elasticity are determined by their constituents elastin and collagen [Loscalzo *et al.* 1996; Tzaphlidou 2004]. We are led to believe that there is a relationship between skin elasticity and arterial elasticity by the observation that an increase in both arterial and skin elasticity has been found in certain hereditary disorders such as Ehlers-Danlos syndrome, Marfan syndrome and PXE [Gogly *et al.* 1998; Kornet *et al.* 2004; Pope *et al.* 1975] as well as in patients with intracranial aneurysms [Grond-Ginsbach *et al.* 2002] and cervical artery dissections [Brandt *et al.* 2001]. In contrast, a decrease in arterial and skin elasticity has been found in women with a history of pre-eclampsia [Elvan-Taspnar *et al.* 2005].

At this point it is instructive to consider the underlying pathological changes that cause arterial stiffness. In stiff arteries there is increased abnormal collagen, frayed and broken elastin molecules, abnormal or disarrayed endothelial cells, and infiltration of macrophages, mononuclear cells and vascular smooth muscle cells [Johnson *et al.* 2001; Lakatta 2003; Zieman *et al.* 2005]. The age-related increase in collagen and thinning and fragmentation of elastin has only been found in the elastic arteries [Nichols 2005]. Collagen in the aorta doubles in content between the ages of 20 to 70 years. In the skin, a decrease in the voids of spaces between fibres with age results in more compact elastin and collagen. The elastic fibres show signs of elastolysis while collagen bundles are

randomly orientated and their numbers decreased [Tzaphlidou 2004]. Elastin is found to be fragmented with age in both arteries and skin.

In certain other diseases, however, although stiffness of arteries has been observed, no studies have sought to establish a link with skin elasticity. Examples of such diseases are diabetes [Cruickshank *et al.* 2002], hypertension [Boutouyrie *et al.* 2002b], left ventricular hypertrophy [Deague *et al.* 2001], end-stage renal disease [Blacher *et al.* 1999b] and CAD [Lim *et al.* 2004a]. A similarity in both the skin and arteries is that there is degeneration of elastin with a decrease in elasticity. If arterial stiffness or a reduced arterial elasticity is found to correlate positively with reduced skin elasticity, skin elasticity measures may be developed as representative external markers of arterial stiffness and cardiovascular risk. This would also provide a simple non-invasive technique that can be easily applied in a clinical setting. However, there are a number of dissimilarities between the skin and arteries. One such dissimilarity is that in the skin about 70% of dermal collagen is type I collagen and 15% is type III collagen [Silver *et al.* 2001b]. These ratios are reversed in arteries [Champion *et al.* 1998]. In addition, in the arteries collagen content increases with advancing age, however, collagen content in the skin is found to decrease with age [Tzaphlidou 2004].

In this chapter, the association between skin elasticity and arterial elasticity was examined (as arteries become less elastic they are described as having an increase in stiffness). Moreover, one of the intentions of this study was to identify which anatomical

skin region (for example leg, arm or back) showed the strongest relationship with arterial stiffness.

The present study was carried out to determine:

1. if there is an association between arterial stiffness and skin elasticity, independent of age,
2. if there is an association, which measure most accurately matched arterial stiffness variables and this would include anatomical site and type of ratio calculated.

## 4.2 METHODS

### 4.2.1 STUDY SUBJECTS

Healthy volunteers between the ages of 18 – 80 years were recruited through advertisements placed at the local churches and at the Western General Hospital, Edinburgh. Subjects were divided into age groups of 18 – 29, 30 – 39, 40 – 49, 50 – 59, 60 – 69 and 70 – 79. Female subjects were studied on days 7 – 28 of their ovulation cycle to reduce the effect of hormonal changes on skin measurements [Berardesca E *et al.* 1989]. We aimed to recruit 20 subjects in each age group. Exclusion criteria were the presence of skin disorders, sun burn and excessive exposure to ultraviolet radiation. In addition, subjects were excluded if they were taking corticosteroids, on long term medications or had recently taken antibiotics.

### 4.2.2 MEASUREMENTS

Arterial stiffness of each subject was measured using each of the techniques of PWV, PWA and the DVP. The technique of applanation tonometry with a high-fidelity micromanometer (SPC-301, Millar Instruments, Texas, USA) was used to detect the carotid, radial and femoral pressure waves. These waveforms were analysed using the SphygmoCor<sup>®</sup> system software (Mx Aortic Blood Pressure Monitoring System, AtCor Medical, Sydney, Australia, version 6.31) from which PWV and PWA were determined. PWA comprised measurements of central AIx, AIx75, and PAIx. The DVP wave was



determined by the Pulse Trace<sup>®</sup> (Micro Medical, Gillingham, Kent, United Kingdom), a high fidelity finger plethysmography, from which the SI and the RI are derived. Non-invasive blood pressure measurements were carried out on the right arm using a validated oscillometric technique (HEM-705CP; Omron Corporation, Japan)

A Cutometer<sup>®</sup> (Courage and Khazaka, Koln, Germany) which uses a vacuum suction technique was used to measure waveforms of vertical skin deformation as a function of time. An 8 mm diameter probe was used, through which a controlled vacuum force of 45 kPa was applied for 5 seconds and reduced to 0 mbar for 3 seconds. This cycle was repeated 10 times. Ratios R2 (gross elasticity), R5 (neto-elasticity of the skin without viscous deformation), R6 (ratio of visco-elastic to elastic distension) and R7 (biological elasticity) were derived from these waveforms to determine elasticity of the skin as shown in Figure 4-1. Details of methods used can be found in Chapter 2.

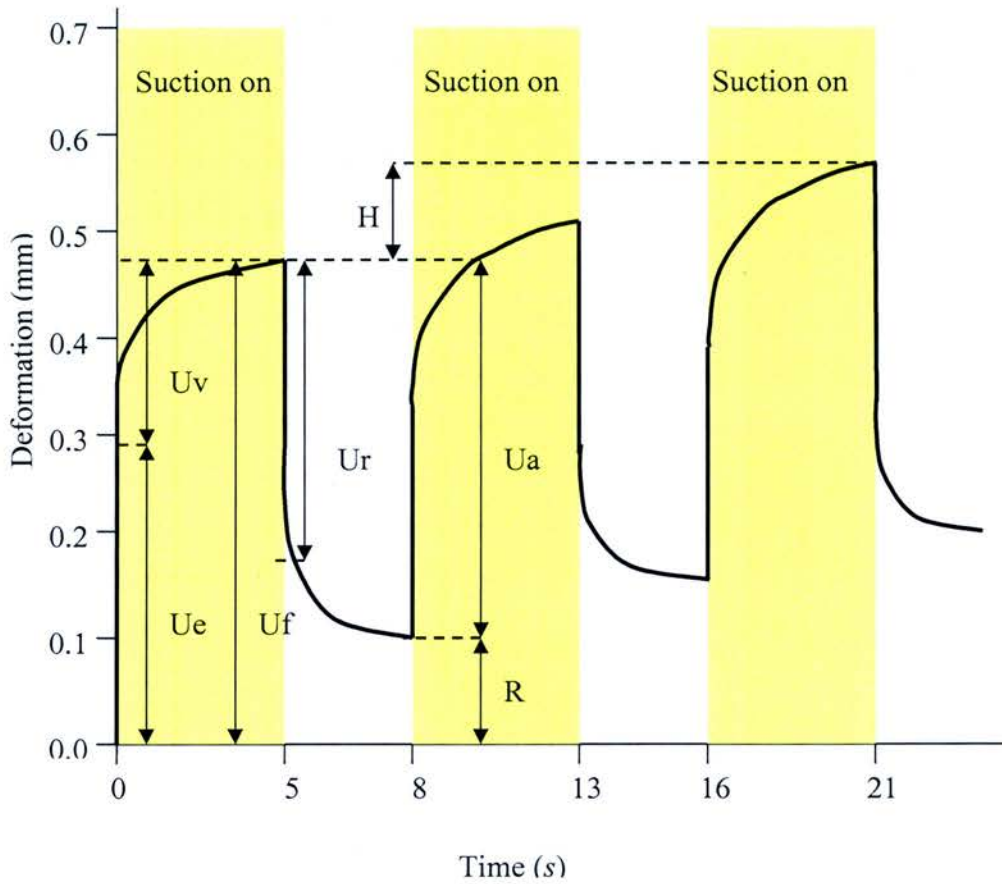


Figure 4-1 Skin elasticity curve derived using a suction probe.

This figure represents skin deformation plotted as a function of time. A negative pressure of 450 mbar is applied for 5s, followed by a 3-s relaxation period. This is repeated 10 times.  $U_e$  denotes immediate distension;  $U_v$ , delayed distension;  $U_f$ , final distension (skin distensibility);  $U_r$ , immediate retraction;  $U_a$ , final retraction and  $R$ , residual deformation at the end of a measuring cycle (resilient distension). The skin elasticity ratios are calculated from the absolute skin elasticity parameters whereby  $R_2 = U_a/U_f$ ,  $R_5 = U_r/U_e$ ,  $R_6 = U_v/U_e$  and  $R_7 = U_r/U_f$ .

#### 4.2.3 STUDY PROTOCOL

All studies were carried out in a quiet temperature controlled room (22 – 25°C) after a brief period (at least 20 minutes) of rest. Subjects were required to abstain from alcohol 24 hours before the study and cigarettes on the day of the study. Subjects were also asked not to use cosmetics, moisturisers or moisturising baths on the day of the study. Three brachial blood pressure recordings were measured 5 minutes apart to give the mean baseline blood pressure. PWV, PWA and DVP recordings were made sequentially at 5 minute intervals with a blood pressure recording before each measurement. This was followed by measurements of skin elasticity on the dorsal forearm, ventral forearm, upper arm, the top third of the upper leg and the upper back above the scapula. The study protocol is summarised in Figure 4-2.

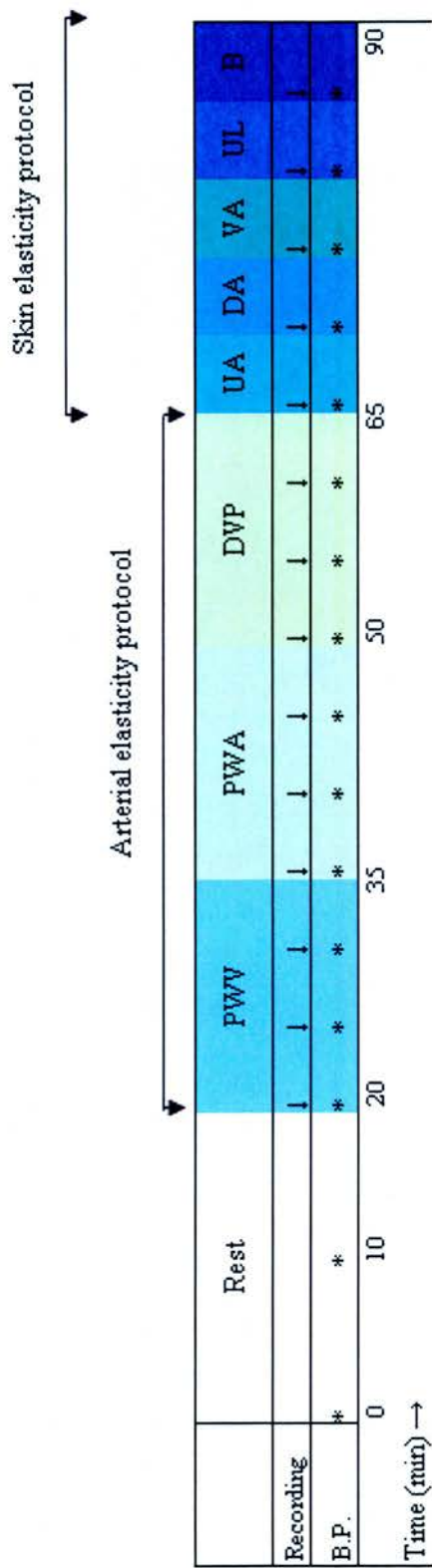


Figure 4-2 Skin and arterial elasticity study protocol.

A schematic of the study protocol carried out in which both skin elasticity and arterial stiffness measurements were made. A downward arrow, ↓, indicates a measurement being taken and an asterisk, \*, indicates measurement of blood pressure. Regions of the body on which skin elasticity measurements were made are denoted by UA for the upper arm; DA, dorsal arm; VA, ventral arm; UL, upper leg; and B for the back.

#### 4.2.4 BLOOD SAMPLING AND ASSAYS

Thirty mL of venous blood was obtained on the day of the study for routine biochemical analysis. Blood samples were collected into tubes containing serum gel and potassium EDTA (Monovette®, Sarstedt, Nümbrecht, Germany), and kept on ice for less than 10 minutes before being centrifuged at 2,000 *g* for 30 minutes at 4°C. The serum was decanted and stored at -80°C before being assayed by the clinical biochemistry laboratory. LDL-C concentration was calculated using the Friedewald equation [Friedewald *et al.* 1972].

#### 4.2.5 DATA ANALYSIS

Statistical analysis was performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± SD. In addition to this, each subject's arterial stiffness measure was calculated as the average of 3 individual measurements. Correlation between variables was evaluated using Pearson's correlation coefficients. Variables that were related in univariate analysis were further analysed in multivariate analysis using stepwise multiple regression analysis. All testing was 2 sided and significance was taken at the 5% level.

### 4.3 RESULTS

Baseline subject characteristics are shown in Table 4-1. Twenty people were recruited in the age groups 18 – 29, 30 – 39, 40 – 49 and 50 – 59, while 18 people were recruited in the 60 – 69 age group and 5 people in the 70 – 79 age group. Nine subjects were on hormone replacement therapy and more than two thirds of subjects were non-smokers.

Skin elasticity ratios correlated well with stiffness measures as shown in Table 4-2 and were also found to correlate with central mean blood pressure and LDL-C concentration. When these variables were entered into multiple regression analysis, the upper leg R6 and R2 ratios and the ventral arm R6 value were the only determinants of arterial stiffness measures. The upper leg R6 ratio was a consistent but weak determinant of PWV, AIx, PAIx and RI. The upper leg R2 ratio was found to determine PWV and the ventral arm R6 ratio was found to determine PAIx, but again both were weak contributors. The regression equations and the respective scatter plots are shown in Figure 4-3, Figure 4-4 and Figure 4-5.

Table 4-1 Baseline subject characteristics of the skin elasticity study.

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**Demographics**

Age, y	44.6 ± 15.8
Men/Women	58/45
Height, cm	171.4 ± 9.9
Weight, kg	70.9 ± 12.2
Body mass index, kg/m <sup>2</sup>	24.0 ± 3.1
Smokers (Non-smokers/current and ex-smokers )	72/31

**Biochemistry**

Serum creatinine, umol/L	76 ± 14
Total cholesterol, mmol/L	4.8 ± 1.1
HDL cholesterol, mmol/L	1.6 ± 0.4
LDL-C, mg/dL	106 ± 37
Triglycerides, mmol/L	1.2 ± 0.6
Chol:HDLC ratio	3.2 ± 1.1

**Haemodynamics**

Heart rate, beats per minute	59 ± 10
Ejection duration, ms	334 ± 23
Pulse wave velocity, m/s	6.9 ± 1.7
Augmentation index, %	18.4 ± 16.6
Augmentation index at HR75, %	10.7 ± 16.4
Peripheral augmentation index, %	-30.5 ± 22.5
Stiffness Index, m/s	8.0 ± 2.5
Reflection Index, %	76.1 ± 9.2

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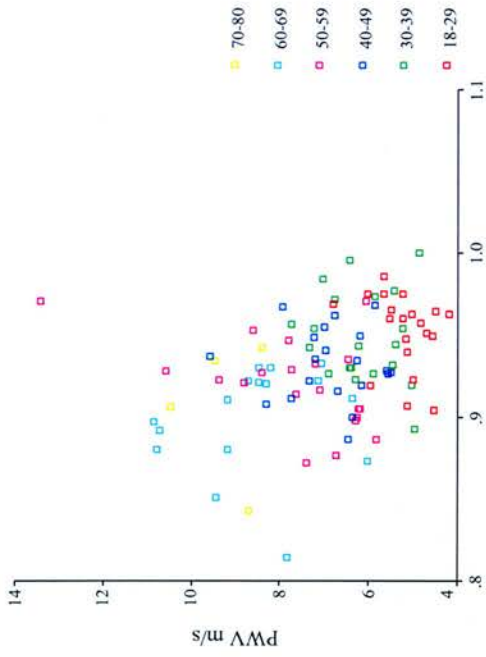
N=103

Table 4-2 Correlation between skin elasticity ratios and arterial stiffness measures.

Area of skin	Ratio	PWV	AIx	AIx75	PAIx	SI	RI
<b>Back</b>	R2	-0.25*	-0.27**	-0.29 **	-0.23*	-0.33**	-0.08
	R5	-0.56**	-0.54**	-0.60 **	-0.51**	-0.57**	-0.14
	R6	0.37**	0.42**	0.42 **	0.38**	0.45**	0.23*
	R7	-0.58**	-0.57**	-0.62 **	-0.54**	-0.60**	-0.17
<b>Dorsal arm</b>	R2	-0.46**	-0.46**	-0.48 **	-0.45**	-0.42**	-0.14
	R5	-0.45**	-0.51**	-0.52 **	-0.49**	-0.46**	-0.14
	R6	0.33**	0.31**	0.32 **	0.30**	0.26**	0.19
	R7	-0.49**	-0.54**	-0.55 **	-0.51**	-0.48**	-0.18
<b>Ventral arm</b>	R2	-0.41**	-0.43**	-0.42 **	-0.43**	-0.41**	-0.18
	R5	-0.51**	-0.49**	-0.52 **	-0.49**	-0.45**	-0.14
	R6	0.31**	0.43**	0.39 **	0.46**	0.41**	0.31**
	R7	-0.53**	-0.54**	-0.56 **	-0.55**	-0.50**	-0.20*
<b>Upper arm</b>	R2	-0.39**	-0.48**	-0.48 **	-0.46**	-0.41**	-0.23*
	R5	-0.47**	-0.59**	-0.58 **	-0.57**	-0.50**	-0.27**
	R6	0.30**	0.22*	0.24 *	0.23*	0.29**	0.14
	R7	-0.49**	-0.58**	-0.58 **	-0.56**	-0.52**	-0.26**
<b>Upper leg</b>	R2	-0.31**	-0.43**	-0.41 **	-0.42**	-0.39**	-0.23*
	R5	-0.52**	-0.52**	-0.55 **	-0.52**	-0.55**	-0.16
	R6	0.31**	0.52**	0.50 **	0.53**	0.39**	0.38**
	R7	-0.54**	-0.60**	-0.61 **	-0.59**	-0.59**	-0.24*

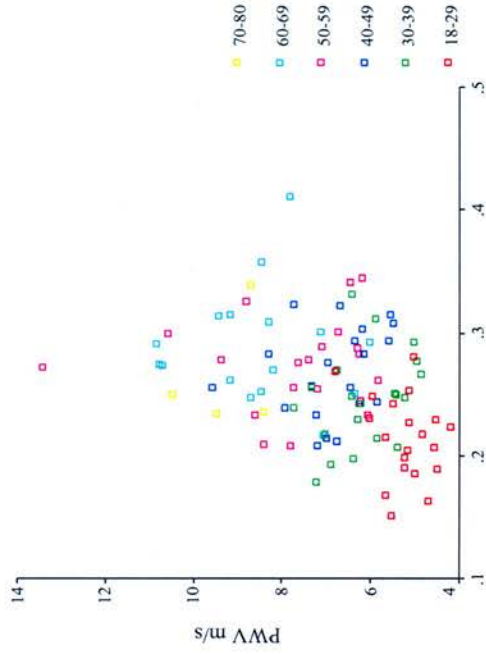
\* P < 0.05, \*\*P < 0.01





(a) Upper Leg R2 ratio

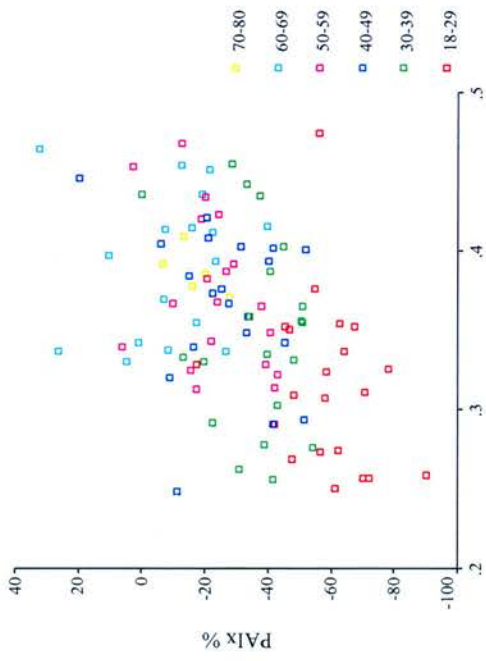
Determinants of upper leg R2		PWV m/s	
	Beta Coefficient		R sq. change
Age, y	0.62†		0.562†
CMBP (mmHg)	0.38†		0.100†
Skin elasticity ratio	-0.16*		0.017*
$(R^2 = 0.68†)$			



(b) Upper Leg R6 ratio

Determinants of upper leg R6		PWV m/s	
	Beta Coefficient		R sq. change
Age, y	0.60†		0.562†
CMBP (mmHg)	0.40†		0.100†
Skin elasticity ratio	0.16*		0.019*
$(R^2 = 0.68†)$			

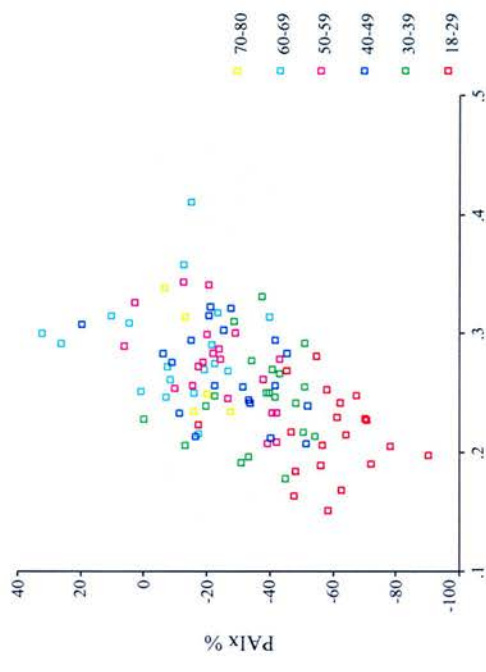
Figure 4-3 Relationship between pulse wave velocity and skin elasticity measures. Scatter plot of PWV versus (a) upper leg R2 measures, (b) upper leg R6 measures. Clinical predictors of PWV (m/s) are shown in the table above. \*  $P < 0.05$ , \*\* $P < 0.01$  † $P < 0.001$



(a) Ventral Arm R6 ratio

Determinants of ventral arm R6		PAIx %	
	Beta Coefficient		R sq. change
Age, y	0.54†		0.500†
LDL-C (mg/dL)	0.18*		0.025*
Skin elasticity ratio	0.18*		0.025*

(R<sup>2</sup> = 0.55†)

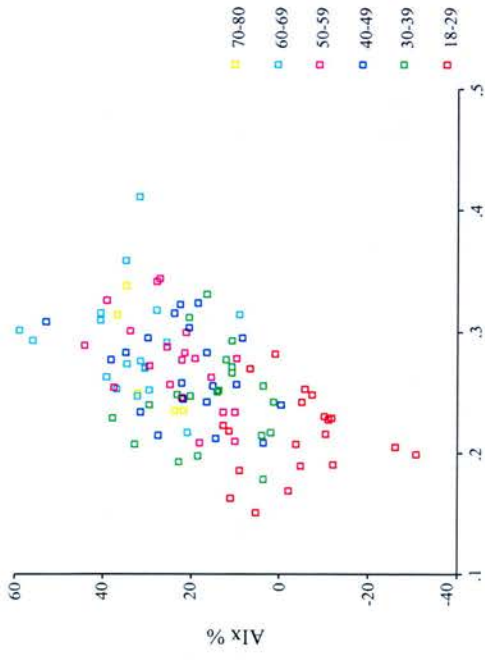


(b) Upper Leg R6 ratio

Determinants of upper leg R6		PAIx %	
	Beta Coefficient		R sq. change
Age, y	0.53†		0.503†
LDL-C (mg/dL)	0.17*		0.028*
Skin elasticity ratio	0.19*		0.023*

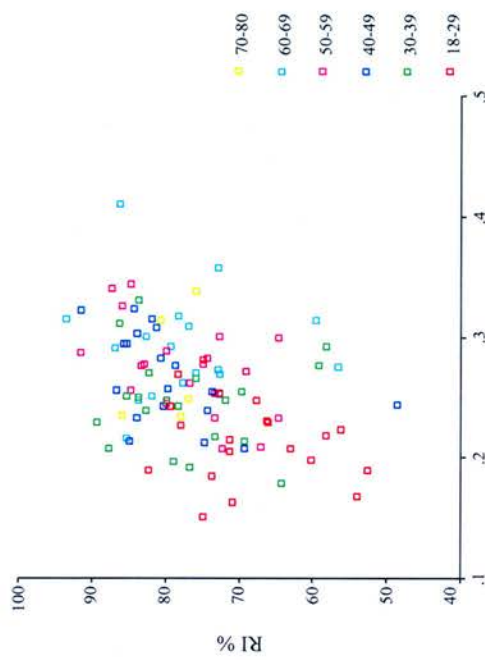
(R<sup>2</sup> = 0.55†)

Figure 4-4 Relationship between peripheral augmentation index and skin elasticity measures. Scatter plot of PAIx % versus (a) ventral arm R6 and (b) upper leg R6 measures. Clinical predictors of PAIx % are shown in the table above. \* P < 0.05, \*\*P < 0.01 †P < 0.001



(a) Upper Leg R6 ratio

Determinants of upper leg R6		AIx %	R sq. change
Age, y	Beta Coefficient	0.63†	0.529†
Skin elasticity ratio	Beta Coefficient	0.19*	0.025*
(R sq = 0.55†)			



(b) Upper Leg R6 ratio

Determinants of upper leg R6		Beta Coefficient	RI %	R sq. change
Skin elasticity ratio	Beta Coefficient	0.38†	0.143†	0.143†
(R sq.= 0.143†)				

Figure 4-5 Relationship between augmentation and reflection index and skin elasticity measures. Scatter plot of (a) AIx % and (b) RI% versus upper leg R6 measures. Clinical predictors of AIx % and RI % are shown in the table above. \* P < 0.05, \*\*P < 0.01 †P < 0.001

#### 4.4 DISCUSSION

Our results show that arterial stiffness is weakly associated with skin elasticity and this is independent of age. By far the biggest predictor of arterial stiffness was age, contributing about 60% of the variance. Measurements of skin from the upper leg represented measurements which most consistently correlated to arterial stiffness measures, independent of age, but it is less convenient as a clinical measure compared to the other regions. Of the 4 skin elasticity ratios, the R6 ratio was a consistent contributor but only by about 2%.

A number of previous studies have investigated relationships between various measures associated with the mechanical properties of both the skin and arteries. One such study by Uiterwaal et al. [Uiterwaal *et al.* 2003] found PP, a partial surrogate for arterial stiffness, to decrease with an increase in skin extensibility and DBP to decrease with an increase in joint mobility. Changes in connective tissue have also been found to occur in both the arteries and the skin of patients with cervical artery dissections [Brandt *et al.* 2001] and intracranial aneurysms [Grond-Ginsbach *et al.* 2002]. However, there have been studies which have not established an association between the pathological change in arteries and skin which are less elastic. One such study found aortic stiffness, in diabetics with CAD, to correlate with collagen linked fluorescence (a measure of advanced glycosylated end products) in the aorta and myocardium but not in the skin [Airaksinen *et al.* 1993]. They found the level of fluorescence, both in the myocardium

and aorta, to be twice as high as that measured in the skin. This difference may partially be explained by the differing ratios of Type I and Type III collagen in the skin and blood vessels. Our study is, however, the first to attempt to investigate the relationship between arterial and skin elasticity.

The dissimilar structural arrangement and response to low strains between the arteries and skin possibly explains its weak (2%) association. At low strains, the elastic component of the aorta in both the transverse and longitudinal direction is greater than the viscous component. However, in the skin the viscous component contributes equally to the elastic component [Dunn *et al.* 1983]. As the strain increases in the aorta there is a decrease in the stress from the elastin component with a shift to an increase in the contribution from the viscous component. The opposite is true for the skin, whereby the stress from the elastin component increases with an increase in strain. Moreover, in both the skin and aorta the curve of the elastin component is exponential and that of the viscous component is linear which may also explain the weak linear relationship in both skin and arterial elasticity. In the aorta, elastic fibres are folded in concentric rings and are connected axially by elastin [Silver *et al.* 2001b]. Collagen and elastin fibres are found in the medial layer. At low strains the elastin lamellae unfold allowing the aorta to deform easily, while at higher strains collagen is sufficiently stretched for it to take most of the stress. In contrast, collagen fibres in the skin are randomly arranged, and deform in the direction of the applied stress [Silver *et al.* 2003]. The large viscous energy in skin allows alignment of the collagen fibres along the stress axis without injury to the fibre-

interfibrillar matrix interfaces. The differences in force contributions from elastin and collagen in the aorta and the skin highlight different structural arrangements in these tissues. Moreover, vascular diseases for example hypertension, may affect the force contributions of either or both the elastic and viscous components at varying strains.

In addition to looking at the relationship between the skin elasticity ratios and arterial elasticity, this study was an attempt to find skin regions which gave the strongest relationship. The upper leg skin measurements were most closely correlated with arterial elasticity measures. The skin of the upper leg is less exposed to extrinsic factors, such as ultraviolet radiation, and therefore changes in its connective tissue may most closely parallel structural changes in the vascular extra-cellular matrix. However, measurements from the upper leg are not a convenient method of assessing skin elasticity in a clinical setting.

Of the 4 skin ratios studied, the R2 and R6 showed a correlation with PWV, but the R6 ratio is considered a better determinant of the two as it correlates with a larger number of stiffness parameters after age has been accounted for. Our findings support those of Cua et al. who have previously shown the R6 ratio, of the 4 ratios, to be the least dependent on age [Cua *et al.* 1990]. The R6 ratio, also called the viscoelastic-to-elastic ratio, compares the delayed to the immediate deformation. The R2, R6 and R7 ratios account for the viscous component of skin deformation unlike the R5 ratio and these ratios may therefore better illustrate elasticity of skin. The elastic component is thought to relate to

the stretching of the collagen and elastic fibres and the viscoelastic part, to the movement of interstitial fluid through the fibrous network [Dobrev 2002].

Associations between skin elasticity and arterial stiffness are more dominant in people who are genetically predisposed to either enzymatic changes in collagen and elastin structure or metabolic changes. The enzymatic changes can be caused by any of the following: growth factors, cytokines [Tayebjee *et al.* 2003], MMPs, TIMPs, hormones and mechanical stress [Kingwell *et al.* 2001; Lakatta 2003]. Metabolic changes can lead to changes in connective tissue as seen, for example, in diabetics [Airaksinen *et al.* 1993] who have high glucose concentrations causing non-enzymatic glycation of proteins. Non-enzymatic glycation of proteins results in the cross-linking of collagen fibres, which can alter the mechanical function of the artery and cause proteins that are normally flexible to become rigid. In these groups of people it is likely that structural changes in the arteries are most strongly related to skin elasticity.

Age plays an important role in the stiffening of arteries and loss of elasticity of the skin. Arterial stiffness has been shown to increase steeply between the ages of 50 to 70 years [Mitchell *et al.* 2004b]. However, there are insufficient data to draw conclusions on the rate of stiffening after the age of 70 years. PP, a surrogate measure of arterial stiffness, has also been shown to increase rapidly between the ages of 50 and 80 years after which it reaches a plateau [Franklin *et al.* 1997]. Central AIX has a curvilinear relationship with age, which flattens out after the age of 60 years [O'Rourke *et al.* 2005]. In contrast, skin

elasticity decreases with age but more rapidly after the age of 70 years [Balin *et al.* 1989]. This effect of age may obscure changes in skin and arterial elasticity due to other pathological causes. Therefore, in older patients skin elasticity may not be an appropriate marker of changes in arterial elasticity.

#### 4.4.1 STUDY LIMITATIONS

There are a number of limitations of our study. First, there were smaller numbers of women in this study in the older age group which may affect interpretation of the relationship between arterial and skin elasticity in this group. Second, there was a difficulty recruiting healthy subjects between the ages of 60 to 80 years because a high proportion of people between these ages were on long term medications. This age group is important to this study in order to evaluate the relationship between skin and arterial elasticity in older patients.



#### 4.5 CONCLUSIONS

Skin elasticity is not a useful predictive means of assessing arterial stiffness indirectly. Moreover, the upper leg dermal skin which was most strongly related to the extracellular matrix of the aorta is not a practical region of the body from which measurements can be made in a clinical setting.

**CHAPTER 5: DETERMINANTS OF ARTERIAL STIFFNESS IN PATIENTS WITH  
CORONARY ARTERY DISEASE**

## 5.1 INTRODUCTION

Arterial stiffness is the rigidity of the vascular wall, which causes the artery to offer more resistance to deformation and flow [Nichols *et al.* 1998]. Arterial stiffness is determined by the structure and function of the vascular wall. The structural elements responsible for the stiffness of the vessel are mainly elastin and collagen [Glasser *et al.* 1997]. Structural modification of the collagen and elastin matrix [Tayebjee *et al.* 2003] leads to changes that alter PWV, wave reflection and vessel compliance [Avolio *et al.* 1998]. Furthermore, vascular smooth muscle cells, neurohumoral factors and endothelium-derived factors can induce dynamic changes in arterial stiffness through functional alterations in vessel tone [Glasser *et al.* 1996].

In young healthy people, blood is ejected from the left ventricle into the aorta which generates a forward-travelling pressure wave or an incident wave [O'Rourke *et al.* 2002]. This incident wave travels towards the periphery and may be reflected at points of discontinuities of the arterial wall, branching points of arterioles or vessel bifurcations. The reflected wave travels back towards the aortic root and overlaps with the diastolic phase of the incident wave. Therefore, the blood pressure wave is a composite of both the incident and reflected pressure waves. However, as the arteries become stiff this leads to the pressure wave propagating at a higher velocity [Nichols *et al.* 1998]. This in turn causes the reflected wave, which would normally return during diastole, to occur earlier during systole. This early return of the pressure wave increases aortic and

ventricular pressures during systole and reduces aortic pressure during diastole [Safar *et al.* 2003]. High central systolic pressures increase the workload of the heart and lead to the development of left ventricular hypertrophy [Deague *et al.* 2001], and low diastolic pressures reduce coronary artery perfusion [Mackenzie *et al.* 2002; Nichols *et al.* 1998].

Arterial stiffening and an early return of wave reflections, with the disappearance of the aortic-to-peripheral pressure amplification, is uncommon in people under 60 years of age, but it is particularly common in patients over 40 years of age with end-stage renal disease (ESRD). Arterial stiffness has been associated with vascular diseases, such as CAD [Gatzka *et al.* 1998], and measures of arterial stiffness, such as PWV, predicts all-cause and cardiovascular mortality in patients with ESRD [Blacher *et al.* 2003; Safar *et al.* 2002] and hypertension [Boutouyrie *et al.* 2002a; Laurent *et al.* 2001; Laurent *et al.* 2003]. However, the relationship between renal function and arterial stiffness in patients with CAD, in the absence of a history of renal disease has not previously been studied. Moreover, the association between non-invasively determined central arterial stiffness, in patients with underlying CAD, with cardiovascular and all-cause mortality, hospitalisation or revascularisation procedures is unknown.

Creatinine clearance, a surrogate measure of renal function, is a predictor of death in patients with acute coronary syndrome [Santopinto *et al.* 2003] and a predictor of renal failure and hospital morbidity in patients undergoing CABG [Noyez *et al.* 2006]. Estimated glomerular filtration rate (eGFR), a more accurate measure of renal function,

is a predictor of mortality in patients experiencing an acute coronary syndrome [Masoudi *et al.* 2004; Schiele *et al.* 2006], and mortality [Hillis *et al.* 2006] or postoperative complications [Cooper *et al.* 2006] following a CABG. However, there is limited information [Beddhu *et al.* 2002] on the association between renal function and adverse fatal and non-fatal cardiovascular related outcomes in patients with CAD.

In a cohort of patients with CAD on treatment, we aimed to determine:

1. the relationship between arterial stiffness and measures of cardiovascular risk including renal function.
2. the relationship between the presence of CAD, renal function, and arterial stiffness, with major adverse cardiovascular events defined as cardiovascular and all-cause mortality, hospitalisation due to cardiovascular causes (including non-fatal myocardial infarct or stroke) and coronary revascularisation procedures (PTCA or CABG).

## 5.2 METHODS

### 5.2.1 STUDY SUBJECTS

Patients who had undergone elective cardiac catheterisation at the Western General Hospital and the Royal Infirmary, Edinburgh were recruited into this study. Patients were excluded if they had either marked left ventricular impairment (ejection fraction < 25%), significant valvular heart disease, critical aortic stenosis or a history of renal disease. All studies were carried out at the Wellcome Trust Clinical Research Facility at the Western General Hospital and the Royal Infirmary, Edinburgh. Subjects who participated in this study attended a visit a week after cardiac catheterisation or at least 3 months after a coronary revascularisation procedure, such as CABG or percutaneous transluminal coronary angiography (PTCA).

### 5.2.2 MEASUREMENTS

PWV and PWA were carried out using the SphygmoCor<sup>®</sup> system (SphygmoCor<sup>®</sup> Mx, AtCor Medical, Sydney, Australia, version 6.31). This system uses the technique of applanation tonometry, in which a high-fidelity micromanometer (SPC-301, Millar Instruments, Texas, USA) is used to determine carotid-femoral PWV. For PWA a probe containing piezoelectric pressure sensors (Colin<sup>®</sup> 7000, Colin Medical Technology Corporation, Japan) was used to derive the central aortic pressure waveforms from the radial pressure waveforms. These central waveforms were used to determine central

(Aix and Aix75) and peripheral (PAIx) augmentation indices. The SI and RI were calculated from the DVP recorded by Pulse Trace<sup>®</sup> (Micro Medical, Gillingham, Kent, United Kingdom), which uses a high fidelity finger photoplethysmograph. Non-invasive blood pressure measurements were carried out on the right arm using a validated oscillometric technique (HEM-705CP, Omron Corporation, Japan). Details of the methods used are described in Chapter 2.

### 5.2.3 STUDY PROTOCOL

Subjects were asked to omit their morning medications on the day of the study. Three brachial blood pressure recordings were made 5 minutes apart to give the mean baseline blood pressure. This was followed by PWV, DVP and PWA measurements made sequentially (Figure 5-1) and the mean of three measurements taken.

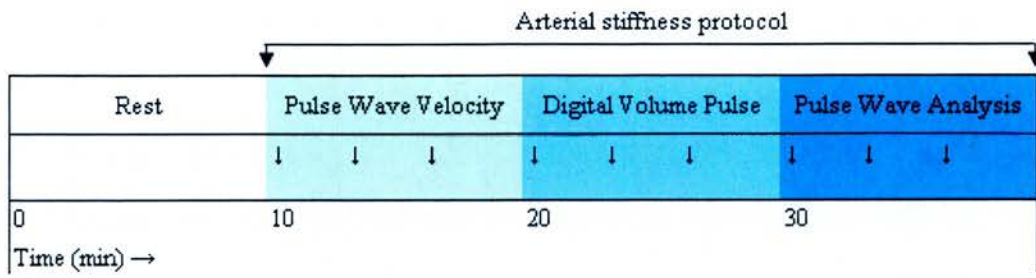


Figure 5-1 A schematic of the arterial stiffness study protocol. The downward arrow, ↓, denotes an arterial stiffness measurement.



#### 5.2.4 OUTCOMES

Subjects were followed up for fatal and non-fatal events as described in Section 2.6.

#### 5.2.5 BLOOD SAMPLING AND ASSAYS

Ten mL of venous blood was obtained on the day of the study and collected into tubes containing potassium EDTA (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany). Routine clinical biochemistry analyses were performed by the regional clinical laboratories. LDL-C concentration was calculated using the Friedewald equation [Friedewald *et al.* 1972].

#### 5.2.6 DATA ANALYSIS

Each arterial stiffness datum point is the mean of 3 measurements. Statistical analyses were performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). There were no violations in the assumptions of normality, linearity, and homogeneity of variances. Data are expressed as mean  $\pm$  SD. All parametric data were analysed using Student's *t*-test, ANOVA, and ANCOVA where appropriate. Subjects had their renal function assessed based on serum creatinine concentrations from biochemical blood analysis and estimated glomerular filtration rate calculated using the Cockcroft and Gault equation [Cockcroft *et al.* 1976] ( $eGFR_{CG}$ )

$$eGFR_{CG} = k[[140 - \text{age (yr)}] \times \text{weight (kg)}] / \text{SrCr } (\mu\text{mol/L})$$

where SrCr is serum creatinine concentration, and  $k$  is a value of 1.22 for males and 1.04 for females. The  $GFR_{CG}$  was corrected for body surface area (BSA) using the estimated BSA according to Du Bois and Du Bois [Du Bois *et al.* 1916] by the formula

$$BSA = \text{weight (kg)}^{0.425} \times \text{height (m)}^{0.725} \times 0.20247$$

$$\text{Corrected eGFR} = eGFR_{CG}/BSA \times 1.73$$

Subjects were grouped into eGFR groups based on the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines of staging eGFR in chronic kidney disease (CKD). Subjects were classed into eGFR stages 1 to 3 as none of the subjects had eGFR values less than 30ml/min. GFR calculated using the Cockcroft and Gault equation has been shown to be more accurate than the Modification of Diet in Renal Disease (MDRD) equation in patients not known to have chronic kidney disease [Poggio *et al.* 2005; Rule *et al.* 2004]. Serum creatinine concentrations (range 52 – 165  $\mu\text{mol/L}$ ) and eGFR (38.2 – 122.8 ml/min) were available in 282 subjects.

### 5.3 RESULTS

Of the 288 participants, 4 were excluded due to technical difficulties. The augmentation, stiffness and reflection indices were available in 284 subjects. The remaining 284 participants (210 men and 74 women with a mean age of  $62 \pm 8$  years) comprised 35 (12.6%) subjects with angiographically normal coronary arteries (Table 5-1 and Table 5-2). Satisfactory PWV recordings were only available in 245 subjects.

#### 5.3.1 DEMOGRAPHIC AND ANTHROPOMETRIC FACTORS

In univariate analysis, PWV and AIX increased with age (Table 5-3). Women had higher augmentation indices (AIX,  $39 \pm 9$  vs  $33 \pm 9$ ,  $P < 0.001$ ; AIX75,  $32 \pm 8$  vs  $24 \pm 8$ ,  $P < 0.001$ ; and, PAIX,  $-5 \pm 14$  vs  $-9 \pm 13$ ,  $P < 0.05$ ) although men had higher SI values ( $11 \pm 3$  vs  $9 \pm 2$ ,  $p=0.001$ ). No difference in PWV was found between men and women. BMI positively correlated with PWV but negatively correlated with AIX, AIX75, PAIX, SI and RI.

#### 5.3.2 HAEMODYNAMIC FACTORS

Heart rate positively correlated with PWV ( $r = 0.20$ ,  $P < 0.01$ ) and inversely correlated with AIX ( $r = -0.39$ ,  $P < 0.001$ ), PAIX ( $r = -0.38$ ,  $P < 0.01$ ) and RI ( $r = -0.38$ ,  $P < 0.01$ ). Subjects with high systolic and mean blood pressures had high PWV, AIX, AIX75 and PAIX measures. In addition, subjects with high PWV, AIX75 or SI measures had high diastolic pressures.

Table 5-1 Baseline subject characteristics of the arterial stiffness study.

	No CAD (N=35)	CAD (N=249)	Total (N=284)
<b>Demographics</b>			
Age, y (range 41-80)	59 ± 9	62 ± 8	62 ± 8
Men/Women	15/20	195/54	210/74
Height, cm	169 ± 9	170 ± 9	169 ± 9
Weight, kg	82 ± 14	82 ± 15	82 ± 14
Body mass index, kg/m <sup>2</sup>	28 ± 4	29 ± 5	29 ± 4
<b>Biochemistry</b>			
Serum creatinine, μmol/L (52 – 165)	85 ± 12	95 ± 18**	94 ± 18
Estimated glomerular filtration rate, ml/min (range 38.2 – 122.8)	76 ± 14	73 ± 17	74 ± 17
Total cholesterol, mmol/L	5 ± 1	5.0 ± 1.1*	5 ± 1.1
HDL cholesterol, mmol/L	1.5 ± 0.4	1.2 ± 0.3†	1.2 ± 0.3
LDL-C, mg/dL	111 ± 39	98 ± 37	100 ± 37
Triglycerides, mmol/L	1.5 ± 0.7	1.9 ± 1.0*	1.9 ± 1.0
Chol:HDLC ratio	3.7 ± 1.2	4.0 ± 1.1	4.0 ± 1.1
<b>Haemodynamics</b>			
Peripheral SBP	132 ± 18	133 ± 17	133 ± 17
Peripheral DBP	76 ± 9	75 ± 10	75 ± 10
Peripheral MBP	96 ± 12	96 ± 12	96 ± 12
Central SBP	125 ± 18	126 ± 17	126 ± 17
Central DBP	77 ± 9	76 ± 10	76 ± 10
Central MBP	76 ± 12	96 ± 12	96 ± 12
Heart rate, bpm (40-89)	60 ± 7	58 ± 9	58 ± 9
Pulse wave velocity, m/s	9.2 ± 3.1	9.6 ± 2.2	9.5 ± 2.4
Augmentation index, %	34 ± 9	34 ± 9	34.4 ± 9.2
Augmentation index at a HR75, %	27 ± 9	26 ± 8	26.3 ± 8.5
Peripheral augmentation, %	-10 ± 12	-7.4 ± 13.5	-7.7 ± 13.3
Stiffness Index, m/s	10 ± 3	10.5 ± 3.3	10.5 ± 3.3
Reflection Index, %	77 ± 9	79.5 ± 14.0	79.2 ± 13.5

Values are means ± SD. SBP denotes systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. \* P < 0.05, \*\*P < 0.01, † P < 0.001 versus no CAD.

Table 5-2 Clinical and therapeutic profile of the subjects.

	No CAD (N=35)	CAD (N=249)	Total (N=284)
<b>Canadian Cardiovascular Society Classification</b>			
Class 1	71.4 (25)	51.4 (128)	53.9 (153)
Class 2	20 (7)	35.3 (88)	33.5 (95)
Class 3	8.6 (3)	11.6 (29)	11.3 (32)
Class 4	0	1.6 (4)	1.4 (4)
<b>Vessel Disease</b>			
Normal/no plaque	100 (35)		12.3 (35)
1 vessel		28.5 (71)	25.0 (71)
2 vessels		37.8 (94)	33.1 (94)
3 vessels		33.7 (84)	29.6 (84)
<b>Past medical history</b>			
Current smokers	14.3 (5)	15.3 (38)	15.1 (43)
Ex-smokers	45.7 (16)	47.8 (119)	47.5 (135)
Non-smokers	40(14)	36.9 (92)	37.3 (106)
Hypercholesterolaemia	77.1 (27)	81.9 (204)	81.3 (231)
Diabetes			
Type I	0	1.2 (3)	1.1 (3)
Type II	2.9 (1)	10.8 (27)	9.8 (28)
Hypertension	48.6 (17)	55.8 (139)	54.9 (156)
Myocardial Infarct	17.1 (6)	36.5 (91)	34.2 (97)
Heart failure	0	4.8 (12)	4.2 (12)
Stroke	8.6 (3)	5.6 (14)	34.2 (97)
<b>Drug Therapy</b>			
Diuretics	25.7(9)	21.7 (54)	22.2 (63)
$\beta$ -blocker	40 (14)	70.3 (175)	66.5 (189)
Nitrates	40 (14)	73.9 (184)	69.7 (198)
ACE inhibitors	17.1 (6)	38.6 (96)	35.9 (102)
Calcium channel blocker	40 (14)	28.1 (70)	29.6 (84)
Angiotensin receptor blocker	5.7 (2)	4.4 (11)	4.6 (13)
Nicorandil	2.9 (1)	19.3 (48)	17.3 (49)
Statin	60 (21)	91.2 (227)	87.3 (248)
HRT	5.7 (2)	2.4 (6)	2.8 (8)

Hypercholesterolaemia was indicated by a previous diagnosis or the use of a cholesterol-lowering agent. Patients were classified as having diabetes, hypertension, ischaemic heart disease, myocardial infarct or heart failure indicated by a previous clinical diagnosis. Values shown are in % (n).

Table 5-3 Correlations between anthropometric and biochemical variables with measures of arterial stiffness.

	Age	BMI	Height	Serum creatinine concentration	eGFR	Total Chol.	HDLC	Total Chol. to HDLC ratio
Pulse wave velocity, m/s	0.47**	0.13*	0.11	0.22**	-0.31**	0.07	0.03	-0.09
Augmentation index, %	0.12*	-0.23**	-0.35**	-0.18**	-0.11	0.04	0.09	-0.04
Augmentation index at a HR75, %	0.06	-0.18**	-0.42**	-0.24**	-0.07	0.10	0.18**	-0.08
Peripheral augmentation, %	0.09	-0.24**	-0.25**	-0.1	-0.11	0.01	0.05	-0.04
Stiffness Index, m/s	0.09	-0.09	0.18**	0.01	-0.03	0.07	0.04	0.02
Reflection Index, %	-0.03	-0.13	0.08	0.04	-0.03	-0.01	-0.15*	0.10

Values are Pearson's correlation coefficients (r). HR75 indicates a correction to a heart rate of 75 beats per minute; Chol., cholesterol and HDLC, high density lipoprotein cholesterol. \* P < 0.05, \*\*P < 0.01

### 5.3.3 RENAL FUNCTION

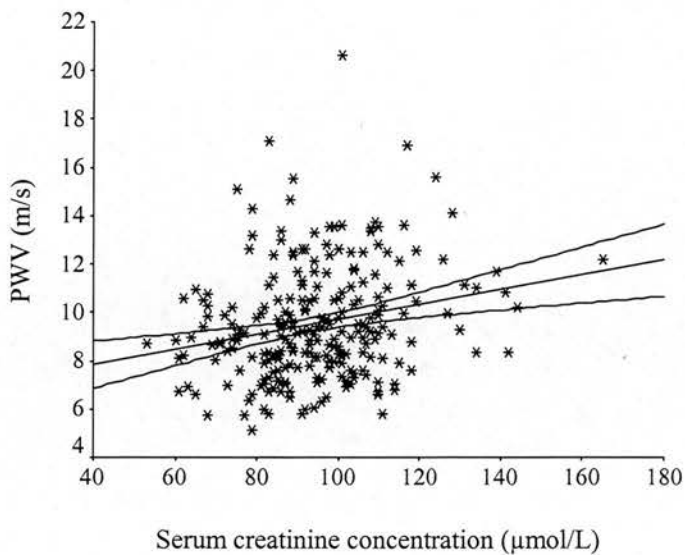
PWV correlated inversely with eGFR and positively with serum creatinine concentration (Figure 5-2 and Figure 5-3). The inverse relationship between PWV and eGFR ( $r = 0.04$ ,  $P < 0.01$ ) persisted in subjects with normal renal function (eGFR  $\geq 60$ ml/min). These correlates with PWV were not found with augmentation, stiffness or reflection indices.

### 5.3.4 DIABETES

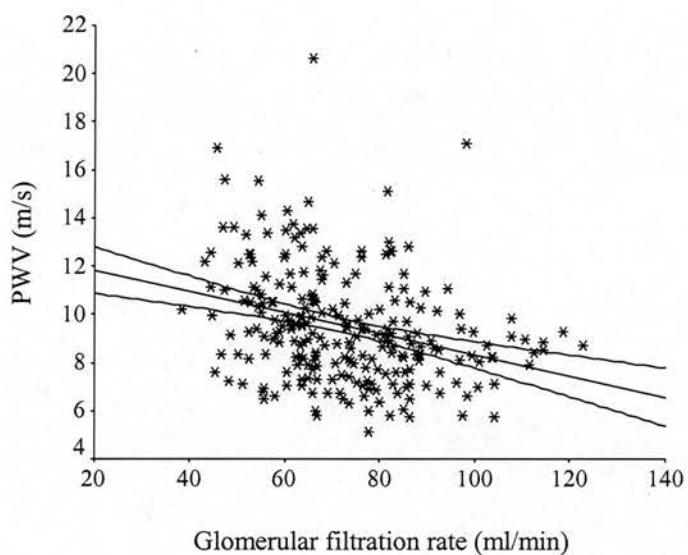
Subjects with diabetes had a higher mean PWV compared to subjects without diabetes ( $10.5 \pm 2.4$  vs  $9.4 \pm 2.3$ ), but had a lower mean AIX ( $30.9 \pm 11.1$  vs  $34.8 \pm 8.8$ ), and RI ( $72.9 \pm 17.1$  vs  $80.0 \pm 12.8$ ) after controlling for mean blood pressure ( $P < 0.05$ ; ANCOVA). The ejection duration was shorter for those subjects with diabetes ( $355 \pm 28$  vs  $340 \pm 28$ ;  $P < 0.01$ ; ANCOVA).

### 5.3.5 ANGINA SEVERITY AND EXTENT OF CORONARY ARTERY DISEASE

Subjects with or without CAD had very similar profiles (Table 5-1). Subjects with CAD had a lower mean HDL-C concentration (Table 5-1). There were no differences in serum creatinine concentrations or eGFR between groups (Figure 5-4). There were no differences in PWV and no consistent relationships between augmentation (Figure 5-5), stiffness and reflection indices with symptoms or severity of CAD.



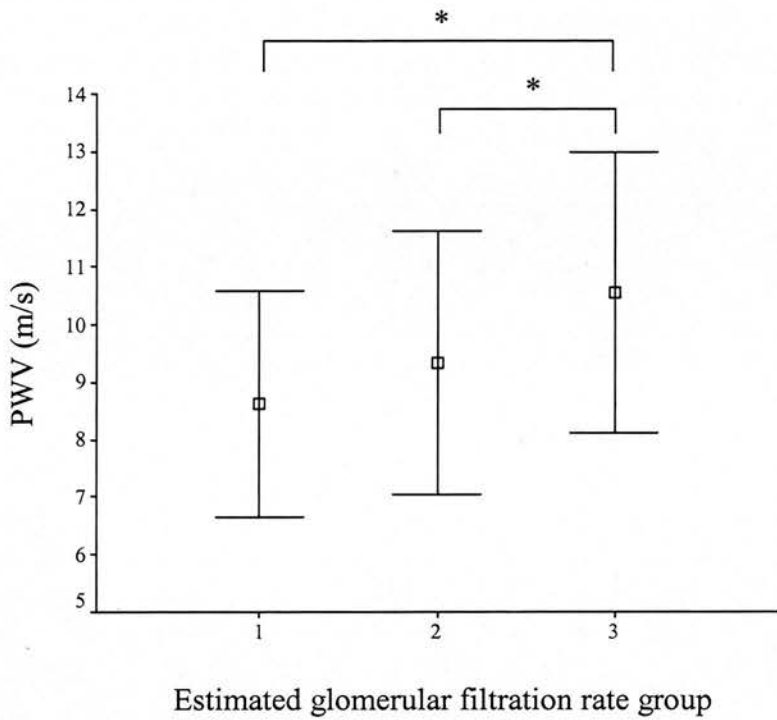
(a)



(b)

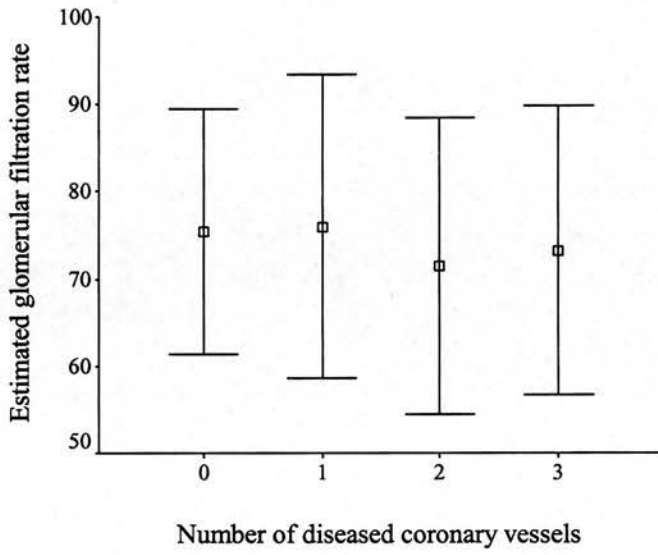
Figure 5-2 Arterial stiffness and renal function in all subjects. Scatterplot of PWV versus (a) serum creatinine concentration and (b) glomerular filtration rate for all subjects.



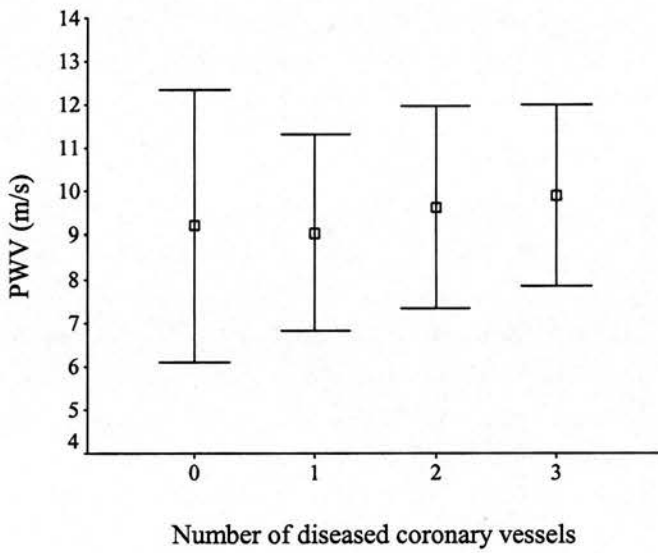


eGFR group 1 - 14.4 % (N=41)  
 eGFR group 2 - 63.4 % (N=180)  
 eGFR group 3 - 19 % (N=54)

Figure 5-3 Relationship between arterial stiffness and renal function groups. PWV for subjects grouped according to tertiles of glomerular filtration rate. The glomerular filtration rate for group 1,  $\geq 90$  ml/min; group 2, 60 – 89 ml/min; and group 3, 30 – 59 ml/min. Values are mean values  $\pm$  SD. \*  $P < 0.05$



(a)



(b)

Figure 5-4 (a) Estimated glomerular filtration rate and (b) pulse wave velocity measures for subjects divided according to severity of CAD.

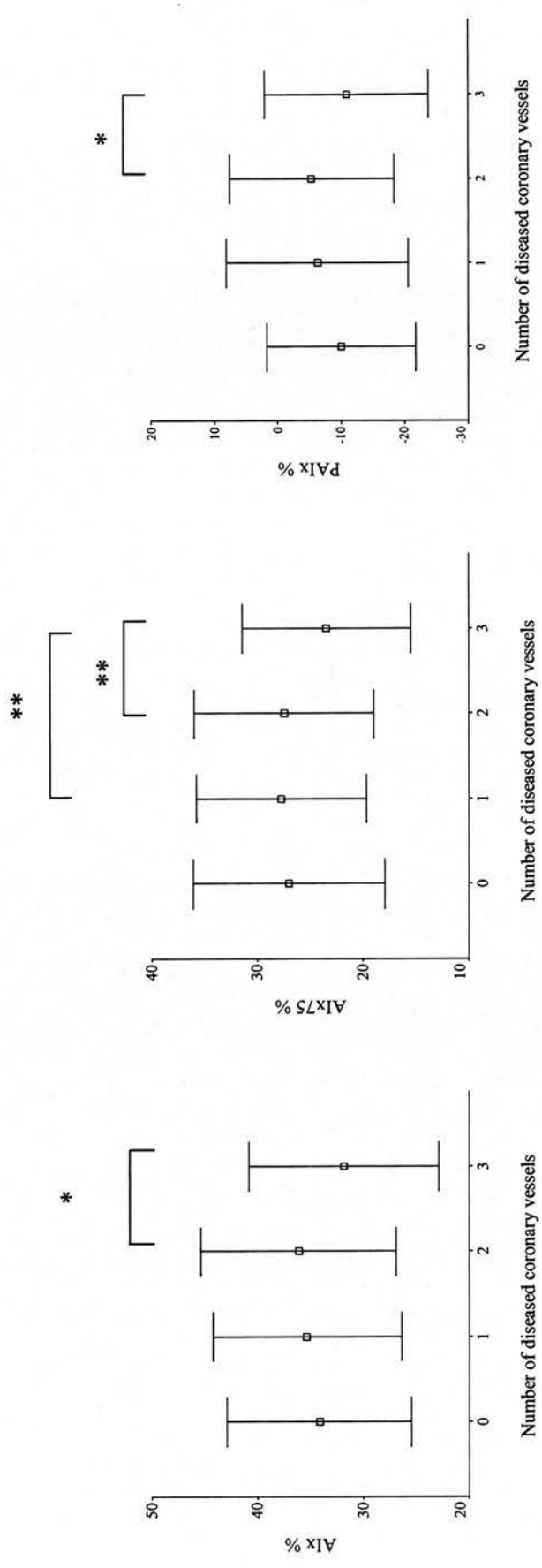


Figure 5-5 Arterial stiffness measures for subjects divided according to severity of CAD. Plots of mean of Aix %, Aix75 % and PAIx % ( $\pm$  SD) against number of diseased coronary vessels. \*  $P < 0.05$ , \*\* $P < 0.01$

### 5.3.6 DRUG EFFECTS ON ARTERIAL STIFFNESS MEASURES

Nitrates,  $\beta$ -blockers, calcium channel blockers, ACE inhibitors, angiotensin receptor blockers, hormone replacement therapy (HRT) and statins did not affect arterial stiffness measures. After adjusting for central mean blood pressure, total cholesterol, and serum creatinine, patients on nicorandil had higher PWV ( $9.32 \pm 0.2$  vs  $10.45 \pm 0.3$ ;  $P < 0.01$ ; ANCOVA).

### 5.3.7 MULTIPLE REGRESSION ANALYSIS

Variables that were found to be correlated in univariate analysis were further analysed in multivariate analysis to determine the interaction of other variables. Aortic PWV positively correlated with age, heart rate, central SBP, BMI and serum creatinine concentration. These variables explained 38% of the variance in PWV (Stepwise multiple linear regression analysis). PWV was negatively correlated with central and peripheral augmentation indices in both univariate and multivariate analysis. The determinants of AIX, AIX75, and PAIX are shown in Table 5-4. None of the independent variables were related with either SI or RI in multivariate analysis.

Table 5-4 Multivariate analysis of arterial stiffness measures.

Arterial stiffness measures	Regression Coefficients	Standard error
<b>Pulse Wave Velocity</b> ( $R^2=0.380$ , $P < 0.001$ )		
Age, y	0.12†	0.02
Heart rate, bpm	0.07†	0.01
Central systolic blood pressure, mmHg	0.03†	0.01
BMI, kg/m <sup>2</sup>	0.10**	0.03
Serum creatinine concentration, μmol/L	0.02*	0.01
<b>Augmentation Index</b> ( $R^2=0.321$ , $P < 0.01$ )		
Heart rate, bpm	-0.20**	0.06
Central systolic blood pressure, mmHg	0.29†	0.05
BMI, kg/m <sup>2</sup>	-0.37**	0.12
Serum creatinine concentration, μmol/L	-0.08*	0.03
Pulse wave velocity, m/s	-0.75**	0.24
Central diastolic blood pressure, mmHg	-0.23**	0.08
<b>Augmentation Index at a HR75</b> ( $R^2=0.20$ , $P < 0.001$ )		
Central systolic blood pressure, mmHg	0.18†	0.03
Pulse wave velocity, m/s	-0.46**	0.23
BMI, kg/m <sup>2</sup>	-0.41**	0.13
Serum creatinine concentration, μmol/L	-0.10*	0.03
<b>Peripheral Augmentation Index</b> ( $R^2=0.242$ , $P < 0.001$ )		
Ejection duration, ms	0.16†	0.03
BMI, kg/m <sup>2</sup>	-0.66**	0.19
Peripheral systolic blood pressure, mmHg	0.20†	0.05
Pulse wave velocity, m/s	-1.10**	0.35

\*  $P < 0.05$ , \*\* $P < 0.01$ , † $P < 0.001$

### 5.3.8 OUTCOMES

Follow-up was carried out in 284 subjects and ended on July 2, 2005 (mean follow-up of 1.5 years). There were 6 deaths, 3 of which were due to a cardiovascular cause and the remaining 3 were due to cancer. One subject had a stroke, 3 subjects had an AMI and 99 subjects had CHD-related admissions. Age, sex, smoking status, blood pressure, serum lipid or cholesterol concentrations were not associated with fatal and non-fatal events. Subjects who had an event were older, had a lower eGFR and a higher PWV compared to subjects who did not have an event.

#### 5.3.8.1 Coronary artery disease severity as a predictor of outcomes

Subjects with ischaemic heart disease (IHD) had a shorter time to a composite end point compared to subjects without IHD (Figure 5-6). Moreover, subjects with a higher number of diseased coronary vessels had a shorter time to an event. The severity of coronary vessel disease contributed to a shorter time to events (Table 5-6).

#### 5.3.8.2 Renal function as a predictor of outcomes

Subjects who suffered an event had a lower eGFR ( $69 \pm 16$  vs  $76 \pm 16$  mL/min;  $P < 0.001$ ) and a higher serum creatinine concentration ( $98.1 \pm 19.2$  vs  $92.2 \pm 16.3$   $\mu\text{mol/L}$ ;  $P < 0.01$ ). Subjects who underwent coronary revascularisation had a higher serum creatinine concentration ( $101.6 \pm 19.2$  vs  $93.7 \pm 17.4$   $\mu\text{mol/L}$ ;  $P < 0.05$ ; *t*-test) but there were no differences in eGFR. When subjects were divided into stages of eGFR, subjects with a lower eGFR had a shorter time to a composite end point ( $P < 0.01$ ) (Figure 5-7). EGFR, in addition to coronary vessel disease severity, determined

a shorter time to events (Table 5-6). Subjects in eGFR group 3 were older and had higher peripheral and central SBP compared to eGFR group 2 and 3 (Table 5-7). However, there was roughly an even distribution of subjects with varying severity of CAD across the 3 groups (Table 5-8). A higher percentage of subjects in eGFR group 3 were on ACE inhibitors. However, the percentage of subjects on other classes of drugs were also roughly evenly distributed across the 3 groups. In subjects with normal renal function (eGFR  $\leq 60$  ml/min) both the severity of coronary vessel disease and eGFR ( $R^2 = 0.064$ ,  $P < 0.01$ ) remained determinants of a shorter time to fatal and non-fatal outcomes.

#### 5.3.8.3 Arterial stiffness as a predictor of outcomes

Subjects who suffered an event had a higher PWV ( $9.9 \pm 2.3$  vs  $9.3 \pm 2.4$  m/s;  $P < 0.05$ ) compared to those without an event. Subjects with PWVs above the median had a shorter time to an event compared to those with PWVs below the median ( $P < 0.05$ ) (Figure 5-8)

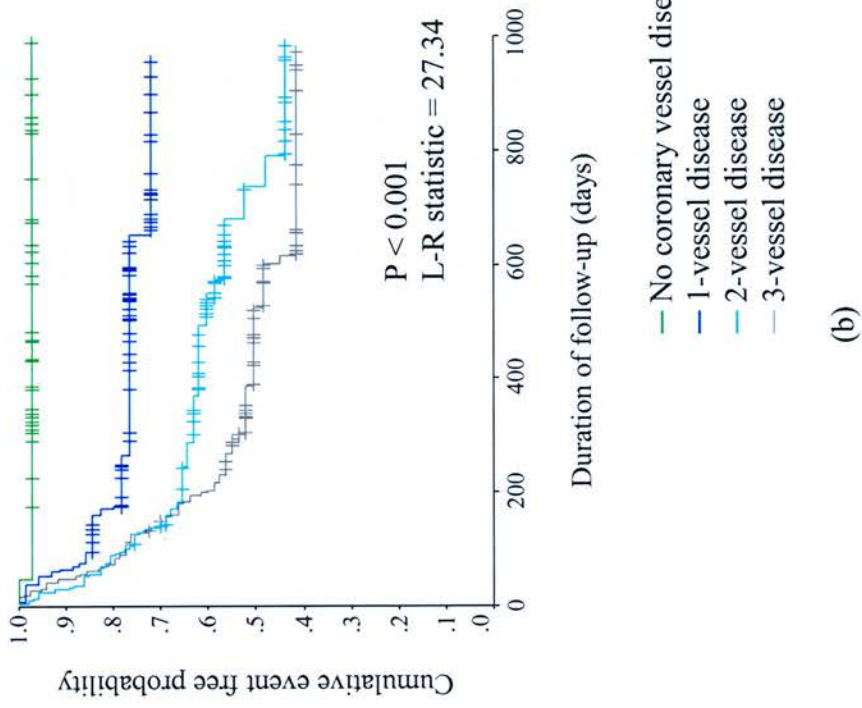
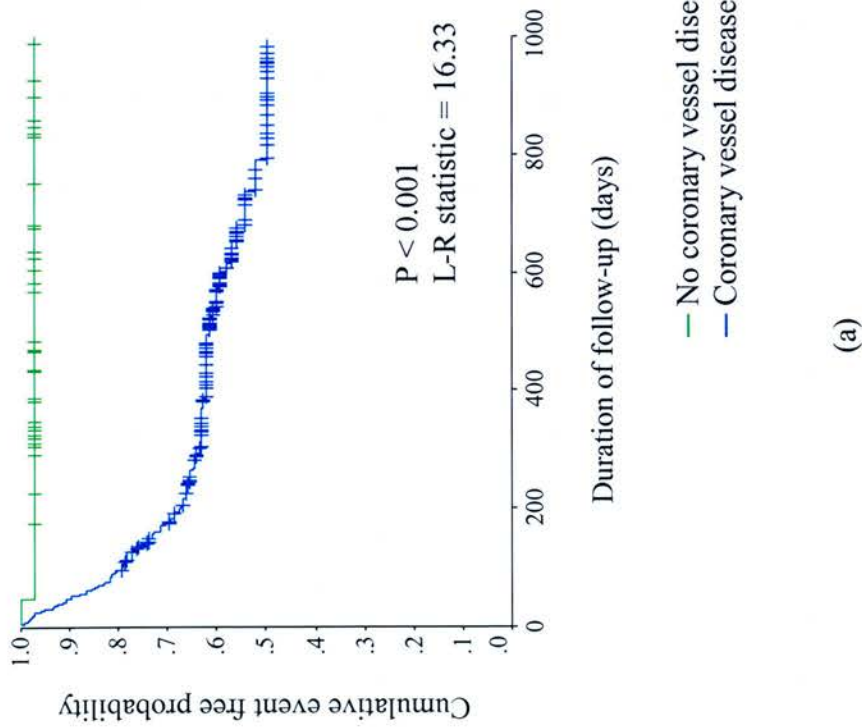


Figure 5-6 Kaplan Meier plots of coronary artery disease severity versus time to a composite end-point. Kaplan meier plots of time to a composite end point for (a) subjects with and without coronary artery disease, and (b) for subjects grouped according to numbers of diseased coronary vessels.



Table 5-5 Variables for subjects with and without fatal and non-fatal outcome.

Variables	No events (n=181)	Events (103)
Age	61 ± 9**	64 ± 8
BMI	29 ± 5	28 ± 4
eGFR	76 ± 16†	69 ± 16
Central SBP	126 ± 17	125 ± 17
Central DBP	76 ± 10	75 ± 11
Central MBP	96 ± 12	95 ± 13
PWV	9.3 ± 2.4*	9.9 ± 2.3

P < 0.05, \*\*P < 0.01

Table 5-6 Determinants of time to a composite event.

Independent variables	Unstandardised coefficient (B)	Standard Error
Numbers of diseased coronary vessels	-54.7**	17.8
Estimated glomerular filtration rate	3.0**	1.1

(R<sup>2</sup> = 0.074, P < 0.001) \*\*P < 0.01

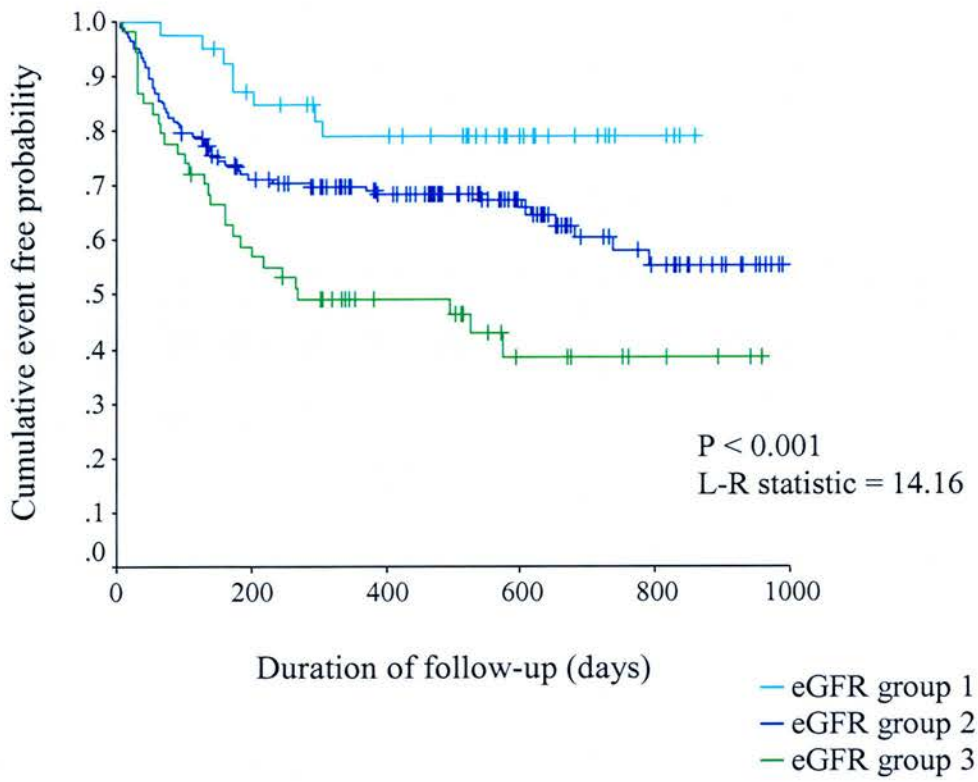


Figure 5-7 Kaplan Meier plots of time to composite end point for all subjects grouped by estimated glomerular filtration rate.

Table 5-7 Demographic variables and blood pressures between estimated glomerular filtration groups.

<b>Variables</b>	<b>eGFR group 1 (n=41)</b>	<b>eGFR group 2 (n=180)</b>	<b>eGFR group 3 (n=54)</b>
Age	52 ± 8 <sup>#+</sup>	62 ± 8 <sup>#</sup>	69 ± 5 <sup>+</sup>
Peripheral SBP	123 ± 13 <sup>#+</sup>	134 ± 17	136 ± 19
Peripheral DBP	72 ± 8 <sup>+</sup>	76 ± 10	73 ± 10
Peripheral MBP	91 ± 9 <sup>+</sup>	97 ± 12	95 ± 13
Central SBP	116 ± 13 <sup>#+</sup>	127 ± 17	129 ± 19
Central DBP	73 ± 8	77 ± 10	74 ± 11
Central MBP	91 ± 9 <sup>+</sup>	97 ± 12	95 ± 13

<sup>+</sup>, a significant difference to eGFR group 2; <sup>#</sup> denotes a significant difference to eGFR group 3

Table 5-8 Severity of vessel disease and distribution of drugs according to estimated glomerular filtration groups.

	eGFR group 1 % (n)	eGFR group 2 % (n)	eGFR group 3 % (n)
Numbers of diseased coronary vessels			
0 vessel	12.2 (5)	13.7 (25)	5.6 (3)
1-vessel	29.3 (12)	24.7 (45)	24.1 (13)
2-vessel	29.3 (12)	31.3 (57)	44.4 (24)
3-vessel	29.3 (12)	30.2 (55)	25.9 (14)
Drug therapy			
$\beta$ -blocker	56.1 (23)	70.9 (129)	57.4 (31)
Nitrate	78 (32)	70.3 (128)	63 (34)
HRT	9.8 (4)	1.6 (3)	1.9 (1)
Statin	87.8 (36)	87.4 (159)	87 (47)
ACE-Inhibitor	29.3 (12)	35.2 (64)	44.4 (24)
Angiotensin receptor blocker	2.4 (1)	5.5 (10)	3.7 (2)
Calcium channel blocker	39 (16)	25.3 (46)	35.2 (19)
Nicorandil	17.1 (7)	15.9 (29)	18.5 (10)
Diuretics	17.1 (7)	22.5 (41)	24.1 (13)

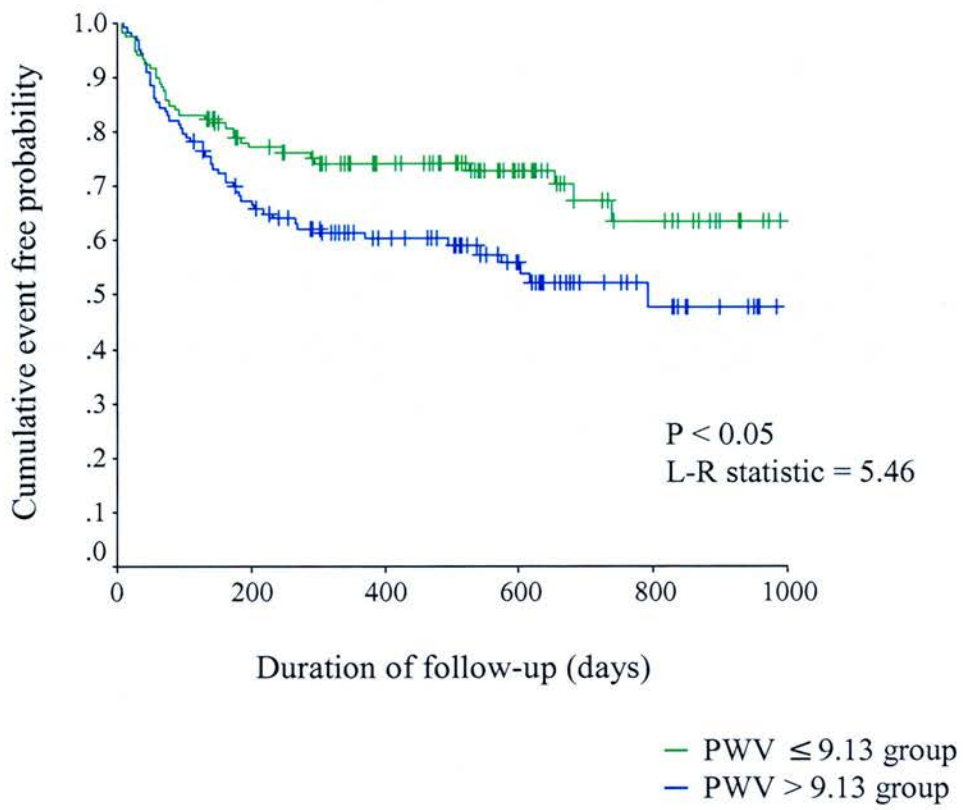


Figure 5-8 Kaplan Meier plots of time to composite end point for subjects grouped according to pulse wave velocities above and below the median pulse wave velocity.

## 5.4 DISCUSSION

Two hundred and eighty four subjects with varying degrees of CAD were studied to assess the determinants of central arterial stiffness in this group of patients. PWV, a “gold standard” of measuring arterial stiffness, was related to age, heart rate, central SBP and serum creatinine concentration. Whilst standard cardiovascular risk factors did not appear to be a major determinant in this treated group of patients with CAD, serum creatinine concentration independently contributed to arterial stiffness in these patients who did not have a history of renal disease. The presence and extent of CAD was a predictor of adverse cardiovascular events, which is consistent with previous findings. In addition, PWV and eGFR have been identified as predictors of adverse cardiovascular outcome. Moreover, even for those subjects with normal renal function eGFR remained an important determinant of cardiovascular outcome.

### 5.4.1 AGE

In this study, PWV was strongly determined by age. This supports findings from previous studies [Avolio AP *et al.* 1983; Mitchell *et al.* 2004a] and a recent large study (N = 998) in healthy subjects [McEniery *et al.* 2005], where a larger increase in PWV was found in older subjects whilst AIx was found to lose its discriminative power in older subjects and a larger increase in AIx was found in younger subjects (Figure5-9). PWV in older subjects is largely determined by a decrease in aortic compliance or an increase in forward pressure wave amplitude rather than a change in the reflected wave [Mitchell *et al.* 2004b]. Although in a small group of healthy men SI appeared to be associated [Millasseau *et al.* 2003] there are no previous data on the association between SI and age in subjects with CAD.

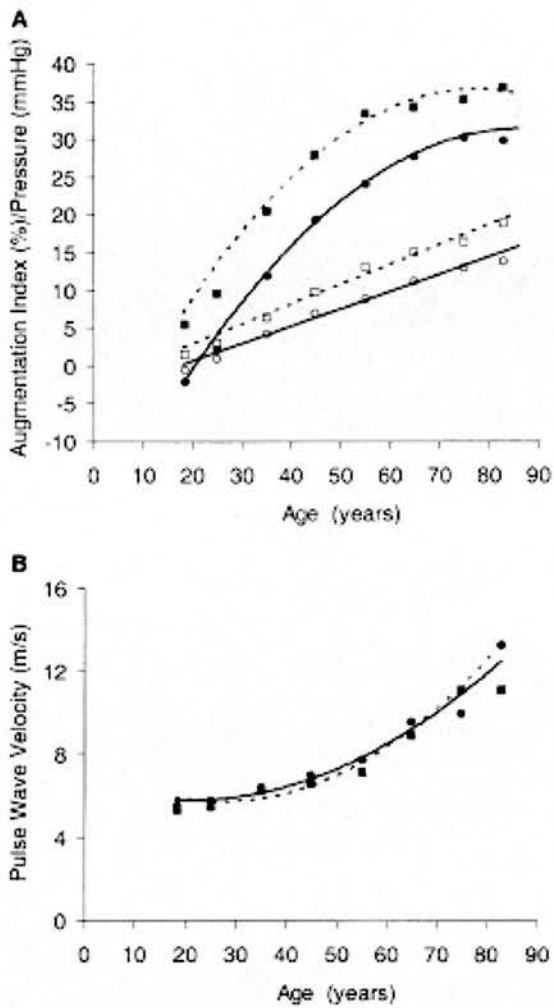


Figure 5-9 Relationship of the AIX and PWV with age.

Regression curves representing the effect of age on parameters of arterial stiffness and wave reflection for males (circles, solid lines) and females (squares, dashed lines). Panel A represents augmentation pressure (open circles/open squares) and augmentation index (closed circles/closed squares). Panel B represents aortic pulse wave velocity. Data points are the group means for each decile of age. Taken from McEniery et al. Normal Vascular Ageing: Differential Vascular Effects on Wave Reflection and Aortic Pulse Wave Velocity. *Journal of American College of Cardiology*. 2005.

#### 5.4.2 SEX

Whilst there were no differences in PWV, augmentation and stiffness indices were related to sex. Consistent with our findings McEniery *et al.* found no difference in PWV between men and women, whereas AIx was consistently higher in women in all age groups [McEniery *et al.* 2005]. It is well established that the differences in central augmentation pressure [Gatzka *et al.* 2001b; Smulyan *et al.* 2001] between males and females are greater than the difference in PWV [Mitchell *et al.* 2004b] between sexes. The disparity between PWV and the augmentation and stiffness indices may be explained by a number of factors. First, the augmentation indices are themselves determined by PWV. An increase in PWV increases the augmentation indices because a greater proportion of the reflected waves arrive earlier during systole. Second, females on average are shorter than males [Hayward *et al.* 1997]. Therefore the distance between the heart and sites of wave reflection, in females, is shorter and leads to the reflected wave arriving earlier during the systolic phase. In a previous study [McEniery *et al.* 2005], when AIx was corrected for height, females were still found to have a higher AIx compared to men highlighting that other factors also contribute [Gatzka *et al.* 2001b]. Third, left ventricular contractility, ejection duration and the size and elasticity of small muscular arteries [Gatzka *et al.* 2001b; Waddell *et al.* 2001] determine the intensity of wave reflections [Kelly *et al.* 2001]. Women have a smaller aortic diameter which results in a longer ejection time and prolonged time to the systolic peak therefore resulting in an earlier overlapping of the forward and reflected waves [Gatzka *et al.* 2001b].



#### 5.4.3 BLOOD PRESSURE AND HEART RATE

Despite previous findings showing aortic stiffness not to be associated with heart rate [Wilkinson *et al.* 2002c], in this study subjects with high PWV measures had high heart rates. There are a possible number of explanations for this discrepancy. First, the high heart rates and the high BMI in subjects with high PWV measures may be due to low physical fitness rather than being a cause of increased PWV. Second, sympathetic activation may have resulted in the high heart rate and arterial stiffness measures. In addition, cyclical stress, due to high heart rates, may result in fragmentation of elastic fibres and replacement by collagen fibres [O'Rourke 1995]. This in turn may cause the arterial lumen to be dilated with a loss of elastic recoil resulting in increased stiffness of the arterial wall [Nichols *et al.* 1998]. However, this study, unlike that of Wilkinson *et al.* [2002], uses epidemiological rather an interventional approach and therefore cannot explain the underlying mechanistic effect of heart rate on PWV. Other studies [Albaladejo *et al.* 2003; Lantelme *et al.* 2002] have shown an association between high PWV and high heart rates. However, the relative benefits of calculating transit time between the different methodologies as a cause of this relationship has been debated [Millasseau *et al.* 2005].

A lower AIx with higher heart rates, in this study, is consistent with previous studies [Albaladejo *et al.* 2001; Wilkinson *et al.* 2000a] which report that an increase in heart rate causes a decrease in the systolic duration with the reflected wave arriving during the diastolic phase resulting in a decrease in AIx.

#### 5.4.4 RENAL FUNCTION

Patients with ESRF who have stiffer arteries are predisposed to cardiovascular related deaths [Blacher *et al.* 2003]. PWV is higher in subjects with low eGFRs and it increases with the severity of CKD [Wang *et al.* 2005]. A high serum creatinine concentration and lower creatinine clearance has been described in subjects with cardiovascular disease [Bortolotto *et al.* 1999b]. More recently, high PWV measures have been found in untreated hypertensive subjects with low creatinine clearance [Mourad *et al.* 2001] and treated hypertensive subjects with high serum creatinine concentrations [Benetos *et al.* 2002; Blacher *et al.* 1999a]. Taken together, these studies suggest there may be a relationship between renal function and arterial stiffness in patients with underlying CAD, which I have investigated in this study.

The main finding of this analysis is that serum creatinine concentration and eGFR, in patients with CAD, is associated with stiffening of the large central elastic arteries. A number of explanations may be given for the causality of this relationship, however, the association above cannot be assumed to establish a causal relationship. The high stiffness measures may be a cause or effect of reduced renal function. In the former, an increase in arterial stiffness leads to an increase in SBP resulting in high PP. The high pressure waves generated can cause injury of the renal arteries or lower glomerular numbers resulting in renal disease [Loutzenhiser *et al.* 2002; Mitchell 2004; Pedrinelli *et al.* 2000]. In the latter case, impaired renal function may lead to stiffening of the large arteries through accumulation of advanced glycosylation end-products due to its reduced elimination by the kidneys [Makita *et al.* 1991; Makita *et al.* 1994], increased collagen cross-linking [Sell *et al.* 1990], sodium and fluid imbalance [Yu *et al.* 1998] or increased activation of the renin–angiotensin–

aldosterone system (RAAS) [Blacher *et al.* 1997]. More recently, renal function, assessed by eGFR, has been found to positively predict cardiovascular death or hospitalisation in patients with CHF [Hillege *et al.* 2006] and in CHF patients who have CAD [Smilde *et al.* 2004]. These studies highlight the prognostic significance of eGFR in not only renal patients but in patients with cardiovascular disease.

The majority of subjects in this study had normal serum creatinine concentrations which may be a result of reduced clearance of serum creatinine. Therefore the corrected eGFR represented a better measure of renal function which accounted for age, gender and calculated body surface area for individual subjects.

#### 5.4.5 CARDIOVASCULAR AND METABOLIC RISK FACTORS

No relationships were found with metabolic and established cardiovascular risk factors. The lack of relationship between CAD and cholesterol and lipid concentrations is very likely a reflection of the high use of statins, in approximately 90% of subjects. In this and other studies, patients with diabetes had a higher PWV compared to patients without diabetes [Schram *et al.* 2004]. However, in this study, subjects with diabetes had a surprisingly lower AIx and RI. When AIx was corrected for heart rate no difference was found between subjects with diabetes and those without diabetes. A study by Lacy *et al.* found subjects with diabetes to have an increase in PWV but no difference in AIx compared to subjects without diabetes [Lacy *et al.* 2004] and this was also shown by Aoun *et al.* in a large study [Aoun *et al.* 2001]. These differences in PWV and augmentation indices may be explained by shorter ejection duration, as was found in this study, or reduced wave reflection in diabetics. Arterial pressure waveforms in diabetics may also be altered by structural

changes such as changes in connective tissue composition or increased collagen cross-linking due to advanced glycosylation end product (AGE) binding [Airaksinen *et al.* 1993]. In contrast, other studies [Brooks *et al.* 2001; Wilkinson *et al.* 2000b] have shown AIx to be higher in diabetics. However, these studies have been carried out in either small sample numbers or in younger subjects. Arterial stiffness not only correlates positively with coronary risk factors [McVeigh 2003; Nichols *et al.* 2002; O'Rourke *et al.* 2002] but also with the presence of CAD [Hayashi *et al.* 2002; Weber *et al.* 2004]. In the present study, PWV was unrelated to the presence of or extent of CAD while the augmentation indices were surprisingly negatively related with the numbers of vessel disease. Atherosclerosis is a systemic disease but it may affect different vascular beds to varying degrees. From these results, we can assume that disease of the coronary arteries is not a measure of widespread atherosclerotic plaque in the body or aortic atherosclerotic disease. The difference in association between CAD with PWV and the augmentation measures may be explained by factors such as heart rate, ejection duration, and wave reflection having a more profound effect on augmentation measures.

#### 5.4.6 OUTCOMES

##### 5.4.6.1 Presence and severity of CAD as a predictor of outcomes

The positive association between the presence and severity of CAD and adverse cardiovascular outcomes provides reassurance that the cohort of subjects studied in the present study was representative of patients with CAD. Moreover, these results support previous findings [Harris *et al.* 1980; Proudfit *et al.* 1983; Waters *et al.* 1993] that the severity of coronary vessel disease as determined by coronary

catheterisation is a prognostic indicator of adverse cardiovascular outcomes in patients with CAD.

#### 5.4.6.2 Renal function as a predictor of outcomes

An interesting finding was that a lower eGFR in these subjects, who do not have a history of renal disease, predicted a shorter time to fatal and non-fatal events and revascularisation procedures. More importantly, eGFR in subjects with normal renal function was a determinant of a shorter time to adverse cardiovascular events. These results indicate the importance of surrogate measures of renal function as prognostic indicators in patients with CAD. The roughly even distribution of subjects according to severity of CAD and distribution of drugs across the eGFR tertiles reinforces that eGFR is an important prognostic indicator of fatal and non-fatal events in the subjects who may be mistaken as being at a lower risk when assessing subjects purely on severity of vessel disease.

Our findings support a previous study [Beddhu *et al.* 2002] carried out in patients with CAD whereby eGFR was found to be a predictor of death, MI or a composite of both death and MI. The latter study, however, comprised of patients who had stable or unstable angina as well as subjects who had an acute MI. The present study built on these findings by assessing the association between renal function and fatal and non-fatal cardiovascular events, including hospitalisation in subjects with stable angina. Renal disease is known to be a risk factor for adverse fatal and non-fatal cardiovascular outcomes in subjects who experienced an acute MI [Anavekar *et al.* 2004]. However, they included patients who also had heart failure or left ventricular systolic dysfunction which may have confounded the predictive ability of eGFR in

patients with stable angina. Recently, a study in a small number of patients with CAD, who had normal GFRs, found a reduction in renal perfusion to be a marker of progressive renal dysfunction [Fuiano *et al.* 2005]. Subjects in our study had low eGFRs and may have had impaired renal function. However, neither renal perfusion nor GFR was measured directly and therefore it is not known whether these subjects did have renovascular disease.

In the present study, the presence of a lower eGFR may have been a cause or an effect of atherosclerosis of the systemic and coronary arteries. The presence of a low eGFR in some subjects, for example, may be an indicator of the presence of atherosclerosis in the renal vascular bed. Renal function may have implications on deteriorating cardiovascular function through increased arterial calcification, anaemia, sodium and fluid imbalance, imbalance in calcium-phosphate homeostasis, activation of the renin-angiotensin-aldosterone system (RAAS) and promotion of coagulation. It is also possible that the presence of atherosclerosis or stiffening of the systemic arteries may have lead to reduced perfusion of the renal arteries which in turn may have indirectly had an effect on the vascular system via the above mechanisms.

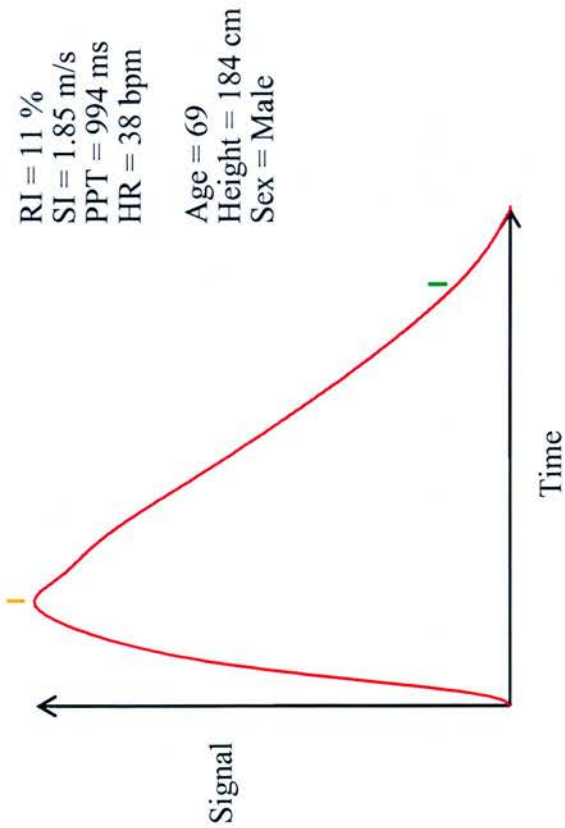
#### 5.4.6.3 Arterial stiffness as a predictor of outcomes

When subjects were grouped above and below the median PWV, those subjects with PWV values above the median were more likely to have a lower event-free probability. Atherosclerotic changes in the aorta may lead to aortic stiffening as a result of fibrosis, calcifications, diffusion of macromolecules into arterial wall and fragmentation of elastin fibres due to cyclical stress. An increase in aortic stiffness

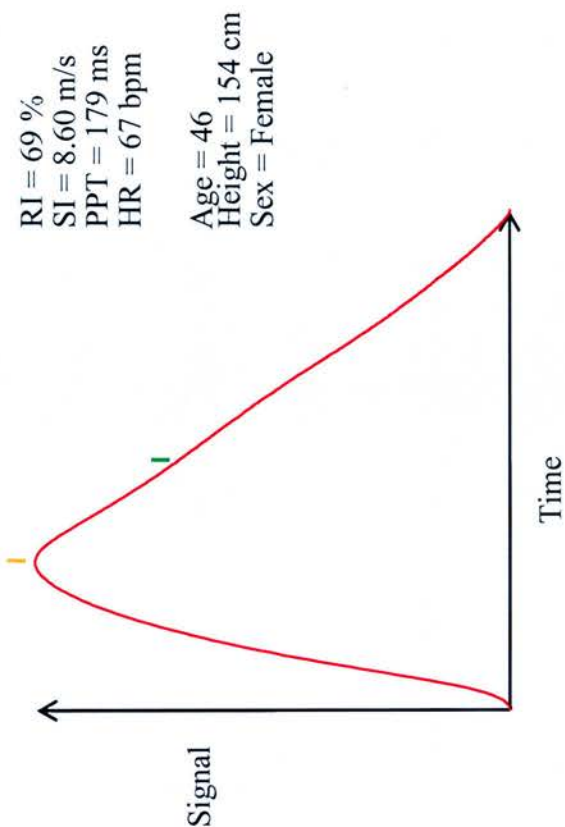
can in turn increase the work the heart needs to undergo to pump blood from the left ventricle into the aorta. Moreover, there is a reduction in the diastolic pressure, which is necessary for perfusion of the coronary arteries, which may have resulted in subjects with higher PWVs being more likely to suffer a cardiovascular event.

#### 5.4.7 ARTERIAL STIFFNESS MEASURES

The augmentation indices, SI and RI did not add any information to that provided by PWV. In the measurement of the augmentation indices and RI it can be difficult to detect the first and second systolic peaks of the arterial pressure waveform, which in turn can hamper the accurate calculation of these indices [O'Rourke *et al.* 2004]. PWV, in the present study, was solely determined by characteristics of the large artery whereas the augmentation, SI and RI measures are affected by the geometry of smaller arteries. Previous studies [Millasseau *et al.* 2000] on healthy subjects and patients with hypertension have shown SI and RI to be useful measures of arterial stiffness. The patients in our study, far from being healthy, had CAD and it was found that the incident and reflected wave overlapped sufficiently for the peaks to coincide and this gives a triangular waveform from which accurate measurements of SI and RI could not be derived (Figure 5-10).



(a)



(b)

Figure 5-10 Digital volume pulse waveforms.

The DVP waveforms above are averaged from 3 separate measurements. The RI and SI in (a) and (b) are calculated based on the orange and green markers representing the incident and reflected waves respectively. Inconsistent measurements in some subjects were due to difficulties in the equipment identifying the incident and reflected peaks on “triangular” shaped waveforms.



#### 5.4.8 STUDY LIMITATIONS

This study had a number of limitations. First, BMI is not an ideal measure of adiposity as it does not take into account the contribution from muscle mass. An alternative for future studies would be to measure the ratio of waist to hip circumference which is a better indicator of body fat distribution [Peiris *et al.* 1989]. Second, eGFR calculated from serum creatinine based equations may not be an accurate measure of GFR. However, it has been shown to correlate strongly with measured GFR [Froissart *et al.* 2005]. Third, the comparator group was relatively small as less than 10% of subjects had angiographically normal coronary arteries. It is well known that underlying intimal atherosclerotic plaque that does not encroach on the lumen can produce a normal looking artery on coronary angiography [Escolar *et al.* 2006]. However, more advanced techniques such as intravascular ultrasound (IVUS) and magnetic resonance imaging (MRI) are able to detect occult disease in angiographically normal sites [Nissen *et al.* 2001]. Therefore, this may be one explanation of why there were no significant differences in the majority of the arterial stiffness measures between subjects with CAD and those with angiographically normal coronary arteries, because these patients with normal angiograms still have widespread atherosclerosis. In addition, subjects who do not have CAD may have aortic, peripheral or renal disease which may explain why there were no differences in arterial stiffness measures between those with and without CAD. Finally, the number of deaths, AMIs and strokes were too small to be assessed as separate end-points. However, due to the short period of follow-up to date a composite

measure consisting of fatal and non-fatal events represented a good measure of cardiovascular risk (see methods Section 2.6).

## 5.5 CONCLUSIONS

In a treated population of patients with CAD, cardiovascular risk factors do not determine arterial stiffness, whereas, renal function is an important determinant of arterial stiffness. Estimated GFR was associated with PWV, even within the normal range, suggesting that it would be useful to use eGFR as an additional risk marker in patients, even within the normal range. However, more research is needed to verify renal function as a marker of risk in patients with CAD. Alternative measures of arterial stiffness were inferior to PWV and did not give similar information. The presence and severity of CAD, arterial stiffness and renal function are important predictors of a shorter time to fatal and non-fatal cardiovascular events.

**CHAPTER 6: NON-INVASIVE ASSESSMENT OF ENDOTHELIAL FUNCTION  
IN PATIENTS WITH CORONARY ARTERY DISEASE**

## 6.1 INTRODUCTION

The endothelium is a single cell layer that regulates vasomotor tone, anticoagulant activity, antiplatelet activity, blood flow and vascular smooth muscle cell proliferation [Davignon *et al.* 2004]. Vasomotor tone is regulated by both contracting (endothelin-1, angiotensin II) and relaxing factors (NO, endothelium-derived hyperpolarising factor (EDHF), prostacyclin). The endothelium is activated by a number of substances such as autocooids and catecholamines, as well as shear stress produced by increased blood flow [Harris *et al.* 2004]. Traditional risk factors such as hypercholesterolaemia, hypertension, diabetes, and smoking have been associated with an impairment in endothelium-dependent vasomotor function [Vita *et al.* 1990]. Endothelial dysfunction is found in the presence of mild and advanced CAD [Ludmer *et al.* 1986] and is present early on in life in children with risk factors for atherosclerosis [Celermajer *et al.* 1992].

Endothelial function can be assessed by exposing blood vessels to drugs that mediate their vasodilatory effect through the release of NO from the endothelial cell layer. For example, ACh is thought to act on the muscarinic M<sub>3</sub> receptor present on endothelial cells [Bruning *et al.* 1994]. The M<sub>3</sub> receptors couple with the heterotrimeric G protein G<sub>q</sub> which leads to an increase in intracellular calcium through the stimulation of phospholipase C [Eglen *et al.* 2001] and the release of inositol (1,4,5)-triphosphate, which results in the release of NO. However, if endothelial function is impaired there is reduced production of NO, increased degradation of NO or both. Reduced production of

NO causes vasodilatation, or increased vasoconstriction, of the coronary circulation in response to ACh. In addition, degradation of NO may be accelerated through its interaction with superoxide anions [Tentolouris *et al.* 2000] resulting in less NO available to smooth muscle cells, which in turn leads to reduced vasodilatation.

As detailed in Chapter 1, there are a number of invasive [Ludmer *et al.* 1986; Vita *et al.* 1990] and non-invasive [Celermajer *et al.* 1992; Lauer *et al.* 2005] techniques that can be used to assess endothelial function. In this study, endothelial function was investigated using the technique of applanation tonometry in combination with the administration of GTN and salbutamol. As blood is ejected from the left ventricle, the pressure pulse wave generated travels along the aorta and the small muscular arteries. These small muscular arteries offer resistance and cause reflection of these pressure waves back to the aorta. Vasodilators such as GTN are able to reduce the amount of wave reflection by decreasing the resistance of the small muscular arteries and causing the reflected wave to return later during diastole. The reduction in resistance of the small muscular arteries is mediated by denitration of GTN in the smooth muscle cells which results in the release of NO from GTN [Abou-Mohamed *et al.* 2000]. This in turn activates smooth muscle guanylyl cyclase and subsequently leads to the accumulation of cyclic guanosine monophosphate (cGMP) causing relaxation of smooth muscle cells. Therefore, GTN causes vasodilatation through its action on the smooth muscle cells. However, the vasodilatory effect of GTN is dependent on the sensitivity of smooth muscle cells to NO [Gori *et al.* 2001], and the bioavailability and degradation of NO

[Abou-Mohamed *et al.* 2000]. Salbutamol is another vasodilator that reduces wave reflections by reducing the resistance of small muscular arteries. Salbutamol induced reduction in wave reflection is mediated through the endothelium and is, therefore, a measure of endothelial function. Salbutamol, a selective  $\beta_2$ -agonist, reduces wave reflection in part by activation of the L-arginine/NO pathway [Dawes *et al.* 1997; Hayward *et al.* 2002] which leads to the release of NO from endothelial cells which in turn activates smooth muscle guanylyl cyclase and causes vasodilatation [Chowienczyk *et al.* 1999; Dawes *et al.* 1997]. Endothelial dysfunction of the coronary [Halcox *et al.* 2002b; Schachinger *et al.* 2000; Suwaidi *et al.* 2000] and peripheral arteries [Heitzer *et al.* 2001; Neunteufl *et al.* 2000; Perticone *et al.* 2001b] is a marker of future cardiovascular events and coronary revascularisation procedures in patients with CAD. However, the association between non-invasively determined endothelial dysfunction, in patients with underlying CAD, with cardiovascular and all-cause mortality or revascularisation procedures is unknown.

Arterial stiffness and endothelial dysfunction are known to co-exist in a number of disease states, such as diabetes [Wilkinson *et al.* 2000b], hypercholesterolaemia [Yacine *et al.* 2000] and cardiovascular disease [Nigam *et al.* 2003], and appear to be determined by common risk factors, for example obesity [Tounian *et al.* 2001]. This study sought to determine the relationship between non-invasive measures of arterial stiffness and endothelial function.

This chapter is aimed at assessing, in patients with CAD:

1. the determinants of endothelial function, particularly the influence of severity of CAD and cardiovascular risk factors, using the technique of non-invasive PWA.
2. the association between arterial stiffness and endothelial dysfunction.
3. the relationship between the presence of endothelial dysfunction with major adverse cardiovascular events defined as cardiovascular and all-cause mortality, hospitalisation due to cardiovascular causes (including non-fatal MI or stroke) and coronary revascularisation procedures (PTCA or CABG).



## 6.2 METHODS

### 6.2.1 STUDY SUBJECTS

Subjects who participated in the study of arterial stiffness described in Chapter 5, were recruited into this study. All studies were carried out in the Wellcome Trust Clinical Research Facility at the Western General Hospital and at the Royal Infirmary, Edinburgh.

### 6.2.2 MEASUREMENTS

All subjects had their morning medications withheld following an overnight fast. Brachial artery blood pressure was measured using an integrated automated sphygmomanometer linked to an automated tonometric device containing piezo-electric pressure sensors (Colin<sup>®</sup> CBM-7000, Colin Medical Technology Corporation, Japan) coupled to a SphygmoCor<sup>®</sup> system (SphygmoCor<sup>®</sup> Mx Aortic Blood Pressure Monitoring System, AtCor Medical, Australia). This automated tonometric device was used to calculate the augmentation indices (AIx, AIx75, and PAIx) from centrally derived and radial pressure waveforms.

The arterial stiffness measures – PWV, AIx, AIx75, PAIx, SI and RI — were assessed by comparing them with endothelial function measures of peak values and area under the curves (AUC) of the augmentation indices (AIx, AIx75 and PAIx) following the

administration of GTN and salbutamol. Details of the methods used to measure arterial stiffness are detailed in Chapters 2 and 5.

### 6.2.3 STUDY PROTOCOL

All subjects were rested for 10 minutes during which three baseline recordings were made. Administration of GTN was recorded as time = 0 where 500  $\mu\text{g}$  of sublingual GTN was administered under the tongue for a period of 3 minutes. Following this, pressure wave recordings were taken every minute for 10 minutes and every 5 minutes for the next 20 minutes. GTN has a short duration of action with haemodynamic changes returning to baseline values within 20 minutes [Greig *et al.* 2005; Wilkinson *et al.* 2002b]. Therefore, in the present study salbutamol was administered 30 minutes after the administration of GTN to allow the augmentation indices to return to baseline values. A dose of 400  $\mu\text{g}$  of salbutamol was then administered over a period of 1 minute via a Volumatic device at time = 33 minutes. Following this, measurements were taken every minute for the next 10 minutes and every 5 minutes for the following 20 minutes. In a previous study, the effect of salbutamol was shown to return to baseline within 30 minutes and therefore measurements of augmentation indices were recorded for a period of 30 minutes. The study protocol is outlined in Figure 6-1.

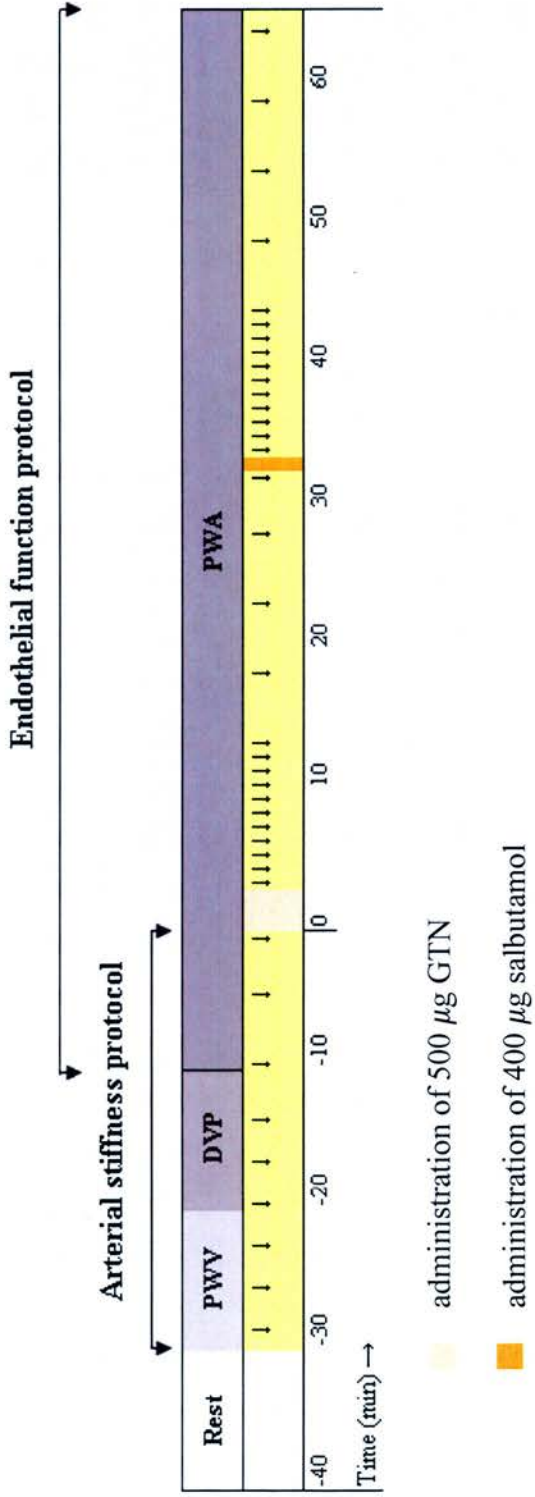


Figure 6-1 Endothelial function protocol.  
A schematic of the study protocol undertaken. The downward arrow, ↓, denotes the recording of a measurement.

#### 6.2.4 OUTCOMES

Follow-up data obtained in 5.2.4 were used to determine whether endothelial function is a predictor of fatal and non-fatal events.

#### 6.2.5 BLOOD SAMPLING AND SERUM ASSAYS

Biochemical blood results obtained in Section 5.2.5 were used to analyse the relationship between endothelial function and biochemical variables in both univariate and multivariate analyses.

#### 6.2.6 DATA ANALYSIS

Statistical analysis was performed using Microsoft Excel 2002 and SPSS version 11.5 for Windows program (SPSS, Inc., Chicago, IL, USA). Box-plots of the overall change in augmentation indices were computed using the R statistical programming language 2003 (R Foundation for Statistical Computing, R Development Core Team, Vienna, Austria) [R Development Core Team 2003]. There were no violations in the assumptions of normality, linearity, and homogeneity of variances. Data are given as mean  $\pm$  SD. The first three baseline AIx measurements were averaged and this was taken as the final baseline measurement. The final baseline measurement was subtracted from subsequent measurements following GTN and salbutamol administration. Thus, all values of augmentation indices for each subject were normalised to their respective averaged baseline values. This procedure was repeated for AIx75 and PAIx. These augmentation values were plotted as a function of time from which the maximal negative change in the augmentation indices were determined, denoted as the peak value, and to calculate the area under the curves

(AUC) using the trapezoid rule. Peak values are defined as the largest negative excursion (deviation) from baseline. Larger negative peak values imply larger vasodilatation to the drugs.

The AUC gives a measure of the AUCs in augmentation indices as a function of time, which is given by the following formula

$$AUC_{a-b} = [\Delta AIX_{ta} + \Delta AIX_{tb}] (t_b - t_a) / 2$$

where  $\Delta AIX_{ta}$  is the difference in AIX from baseline at time  $t=a$ ,  $\Delta AIX_{tb}$  is the difference in AIX from baseline at time  $t=b$  and  $AUC_{a-b}$  is the AUC between the segment at  $t=a$  and  $t=b$ .

The AUC for each drug was calculated as the sum of the individual segments for the respective drugs. These are given by the following formulas

$$AUC_{GTN} = \Sigma AUC_{0-33}$$

$$AUC_{salbutamol} = \Sigma AUC_{33-64}$$

where  $AUC_{GTN}$  is the area under the curve for the response to GTN between  $t = 0$  and  $t = 33$  minutes and  $AUC_{Salbutamol}$  is the area under the curve for the response to salbutamol between  $t = 33$  and  $t = 64$  minutes. In this study area under the curves were observed to be negative with vasodilatation and positive due to vasoconstriction.

The boxplot of AIX over time for each subject was plotted by taking the average AIX from times measurement at  $t = -10$ mins,  $-5$ mins, and  $0$  minutes to give an overall baseline average. This baseline average was deducted from each data measurement

including each individual baseline measurement for that subject. Thus each time-point is translated to bring all individual subject data curves to a common baseline. This common baseline is labelled as  $AIx = 0$ . This was repeated for  $AIx75$  and  $PAIx$ . The boxes on the boxplots contain 50% of the data; the lines splitting each box are at the positions of the medians or 50% quartiles; the lines representing the lower and upper surfaces of the boxes are at the lower and upper quartiles, or the 25% and 75% quartiles, respectively. The upper whiskers are at positions 1.5 times the interquartile range higher than the upper quartile; and the lower whiskers are at positions 1.5 times the interquartile range lower than the lower quartile. The points represent data outside these ranges.

Follow-up time was calculated as days between the first study visit and date of the first cardiovascular event or date of last follow-up for censored subjects. The primary pre-defined outcome of this study was a composite end-point consisting of death, AMI, stroke and hospitalisation due to CHD while the post-hoc outcome studied was future coronary intervention (CABG or PTCA). Cumulative survival and event-free probabilities were determined using the Kaplan-Meier product-limit method and compared by the Mantel (log-rank) test. Testing was two-sided and significance was taken at the 5% level.

Univariate correlation coefficients were given as Pearson's  $r$ , and stepwise linear regression was used for multivariate analysis. All parametric data were analysed using Student's  $t$ -test, ANOVA and ANCOVA where appropriate. The Bonferroni correction was used for post-hoc comparisons. All testing was two-sided and significance was taken at the 5% level.

### 6.3 RESULTS

Out of 288 subjects, 10 subjects had to be excluded due to difficulties in obtaining good quality radial pressure pulse wave recordings. The remaining 278 subjects formed the study population. The change in AIx with the administration of GTN and salbutamol is outlined in Figure 6-2. From the figure it is clear that the value of AIx changed after the administration of GTN and salbutamol. Following the administration of GTN changes in AIx returned to baseline after 30 minutes. Salbutamol, however, still had an effect 30 minutes after its administration. A similar trend was found for AIx75 and PAIx. The peak changes in the augmentation indices to GTN were bigger compared to those of salbutamol.

#### 6.3.1 DEMOGRAPHIC VARIABLES

Older subjects were found to have a smaller peak value and AUC of the augmentation indices following the administration of GTN. However, no relationship was found between age and peak value or AUC of the augmentation indices following salbutamol (Table 6.1). Subjects with a higher BMI had a smaller peak value and AUC of the augmentation indices due to GTN and a smaller AUC in AIx due to salbutamol. Sex was only found to contribute to a larger AUC in differences in PAIx due to GTN in men compared to women ( $-178 \pm 173$  vs  $-129 \pm 196$ ;  $P < 0.05$ ; independent *t*-test) while no association was found with smoking status. Neither sex nor smoking status was associated with endothelium-dependent changes in augmentation indices.

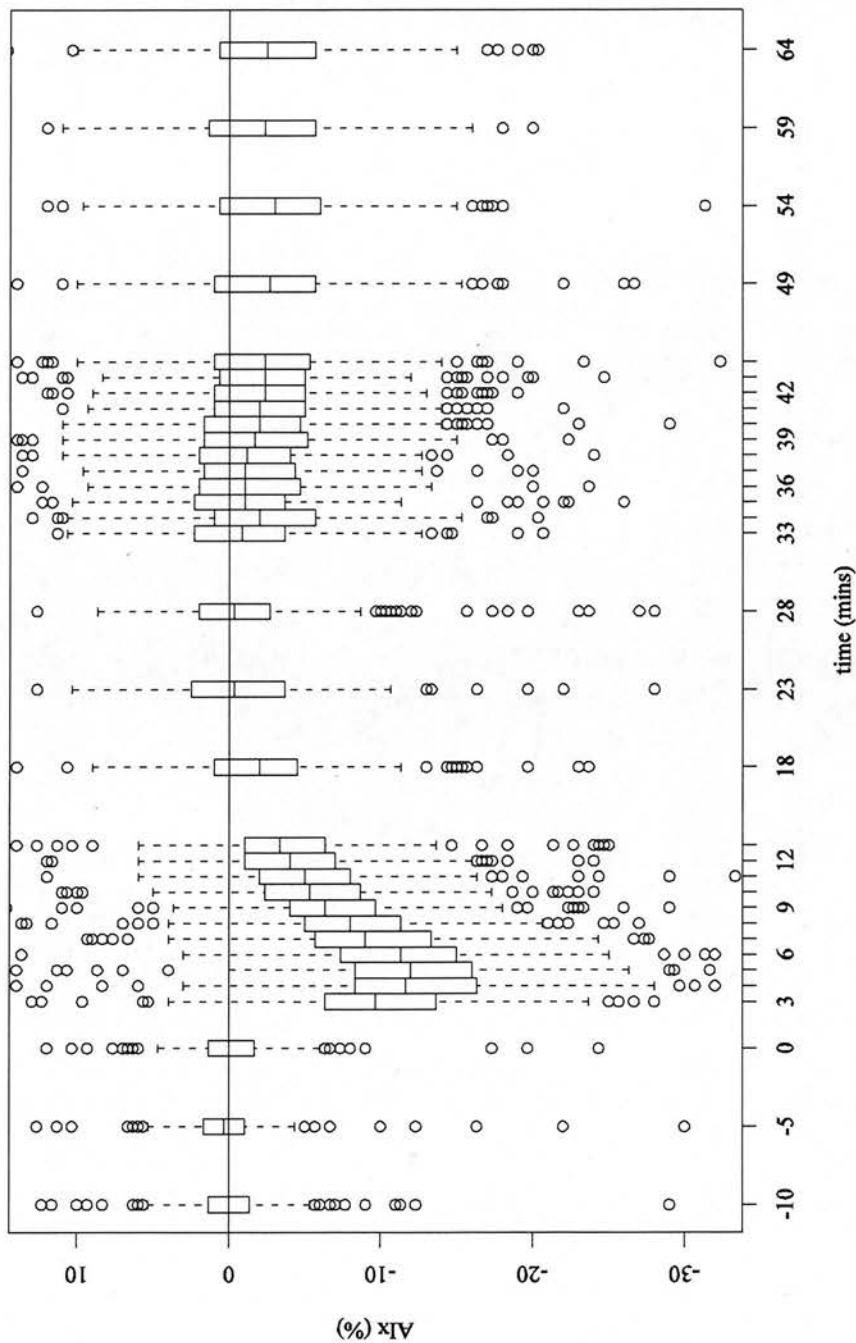


Figure 6-2 Boxplot of A1x following the administration of glyceryl trinitrate and salbutamol. GTN was administered at time = 0 minutes and salbutamol was administered at time = 33 minutes.



Table 6-1 Correlations between demographic variables and measures of endothelial dysfunction.

Parameters		Age	BMI
Peak value			
GTN	Augmentation index	0.22†	0.14*
	Augmentation index at HR 75 bpm	0.20**	0.14*
	Peripheral augmentation index	0.18**	0.16**
Salbutamol	Augmentation index	0.06	0.02
	Augmentation index at HR 75 bpm	0.04	-0.02
	Peripheral augmentation index	0.04	0.06
AUC			
GTN	Augmentation index	0.16**	0.17**
	Augmentation index at HR 75 bpm	0.18**	0.14*
	Peripheral augmentation index	0.12*	0.16**
Salbutamol	Augmentation index	-0.02	0.12*
	Augmentation index at HR 75 bpm	-0.02	0.10
	Peripheral augmentation index	-0.01	0.06

N=278 Values are Pearson's correlation coefficients (*r*) and are represented as means ± SD. \* P < 0.05, \*\*P < 0.01 † P < 0.001

### 6.3.2 BIOCHEMICAL VARIABLES

High serum creatinine concentrations and low GFR were associated with a smaller peak change in AIX ( $r = 0.13, P < 0.05$ ;  $r = -0.14, P < 0.05$ ) and AIX75 ( $r = 0.13, P < 0.05$ ;  $r = -0.14, P < 0.05$ ) due to GTN. No association was found between serum creatinine concentrations or GFR with changes in augmentation values due to salbutamol. Surprisingly, higher serum triglyceride concentrations were associated with a larger peak change in AIX75 to salbutamol ( $r = -0.13, P < 0.05$ ) while the Chol:HDL-C ratio was associated with a smaller AUC in AIX to GTN ( $r = 0.15, p = 0.05$ ).

### 6.3.3 DETERMINANTS OF VASCULAR FUNCTION

#### 6.3.3.1 Endothelium-independent vasodilatation

Variables that were associated with endothelial function in univariate analysis were further analysed in multivariate analysis and the results are shown in Table 6-2. The R square value explains how much of the variance in the dependent variable is explained by the model while the regression coefficients are used to construct the regression equation for the model. From these results it is clear that age and BMI were weak determinants of the peak value and AUC in AIX and AIX75 to GTN. Peak changes in PAIX due to GTN were determined by age, heart rate and BMI, but only BMI weakly determined AUC of PAIX.

#### 6.3.3.2 Endothelium-dependent vasodilatation

Peak values of AIX75 were weakly determined by ejection duration and serum triglyceride concentration while the peak values of PAIX due to salbutamol were

weakly determined by heart rate. The AUC of AIx for salbutamol was weakly determined by central SBP while the AUC of AIx75 was determined by CSBP and weight. The AUC of PAIx for salbutamol was not determined by any of the variables.

#### 6.3.4 CORONARY ARTERY DISEASE

There were no differences in peak or AUC of the augmentation indices between subjects with and without symptoms or presence of CAD (Table 6-3) nor was there a relationship between the number of coronary vessels affected (Figure 6-3).

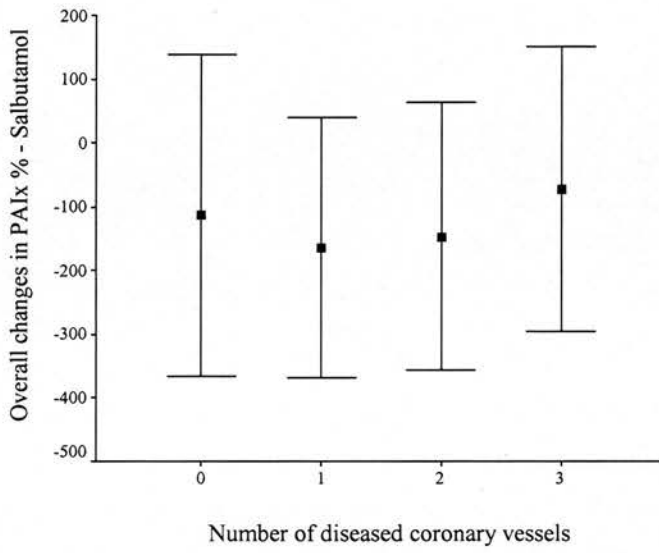


Figure 6-3 Mean AUC of peripheral augmentation index against numbers of diseased coronary vessels. Values are means  $\pm$  SD.

Table 6-2 Multiple regression analysis of endothelial function.  
Determinants of peak and AUC in augmentation indices to GTN and salbutamol.

Parameters	Determinants	Regression coefficient	Standard error	
<b>Peak change</b>				
GTN	<b>AIx</b> ( $R^2=0.089$ , $P < 0.001$ )			
	Age, years	0.2	0.05†	
	BMI, $\text{kg/m}^2$	0.2	0.09**	
	Central mean blood pressure, mmHg	0.07	0.03*	
	<b>AIx75</b> ( $R^2=0.067$ , $P < 0.001$ )			
	Age, years	0.2	0.05†	
	BMI, $\text{kg/m}^2$	0.2	0.08**	
	<b>PAIx</b> ( $R^2=0.093$ , $P < 0.001$ )			
	Age, years	0.2	0.06†	
	HR, beats per minute	0.2	0.06**	
	BMI, $\text{kg/m}^2$	0.3	0.1**	
	Salbutamol	<b>AIx75</b> ( $R^2=0.042$ , $P < 0.01$ )		
		ED, seconds	-0.04	0.01**
		Triglycerides	-0.8	0.4*
		<b>PAIx</b> ( $R^2=0.029$ , $P < 0.01$ )		
HR, beats per minute		0.2	0.06†	
<b>AUC</b>				
GTN	<b>AIx</b> ( $R^2=0.091$ , $P < 0.001$ )			
	BMI, $\text{kg/m}^2$	6.24	1.78†	
	Age, years	2.72	0.97**	
	Chol:HDL ratio	16.01	7.38*	
	<b>AIx75</b> ( $R^2=0.06$ , $P < 0.01$ )			
	Age, years	5.04	1.45†	
	BMI, $\text{kg/m}^2$	7.74	2.80**	
	<b>PAIx</b> ( $R^2=0.02$ , $P < 0.01$ )			
	BMI	6.23	2.38**	
	Salbutamol	<b>AIx</b> ( $R^2=0.020$ , $P < 0.05$ )		
		Central systolic blood pressure, mmHg	-1.20	0.52*
		<b>AIx75</b> ( $R^2=0.035$ , $P < 0.01$ )		
Central systolic blood pressure, mmHg		-1.10	0.46*	
	Weight, kg	1.09	0.54*	

\*  $P < 0.05$ , \*\* $P < 0.01$ , † $P < 0.001$

Table 6-3 Endothelial function measurements.  
 Baseline augmentation indices, peak values and AUC of augmentation indices for subjects with and without CAD.

Parameters	Augmentation Indices	Angiographically normal coronary arteries (N=34)	CAD (N=244)	Total (N=278)
<b>Baseline values</b>				
	Augmentation Index	32.5 ± 8.5	34.6 ± 9.2	34.4 ± 9.1
	Augmentation index at HR 75 bpm	26.4 ± 7.5	26.3 ± 8.6	26.3 ± 8.5
	Peripheral augmentation index	-13.3 ± 11.9	-7.3 ± 13.4*	-7.8 ± 13.3
<b>Peak value</b>				
Glyceryl trinitrate	Augmentation Index	-14.6 ± 5.9	-16.1 ± 6.8	-15.9 ± 6.7
	Augmentation index at HR 75 bpm	-13.6 ± 5.4	-14.8 ± 6.4	-14.7 ± 6.4
	Peripheral augmentation index	-18.0 ± 6.5	-20.4 ± 9.0	-20.2 ± 8.9
Salbutamol	Augmentation Index	-10.0 ± 8.6	-8.9 ± 6.6	-9.0 ± 6.8
	Augmentation index at HR 75 bpm	-8.5 ± 7.6	-7.1 ± 6.2	-7.2 ± 6.3
	Peripheral augmentation index	-11.8 ± 11.1	-13.2 ± 8.3	-13.1 ± 8.6
<b>AUC</b>				
Glyceryl trinitrate	Augmentation Index	-116 ± 82	-130 ± 130	-128 ± 126
	Augmentation index at HR 75 bpm	-125 ± 77	-133 ± 223	-132 ± 215
	Peripheral augmentation index	-137 ± 130	-168 ± 184	-165 ± 180
Salbutamol	Augmentation Index	-72 ± 148	-73 ± 149	-73 ± 149
	Augmentation index at HR 75 bpm	-47 ± 115	-25 ± 134	-27 ± 133
	Peripheral augmentation index	-87 ± 273	-129 ± 215	-125 ± 220

Values are means ± SD. \* P < 0.05 \*\*P < 0.01 † P < 0.001

### 6.3.5 DRUG EFFECTS

There were no differences in peak values or AUC of the augmentation indices due to GTN or salbutamol between subjects who were receiving and those who were not receiving nitrates, calcium channel blockers (CCB) or ACE inhibitors after adjusting for central mean blood pressure, total cholesterol and serum creatinine concentrations. Patients on a  $\beta$ -blocker had a *larger* peak change in PAIx to salbutamol than those not on a  $\beta$ -blocker ( $-13.7 \pm 8.9$  vs  $-11.6 \pm 8.9$ ;  $P < 0.05$ ; ANCOVA). Patients receiving nicorandil had a smaller AUC of PAIx due to GTN and salbutamol compared to those not on nicorandil ( $-115 \pm 178$  vs  $-178 \pm 179$ ;  $P < 0.05$ ;  $-60 \pm 220$  vs  $-140 \pm 219$ ; ANCOVA).

### 6.3.6 ENDOTHELIAL FUNCTION AND SMOOTH MUSCLE DYSFUNCTION

The relationship between the endothelium-dependent and -independent changes in augmentation indices was assessed to determine whether reduced endothelium-dependent changes in augmentation indices were confounded by underlying underlying smooth muscle dysfunction (Figure 6-4). Subjects who had small peak values or AUC of AIx due to salbutamol also had small peak ( $r = 0.35$ ,  $P < 0.000$ ) and AUC ( $r = 0.52$ ,  $P < 0.000$ ) of AIx due to GTN respectively. These trends were also seen for AIx75 ( $r = 0.44$  for peak changes;  $r = 0.36$  for AUCs;  $P < 0.000$ ) and PAIx ( $r = 0.45$  for peak changes;  $r = 0.61$  for AUCs;  $P < 0.000$ ).

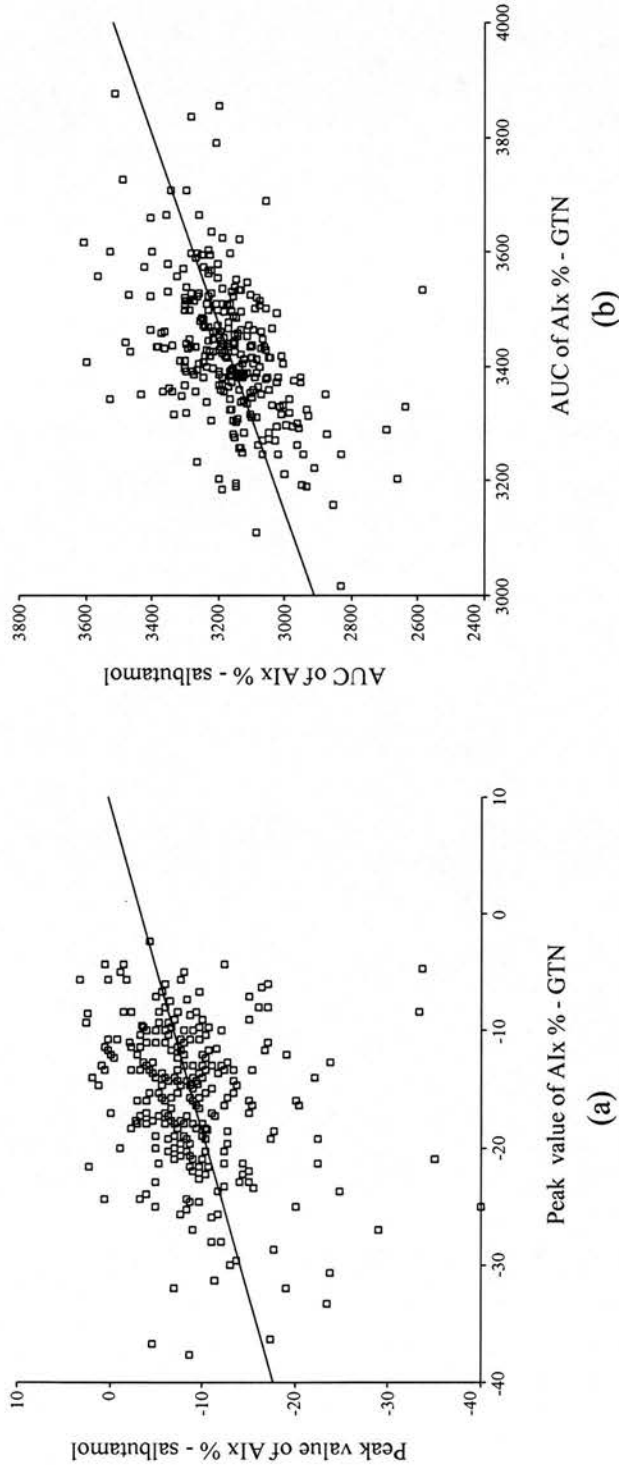


Figure 6-4 Scatterplots and linear regression of (a) peak changes in A1x and (b) AUC of A1x due to GTN and salbutamol.



### 6.3.7 OUTCOMES

Follow-up data was obtained from Section 5.3.8.

#### 6.3.7.1 Endothelium-independent and -dependent changes in augmentation indices as predictors of outcomes

A smaller peak change in PAIx due to GTN was found in subjects who suffered an event compared to subjects who did not suffer an event ( $-18.5 \pm 7.6$  vs  $-21.1 \pm 9.4$  %;  $P < 0.05$ ; *t*-test). When subjects were divided into quintiles of peak or AUCs in the augmentation indices due to GTN, there were no differences between the groups. There were also no differences in the quintiles of peak and AUC of the augmentation indices following salbutamol.

### 6.3.8 ARTERIAL STIFFNESS AND ENDOTHELIAL FUNCTION

Arterial stiffness and endothelial function were analysed in 278 subjects. PWV measures were only available in 238 subjects of the 278 subjects. Subjects with high PWVs had smaller peak response to GTN as well as a smaller AUC in augmentation indices. There were, however, no correlations between PWV and measures of augmentation indices following salbutamol (Table 6-4).

Baseline AIX, AIX75 and PAIX negatively correlated with peak changes in augmentation indices following GTN and salbutamol. That is to say, subjects with the higher stiffness as measured by AIX, AIX75 and PAIX had a higher peak response to GTN and salbutamol. Higher AIX, AIX75 and PAIX values were also correlated with a larger AUC in augmentation indices following GTN and salbutamol.

However, arterial stiffness as measured by SI and RI, showed no correlation with measures of endothelial function.

Table 6-4 Correlations between measures of arterial stiffness and endothelial function measures.

	PWV	AIx	AIx75	PAIx	CPP	PPP
<b>Peak changes</b>						
<b>Glyceryl trinitrate</b>						
Augmentation index	0.20**	-0.29**	-0.29**	-0.27**	0.13*	0.19**
Augmentation corrected to a HR75	0.25**	-0.35**	-0.34**	-0.31**	0.10	0.17**
Peripheral augmentation index	0.22**	-0.21**	-0.14*	-0.27**	0.06	0.14*
<b>Salbutamol</b>						
Augmentation index	0.05	-0.21**	-0.21**	-0.21**	-0.05	-0.03
Augmentation corrected to a HR75	0.05	-0.25**	-0.27**	-0.25**	-0.08	-0.05
Peripheral augmentation index	0.07	-0.21**	-0.14*	-0.29**	-0.12	-0.06
<b>AUC</b>						
<b>Glyceryl trinitrate</b>						
Augmentation index	0.15*	-0.34**	-0.32**	-0.22**	-0.05	-0.12*
Augmentation corrected to a HR75	0.16*	-0.26**	-0.24**	-0.20*	-0.05	-0.10
Peripheral augmentation index	0.18**	-0.22**	-0.17**	-0.25**	-0.07	-0.14*
<b>Salbutamol</b>						
Augmentation index	0.05	-0.34**	-0.35**	-0.26**	0.11	0.05
Augmentation corrected to a HR75	0.06	-0.37**	-0.40**	-0.26**	0.10	0.05
Peripheral augmentation index	0.08	-0.23**	-0.19**	-0.27**	0.09	0.03

CPP denotes central pulse pressure and PPP, peripheral pulse pressure.

\* P < 0.05, \*\*P < 0.01 † P < 0.001

#### 6.4 DISCUSSION

Both GTN and salbutamol reduced the augmentation indices as a function of time with GTN having a shorter duration of action but a greater peak effect compared to salbutamol. Both the endothelium-dependent and independent changes in augmentation indices were weakly explained by blood pressure and BMI but not by the presence or severity of CAD, renal function or serum cholesterol and lipid concentrations. Moreover, they did not predict fatal and non-fatal cardiovascular outcomes. Our findings suggest that non-invasive assessment of endothelial function using measures of arterial stiffness do not correlate with established markers of cardiovascular risk nor predict outcome in patients with CAD.

The age-associated impairment in endothelium-independent vasodilatation may reflect underlying impairment of relaxation of smooth muscle cells. However, there was a lack of association between endothelium-dependent changes in augmentation indices and age. This is in contrast to large studies [Celermajer *et al.* 1994a; Herrington *et al.* 2001] that have shown an inverse relationship between endothelial function and age. This difference may be explained by the fact that these former studies have mainly been carried out in healthy subjects [Celermajer *et al.* 1994b] or have not assessed endothelium-independent vasodilator response [Herrington *et al.* 2001]. Age explains only a small percentage of the variance in endothelium-dependent vasodilatation [Benjamin *et al.* 2004] and we cannot exclude a small effect. Other factors than age may

have had a larger role in the development of endothelial dysfunction. The peak augmentation indices and AUC for GTN were similarly associated with age and BMI. Therefore, either the peak value or AUC may similarly reflect the extent of endothelium-independent vasodilatation. However, the different associations between salbutamol-mediated peak augmentation values and AUC with age or BMI may have resulted from incomplete capture of measurements and hence an underestimation of the AUC for the salbutamol response which persisted 30 minutes following salbutamol administration.

A low BMI was also associated with larger endothelium-independent peak and AUCs of the augmentation indices but not endothelium-dependent peak and AUCs. This is consistent with previous findings of no association between changes in AIx due to salbutamol and weight or BMI, using the technique of peripheral waveform analysis [Hayward *et al.* 2002]. Subjects in the present study were not given weight adjusted doses of salbutamol which may have resulted in varying volume of distributions (normally 7.2 – 9 L/Kg) of the drug. Moreover, BMI values are confounded by muscle mass. Therefore visceral fat distribution, a measure of adiposity, may be a more useful determinant of endothelium-dependent changes in augmentation indices as shown in a study of obese premenopausal women [Suh *et al.* 2005]. Abdominal fat distribution has been shown to be correlated with other measures of endothelial function [Perticone *et al.* 2001a; Tounian *et al.* 2001].

#### 6.4.1 BIOCHEMICAL PARAMETERS

Hypercholesterolaemia increases the risk of CAD [Castelli 1984] and is associated with impaired endothelial function [Cardillo *et al.* 2000; Casino *et al.* 1995; Lewis *et al.* 1999], even in angiographically normal coronary arteries [Quyyumi *et al.* 1997]. Unlike previous findings of an association between endothelial dysfunction and hypercholesterolaemia, the lack of relationship between serum cholesterol and lipid concentrations with endothelial function in this study may be a reflection of the large numbers of subjects on cholesterol lowering therapy. This may have resulted in well controlled cholesterol and lipid concentrations which obscured the relationship with measures of endothelium-dependent and independent changes in augmentation indices. Moreover, total serum triglyceride concentrations may not represent the atherogenic triglyceride-containing lipoproteins such as VLDL or chylomicron remnants [Jeppesen *et al.* 1998].

Patients with chronic renal failure [Thambyrajah *et al.* 2000] or hypertension who have reduced renal function [Perticone *et al.* 2004] have been shown to have impaired endothelium-dependent dilatation. In the present study, subjects with a lower renal function values had lower endothelium-independent changes in augmentation indices but no association was found with endothelium-dependent changes in augmentation indices. The relationship between endothelium-independent changes in augmentation indices and renal function, however, was weak and did not persist in multivariate analysis. Therefore, in the present cohort of subjects with CAD, other factors may play a larger

role in the development of systemic endothelial dysfunction, rather than impaired renal function, such as increased oxidative stress.

#### 6.4.2 BLOOD PRESSURE AND CARDIOVASCULAR RISK

A number of earlier studies [Gokce *et al.* 2001; Panza *et al.* 1993] have shown an association between endothelial dysfunction and hypertension. In the present study, central SBP was found to be a small determinant (2%) of endothelium-dependent changes in augmentation indices and, in contrast, a higher SBP was associated with a larger AUC for salbutamol. However, this was a weak association which did not persist on assessment of peripheral SBP. This association may be a result of an underestimation of the AUC for salbutamol.

The lack of relationship between the presence or absence of CAD and endothelial dysfunction may be due to subjects with angiographically normal coronary arteries having underlying coronary microvascular dysfunction [Quyyumi *et al.* 1992], or segments of coronary arteries which are functionally abnormal [Tentolouris *et al.* 2000] (as for example, in patients with syndrome X) which is not detectable by angiography. Moreover, impaired endothelial function resulting from early atherosclerosis has been previously shown to be present in each of the following groups of patients: those with normal coronary arteries, those presenting with chest pain, and those presenting with risk factors for atherosclerosis [Tentolouris *et al.* 2000; Tousoulis *et al.* 1996].

When assessing symptoms and severity of CAD, no association was found with the presence of endothelial dysfunction nor was there a difference between subjects with and without CAD. There are four possible reasons why we did not find an association. First, these patients had multiple risk factors such as hypertension, diabetes and hypercholesterolaemia. Therefore, it is not known to what extent these risk factors contributed to endothelial dysfunction and the cumulative effect of these risk factors on the endothelium. Second, coronary angiography does not accurately quantify the burden of atherosclerosis of the coronary or systemic arteries. The severity of endothelial dysfunction of the systemic arteries may not parallel the severity of endothelial dysfunction of the coronary arteries. Third, the majority of subjects were on long-term multiple drug therapies which may have influenced endothelial function. Fourth, technical problems with the technique may have undermined the association between endothelial dysfunction and CAD severity. The period between the administration of GTN and that of salbutamol may not have completely precluded any persistent effect of GTN in some subjects. The peak effect may have been missed due to insufficient measurements after the first 11 minutes following salbutamol administration. In addition, changes in augmentation indices following salbutamol persisted following a period of 30 minutes and therefore the complete response to salbutamol is not known. Our findings would suggest that the technique used in this study, is not a good test of endothelial function in patients with CAD.



Drug therapy was assessed to determine its effects on endothelial function measures but is inconclusive. Subjects in the present study were on multiple drug therapy making it difficult to determine whether any one particular drug is associated with better endothelial function measures.

#### 6.4.3 ENDOTHELIUM-DEPENDENT AND -INDEPENDENT RESPONSE

GTN is denitrated in smooth muscle cells causing NO to be released and resulting in vasodilatation [Abou-Mohamed *et al.* 2000]. In contrast, the vasodilatory effect of salbutamol is only partly mediated by NO [Dawes *et al.* 1997; Wilkinson *et al.* 2002b]. Therefore, salbutamol-mediated vasodilatation may be additionally caused by other mechanisms such as endothelium-dependent hyperpolarising factor which acts through calcium-activated potassium channels on smooth muscle cells resulting in smooth muscle cell hyperpolarisation and relaxation [Bellien *et al.* 2005]. NO-independent vasodilatation may also be caused by prostanoids such as prostacyclin increasing cAMP [Lamping 2001]. This implies that the changes in the augmentation indices to salbutamol may not be a specific measure of the severity of impaired NO-mediated vasodilatation of smooth muscle cells.

The changes in augmentation indices to salbutamol may be explained by polymorphisms of the human  $\beta_2$  adrenoceptor responsible for interindividual variation in expression, regulation and function of the  $\beta_2$  adrenoceptor [Green *et al.* 1994; Johnson 2006; Reihnsaus *et al.* 1993]. A coding region on the  $\beta_2$  adrenoceptor gene can have several

genetic polymorphisms [Cho *et al.* 2005; Joos *et al.* 2002]. The effect of these polymorphisms on the reduction in AIx to inhaled salbutamol is currently not known. Therefore, there is a potential that this polymorphism may result in a degree of variability in augmentation measures which is not determined by endothelial function.

In the present study, both the endothelium-dependent and independent changes in augmentation indices are strongly and positively correlated. Patients with CAD have previously been shown to have a reduced response to endothelium derived [Adams *et al.* 1998] as well as exogenous nitrovasodilators [Raitakari *et al.* 2001]. A number of studies also suggest that the endothelium-independent response is an important predictor of adverse cardiovascular events. A lower vasodilatory response to GTN has been shown to predict adverse cardiovascular events, in patients with CAD [Schachinger *et al.* 2000], while a study of patients with chest pain [Neunteufl *et al.* 2000] found a larger GTN-mediated vasodilatory response to be associated with an increase in myocardial infarction and to predict adverse cardiovascular events. The results of the latter study may be a result of hypersensitivity of the artery to GTN due to deficient endothelial NO activity or patients at high risks of MI having an increased vasoconstrictor response to endogenous vasoconstrictors. However, both these studies highlight the importance of the endothelium-independent response as predictors of cardiovascular outcomes. In the present study, however, it is difficult to distinguish whether the endothelium-dependent changes in augmentation indices were due to the presence of endothelial dysfunction, smooth muscle dysfunction or a combination of both.

#### 6.4.4 ARTERIAL STIFFNESS AND ENDOTHELIUM-INDEPENDENT AND -DEPENDENT CHANGES IN AUGMENTATION INDICES

In this study, arterial stiffness was unrelated to endothelial function but associated with endothelium-independent changes in augmentation indices. One explanation of this finding is that the artery may be very stiff and therefore it restricts the endothelium-dependent and independent vasodilatory response. In addition, salbutamol may be a less potent vasodilator and therefore the endothelium-dependent vasodilatory response may have only been partially stimulated.

#### 6.4.5 ENDOTHELIUM-INDEPENDENT AND -DEPENDENT CHANGES IN AUGMENTATION INDICES AS PREDICTORS OF OUTCOMES

The severity of coronary atherosclerosis is associated with the severity of coronary endothelial dysfunction in patients with angiographically normal coronary arteries, assessed by intravascular ultrasonography [Matsuda *et al.* 2003] and in patients with atherosclerotic coronary arteries [Matsubara *et al.* 2003], and predicts cardiovascular outcome [Halcox *et al.* 2002a; Suwaidi *et al.* 2000; Vita *et al.* 1990]. Although the coronary circulation is of most relevance, peripheral endothelial dysfunction has also been shown to be associated with coronary endothelial function [Anderson *et al.* 1995b; Takase *et al.* 2005] and CAD severity [Thanyasiri *et al.* 2005; Wu *et al.* 2005]. In addition, peripheral endothelial function predicts cardiovascular outcome [Gokce *et al.* 2002; Heitzer *et al.* 2001; Kuvin *et al.* 2001; Neunteufl *et al.* 2000].

The endothelium has important antiatherogenic effects which are mediated by NO such as inhibition of smooth muscle proliferation, platelet aggregation, oxidation of LDL-C, adhesion of leukocytes and it counteracts vasoconstrictor forces. The studies above allude towards an association between the severity of endothelial dysfunction in the peripheral and coronary circulation and with coronary atherosclerosis. However, in the current study, endothelial dysfunction was neither associated with CAD severity (as detailed in Chapter 6) nor was it associated with cardiovascular outcomes. This difference may be due to coronary structure being more predictive of outcome rather than functional measurements (endothelial function) [Asselbergs *et al.* 2004; Schachinger *et al.* 2000]. Atherosclerosis is also known to less commonly develop in the forearm as compared to the coronary arteries [Anderson *et al.* 1995b] and therefore atherosclerosis in the forearm and the coronary circulation may not be linearly associated.

#### 6.4.6 STUDY LIMITATIONS

There are a number of limitations of this study: first, subjects classified as not having CAD were not a true healthy population and therefore were not an ideal comparator group. Second, the majority of subjects were on long-term drug therapies which may have modified endothelial function and resulted in the lack of association with the presence or severity of CAD. Third, this study was not designed to assess mechanisms that underlie the reduced change in the augmentation indices to GTN or salbutamol.

Fourth, subjects were on long-term nitrates and/or  $\beta$ -blockers which may have underestimated the vasodilatory effects to GTN and salbutamol respectively. Long-term nitrate therapy can lead to the development of nitrate tolerance causing a lower vasodilatory response to GTN and an underestimation of the endothelium-independent peak and AUC values. Fifth, there are limitations with the technique. The vasodilatory action of salbutamol is only partly mediated by NO, and therefore there may be vasodilatation in the presence of a dysfunctional endothelium. In addition, there may be some persistent residual dilatation due to GTN during the administration of salbutamol which may have led to an overestimation of the vasodilatory response. Sixth, it is difficult to determine to what extent endothelial function measurements have been confounded by smooth muscle dysfunction or loss of smooth muscle sensitivity to NO. This may have affected the associations between arterial stiffness and endothelial function. Finally, the small numbers of fatal events were small and therefore limited the assessment of endothelial function as a predictor of cardiovascular and all-cause mortality.

## 6.5 CONCLUSIONS

The technique of pressure PWA in combination with the administration of GTN and salbutamol is not a good test of endothelial function in patients with CAD and therefore its association with standard cardiovascular risk factors and renal function cannot be determined. Moreover, endothelial function measured using this technique does not provide any valuable information on fatal and non-fatal outcomes.

## CHAPTER 7: CONCLUSIONS AND FUTURE WORK

## 7.1 SUMMARY

In this thesis, I describe how I assessed the reproducibility of skin elasticity measurements and endothelial function in healthy subjects and subjects with CAD respectively. Having established both techniques as being reproducible, I went on to assess skin elasticity as an alternative marker of arterial elasticity in a healthy group of subjects. Skin elasticity was not a reliable marker of arterial elasticity, and therefore I went on to assess arterial stiffness using conventional and a newer non-invasive methodology (DVP analysis) in patients with CAD. I also incorporated the newer non-invasive technique of pressure PWA with salbutamol, as a test of endothelial function, in this cohort of subjects. Endothelial function was measured using an automated tonometric device, Colin<sup>®</sup> 7000, coupled to a SphygmoCor<sup>®</sup> system which was found to be in good agreement with a more commonly used device, the Millar micromanometer. Next, the relationship between arterial stiffness and endothelial function with the presence and severity of CAD, and cardiovascular risk factors was assessed. Finally, I examined the relationship between arterial stiffness and endothelial function in subjects with CAD and the survival of subjects from cardiovascular morbidity and all-cause mortality as determined by the severity of CAD, arterial stiffness, renal and endothelial function.



## 7.2 TECHNICAL IMPROVEMENTS

### 7.2.1 REPRODUCIBILITY STUDIES

#### 7.2.1.1 Skin elasticity

Before studying the relationship between skin and arterial elasticity, it was necessary to assess the reproducibility of skin measurements in order to ensure that this methodology would produce valid consistent measurements in a clinical setting. It was also necessary to determine the region of the skin which may be least affected by external factors and the most closely associated with changes in the arteries. Skin elasticity measurements were carried out in the leg, arm and back and involved the R2, R5, R6 and R7 skin elasticity ratios. This study showed skin elasticity measurements were most reproducible in the upper leg and upper arm and the most reproducible skin elasticity ratio was the R6 ratio.

Whilst the above results showed good reproducibility, reproducibility of skin elasticity measurements may be improved by pre-tensioning [Serup *et al.* 1995] the skin by applying suction for a short period of time on the surface of the skin before measurements of vertical deformation. This pre-conditioning reduces the contribution of lateral displacement to the vertical displacement measurements.

In any future studies the absolute skin elasticity parameters, which have been adjusted for dermal skin thickness, should be studied where possible as they are measures of

individual absolute parameters rather than ratios of absolute parameters (relative parameters) [Dobrev 2002]. The individual absolute parameters would give more information on the contribution of specific components such as the elastic and viscous components. Skin thickness can be measured using the method of ultrasonic echography as described by Alexander and Miller [Alexander *et al.* 1979; Escoffier *et al.* 1989], however, it is currently not a clinically useful method.

#### 7.2.1.2 Endothelial Function

The non-invasive assessment of endothelial function using the technique of PWA has previously been carried out in a small number of patients with hypercholesterolaemia [Wilkinson *et al.* 2002b] and CAD [Hayward *et al.* 2002]. In this thesis I aimed to investigate the reproducibility of the PWA technique in subjects with CAD, whereby GTN was administered first followed by the administration of salbutamol 30 minutes later, as a potential tool for clinical risk assessment. This study showed that the changes in augmentation indices due to salbutamol were reproducible. However, the reproducibility of this technique may have been improved by a longer training period, prior to undertaking the study, of inhaling salbutamol via a Volumatic device.

#### 7.2.2 SKIN ELASTICITY AS A MARKER OF ARTERIAL ELASTICITY

Skin and arterial elasticity were weakly associated, with age being the largest determinant of arterial elasticity. However, the differences in the mechanistic effect of skin and arterial elasticity measures, could not be addressed, as there is no means of

determining the stress-strain curves of elastin and collagen in the aorta to be compared with that of the skin. We, however, do not know if skin elasticity and arterial elasticity are more closely associated in patients with vascular diseases, in whom other factors may be a more important determinant than age.

### 7.2.3 DETERMINANTS OF ARTERIAL STIFFNESS IN CAD PATIENTS

PWV was a more useful correlate of arterial stiffness than the augmentation, stiffness and reflection indices. Renal function, rather than standard cardiovascular risk factors, was associated with the stiffness of arteries in this cohort of subjects who had underlying CAD. Importantly, renal function, within the normal range, remained an independent predictor of arterial stiffness. This study, however, did not assess the underlying mechanism of the association between arterial stiffness and renal function, and therefore it is not known whether reduced renal function is a cause or effect of arterial stiffness in this group of subjects. When subjects were followed up for a period of 18 months, the presence of CAD, severity of CAD, stiffness of arteries and eGFR were predictive of cardiovascular events including death. Subjects who participated in this study were stable and are, therefore, more likely to have a low number of events. Therefore, extending the follow-up of subjects to 5 years is more appropriate, to investigate the key determinants of long term cardiovascular outcomes in this cohort of subjects.

#### 7.2.4 DETERMINANTS OF ENDOTHELIAL FUNCTION IN CAD PATIENTS

Subjects with CAD had a smaller change in augmentation indices to salbutamol compared to GTN. However, the salbutamol mediated changes in AIs were not dependent on standard cardiovascular risk factors or renal function. Moreover, endothelial dysfunction was not predictive of fatal and non-fatal outcomes. Changes in the augmentation indices following GTN were associated with changes in augmentation indices following salbutamol. However, we do not know whether this association is mediated by the presence of endothelial dysfunction and/or the presence of smooth muscle dysfunction. In addition, the association between arterial stiffness and changes in augmentation indices due to GTN but not salbutamol may also be affected by smooth muscle dysfunction.

The time required for endothelium-dependent changes in augmentation indices to return to baseline will need to be evaluated by carrying out a time response curve. This will lead to more accurate measurements of the AUC of the augmentation indices following salbutamol administration in future studies. Furthermore, one approach that may be useful, in future studies, would be to investigate the ratio of changes in augmentation indices of salbutamol following GTN, which reflects normal endothelial function, and the relationship of this ratio with the severity of vascular diseases such as hypercholesterolaemia, diabetes or renal disease.

### 7.3 CONCLUSIONS

Skin elasticity is not a marker of arterial elasticity and the very weak association does not justify further investigation, particularly in healthy volunteers. In treated patients with CAD, PWV is not determined by the severity of CAD or traditional CV risk factors. However, renal function is an important contributor to the variance in PWV. The severity of CAD, PWV and renal function are important predictors of fatal and non-fatal cardiovascular events.

The administration of salbutamol in combination with pressure wave analysis is not a useful test of endothelial function and does not provide any prognostic information that will be useful in the clinical management of patients with CAD on pre-existing treatments. Moreover, endothelial function is not associated with arterial stiffness in this treated population. However, in order to justify the lack of prognostic information and association with arterial stiffness, endothelial function should be assessed prior to starting treatment in CAD patients. The endothelial function protocol should also be revised to carry out measurements of the endothelium-dependent and -independent changes in augmentation indices on separate occasions to avoid any overlapping influence of either drug. In addition, more measurements should be taken to identify the peak effect and a longer time frame of the salbutamol-mediated response should be assessed in order to gather complete AUCs.

#### 7.4 FUTURE DIRECTIONS

In order to better understand if there is a pathological relationship between skin elasticity and arterial stiffness it is proposed that skin and arterial elasticity be assessed in diabetic patients who are predisposed to accelerated stiffening of arterial connective tissue. Patients with diabetes would be an ideal study population as intrinsic (pathological or genetically mediated factors), rather than extrinsic factors (external factors such as ultraviolet light, temperature and humidity), would play a larger role. They may also present with changes in connective tissue at an earlier age [Braverman *et al.* 1984] and therefore more remarkable changes in skin elasticity and arterial stiffness may be seen within a narrower age range. In addition, there are a large number of alternative techniques (e.g. high-frequency B-mode ultrasound) available to measure skin elasticity that are promising [de Rigal *et al.* 1989; Serup *et al.* 1995]. To date, however, there is a lack of standardisation of their application for measuring biophysical properties of the skin.

A suggestion for further research is to investigate the underlying relationship between deteriorating renal function and stiffening of the large elastic arteries in patients with CAD who have not been started on drug treatment. This will eliminate any confounding effect that drugs may have on arterial structure and function, and on renal function. Large longitudinal studies are required to determine the relationship and progression of arterial stiffness with renal function in patients with CAD. Moreover, large

interventional studies are needed to delineate which drug classes most influence arterial stiffness and renal function. For example, by aggressively lowering blood pressure in patients with CAD, we could evaluate the role of blood pressure in impairment of renal function through its damaging effect on the glomeruli. Another study for the future would be to evaluate the effect of antioxidants, such as vitamins C and E [Sies 1997], on vascular and renal function. Oxidative stress is known to be associated with atherosclerotic disease in patients with diabetes, renal disease and hypercholesterolaemia [Heistad 2006]. Importantly, oxidative stress results in an impairment of renal function via a reduction in glomerular filtration capacity and/or an increase in renal vasoconstriction and it is a marker of cardiovascular events [Himmelfarb 2005; Locatelli *et al.* 2003]. Therefore, it would be important to evaluate the effect of antioxidants on the progression or improvement of arterial stiffness and renal dysfunction. In addition, analysis of markers of reactive advanced glycation end-product (AGE) formation (urinary pentosidine and pyrraline), a measure of carbohydrate oxidation, may explain the high stiffness in patients with CAD who have low eGFRs [Falcone *et al.* 2005; Hudson *et al.* 2005; Kalousova *et al.* 2006]. AGEs are formed through the non-enzymatic reaction between glucose and reducing sugars with protein amino groups which results in the formation of stable Amadori products [Bucala *et al.* 1995; Tsukahara *et al.* 2003]. A small proportion of this product is transformed into AGEs irreversibly. AGEs are found in collagenous structures, such as basement membranes and vascular wall collagen and lead to collagen cross-linking. Moreover, AGEs increase

endothelial permeability and bind to AGE-specific receptors, for example on endothelial cells, vascular smooth muscle cells, and glomerular mesangial cells resulting in increased inflammation, oxidative stress, oxidation of LDL and a reduction in the vasodilator and antiproliferative effects of NO.

An important factor in future studies is the analysis of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NO synthase [Vallance *et al.* 2004]. ADMA is partially excreted by the kidneys and, therefore, the plasma concentration of ADMA is increased in patients with renal failure [Fliser *et al.* 2005; Vallance *et al.* 1992]. Moreover, plasma ADMA concentrations predict all-cause mortality and cardiovascular outcomes in patients on haemodialysis and in ESRD [Valkonen *et al.* 2001; Zoccali *et al.* 2001]. Therefore, by measuring plasma ADMA concentrations in future studies we will be able to assess its role as a risk factor in patients with CAD and its role as an important prognostic indicator in patients with CAD.



## CHAPTER 8: REFERENCES

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